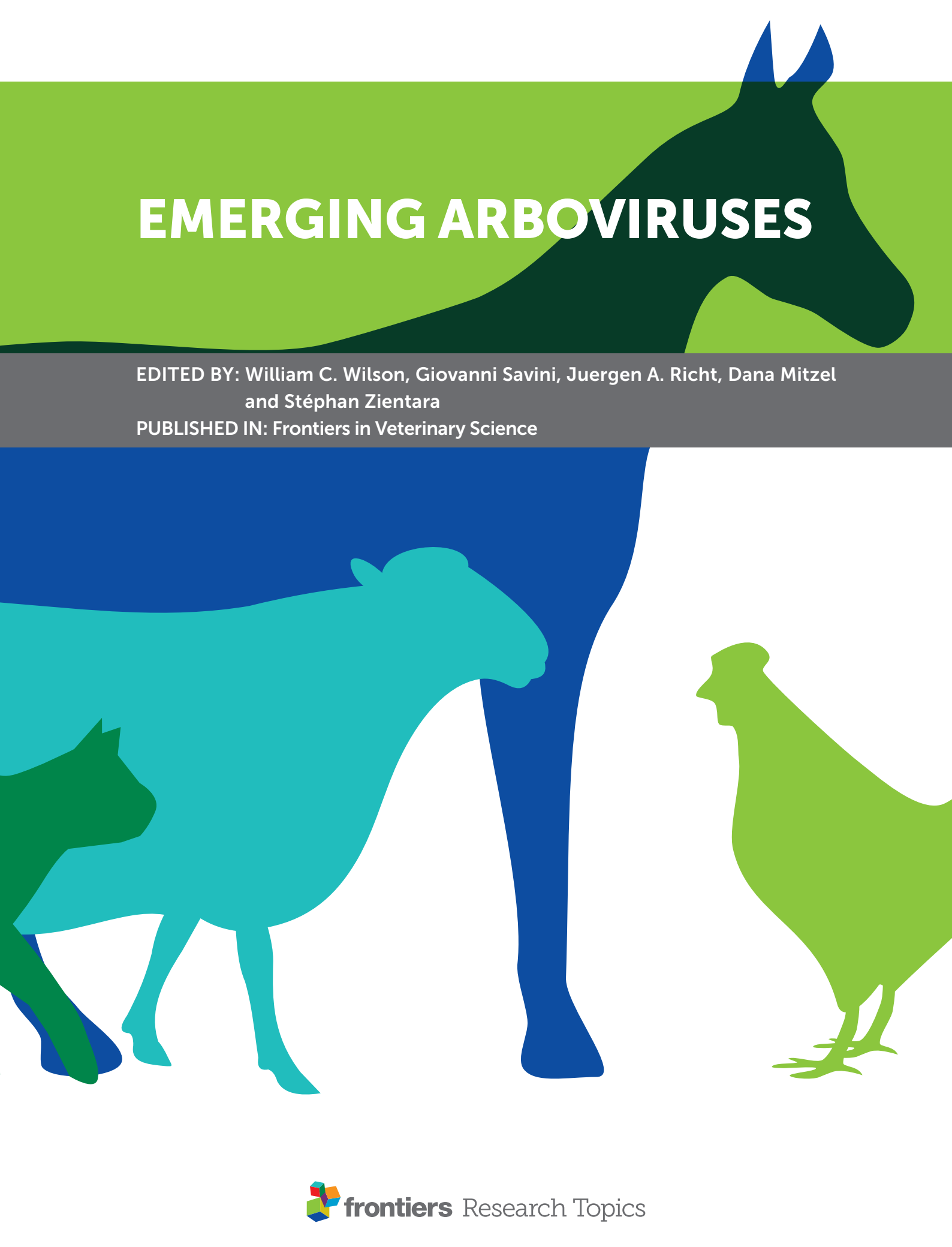


The cover features stylized silhouettes of animals in various colors. At the top, a dark green horse head is set against a light green background. Below this, a grey band contains the editors' names. The lower half of the cover is white, featuring a large blue silhouette of a cow, a smaller teal silhouette of a cat, and a light green silhouette of a chicken.

EMERGING ARBOVIRUSES

EDITED BY: William C. Wilson, Giovanni Savini, Juergen A. Richt, Dana Mitzel
and Stéphan Zientara

PUBLISHED IN: *Frontiers in Veterinary Science*



frontiers

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence.

The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714

ISBN 978-2-88966-427-6

DOI 10.3389/978-2-88966-427-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

EMERGING ARBOVIRUSES

Topic Editors:

William C. Wilson, United States Department of Agriculture, United States

Giovanni Savini, Experimental Zooprophyllactic Institute of Abruzzo and Molise G. Caporale, Italy

Juergen A. Richt, Kansas State University, United States

Dana Mitzel, United States Department of Agriculture, United States

Stéphan Zientara, Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), France

Citation: Wilson, W. C., Savini, G., Richt, J. A., Mitzel, D., Zientara, S., eds. (2021).

Emerging Arboviruses. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-88966-427-6

Table of Contents

- 06 Editorial: Emerging Arboviruses**
William C. Wilson, Dana Mitzel, Giovanni Savini, Stéphan Zientara and Juergen A. Richt
- 09 Prospects of Next-Generation Vaccines for Bluetongue**
Piet A. van Rijn
- 24 Failure to Remove Bluetongue Serotype 8 Virus (BTV-8) From in vitro Produced and in vivo Derived Bovine Embryos and Subsequent Transmission of BTV-8 to Recipient Cows After Embryo Transfer**
Andy Haegeman, Leen Vandaele, Ilse De Leeuw, André P. Oliveira, Hans Nauwynck, Ann Van Soom and Kris De Clercq
- 33 Emerging Trends in the Epidemiology of West Nile and Usutu Virus Infections in Southern Europe**
Tatjana Vilibic-Cavlek, Vladimir Savic, Tamas Petrovic, Ivan Toplak, Ljubo Barbic, Dusan Petric, Irena Tabain, Ivana Hrnjakovic-Cyjetkovic, Maja Bogdanic, Ana Klobucar, Anna Mrzljak, Vladimir Stevanovic, Petra Dinjar-Kujundzic, Luka Radmanic, Federica Monaco, Eddy Listes and Giovanni Savini
- 43 Detection of Neutralizing Antibodies to Tembusu Virus: Implications for Infection and Immunity**
Junfeng Lv, Lixin Yang, Shenghua Qu, Runze Meng, Qingxiangzi Li, Huicong Liu, Xiaoyan Wang and Dabing Zhang
- 53 Rift Valley Fever Virus, Japanese Encephalitis Virus, and African Swine Fever Virus: Three Transboundary, Vector-Borne, Veterinary Biothreats With Diverse Surveillance, and Response Capacity Needs**
Rebekah C. Kading, Edward O. Abworo and Gabriel L. Hamer
- 66 Potential of Using Capripoxvirus Vectored Vaccines Against Arboviruses in Sheep, Goats, and Cattle**
Mahder Teffera and Shawn Babiuk
- 78 Magnetic Nanotrap Particles Preserve the Stability of Venezuelan Equine Encephalitis Virus in Blood for Laboratory Detection**
Ivan Akhrymuk, Shih-Chao Lin, Mei Sun, Anurag Patnaik, Caitlin Lehman, Louis Altamura, Timothy Minogue, Ben Lepene, Monique L. van Hoek and Kylene Kehn-Hall
- 89 Serological Evidence for Japanese Encephalitis and West Nile Virus Infections in Domestic Birds in Cambodia**
Heidi Auerswald, Anne-Sophie Ruget, Helena Ladreyt, Saraden In, Soktheaom Mao, San Sorn, Sothyra Tum, Veasna Duong, Philippe Dussart, Julien Cappelle and Véronique Chevalier
- 98 Emerging Threats to Animals in the United Kingdom by Arthropod-Borne Diseases**
Arran J. Folly, Daniel Dorey-Robinson, Luis M. Hernández-Triana, L. Paul Phipps and Nicholas Johnson

- 117 ***Susceptibility of Chicken Embryos, Sheep, Cattle, Pigs, and Chickens to Zika Virus Infection***
Aruna Ambagala, Thang Truong, Colleen Cottam-Birt, Yohannes Berhane, Volker Gerdts, Uladzimir Karniychuk, David Safronetz and Shawn Babiuk
- 125 ***Perspectives Regarding the Risk of Introduction of the Japanese Encephalitis Virus (JEV) in the United States***
Ana R. S. Oliveira, Lee W. Cohnstaedt, Leela E. Noronha, Dana Mitzel, D. Scott McVey and Natalia Cernicchiaro
- 133 ***Identification and Characterization of Bluetongue Virus Serotype 14 in Russia***
Andrei Koltsov, Sodnom Tsybanov, Andrey Gogin, Denis Kolbasov and Galina Koltsova
- 141 ***Bluetongue Serotype 3 in Israel 2013–2018: Clinical Manifestations of the Disease and Molecular Characterization of Israeli Strains***
Natalia Golender, Velizar Bumbarov, Avi Eldar, Alessio Lorusso, Gabriel Kenigswald, Joseph Seffi Varsano, Dan David, Shani Schainin, Ilan Dagoni, Iosef Gur, Alon Kaplan, Anna Gorohov, Ori Koren, Eldad Oron, Yevgeny Khinich, Ily Sclamovich, Abraham Meir and Giovanni Savini
- 155 ***Endemic and Emerging Arboviruses in Domestic Ruminants in East Asia***
Tohru Yanase, Katsunori Murota and Yoko Hayama
- 174 ***Ecological Dynamics Impacting Bluetongue Virus Transmission in North America***
Christie Mayo, Emily McDermott, Jennifer Kopanke, Mark Stenglein, Justin Lee, Candace Mathiason, Molly Carpenter, Kirsten Reed and T. Alex Perkins
- 187 ***A Duplex Quantitative Real-Time Reverse Transcription-PCR for Simultaneous Detection and Differentiation of Flaviviruses of the Japanese Encephalitis and Ntaya Serocomplexes in Birds***
Maia Elizalde, Cristina Cano-Gómez, Francisco Llorente, Elisa Pérez-Ramírez, Laia Casades-Martí, Pilar Aguilera-Sepúlveda, Francisco Ruiz-Fons, Miguel Ángel Jiménez-Clavero and Jovita Fernández-Pinero
- 199 ***Development of a Digital RT-PCR Method for Absolute Quantification of Bluetongue Virus in Field Samples***
Angela M. Rocchigiani, Maria G. Tilocca, Ottavio Portanti, Bruna Vodret, Roberto Bechere, Marco Di Domenico, Giovanni Savini, Alessio Lorusso and Giontonella Puggioni
- 208 ***Increased Susceptibility of Cattle to Intranasal RVFV Infection***
Andrea L. Kroeker, Valerie Smid, Carissa Embury-Hyatt, Brad Collignon, Mathieu Pinette, Shawn Babiuk and Bradley Pickering
- 219 ***Enhanced West Nile Virus Circulation in the Emilia-Romagna and Lombardy Regions (Northern Italy) in 2018 Detected by Entomological Surveillance***
Mattia Calzolari, Paola Angelini, Luca Bolzoni, Paolo Bonilauri, Roberto Cagarelli, Sabrina Canziani, Danilo Cereda, Monica Pierangela Cerioli, Mario Chiari, Giorgio Galletti, Giovenale Moirano, Marco Tamba, Deborah Torri, Tiziana Trogu, Alessandro Albieri, Romeo Bellini and Davide Lelli

232 *African Swine Fever Virus: An Emerging DNA Arbovirus*

Natasha N. Gaudreault, Daniel W. Madden, William C. Wilson,
Jessie D. Trujillo and Juergen A. Richt

**249 *Protection of Cattle Elicited Using a Bivalent Lumpy Skin Disease
Virus-Vectored Recombinant Rift Valley Fever Vaccine***

David B. Wallace, A. Mather, P. D. Kara, Leeann Naicker,
Nobalanda B. Mokoena, A. Pretorius, T. Nefefe, N. Thema and Shawn Babiuk

**262 *Livestock Challenge Models of Rift Valley Fever for Agricultural Vaccine
Testing***

Andrea Louise Kroeker, Shawn Babiuk, Bradley S. Pickering, Juergen A. Richt
and William C. Wilson



Editorial: Emerging Arboviruses

William C. Wilson^{1*}, Dana Mitzel¹, Giovanni Savini², Stéphan Zientara³ and Juergen A. Richt⁴

¹ Arthropod-Borne Animal Diseases Research Unit, United States Department of Agriculture (USDA), Agricultural Research Service, Manhattan, KS, United States, ² OIE Reference Laboratory for Bluetongue, IZS Istituto Zooprofilattico Sperimentale, Teramo, Italy, ³ French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Maisons Alfort, France, ⁴ Department of Diagnostic Medicine/Pathobiology and Center of Excellence for Emerging and Zoonotic Animal Diseases, College of Veterinary Medicine, Kansas State University, Manhattan, KS, United States

Keywords: emerging, arbovirus, diagnostic, vaccine, risk model

Editorial on the Research Topic

Emerging Arboviruses

INTRODUCTION

The current COVID-19 pandemic has highlighted the well-known fact that debilitating infectious diseases can emerge naturally from an animal source. The estimates range from 60 to 75% of emerging infectious diseases in humans are of zoonotic origin. Up to one third of these emerging infectious diseases are caused by vector-borne pathogens, which cause more than 700,000 deaths annually (1). In the last two decades, there are multiple examples of the emergence of vector-borne pathogens affecting both animal and public health. This includes animal pathogens such as the introduction and re-emergence of Bluetongue virus serotype 8 into ruminant livestock in Europe (2) and the introduction of African Swine fever virus to Europe and Asia (Gaudreault et al.). On the zoonotic side, there is West Nile virus that was introduced into the United States in 1999 (3), Rift Valley fever which emerged in the Arabian Peninsula outside of its endemic area in Sub-Saharan Africa (4), and the displacement of one genotype of Japanese encephalitis virus by another in Asia (5) as well as its potential emergence in Australia (6); these are all examples of the recent emergence of arboviruses into animal and human populations. Emerging or re-emerging vector-borne diseases are an important *Global One Health* concern. A detailed understanding of the virus-vector-host interactions in its natural environment is critical to develop effective diagnostics, vaccines, and other control strategies. The present collection of manuscripts was developed to provide an unique compilation of recent advances in research and mitigation strategies for emerging and re-emerging arboviruses of veterinary/agricultural and public health concern such as viruses from the families *Asfarviridae*, *Flaviviridae*, *Phenuiviridae*, *Reoviridae*, and *Togaviridae*. This manuscript collection will provide scientists up-to-date information on these diverse pathogens and their respective insect vectors. It is organized with an initial series of articles reviewing risk assessments, which is followed by articles reviewing the state-of-the-art in epidemiology, diagnostics, vaccines, and other control methods.

ORGANIZATION OF THE SPECIAL EDITION

This Special Edition includes three several comprehensive reviews describing various emerging arthropod-borne disease threats in animals, each from a different continent with a different perspective. The first review is provided by Folly et al. and provides an overview of mosquitoes and mosquito-borne diseases and their possible risk of introduction into the UK. It also discusses ticks and tick-borne diseases including Louping ill virus, which is endemic in the UK, and biting

OPEN ACCESS

Edited by:

Michael Kogut,
United States Department of
Agriculture, United States

Reviewed by:

Fernando Bauermann,
Oklahoma State University,
United States

*Correspondence:

William C. Wilson
william.wilson2@usda.gov

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 11 August 2020

Accepted: 23 September 2020

Published: 06 November 2020

Citation:

Wilson WC, Mitzel D, Savini G,
Zientara S and Richt JA (2020)
Editorial: Emerging Arboviruses.
Front. Vet. Sci. 7:593872.
doi: 10.3389/fvets.2020.593872

midge-borne viruses, two of which emerged in Europe since 2006. The need for surveillance of these pathogens in domestic pets, livestock and wildlife is emphasized. The second review by Yanase et al. addresses endemic and emerging mosquito, tick-, and midge-borne arboviruses that affect domestic ruminants in East Asia. The midge-borne arboviruses include Akabane, Aino, and Chuzan viruses associated with reproductive losses in domestic ruminants in East Asia. The relevance in other Culicoides-transmitted viruses such as Bluetongue and the emergence of Epizootic Hemorrhagic Disease virus, serotype 6, in East Asia and Japan are also discussed. The final emerging disease review by Kading et al. identifies potential pathogen threats of concern to the United States. African Swine fever, Japanese encephalitis and Rift Valley fever as the top three arboviral threats are discussed in detail. In addition, currently available surveillance and diagnostic tools are summarized including useful information for the development of detection and response plans for these arboviral threats.

The above described reviews are followed by a series of manuscripts which are described below in virus-based alphabetic order. The review on the emerging DNA arbovirus, African Swine fever virus (ASFV), by Gaudreault et al. summarizes the threat of this pathogen to the swine industry worldwide. ASF has been a concern in Africa for more than a century and first emerged in Europe in the 1950s and again in the 1960s, and to Russia, Caribbean Islands and South America in the 1970's; it was controlled by the mid-1990's. The second emergence started in 2007 in the Caucasus region and since then the virus has spread across Eastern Europe and Southeast Asia. This review provides an overview of the epidemiology, biology, diagnostic, and mitigation strategies for this rather complex arboviral threat pathogen.

The next series of manuscripts describe various aspects of Bluetongue virus (BTV) that a long history of causing disease and economic losses. A description of the clinical disease associated with the incursion of BTV serotype 3 in Israel is provided by Golender et al., and the detection and characterization of BTV serotype 14 in Russia by Koltsov et al.. In 2006, BTV serotype 8 was introduced into Europe and caused substantial clinical disease and also transplacental virus transmission. The failure to remove BTV serotype 8 from bovine embryos with subsequent spread of the virus via embryo transfers demonstrated the need for screening of embryo donors and embryos for BTV in endemic areas (Haegeman et al.). This is critical since embryo transfer techniques have been used as a way to safely transfer genetic materials from a BTV endemic area to non-endemic regions. Since 1999, 11 exotic BTV serotypes have been isolated in the Southeastern United States but only one has been found outside of the Southeastern region (7). An overview of the ecology and epidemiology of BTV in North America by Mayo et al. provides insights into the environmental factors that drive virus transmission. Sensitive and early detection of BTV is essential to rapidly detect BTV and to facilitate mitigation strategies. Rocchigiani et al. describe the development of sensitive digital RT-qPCR for accurate quantification of BTV in field samples. This field-deployable RT-qPCR assay demonstrates

similar sensitivity and specificity to a previously established laboratory RT-qPCR assay.

Flaviviruses are discussed in the next series of manuscripts including Japanese encephalitis, Tembusu, Usutu, West Nile, and Zika viruses. Endemic circulation of Japanese encephalitis virus (JEV) in Southeast Asia is well-established; this is also an area where West Nile virus (WNV) is known to circulate. A seroepidemiological study by Auerswald et al. demonstrates clear evidence of current circulation of these viruses in Cambodian domestic birds; these findings necessitate the need for increased surveillance for these viruses in this region. The factors affecting the risks of JEV introduction into the United States were assessed by Oliveira et al.; this was done by qualitative risk assessment. WNV emerged in Europe in the early 2000's and another flavivirus, Usutu virus (USUV), which was originally found in Africa, was identified for the first time in the 1996 in Italy. Vilibic-Cavlek et al. summarize the epidemiology of these two mosquito-borne flaviviruses (WNV and USUV) in Southern Europe. Entomological surveillance by Calzolari et al. demonstrates increased circulation of WNV in Northern Italy and the importance of temperature on WNV infection of mosquitoes. A less known flavivirus, Tembusu virus which causes an egg-drop syndrome in ducks, emerged in 2010 in China. Vaccines against Tembusu virus have been generated but understanding of the neutralizing immune response to infection or vaccination is minimal. Lv et al. describe the development of a plaque reduction neutralization titration assay to detect antibodies to Tembusu virus and found long-lasting neutralizing antibodies in sera from infected and vaccinated flocks. The Japanese serocomplex includes JEV, USUV and WNV; whereas, Tembusu is grouped within the Ntaya serocomplex. The Ntaya complex also includes the Bagaza/Israel turkey meningoencephalomyelitis virus. The development of a duplex RT-qPCR assay to detect and distinguish between the Japanese and Ntaya serocomplexes is reported by Elizalde et al.. Zika virus is the most recent emerging mosquito-borne virus for which there are limited animal models available. Ambagala et al. conducted experimental infections with Zika virus in cattle, chickens, pigs, sheep and chicken embryos. None of the animals were susceptible to experimental infection except for the chicken embryos; this could provide an additional tool for Zika virus investigations.

The next series of manuscripts focusses on Rift Valley fever (RVF) which is a mosquito-borne zoonotic disease endemic in Sub-Saharan Africa. RVF is a significant public and animal health concern and reliable RVF virus (RVFV) infection models are badly needed to develop advanced diagnostic tools and control strategies. A review on presently available livestock models (cattle, sheep and goats) for experimental RVF virus infections is provided by Kroeker et al. Although RVFV is primarily transmitted by mosquito bites it is known that it can also be transmitted by aerosol (8). In addition, contact transmission has been demonstrated in one study of experimental RVFV infection of white-tailed deer (9). A comparison of different routes of experimental RVFV infection is reported by Kroeker et al.. Approaches to RVFV diagnostics and vaccines have been reviewed previously (10, 11); however,

a perhaps less known vector-based vaccine approach for RVFV is using capripoxvirus; the use of this virus vector for vaccines against different arboviruses affecting ruminant livestock is addressed in a review by Teffera and Babiuk. A similar approach is described by Wallace et al. with the development of a bivalent Lumpy Skin Disease-vectored Rift Valley fever virus vaccine.

An important part of a successful mitigation strategy for emerging and endemic pathogens is the ability to detect the presence of the agent in a rapid, sensitive and specific manner. The final paper in this Special Edition describes a novel approach using nanoparticles to preserve arboviral RNA in blood samples of infected animals thus increasing the ability for detection. Akhrymuk et al. discuss the development of this approach using Venezuelan equine encephalitis virus (VEEV), an often neglected but important zoonotic viral pathogen that can cause high mortality in horses.

CONCLUSION

The emergence of a new viral disease could affect public and animal health as well as the economy of many countries worldwide as demonstrated by the current COVID-19 pandemic. Therefore, we cannot forget that other known and unknown animal and zoonotic pathogens could also pose considerable

threats to animal and public health globally. Arthropod-borne pathogens have emerged and are re-emerging in increased numbers in the last decades. This Special Edition provides overviews of these arboviral threats and discusses current and novel strategies for their detection and control.

AUTHOR CONTRIBUTIONS

WW, DM, and JR wrote the editorial with careful editing and comments from SZ and GS. All authors contributed to the article and approved the submitted version.

FUNDING

We acknowledge the support of NBAF Transition Funds from the State of Kansas and the Department of Homeland Security support for the Center of Excellence for Emerging and Zoonotic Animal Disease (CEEZAD), grant number DHS HSHQDC-16-A-B0006 to JR.

ACKNOWLEDGMENTS

We also acknowledge the support of the United States Department of Agriculture, Agricultural Research Service for DM and WW.

REFERENCES

1. WHO. *Vector-Borne Diseases*. (2017). Available online at: <http://www.who.int/mediacentre/factsheets/fs387/en/>
2. Sailleau C, Breard E, Viarouge C, Vitour D, Romey A, Garnier A, et al. Re-emergence of bluetongue virus serotype 8 in France, 2015. *Transb Emerg Dis*. (2017) 64:998–1000. doi: 10.1111/tbed.12453
3. Kramer LD, Styer LM, Ebel GD. A global perspective on the epidemiology of west Nile virus. *Ann Rev Ent*. (2008) 53:61–81. doi: 10.1146/annurev.ento.53.103106.093258
4. Miller BR, Godsey MS, Crabtree MB, Savage HM, Al-Mazrao Y, Al-Jeffri MH, et al. Isolation and genetic characterization of Rift Valley fever virus from *Aedes vexans arabiensis*, Kingdom of Saudi Arabia. *Emerg Infect Dis*. (2002) 8:1492–4. doi: 10.3201/eid0812.020194
5. Karna AK, Bowen RA. Experimental evaluation of the role of ecologically-relevant hosts and vectors in Japanese encephalitis virus genotype displacement. *Viruses*. (2019) 11:32. doi: 10.3390/v11010032
6. van den Hurk AF, Ritchie SA, Johansen CA, Mackenzie JS, and Smith GA. Domestic Pigs and Japanese Encephalitis Virus Infection, Australia. *Emerg Infect Dis*. (2008) 14:1736–8. doi: 10.3201/eid1411.071368
7. Schirtzinger EE, Jasperson DC, Ostlund EN, Johnson DJ, and Wilson WC. Recent US bluetongue virus serotype 3 isolated found outside of Florida indicate evidence of reassortment with co-circulating endemic serotypes. *J. Gen. Virol*. (2018) 99:157–168. doi: 10.1099/jgv.0.000965
8. Reed C, Lin K, Wilhelmsen C, Friedrich B, Nalca A, Keeney A, et al. Aerosol exposure to Rift Valley fever virus causes earlier and more severe neuropathology in the murine model, which has important implications for therapeutic development. *PLoS Negl Trop Dis*. (2013) 7:e2156. doi: 10.1371/journal.pntd.0002156
9. Wilson WC, Kim JJ, Trujillo JD, Sunwoo SY, Noronha LE, Urbaniak K, et al. Susceptibility of white-tailed deer to rift valley fever virus. *Emerg Infect Dis*. (2018) 24:1717–9. doi: 10.3201/eid2409.180265
10. Dungu B, Lubisi BA, Ikegami T. Rift Valley fever vaccines: current and future needs. *Curr Opin Virol*. (2018) 29:8–15. doi: 10.1016/j.coviro.2018.02.001
11. LaBeaud AD, LaBeaud AD, Kazura JW, Kazura JW, King CH, King CH. Advances in Rift Valley fever research: insights for disease prevention. *Curr Opin Infect Dis*. (2010) 23:403–8. doi: 10.1097/QCO.0b013e32833c3da6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Wilson, Mitzel, Savini, Zientara and Richt. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Prospects of Next-Generation Vaccines for Bluetongue

Piet A. van Rijn^{1,2*}

¹ Department of Virology, Wageningen Bioveterinary Research, Lelystad, Netherlands, ² Department of Biochemistry, Centre for Human Metabolomics, North-West University, Potchefstroom, South Africa

OPEN ACCESS

Edited by:

Stéphan Zientara,
National Agency for Sanitary Safety of
Food, Environment and Labor
(ANSES), France

Reviewed by:

Emmanuel Breard,
National Agency for Sanitary Safety of
Food, Environment and Labor
(ANSES), France
Javier Ortego,
Instituto Nacional de Investigación y
Tecnología Agraria y Alimentaria
(INIA), Spain

*Correspondence:

Piet A. van Rijn
piet.vanrijn@wur.nl

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 27 September 2019

Accepted: 01 November 2019

Published: 21 November 2019

Citation:

van Rijn PA (2019) Prospects of
Next-Generation Vaccines for
Bluetongue. *Front. Vet. Sci.* 6:407.
doi: 10.3389/fvets.2019.00407

Bluetongue (BT) is a haemorrhagic disease of wild and domestic ruminants with a huge economic worldwide impact on livestock. The disease is caused by BT-virus transmitted by *Culicoides* biting midges and disease control without vaccination is hardly possible. Vaccination is the most feasible and cost-effective way to minimize economic losses. Marketed BT vaccines are successfully used in different parts of the world. Inactivated BT vaccines are efficacious and safe but relatively expensive, whereas live-attenuated vaccines are efficacious and cheap but are unsafe because of under-attenuation, onward spread, reversion to virulence, and reassortment events. Both manufactured BT vaccines do not enable differentiating infected from vaccinated animals (DIVA) and protection is limited to the respective serotype. The ideal BT vaccine is a licensed, affordable, completely safe DIVA vaccine, that induces quick, lifelong, broad protection in all susceptible ruminant species. Promising vaccine candidates show improvement for one or more of these main vaccine standards. BTV protein vaccines and viral vector vaccines have DIVA potential depending on the selected BTV antigens, but are less effective and likely more costly per protected animal than current vaccines. Several vaccine platforms based on replicating BTV are applied for many serotypes by exchange of serotype dominant outer shell proteins. These platforms based on one BTV backbone result in attenuation or abortive virus replication and prevent disease by and spread of vaccine virus as well as reversion to virulence. These replicating BT vaccines induce humoral and T-cell mediated immune responses to all viral proteins except to one, which could enable DIVA tests. Most of these replicating vaccines can be produced similarly as currently marketed BT vaccines. All replicating vaccine platforms developed by reverse genetics are classified as genetic modified organisms. This implies extensive and expensive safety trails in target ruminant species, and acceptance by the community could be hindered. Nonetheless, several experimental BT vaccines show very promising improvements and could compete with marketed vaccines regarding their vaccine profile, but none of these next generation BT vaccines have been licensed yet.

Keywords: bluetongue, safety, efficacy, affordability, DIVA, acceptance, marketed vaccine, experimental vaccine

INTRODUCTION

Bluetongue Disease

Bluetongue (BT) is a hemorrhagic disease of wild and domestic ruminants caused by bluetongue virus (BTV) (1, 2). BT is one of the main veterinary diseases worldwide causing significant economic losses (3, 4). The outcome of BTV infection varies and depends on the pathogenicity of the virus strain and the susceptibility of the ruminant host. Indigenous ruminants in BT endemic areas, goats, and cattle are less susceptible than many sheep breeds from BT-free areas (5). Sheep can induce severe clinical disease (6, 7), whereas cattle rarely show clinical disease but are readily infected and are an epidemiologically important BTV reservoir. BTV is not contagious but transmitted by biting competent *Culicoides* midges (8), whereas several recently discovered BTV serotypes spread without midges by direct contact transmission (9–11). Virulent BTV can also spread oronasally or vertically (12, 13) and have been reported in the field (14–16). A role of transplacental transmission in overwintering has been hypothesized (17), and trade of pregnant heifers can transport infectious BTV over long distances potentially causing outbreaks in former BT-free areas by delivery of viremic fetuses (18).

Bluetongue Virus

BTV is the prototype orbivirus within the genus *Orbivirus* of the family *Reoviridae* (19). Orbiviruses are non-enveloped viruses and consist of a three-layered icosahedral capsid containing a segmented genome. Ten double stranded RNA genome segments S1–10 encode seven structural proteins VP1–7 and at least 4 non-structural proteins NS1–4 (19–22). BTV infection results in a transcriptionally active core particle producing mRNAs of all ten segments which are released into the cytoplasm (23). BTV was recovered from core-derived mRNAs about 20 years later (24), and BTV was rescued by double transfection of ten synthetic RNA run-off transcripts from cDNAs, which is known as reverse genetics (25). Reverse genetics has opened endless possibilities to study viral functions in the BTV infected cell, in particular of non-structural proteins (26, 27). The BTV species or serogroup consists of many neutralization groups hardly showing cross-neutralizing antibodies and poor cross-protection (28, 29) (Figure 1).

BTV Serotypes

BTV serotypes 1–24 have been recognized by cross neutralization assays and have been confirmed by phylogenetic analysis of S2, which encodes the serotype specific and immunodominant VP2 protein of the outer shell (34) (Figure 1). Eastern and western topotypes of many serotypes are recognized, suggesting segregation a long time ago (35). In the last decade, at least five new BTV serotypes have been discovered (30, 32, 33, 36–38) (Figure 1). BTV25–27 are known as “atypical BTV,” because, in contrast to typical BTV1–24, these are exclusively found in small ruminants, are not pathogenic, spread by direct contact transmission, and cannot be cultured in *Culicoides* cells (9, 10, 39–41). BTV28 is also transmitted by in-contact transmission but causes clinical disease and its VP2 is closest related to the

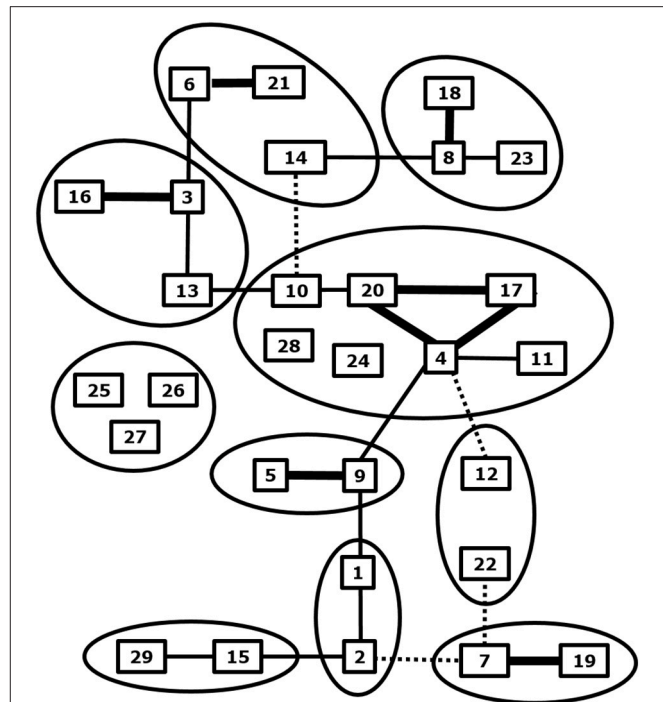


FIGURE 1 | Phylogenetic and neutralization relationship between BTV serotypes. Related BTV serotypes based on genome segment 2 expressing serotype specific immunodominant outer shell protein VP2 are grouped by circles. Cross neutralization between BTV serotypes is indicated by lines; strong (thick), some (normal) and weak neutralization (dashed). Adapted from Erasmus et al. (28), and Maan et al. (30) and updated for BTV27–29 from Bumbarov et al. (31), Wright (32), and Zientara (33).

main BTV genotype group consisting of serotypes 4, 10, 11, 17, 20, and 24 (31). BTV29 has been isolated from Alpaca in South Africa and is closely related to serotype15 based on phylogenetic and cross neutralization analysis (32). Two recently found BTVs are not studied in detail yet, but are proposed as new serotypes according to phylogenetic analysis of S2 sequences (37, 38). Discovery of more typical and atypical BTV serotypes in livestock and wild ruminant species can be expected by intensified surveys with more sensitive and new technologies (42). These “to-be-discovered” BTVs are likely not pathogenic but could become of concern, since mutations and reassortment with virulent BTV serotypes quickly change virus characteristics, including pathogenicity and the epidemiology.

Epidemiology

For a long time, BT has been widespread in tropical and subtropical regions all over the world, but is restricted and dependent on the local presence of specific competent biting midges in different parts of the world (43). At the end of the twentieth century, BT-affected areas had started to expand to former BT-free areas with a moderate climate, and outbreaks caused by emerging BTV serotypes have been frequently reported since then (44, 45).

BTV1, 2, 4, 9, and 16 entered southern Europe associated with expansion of the *Culicoides imicola* vector from northern Africa. In 2006, BTV8 (BTV8/net06) emerged in north-western Europe (15, 46), and was spread by indigenous midge species of the *Culicoides obsoletus* complex (47–49). Likely, global warming favors expansion of well-known competent midges species as well as increases the vector competence of some midge species. Subsequently, BTV8/net06 survived the inter-seasonal “vector-free” period known as “overwintering” and was spread to many European countries resulting in the largest recorded BT outbreak. Vaccination campaigns eradicated BTV8 in most European countries but BTV8 re-emerged in France in 2015 after 5 years of silence (50). This virus variant, BTV8/fr15, caused a lower viremia, less severe disease and virus transmission was much slower suggesting a lower vector competence. Likely, one or more amino acid changes in BTV8/fr15 are involved in this changed phenotype (51). In 2014, a new BTV reassortant of serotype 4 emerged in south-eastern Europe (52), and expanded to a wide area into Italy and mainland France in following years. Like BTV9 in this area, this BTV reassortant is likely spread by *C. obsoletus*, since *C. imicola* has not been found in the Balkan region. In 2017–18, BTV3 “jumped” from Tunisia to the Italian islands Sicily and Sardinia (53–55).

Many serotypes are endemic in Northern Australia but BTV5 emerged in 2015 for the first time (56). Additionally, the Australian authorities have moved the installed border of the BT-free area, including quarantine centers, southwards due to expansion of the BTV affected area (<https://animalhealthaustralia.com.au>). In large parts of the USA, serotypes 2, 10, 11, 13, and 17 are endemic and temporarily expand further northwards up to Canada depending on annual environmental conditions (57). In addition, 11 serotypes circulate in south-eastern USA, mainly in Florida, and reassortants of serotype 3 have been recently isolated in several states in the USA (58). Many serotypes are endemic in large parts of South America (59), but little is known of the BT-history on this continent.

BTV constantly evolves by mutations and reassortment events leading to invasion of new variants in areas with susceptible hosts and competent midges (60, 61). Additionally, global warming and climate change likely contribute to expansion of BT affected areas (57, 62). Intensified movements of animals and animal products will also increase the chance on incursions of BT. In conclusion, (re-)emerging BT outbreaks can be expected all over the world. Preparedness on this threatening situation should be of high priority to safeguard the health and production of ruminant livestock in developing and developed countries (63).

Control of Bluetongue

BT control by restrictions on trade and movements and vector control is inadequate, insufficient, non-proportional, and expensive compared to the impact, while destruction of infected ruminants is not acceptable by the community. The failure in disease control is mainly caused by uncontrolled spread of BTV by infected midges. Vaccination is the preferred method for BT control (64–66). Prophylactic and emergency vaccination have contributed to BT control and significantly reduce economic

losses caused by mortality, morbidity, reproduction problems, animal losses and lower milk production (67–69). The success of vaccination campaigns is best demonstrated by the eradication of BT in many European countries after the devastating outbreak caused by BTV8. Eradication of BT strongly depends on participation of animal owners to reach a high vaccination coverage of livestock, the used vaccine, and the field situation, like the presence of wildlife species as BTV reservoir and thus potential re-incursions (70). Still, intensified and repeated monitoring for several years is required to proof the absence of BTV circulation. Serological monitoring in the vaccination population is hindered by lack of specific assays to discriminate between infected and vaccinated animals, but is feasible by testing of non-vaccination sentinel herds or testing of selected newborn (non-vaccinated) animals after maternal antibodies have been disappeared.

VACCINES

Vaccine Profile

The ideal BT vaccine is efficacious, safe, affordable, and has been licensed. Preferably, the vaccine is a DIVA vaccine [Differentiation Infected from Vaccinated individuals (71)] to support eradication and to safely allow trade and movement of DIVA-vaccinated and BT-naïve animals. Each of these main standards for vaccines is the sum of several criteria (Figure 2). Efficacy is divided into protection against disease and blocking of onward virus transmission. Further, protection should be quick and lasting, preferably lifelong. Because of many neutralization groups, the ideal vaccine is broad protective or is tailor-made to anticipate on circulation of multiple serotypes. Safety is subdivided into non-pathogenic and no adverse effects in ruminants of different status, like pregnant and young animals. Further, the vaccine should not spread into the environment, like through uptake and spread by midges or in-contact transmission. Affordability consists of costs/dose and

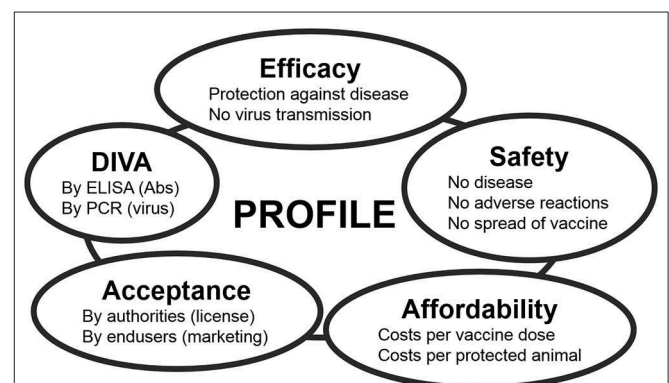


FIGURE 2 | The main standards for modern veterinary vaccines. Each standard can be subdivided into several criteria. The ideal vaccine completely meets all these criteria, but profiles of marketed and experimental vaccines mostly compromise between standards depending on the foreseen aim of vaccination and on the field situation Feenstra and van Rijn (70).

price/protected animal. The costs/dose depends on development costs and production costs, while the price/protected animal also depends on vaccine efficacy and vaccination strategy, like one single vaccination or repeated vaccinations to achieve lasting protection. Consequently, affordability is also associated with the value and lifespan of the susceptible species in a certain country or region. DIVA is subdivided into genetic DIVA to detect acute BTV infections, and serological DIVA to massively monitor (vaccinated) ruminant populations for anti-BTV Abs in order to detect past BTV circulation. Finally, for massive use and success of vaccination campaigns, the ideal BT vaccine should be licensed, and of course, its own success will increase the acceptance by users.

Marketed Vaccines

Currently, two types of marketed BT vaccines are used in large parts of the world, conventionally live-attenuated vaccines (LAVs) and inactivated BT vaccines. Both are based on whole BT-virus, and induce immune responses against immunogenic BTV proteins (**Figure 3A**).

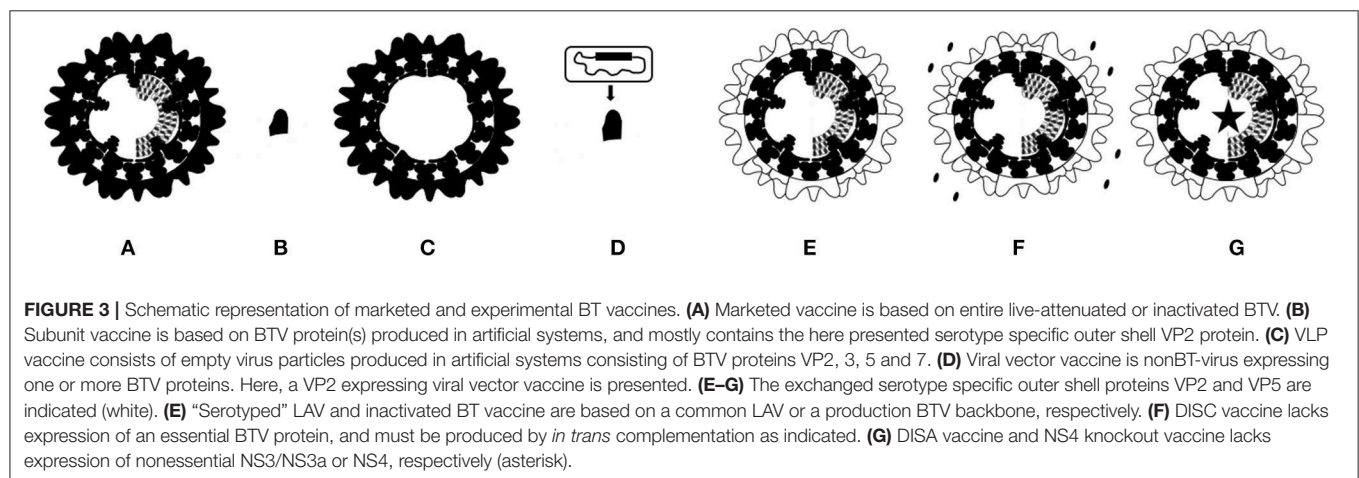
Live-Attenuated Vaccines

Protection by LAV is serotype specific, although some cross neutralization has been noticed (28) (**Figure 1**). A cocktail containing LAVs of 14 serotypes did not result in broad protection of sheep (72). However, multi-serotype LAV cocktails can induce neutralizing antibodies against not-included serotypes, and subsequent vaccinations with three different pentavalent LAV cocktails induce broad protection (73). These pentavalent cocktails contain 15 different serotypes in total; bottle A (serotypes 1, 4, 6, 12, and 14), bottle B (serotypes 3, 8, 9, 10, and 11), and bottle C (serotypes 2, 5, 7, 13, and 19). These LAVs induce some clinical reactions commonly including a transient febrile reaction [reviews; (72, 73)], and may cause teratogenic effects; abortions, stillbirths, fetal malformations, temporary infertility in rams, and ewes, and reduced milk production [(74) and included references]. Further, these LAV cocktails require a correct order of use, since bottles B and C contain under-attenuated LAVs, which could lead to a higher

incidence of disease if used as prime vaccination. Adverse effects have been shown after vaccination with LAVs of serotypes 2, 4, 9, and 16 in the Middle East and after temporarily use in southern Europe [reviewed in (75)]. More importantly, LAV viremia is sufficiently high for uptake by midges and thus onward spread, and these LAVs are no longer used in South Europe (76–79). Nonetheless, vaccination with LAVs prevent severe clinical disease and reduce viremia of wild type BTV (wtBTV) (80). Since the exact mutations and attenuation sites in LAVs are unknown and are likely located on different genome segments for each LAV, reversion to virulence and virulent variants by reassortment are possible (75, 81, 82). Despite of the debatable safety of conventionally live attenuated vaccines, these are used in several parts of the world, since LAVs are cheap and effective, while adverse reactions are marginal in local breeds (83, 84).

Inactivated Vaccines

In the 1970s and 1980s, inactivated BT vaccines have been developed in the USA but have not been licensed (85–88). The emergence of several BTV serotypes in Europe re-activated this approach. Inactivated BT vaccines for some serotypes have been licensed in Europe and are produced at industrial level on request in case of emergency [reviewed in (89)]. Inactivated vaccine cross protects early after vaccination by innate immunity but protection switches to serotype specific protection later on (90). In general, protection by inactivated vaccine is serotype specific, although some heterologous protection against other serotypes can be induced but hard to predict (91), whereas inactivated BT vaccines for serotypes 1 and 4 showed negative interference for serotype 4 (92). Inactivated BT vaccines are completely safe and, although for a limited number of serotypes, the only type of BT vaccine currently registered in Europe. Success of inactivated BT vaccines is the best demonstrated by eradication of serotypes 1, 2, 4, and 8 in several European countries after massive vaccination (75, 93–95). Field application shows very good records and neutralizing antibodies persist for many years (96–98). Details of BTV antigen production, formulation and adjuvant have not been published in detail. The amount of antigen per dose of inactivated BT vaccine typically corresponds to approximately



10^7 TCID₅₀ virus (99), which is about 100 times more than 10^5 TCID₅₀ virus/dose for LAVs. Inactivated BT vaccine is therefore more expensive but safer than LAV. Particularly, inactivated vaccine is more expensive for large ruminants, since booster vaccination is recommended (100). Inactivated BT vaccines are potential DIVA vaccine, since non-structural (NS) proteins can be removed from produced BTV antigen. ELISAs to detect antibodies directed to NS proteins have been published (101–103). However, stringent purification to remove NS proteins from crude extract of produced BTV particles is required and will increase the production costs of inactivated BT vaccine.

In conclusion, LAVs and inactivated BT vaccines are available, although for the latter only for a limited number of serotypes. Despite of several success stories in different parts of the world for these marketed vaccines, both vaccine types have their specific shortcomings. The current choice of vaccine depends on many aspects, including the objective, local legislation, and their vaccine profile taking pros and cons into account. Clearly, there is ample room for improvement of currently used vaccines (70).

Promising Experimental Vaccines

Several experimental BT vaccines are under development, and are divided into; (1) vaccines based on BTV proteins, e.g., VP2 subunit and virus like particles (VLPs); (2) viral vector vaccines based on nonBT-virus expressing one or more BTV proteins, and; (3) vaccine platforms based on BTV (Figure 3). These approaches are subject of vaccine research for many years and show improvements compared to marketed vaccines.

BTV Protein Vaccines

Experimental protein based BT vaccines all include the serotype specific immunodominant VP2 protein (Figure 3B). Protein production has been studied in bacteria (104), in insect cells (105–108), in yeast (109), and in plants (110–112).

VP2 subunit vaccines

A protective dose by VP2 could be reduced 50% by adding VP5 protein, but adding of Freund's adjuvant or other BTV proteins did not further enhance the protective immunity (113). Recently, 150 µg purified VP2, NS1, and NS2 proteins with the immunostimulating complex AbISCO-300 showed a good cellular and humoral immunity in cattle (114). This candidate protects calves 3 weeks after booster vaccination. T-lymphocytes were mainly raised against NS1 and are cross reactive amongst different serotypes because of a higher conservation of NS1 protein. This suggests that the cellular responses to NS1, and likely NS2, can be the fundament of vaccine for other serotypes by varying VP2 protein (115). Other experimental subunit vaccine candidates have been developed and showed promising results but are mostly not tested in the natural ruminant host yet (110). Two domains of VP2 (aa 63–471 and 555–956) and VP5 lacking the first 100 amino acids are produced in bacteria as soluble fusion-proteins with glutathione S-transferase (116). Immunized IFNAR^(-/-) mice expressed neutralizing antibodies and survived homologous challenge without clinical signs after booster vaccination with 15 µg of the VP2 domains and 25 µg VP5. Addition of VP5 protein enhanced the immunity but

addition of VP7 did not. VP2, VP7, and NS1 were incorporated in MuNS microspheres (117). An advantage of these inclusions using the baculovirus expression system is the easy method of purification and their potent adjuvant activity (118). IFNAR^(-/-) mice immunized with these particles without adjuvant induced both humoral and cellular immune responses, and these mice were protected against lethal BTV challenge. VP2 has also been fused to the antigen presenting cell homing (APCH) molecule, and was produced in insect cells (119). APCH fusion has been demonstrated to improve the immune responses induced against many different antigens. This antigen formulated with oil adjuvant Montanide ISA50 showed a good humoral immune response in cattle with a minimal dose of 900 ng, but a BTV challenge has not been performed. IFNAR^(-/-) mice have also been vaccinated and specific CD4+ and CD8+ T cells producing IFNγ following virus stimulation were observed, whereas lower levels were recorded for mice immunized with only VP2. Part of the VP2 gene has also been expressed using *Pichia pastoris* (109). High level of secreted expression was achieved, and the produced protein is immunogenic in rabbits.

VLP vaccines

VLPs are empty virus particles consisting of structural proteins and are investigated as vaccine candidates for decades. BTV VLPs consist of VP3, VP7, VP2, and VP5 which are expressed in insect cells using baculovirus expression (120–124), and by the *Nicotiana benthamiana* plant and the cowpea mosaic virus based HyperTrans plant transient expression vector system (125) (Figure 3C). A cocktail of VLPs for several serotypes 1, 2, 10, 13, and 17 protected against all five serotypes and partially protected against some other serotypes (126). Huge sheep trials with 50–200 sheep per trial showed afforded protection by VLP vaccination against homologous challenge (127). Despite of all these efforts and promising results, VLPs have not been manufactured in that time. Most likely, marketed inactivated BT vaccines are much cheaper to produce, and equally safe. Protein and VLP production in plants have become an increasingly popular alternative for artificial protein production of complex high-value proteins, and might become cost effective.

New inactivated BT vaccines

A vaccine platform for production of inactivated BTVs has been developed (128). Reverse genetics for BTV1 (25) was used to exchange serotype specific outer shell proteins of 18 BTV serotypes (Figure 3E). The prototype “serotyped” inactivated BT vaccine for serotype 8 induces serotype specific neutralizing antibodies and protects sheep against virulent BTV8 challenge. This synthetic biology approach will optimize production and will shorten the time to produce inactivated BT vaccines for new and emerging serotypes.

Summarizing, protein based BT vaccines provide opportunities compared to commercial inactivated BT vaccines. VP2 subunit and VLP vaccines contain specific BTV proteins and are produced in artificial production systems. Therefore, these require minimal biocontainment facilities and can be DIVA compliant. In particular, guaranteed vaccine safety by lack of

infectious BTV or contamination of animal related viruses is a great advantage of protein based BT vaccines.

Viral Vector Vaccines

Different viruses have been explored as vector for the development viral vector vaccines expressing one or more BTV proteins intracellularly and therefore inducing cytotoxic T lymphocyte responses in addition to humoral responses (**Figure 3D**). Replication of viral vector vaccines is abortive, and will not induce clinical signs associated to BTV infection. Canarypox virus expressing both VP2 and VP5 induced sterile immunity in sheep (129), whereas capripox viruses expressing VP2, VP7, NS1, or NS3 induced partial protection (130). Myxomavirus expressing VP2 or both VP2 and VP5 also partially protects sheep against BT (131). Bovine herpes virus expressing VP2 targeted to the cell membrane also induced partial protection in IFNAR^(-/-) mice (132). Immunization of IFNAR^(-/-) mice with equine herpes virus expressing both VP2 and VP5 protects against mortality but mild clinical signs were observed after challenge (133). All these viral vector vaccine candidates require booster vaccination, and most of these did not completely protect mice or the ruminant host. A promising exception with regard to previous research on viral vector vaccines is the wide immunoprotection of IFNAR^(-/-) mice by inoculation with modified vaccinia Ankara virus (MVA) vector expressing an immunodominant epitope on BTV-NS1 protein (134). Research in ruminants is needed to study broad and effective protection in the target species.

The main obstacle of viral vector vaccines is immunity against vector associated antigens by previous exposure [reviewed in (135)]. Priming by DNA vaccination followed by vaccination with viral vector vaccine can partially overcome the disadvantage, and DNA vaccine is a potent inducer of Th1 responses. However, reliability and effectiveness of DNA vaccines are questionable by inefficient delivery and is therefore still limited [reviewed in (136)]. Prime vaccination with BTV1 pCAGGS DNA vaccine (137), followed by recombinant fowlpox virus vaccine for VP2, VP5, or both proteins induced T-cell response in BALB/c mice, and high titres of neutralizing antibodies in both mice and sheep but protection against BTV was not investigated (138). Similar strategies showed protection in IFNAR^(-/-) mice with plasmids encoding VP2, VP5, and VP7 and MVA vector (139), and with NS1 instead of VP5 showed a higher T-cell response and heterologous immunity (140). VP2 expression induced protection to homologous challenge similar as expression of VP2, VP5, and VP7 together, which indicates the importance of serotype specific immunodominant VP2 protein (141). The prime-boost strategy with DNA and viral vector vaccines is promising but more research in the susceptible ruminant host is needed.

Viral vector vaccines are potential DIVA vaccines and safety with regard to lack of infectious BTV is guaranteed. In addition, production of viral vector vaccines requires a permitted (lower) biocontainment level and will lower the production costs. Some viral vectors have been registered and likely further reduces the costs to license these viral vector vaccines for BT.

Replicating BT Vaccines (MLVs)

Development of reverse genetics for orbivirus prototype BTV was a breakthrough in orbivirus research (25), and has been optimized to robustly generate BTV mutants, modified-live vaccines (MLVs) and “synthetic” reassortants (142–144). Reverse genetics has been used for fundamental and applied research to investigate viral functions in the BTV infected cell. Synthetically derived BTV is indistinguishable from its virulent or nonvirulent ancestor BTV (145). The segmented BTV genome is very flexible, and many desired so-named “synthetic” BTV reassortants can be generated easily using a set of 10 selected RNA run-off transcripts (145, 146). One example as used for the here described vaccine platforms is the forced exchange of S2[VP2] and S6[VP5] encoding serotype immunodominant outer shell proteins. In addition, reverse genetics opened possibilities to manipulate viral functions by genetic modification of BTV in order to develop replicating vaccine platforms (MLV platforms).

“Serotyped” live-attenuated vaccines

A new generation of experimental LAVs is based on LAV serotype 6 (BTV6/net08) (35, 147) with exchanged outer shell proteins (**Figure 3E**). This LAV platform has been studied for serotypes 1 and 8 and results in nonvirulent so-named “serotyped” LAV1 and 8, respectively (148). Vaccination with monovalent or a trivalent cocktail of serotyped LAVs protects sheep against virulent BTV and induces serotype specific neutralizing antibodies against included serotypes. To combat multiple serotypes, tailor-made cocktails of serotyped LAVs could be freely applied, since reversion to virulence by reassortment between serotyped LAVs will be negligible because of the common LAV backbone. Consequently, these LAVs share most genome segments and the risk of arise of virulent variants has minimized. Further, negative interference of protection by different serotyped LAVs will be minimized because of the shared replication machinery. Though, reversion to virulence of serotyped LAVs by point mutations is a potential risk (149). Furthermore, elevated body temperature, clinical signs and viremia have been observed after vaccination (148). Therefore, safety of serotyped LAVs is incomplete and debatable as viremia could lead to undesired onward spread of vaccine virus by midges. Altogether, cocktails of serotyped LAVs are safer than cocktails of conventional LAVs, but their safety is still debatable due to the risk of reassortment events with wtBTV.

Disabled infectious single cycle (DISC) vaccines

Reverse genetics has initiated the development of improved vaccines by genetic modification of BTV. The Disabled Infectious Single Cycle (DISC) vaccine platform is based on BTV1 without expression of essential viral helicase VP6 (150). DISC vaccine virus cannot fulfill the virus replication cycle by lack of *de novo* VP6 synthesis, and DISC vaccine viruses must be produced by *in trans* complementation in cells expressing VP6 protein (**Figure 3F**). Consequently, DISC vaccine virus infects cells of the vaccinated ruminant only once, since infectious BTV cannot be assembled. The abortive replication of DISC vaccine virus induce a full blown immune response closely mimicking BTV infection, and results in mRNA synthesis and expression of all BTV proteins, except for VP6. The DISC vaccine platform has

been applied for several serotypes by exchange of the serotype specific outer shell. Monovalent DISC vaccine and some DISC cocktail vaccines have been studied in sheep and cattle (150–152). A single DISC vaccination is protective in both sheep and cattle. In these studies, DISC vaccination contains crude cell lysate with $\pm 1 \times 10^7$ TCID₅₀/ml per DISC vaccine virus, but the minimal protective dose of DISC vaccine has not been determined yet. DISC vaccine virus is completely safe with respect to clinical signs and viremia, although transient positivity by PCR has been observed short after vaccination. Monovalent DISC vaccine for serotype 8 protects sheep against clinical signs and viremia (150). Trivalent DISC vaccine for serotype 2, 4, and 8 completely protects sheep and cattle at 3 weeks post booster vaccination against virulent BTV2, 4 or 8 (151). Hexavalent DISC vaccine for serotypes 1, 2, 4, 8, 13, and 21 also protects against virulent BTV2 or 8 (152). Moreover, hexavalent DISC vaccine induced neutralizing antibodies against all included serotypes after booster vaccination, suggesting protection for all these serotypes. The deletion in S9[VP6/NS4] of DISC vaccine abolishes expression of VP6 but also of recently discovered NS4 protein. NS4 protein is not essential for virus replication *in vitro* but antagonizes Interferon-I expression *in vivo* (26, 153). Likely, lack of NS4 will positively affect the immune response by DISC vaccination, although this has not been studied. So far, the studied DISC vaccine consists of crude cell lysate with minor amounts of complemented VP6 protein. The DIVA potential of the DISC vaccine platform based on VP6 or NS4 has not been investigated yet.

Disabled infectious single animal (DISA) vaccines

The principle of Disabled Infectious Single Animal (DISA) is a blockade on transmission of vaccine virus by midges. The key of DISA vaccine platform is knockout of NS3/NS3a protein by a deletion in S10[NS3/NS3a] (Figure 3G). Both NS3 and NS3a protein are not essential for virus replication *in vitro*, whereas virus release from *Culicoides* cells depends on NS3/NS3a protein (27). DISA vaccine virus cannot propagate in competent midges after intrathoracic inoculation (154). Moreover, a small in-frame deletion of 72 amino acid codons in NS3/NS3a protein leads to the same phenotype (155). Furthermore, DISA vaccine virus cannot pass the midge midgut barrier after blood feeding, and cannot reach the salivary glands, and therefore will not be secreted in saliva (155). It has been proposed that DISA vaccine virus only replicates near the vaccination site (156). Altogether, onward transmission of DISA vaccine has been blocked on uptake as well as on secretion (Figure 4). The DISA vaccine platform has applied for several serotypes by single S2[VP2] exchange (157), by exchange of both outer shell proteins as described (128, 148), and by incorporation of chimeric S2[VP2] of serotype 1 and 16 (157). DISA vaccine can be produced in established vaccine production facilities similar as for production of LAV or BTV antigen.

Virulent BTV8 without NS3/NS3a expression does not cause disease in sheep, indicating that NS3/NS3a is essential for virulence (156). Several deletions in S10[NS3/NS3a] are genetically unstable, but NS3/NS3a expression and pathogenicity of BTV has never been restored (158). Replication of DISA vaccine virus is required for protection but does not cause

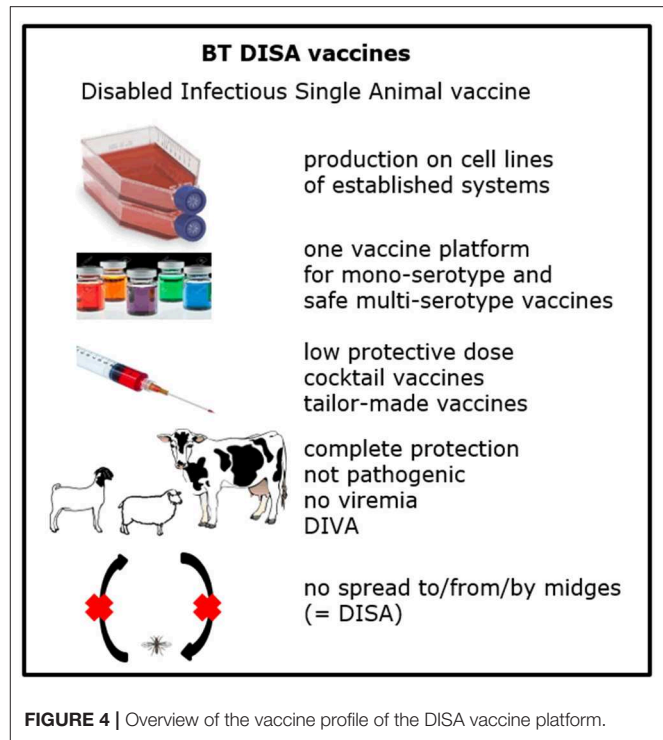


FIGURE 4 | Overview of the vaccine profile of the DISA vaccine platform.

viremia (156). DISA vaccine based on BTV6/net08 (159) is superior to that based on a BTV1 or BTV8/net06 backbone with respect to protection, and completely protects sheep against virulent BTV8 at 3 week post single vaccination (156). Prime-boost DISA vaccination results in lasting serotype specific protection (160). A standardized dose of $2 \times 1 \text{ ml } 10^5$ TCID₅₀/ml DISA vaccine was subcutaneously administered in these studies, however, a 100 times diluted vaccine dose, $2 \times 1 \text{ ml } 10^3$ TCID₅₀/ml, and intramuscular or intravenous vaccination with a standard dose all results in VP7 seroconversion (161). Recent vaccination-challenge studies demonstrate early and serotype specific protection after intramuscular vaccination of cattle with DISA vaccine with the small in-frame deletion (van Rijn et al. personal communication). Further, prime-boost intramuscular vaccination of sheep with a pentavalent cocktail of DISA vaccines for the “European” serotypes 1, 2, 3, 4, and 8 based on the same DISA platform protects against virulent BTV2 or 8, suggesting that sheep are protected for all five serotypes (van Rijn et al. personal communication) (Figure 4). Lack of NS3/NS3a protein likely enhances the interferon mediated immune response, since NS3/NS3a counteracts the innate immune response, and in particular the type I interferon (IFN- α/β) pathway by different mechanisms (162–164).

Finally, DISA vaccine is DIVA compatible with panBTV PCR tests targeting S10 (36, 165–167), since the deletion in S10 partially overlaps their PCR targets (168). Furthermore, BTV infection induces NS3 Abs (102), and DISA vaccine is therefore DIVA compatible with an experimental NS3 competitive ELISA (103). Indeed, the NS3 competitive ELISA differentiates BTV infected from DISA vaccinated animals (156, 160) (Figure 4). Studies in large animal groups, preferably in the field, are

required to determine the final vaccine profile of these DISA vaccines. Although DISA vaccines are scientifically safe and rationally acceptable, the current hurdle is permission to perform field trials with DISA vaccines as these BTVs with a small deletion are classified as GMOs.

NS4 knockout vaccines

BTV without NS4 expression from S9[VP6/NS4] could be an attractive vaccine platform, as NS4 is a determinant of virus virulence (153) (**Figure 3G**). Three silent point mutations in the VP6 open reading frame result in a mutated NS4 start codon and two in-frame stop codons in the open reading frame of NS4 adjacent downstream the NS4 start codon and selectively abolish NS4 expression. The BTV NS4 knockout mutant did not induce elevated body temperature nor clinical signs in sheep, while neutralizing antibodies were raised against the BTV NS4 knockout mutant similar as by wtBTV infection. Unfortunately, viremia was observed after inoculation and lasted for up to 28 days and protection against BTV challenge was not studied. Recovery of NS4 expression, and thus virulence, is minimized by the triple point mutation. However, due to its lasting viremia, and potential onward spread by midges, reversion to virulence cannot be excluded. BTV NS4 knockout mutants are not further explored as potential vaccine yet, but a NS4 knockout mutant of the related African horse sickness virus has shown promising results in horses (169). The BTV NS4 knockout mutant replicates in cell lines as used for BTV propagation, indicating that production of BT NS4 knockout vaccines should be possible in established facilities. Similar to other published BT vaccine platforms, this platform will be applicable for many serotypes by exchange of serotype specific outer shell proteins.

The here described MLV platforms are based on one appropriate virus backbone used to vary one or two segments encoding serotype specific outer shell proteins. Thus, each platform share 8 or 9 out of 10 genome segments including one mutated segment for most of the vaccine platforms (**Figures 3E–G**). Consequently, the vaccinated animal induces humoral as well as T-cell mediated responses directed against all BTV proteins, except for the one encoded by the modified genome segment. The lack of expression leads to attenuation (NS4 knockout platform), abortive replication (DISC platform), or a combined non-transmissibility, non-virulence and DIVA (DISA platform). Importantly, the shared backbone prevents reversion of virulence by reassortment between vaccine viruses. Tailor-made cocktail vaccines or foreseen broad protective vaccines are equally safe as single vaccines. Nonetheless, these modern vaccine platforms based on reverse genetics are genetically modified organisms (GMOs), and more efforts must be invested to proof their complete safety but eventually could allow a lower biosecurity level for vaccine production.

Expectedly, the here described approaches could be combined by improved technologies in the future. Inactivated DISA vaccine combines DIVA and avoids the GMO issue. Reverse genetics for circulating or (re-)emerging wtBTVs will be quickly developed in the future. In combination with the modification according to the described MLV platforms will result in a safe and protective vaccine that will induce an immune response exactly matching to

the field BTV strain. More importantly, this strategy will avoid arise of virulent variants by reassortment events between vaccine strain and wtBTV.

CONCLUDING REMARKS

The main vaccine standards are efficacy, safety, affordability, DIVA, and acceptance by the community. Marketed LAVs and inactivated BT vaccines are both successful to control BT outbreaks but have their specific pros and cons. LAVs are cheap but considered unsafe, while inactivated BT vaccines are safe but more expensive (**Table 1**).

In addition, both marketed BT vaccines lack DIVA and have limitations with regard to safely combat multi-serotype situations in the field. Experimental BT vaccines, such as protein vaccines, viral vector vaccines, and replicating vaccines, have been developed and some are well studied but none have been licensed yet. Nonetheless, new vaccine candidates show improvement for one or more of the vaccine standards. However, their final vaccine profile has not been definitely determined yet, although some can be assumed based on the present data. Because of this incompleteness, comparison of their final (expected) vaccine profiles is hardly possible (**Table 1**).

Efficacy

MLVs are likely more effective than BTV protein vaccines and viral vector vaccines, since replicating BT vaccines can induce humoral and T-cell mediated immune responses against almost every BTV protein, and show protection after single vaccination. Further, broad protection is likely easier to achieve, since more conserved epitopes among BTVs as well as serotype specific epitopes are exposed to the immune system. Furthermore, application for multiple serotypes have been successfully studied for several MLV vaccine platforms.

Safety

BTV protein vaccines and viral vector vaccines are completely safe due by the absence of infectious BTV, although local reactions on the vaccination site could be induced depending on the used adjuvant. Safety of MLVs varies between different platforms. DISC and DISA vaccines do not cause viremia or adverse effects, and are blocked on spread of vaccine virus between animals. “Serotyped” LAVs and NS4 knockout vaccine are not 100% safe, since a significant viremia could lead to onward transmission of vaccine virus by midges and might transmit vertically to the fetus.

Affordability

The price per dose as well as per protected animal is hard to calculate for these experimental vaccines. Expectedly, vector vaccines and MLVs will be cheaper than BTV protein vaccines, since replication of MLVs in the receipt will trigger the immune system better than BTV protein based vaccines. Generally, the protective dose will be lower for replicating vaccines. Eventually, affordability will depend on vaccine efficacy but also on required boost vaccinations.

TABLE 1 | Evaluation of vaccine profiles of marketed and experimental vaccines.

Marketed vaccines	Protection	Safe	Affordable	Acceptable	DIVA	Main advantages	Main disadvantages	Remarks
Live-attenuated vaccine (LAV)	Yes	No ¹	Yes	Yes/No ²	No	Cheap full blown response	No DIVA unsafe, in particular for cocktails	¹ : Virulence (residual and/or reversion) ² : Licensed in African countries, not accepted in other countries
Inactivated vaccine	Yes ¹	Yes	Yes ²	Yes ³	No	Safe	No DIVA expensive	¹ : Most require annual revaccination ² : More expensive than LAVs ³ : Available for limited serotypes
Experimental vaccines	Protection	Safe	Affordable	Acceptable	DIVA	Main advantages	Main disadvantages	Remarks
PROTEIN VACCINES								
VP2 subunit vaccine	Yes ¹	Yes	No ²	Yes	Yes ³	Commercial DIVA test	Expensive late onset	¹ : Requires booster vaccination ² : Likely expensive ³ : Commercial VP7 cELISA
VLP vaccine	Yes ¹	Yes	No ²	Yes	Yes ³	Safe	Expensive late onset	¹ : Requires booster vaccination ² : Likely expensive ³ : Experimental NS ELISAs
"Serotyped" inactivated vaccine	Yes ¹	Yes	Yes ²	Yes	No	Traditional vaccine production	No DIVA	¹ : Requires annual revaccination ² : More expensive than LAVs
VIRAL VECTOR VACCINES								
VP2 expressing vector vaccine	Yes ¹	Yes	? ²	Yes ³	Yes ⁴	Commercial DIVA	Late onset unknown efficacy in ruminants	¹ : Requires booster vaccination ² : Not or marginally tested in ruminants ³ : Safe viral vector but GMO ⁴ : Commercial VP7 cELISA
NS expressing vector vaccine	Yes ¹	Yes	? ²	Yes ³	Yes ⁴	Commercial DIVA test proposed broad protection	Late onset unknown efficacy in ruminants	¹ : Requires booster vaccination ² : Not or marginally tested in ruminants ³ : Safe viral vector but GMO ⁴ : Commercial VP7 cELISA
REPLICATING VACCINES (MLV)								
"Serotyped" LAV	Yes	No ¹	Yes	Yes ²	No	Traditional vaccine production full blown response	No DIVA viremia	¹ : Viremia suggests onward transmission cocktails will be safer than of LAVs ² : BTV reassortant (GMO issue)
DISC vaccine	Yes	Yes ¹	Yes ²	Yes ³	No	Abortive vaccine replication combined with full blown response	No DIVA high dose	¹ : Abortive replication ² : Likely high protective dose ³ : Complemented BTV (GMO issue)
DISA vaccine	Yes	Yes ¹	Yes	Yes ²	Yes ³	No vaccine transmission combined with DIVA and full blown response	GMO	¹ : Not transmittable by midges ² : Deletion BTV (GMO issue) ³ : Experimental NS3 cELISA
NS4 knockout vaccine	Not tested	No ¹	Yes	Yes ²	No	Cheap	No DIVA efficacy unknown	¹ : Viremia suggests onward transmission ² : BTV knockout mutant (GMO issue)

For each vaccine, comments on some main vaccine standards (numbered) are described in the column "Remarks". In addition, the most important advantages and disadvantages of each of the vaccines are indicated based on the current available data.

DIVA

BTV protein vaccines and viral vector vaccines are DIVA compatible with the commercially available and widely used VP7 ELISA if VP7 protein is not part of the vaccine. Therefore, DIVA monitoring will be very easy and cheap by testing bulk milk samples, in particular if combined with other monitoring programs like for Infectious Bovine Rhinotracheitis and Bovine Viral Diarrhea (170, 171). This will support eradication programs in an affordable manner, and will increase the acceptance of DIVA testing. DISA vaccination can have the same advantage, since an experimental NS3 ELISA accompanying the DISA vaccine platform has been developed but this ELISA is not extensively validated and evaluated for milk samples yet.

Acceptance

BTV protein vaccines will be acceptable, since these are completely safe. Even more, unnoticed pathogens, like in contaminated serum used for antigen production, will be inactivated or removed during down processing of antigen. With regard to viral vector vaccines and MLVs, control of used components in advance as well as of produced vaccine batches is extremely important. Many incidences of contaminated batches of replicating vaccines have been reported (172). Complete synthetic culture medium will avoid this disadvantage of replicating vaccines but is still quite expensive. Nevertheless, all here described MLV vaccine platforms are classified as GMOs, and licensing and acceptance will be costly due to extra safety trials. DISC and DISA platforms are based on disabled BTV due to a single deletion, and their safety has been

scientifically predicted and has been proven in many sheep and cattle trials.

Several research groups have developed experimental BT vaccines and BT vaccine platforms showing promising vaccine profiles close to animal trial required for official vaccine registration. Licensing and launching next-generation BT vaccine, however, will mainly depend on the need for better than current vaccines in order to combat Bluetongue in mono-serotype situations to eradicate the disease and in multi-serotype endemic situations to minimize economic losses.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

This work was supported by Project WOT-01-003-015 of the Dutch Ministry of Agriculture, Nature and Food Quality (LNV)(WBVR-project no. 16000013-01), and EU-grant PALE-Blu project REP-727393-1 (WBVR-project no. 1600001501).

ACKNOWLEDGMENTS

The author is grateful to René van Gennip for critical reading of the manuscript. The author thanks PALE-Blu partners for fruitful discussions.

REFERENCES

- Hutcheon D. Malarial catarrhal fever of sheep. *Vet Rec.* (1902) 14:629–33.
- Spreull J. Malarial Catarrhal Fever (Bluetongue) of Sheep in South Africa. *J Comp Pathol Ther.* (1905) 18:321–37. doi: 10.1016/S0368-1742(05)80073-6
- Tago D, Hammit JK, Thomas A, Raboisson D. Cost assessment of the movement restriction policy in France during the 2006 bluetongue virus episode (BTV-8). *Prev Vet Med.* (2014) 117:577–89. doi: 10.1016/j.prevetmed.2014.10.010
- Velthuis AG, Saatkamp HW, Mourits MC, de Koeijer AA, Elbers AR. Financial consequences of the Dutch bluetongue serotype 8 epidemics of 2006 and 2007. *Prev Vet Med.* (2010) 93:294–304. doi: 10.1016/j.prevetmed.2009.11.007
- Dercksen D, Groot Nibbelink N, Pauwe R, Backx A, van Rijn P, Vellema P. First outbreak of bluetongue in goats in The Netherlands. *Tijdschr Diergeneesk.* (2007) 132:786–90. Available online at: <https://www.ncbi.nlm.nih.gov/pubmed/17990633>
- Barratt-Boyes SM, MacLachlan NJ. Dynamics of viral spread in bluetongue virus infected calves. *Vet Microbiol.* (1994) 40:361–71. doi: 10.1016/0378-1135(94)90123-6
- Erasmus BJ. Bluetongue in sheep and goats. *Aust Vet J.* (1975) 51:165–70. doi: 10.1111/j.1751-0813.1975.tb00048.x
- Du Toit RA. The transmission of Blue-Tongue and Horse sickness by culicoides. *Onderstepoort J Vet Sci Anim Ind.* (1944) 19:7–16.
- Batten CA, Henstock MR, Steedman HM, Waddington S, Edwards L, Oura CA. Bluetongue virus serotype 26: infection kinetics, pathogenesis and possible contact transmission in goats. *Vet Microbiol.* (2013) 162:62–7. doi: 10.1016/j.vetmic.2012.08.014
- Breard E, Schulz C, Sailleau C, Bernelin-Cottet C, Viarouge C, Vitour D, et al. Bluetongue virus serotype 27: experimental infection of goats, sheep and cattle with three BTV-27 variants reveal atypical characteristics and likely direct contact transmission BTV-27 between goats. *Transbound Emerg Dis.* (2018) 65:e251–63. doi: 10.1111/tbed.12780
- Chaigat V, Schwermer H, Casati S, Planzer J, Worwa G, Vanzetti T, et al. Occurrence and spatial distribution of Toggenburg Orbivirus in Switzerland. *Small Rumin Res.* (2010) 93:157–64. doi: 10.1016/j.smallrumres.2010.05.016
- Backx A, Heutink R, van Rooij E, van Rijn P. Transplacental and oral transmission of wild-type bluetongue virus serotype 8 in cattle after experimental infection. *Vet Microbiol.* (2009) 138:235–43. doi: 10.1016/j.vetmic.2009.04.003
- Rasmussen LD, Savini G, Lorusso A, Bellacicco A, Palmarini M, Caporale M, et al. Transplacental transmission of field and rescued strains of BTV-2 and BTV-8 in experimentally infected sheep. *Vet Res.* (2013) 44:75. doi: 10.1186/1297-9716-44-75
- Darpe KE, Batten CA, Veronesi E, Williamson S, Anderson P, Dennison M, et al. Transplacental transmission of bluetongue virus 8 in cattle, UK. *Emerg Infect Dis.* (2009) 15:2025–8. doi: 10.3201/eid1512.090788
- De Clercq K, De Leeuw I, Verheyden B, Vandemeulebroucke E, Vanbinst T, Herr C, et al. Transplacental infection and apparently immunotolerance induced by a wild-type bluetongue virus serotype 8 natural infection. *Transbound Emerg Dis.* (2008) 55:352–9. doi: 10.1111/j.1865-1682.2008.01044.x
- Santman-Berends IM, van Wuijkhuise L, Vellema P, van Rijn PA. Vertical transmission of bluetongue virus serotype 8 virus in Dutch dairy herds in 2007. *Vet Microbiol.* (2010) 141:31–5. doi: 10.1016/j.vetmic.2009.08.010

17. Gibbs EP, Lawman MJ, Herniman KA. Preliminary observations on transplacental infection of bluetongue virus in sheep—a possible overwintering mechanism. *Res Vet Sci.* (1979) 27:118–20. doi: 10.1016/S0034-5288(18)32870-4
18. Menzies FD, McCullough SJ, McKeown IM, Forster JL, Jess S, Batten C, et al. Evidence for transplacental and contact transmission of bluetongue virus in cattle. *Vet Rec.* (2008) 163:203–9. doi: 10.1136/vr.163.7.203
19. Attoui H, Mertens P, Becnel J, Belaganahalli S, Bergoin M, Brussaard CP, et al. Virus taxonomy. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz E. editors. *Ninth Report of the International Committee on Taxonomy of Viruses.* Amsterdam: Academic Press (2011).
20. Belhouche M, Mohd Jaafar F, Firth AE, Grimes JM, Mertens PP, Attoui H. Detection of a fourth orbivirus non-structural protein. *PLoS ONE.* (2011) 6:e25697. doi: 10.1371/journal.pone.0025697
21. Firth AE. Bioinformatic analysis suggests that the Orbivirus VP6 cistron encodes an overlapping gene. *Virology.* (2008) 548. doi: 10.1186/1743-422X-5-48
22. Stewart M, Hardy A, Barry G, Pinto RM, Caporale M, Melzi E, et al. Characterization of a second open reading frame in genome segment 10 of bluetongue virus. *J Gen Virol.* (2015) 96:3280–93. doi: 10.1099/jgv.0.000267
23. Van Dijk AA, Huismans H. *In vitro* transcription and translation of bluetongue virus mRNA. *J Gen Virol.* (1988) 69(Pt 3):573–81. doi: 10.1099/0022-1317-69-3-573
24. Boyce M, Roy P. Recovery of infectious bluetongue virus from RNA. *J Virol.* (2007) 81:2179–86. doi: 10.1128/JVI.01819-06
25. Boyce M, Celma CC, Roy P. Development of reverse genetics systems for bluetongue virus: recovery of infectious virus from synthetic RNA transcripts. *J Virol.* (2008) 82:8339–48. doi: 10.1128/JVI.00808-08
26. Ratnien M, Caporale M, Golder M, Franzoni G, Allan K, Nunes SF, et al. Identification and characterization of a novel non-structural protein of bluetongue virus. *PLoS Pathog.* (2011) 7:e1002477. doi: 10.1371/journal.ppat.1002477
27. van Gennip RG, van der Water SG, van Rijn PA. Bluetongue virus nonstructural protein NS3/NS3a is not essential for virus replication. *PLoS ONE.* (2014) 9:e85788. doi: 10.1371/journal.pone.0085788
28. Erasmus BJ. *Virus Infections of Ruminants*, 3. New York, NY: Elsevier Science Publishers (1990).
29. Huismans H, Erasmus BJ. Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort J Vet Res.* (1981) 48:51–8.
30. Maan S, Maan NS, Nomikou K, Batten C, Antony F, Belaganahalli MN, et al. Novel bluetongue virus serotype from Kuwait. *Emerg Infect Dis.* (2011) 17:886–9. doi: 10.3201/eid1705.101742
31. Bumbarov V, Golender N, Jenckel M, Wernike K, Beer M, Khinich E, et al. Characterization of bluetongue virus serotype 28. *Transbound Emerg Dis.* (2019). doi: 10.1111/tbed.13338. [Epub ahead of print].
32. Wright IM. *Serological and Genetic Characterisation of Putative New Serotypes of Bluetongue Virus and Epizootic Haemorrhagic Disease Virus Isolated From an Alpaca*. Ph.D. dissertation, North-West University (2014).
33. Zientara S, Sailleau C, Viarouge C, Hoper D, Beer M, Jenckel M, et al. Novel bluetongue virus in goats, corsica, france, 2014. *Emerg Infect Dis.* (2014) 20:2123–32. doi: 10.3201/eid2012.140924
34. Maan S, Maan NS, Samuel AR, Rao S, Attoui H, Mertens PP. Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. *J Gen Virol.* (2007) 88(Pt 2):21–30. doi: 10.1099/vir.0.82456-0
35. Maan S, Maan NS, Ross-smith N, Batten CA, Shaw AE, Anthony SJ, et al. Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains. *Virology.* (2008) 377:308–18. doi: 10.1016/j.virol.2008.04.028
36. Hofmann MA, Renzullo S, Mader M, Chaignat V, Worwa G, Thuer B. Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. *Emerg Infect Dis.* (2008) 14:1855–61. doi: 10.3201/eid1412.080818
37. Savini G, Puggioni G, Meloni G, Maracci M, Di Domenico M, Rocchigiani AM, et al. Novel putative Bluetongue virus in healthy goats from Sardinia, Italy. *Infect Genet Evol.* (2017) 51:108–17. doi: 10.1016/j.meegid.2017.03.021
38. Sun EC, Huang LP, Xu QY, Wang HX, Xue XM, Lu P, et al. Emergence of a Novel Bluetongue Virus Serotype, China 2014. *Transbound Emerg Dis.* (2016) 63:585–9. doi: 10.1111/tbed.12560
39. Planzer J, Kaufmann C, Worwa G, Gavriel-Widen D, Hofmann MA, Chaignat V, et al. *In vivo* and *in vitro* propagation and transmission of Toggenburg orbivirus. *Res Vet Sci.* (2011) 91:e163–8. doi: 10.1016/j.rvsc.2011.03.007
40. Pullinger GD, Guimera Busquets M, Nomikou K, Boyce M, Attoui H, Mertens PP. Identification of the genome segments of Bluetongue Virus Serotype 26 (isolate KUV2010/02) that restrict replication in a *Culicoides sonorensis* Cell Line (KC Cells). *PLoS ONE.* (2016) 11:e0149709. doi: 10.1371/journal.pone.0149709
41. Schulz C, Breard E, Sailleau C, Jenckel M, Viarouge C, Vitour D, et al. Bluetongue virus serotype 27: detection and characterization of two novel variants in Corsica, France. *J Gen Virol.* (2016) 97:2073–83. doi: 10.1099/jgv.0.000557
42. Marcacci M, Sant S, Mangone I, Gorla M, Dondo A, Zoppi S, et al. One after the other: a novel Bluetongue virus strain related to Toggenburg virus detected in the Piedmont region (North-western Italy), extends the panel of novel atypical BTV strains. *Transbound Emerg Dis.* (2018) 65:370–4. doi: 10.1111/tbed.12822
43. MacLachlan NJ. *Bluetongue: history, global epidemiology, and pathogenesis.* *Prev Vet Med.* (2011) 102:107–11. doi: 10.1016/j.prevetmed.2011.04.005
44. Carpenter S, Wilson A, Barber J, Veronesi E, Mellor P, Venter G, et al. Temperature dependence of the extrinsic incubation period of orbiviruses in *Culicoides* biting midges. *PLoS ONE.* (2011) 6:e27987. doi: 10.1371/journal.pone.0027987
45. MacLachlan NJ. Global implications of the recent emergence of bluetongue virus in Europe. *Vet Clin North Am Food Anim Pract.* (2010) 26:163–71. doi: 10.1016/j.cvfa.2009.10.012
46. ISID Promed mail (2006). *Bluetongue - Europe (1): Bluetongue, ovine, Netherlands, confirmed: ProMED-mail 2006; 18 Aug: 20060818.2311.* <http://www.promedmail.org>.
47. Dijkstra E, van der Ven IJ, Meiswinkel R, Holzel DR, Van Rijn PA, Meiswinkel R. *Culicoides chiopterus* as a potential vector of bluetongue virus in Europe. *Vet Rec.* (2008) 162:422. doi: 10.1136/vr.162.13.422-a
48. Mehlhorn H, Walldorf V, Klimpel S, Schaub G, Kiel E, Focke R, et al. Bluetongue disease in Germany (2007–2008): monitoring of entomological aspects. *Parasitol Res.* (2009) 105:313–9. doi: 10.1007/s00436-009-1416-y
49. Meiswinkel R, van Rijn P, Leijes P, Goffredo M. Potential new *Culicoides* vector of bluetongue virus in northern Europe. *Vet Rec.* (2007) 161:564–5. doi: 10.1136/vr.161.16.564
50. Sailleau C, Breard E, Viarouge C, Vitour D, Romey A, Garnier A, et al. Re-emergence of Bluetongue Virus serotype 8 in France, 2015. *Transbound Emerg Dis.* (2017) 64:998–1000. doi: 10.1111/tbed.12453
51. Flannery J, Sanz-Bernardo B, Ashby M, Brown H, Carpenter S, Cooke L, et al. Evidence of reduced viremia, pathogenicity and vector competence in a re-emerging European strain of bluetongue virus serotype 8 in sheep. *Transbound Emerg Dis.* (2019) 66:1177–85. doi: 10.1111/tbed.13131
52. Sailleau C, Breard E, Viarouge C, Gorlier A, Quenault H, Hirschaud E, et al. Complete genome sequence of bluetongue virus serotype 4 that emerged on the French island of Corsica in December 2016. *Transbound Emerg Dis.* (2017) 65:e194–7. doi: 10.1111/tbed.12660
53. Aguilar-Vega C, Fernandez-Carrion E, Sanchez-Vizcaino JM. The possible route of introduction of bluetongue virus serotype 3 into Sicily by windborne transportation of infected *Culicoides* spp. *Transbound Emerg Dis.* (2019) 66:1665–73. doi: 10.1111/tbed.13201
54. Cappai S, Rolesu S, Loi F, Liciardi M, Leone A, Marcacci M, et al. Western Bluetongue virus serotype 3 in Sardinia, diagnosis and characterization. *Transbound Emerg Dis.* (2019) 66:1426–31. doi: 10.1111/tbed.13156
55. Lorusso A, Guercio A, Purpari G, Camma C, Calistri P, D'Alterio N, et al. Bluetongue virus serotype 3 in Western Sicily, November 2017. *Vet Ital.* (2017) 53:273–5. doi: 10.12834/VetIt.251.520.178
56. White JR, Williams DT, Wang J, Chen H, Melville LF, Davis SS, et al. Identification and genomic characterization of the first isolate of bluetongue virus serotype 5 detected in Australia. *Vet Med Sci.* (2019) 5:129–45. doi: 10.1002/vms3.156

57. Boyer TC, Ward MP, Singer RS. Climate, landscape, and the risk of orbivirus exposure in cattle in Illinois and western Indiana. *Am J Trop Med Hyg.* (2010) 83:789–94. doi: 10.4269/ajtmh.2010.10-0132
58. Schirtzinger EE, Jaspersen DC, Ostlund EN, Johnson DJ, Wilson WC. Recent US bluetongue virus serotype 3 isolates found outside of Florida indicate evidence of reassortment with co-circulating endemic serotypes. *J Gen Virol.* (2017) 99:157–68. doi: 10.1099/jgv.0.000965
59. Legisa DM, Gonzalez FN, Dus Santos MJ. Bluetongue virus in South America, Central America and the Caribbean. *Virus Res.* (2014) 182:87–94. doi: 10.1016/j.virusres.2013.10.030
60. Jacquot M, Rao PP, Yadav S, Nomikou K, Maan S, Jyothi YK, et al. Contrasting selective patterns across the segmented genome of bluetongue virus in a global reassortment hotspot. *Virus Evol.* (2019) 5:vez027. doi: 10.1093/ve/vez027
61. Nomikou K, Hughes J, Wash R, Kellam P, Breard E, Zientara S, et al. Widespread reassortment shapes the evolution and epidemiology of Bluetongue Virus following European Invasion. *PLoS Pathog.* (2015) 11:e1005056. doi: 10.1371/journal.ppat.1005056
62. Purse BV, Nedelchev N, Georgiev G, Veleva E, Boorman J, Denison E, et al. Spatial and temporal distribution of bluetongue and its Culicoides vectors in Bulgaria. *Med Vet Entomol.* (2006) 20:335–44. doi: 10.1111/j.1365-2915.2006.00636.x
63. MacLachlan NJ, Zientara S, Wilson WC, Richt JA, Savini G. Bluetongue and epizootic hemorrhagic disease viruses: recent developments with these globally re-emerging arboviral infections of ruminants. *Curr Opin Virol.* (2019) 34:56–62. doi: 10.1016/j.coviro.2018.12.005
64. MacLachlan NJ, Mayo CE. Potential strategies for control of bluetongue, a globally emerging, Culicoides-transmitted viral disease of ruminant livestock and wildlife. *Antiviral Res.* (2013) 99:79–90. doi: 10.1016/j.antiviral.2013.04.021
65. Papadopoulos O, Mellor PS, Mertens PPC. Bluetongue control strategies. In: Mellor PS, Baylis M, Mertens PPC, editors. *Bluetongue* Amsterdam: Elsevier Academic press (2008). p. 429–52.
66. Roy P, Boyce M, Noad R. Prospects for improved bluetongue vaccines. *Nat Rev Microbiol.* (2009) 7:120–8. doi: 10.1038/nrmicro2052
67. Baetza HJ. Eradication of bluetongue disease in Germany by vaccination. *Vet Immunol Immunopathol.* (2014) 158:116–9. doi: 10.1016/j.vetimm.2013.09.001
68. Pioz M, Guis H, Pleydell D, Gay E, Calavas D, Durand B, et al. Did vaccination slow the spread of bluetongue in France? *PLoS ONE.* (2014) 9:e85444. doi: 10.1371/journal.pone.0085444
69. Sumner T, Burgin L, Gloster J, Gubbins S. Comparison of pre-emptive and reactive strategies to control an incursion of bluetongue virus serotype 1 to Great Britain by vaccination. *Epidemiol Infect.* (2013) 141:102–14. doi: 10.1017/S0950268812000532
70. Feenstra F, van Rijn PA. Current and next-generation bluetongue vaccines: requirements, strategies, and prospects for different field situations. *Crit Rev Microbiol.* (2017) 43:142–55. doi: 10.1080/1040841X.2016.1186005
71. van Oirschot JT. Diva vaccines that reduce virus transmission. *J Biotechnol.* (1999) 73:195–205. doi: 10.1016/S0168-1656(99)00121-2
72. Van Dijk AA. Development of recombinant vaccines against bluetongue. *Biotechnol Adv.* (1993) 11:1–12. doi: 10.1016/0734-9750(93)90407-E
73. Dungu B, Gerdes T, Smit T. The use of vaccination in the control of bluetongue in southern Africa. *Vet Ital.* (2004) 40:616–22. Available online at: <https://www.ncbi.nlm.nih.gov/pubmed/20422597>
74. Breard E, Pozzi N, Sailleau C, Durand B, Catinot V, Sellem E, et al. Transient adverse effects of an attenuated bluetongue virus vaccine on the quality of ram semen. *Vet Rec.* (2007) 160:431–5. doi: 10.1136/vr.160.13.431
75. Savini G, MacLachlan NJ, Sanchez-Vizcaino JM, Zientara S. Vaccines against bluetongue in Europe. *Comp Immunol Microbiol Infect Dis.* (2008) 31:101–20. doi: 10.1016/j.cimid.2007.07.006
76. Ferrari G, De Liberato C, Scavia G, Lorenzetti R, Zini M, Farina F, et al. Active circulation of bluetongue vaccine virus serotype-2 among unvaccinated cattle in central Italy. *Prev Vet Med.* (2005) 68:103–13. doi: 10.1016/j.prevetmed.2004.11.011
77. Monaco F, Camma C, Serini S, Savini G. Differentiation between field and vaccine strain of bluetongue virus serotype 16. *Vet Microbiol.* (2006) 116:45–52. doi: 10.1016/j.vetmic.2006.03.024
78. Monaco F, De Luca N, Morelli D, Pesciella M, Palmarini S, Di Giandomenico M, et al. Field vaccination of cattle using a bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9: effect on milk production. *Vet Ital.* (2004) 40:661–3. Available online at: <https://www.ncbi.nlm.nih.gov/pubmed/20422607>
79. Veronesi E, Hamblin C, Mellor PS. Live attenuated bluetongue vaccine viruses in Dorset Poll sheep, before and after passage in vector midges (Diptera: Ceratopogonidae). *Vaccine.* (2005) 23:5509–16. doi: 10.1016/j.vaccine.2005.07.039
80. Patta C, Giovannini A, Rolesu S, Nannini D, Savini G, Calistri P, et al. Bluetongue vaccination in Europe: the Italian experience. *Vet Ital.* (2004) 40:601–10. Available online at: <https://www.ncbi.nlm.nih.gov/pubmed/20422595>
81. Batten CA, Maan S, Shaw AE, Maan NS, Mertens PP. A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment. *Virus Res.* (2008) 137:56–63. doi: 10.1016/j.virusres.2008.05.016
82. Van den Bergh C, Coetzee P, Venter EH. Reassortment of bluetongue virus vaccine serotypes in cattle. *J S Afr Vet Assoc.* (2018) 89:e1–7. doi: 10.4102/j.sava.v89i0.1649
83. Bhanuprakash V, Indrani BK, Hosamani M, Balamurugan V, Singh RK. Bluetongue vaccines: the past, present and future. *Expert Rev Vaccines.* (2009) 8:191–204. doi: 10.1586/14760584.8.2.191
84. McVey DS, MacLachlan NJ. Vaccines for prevention of Bluetongue and epizootic hemorrhagic disease in livestock: a North American perspective. *Vector Borne Zoonotic Dis.* (2015) 15:385–96. doi: 10.1089/vbz.2014.1698
85. Campbell CH, Barber TL, Knudsen RC, Swaney LM. Immune response of mice and sheep to bluetongue virus inactivated by gamma irradiation. *Prog Clin Biol Res.* (1985) 178:639–47.
86. Parker J, Herniman KA, Gibbs EP, Sellers RF. An experimental inactivated vaccine against bluetongue. *Vet Rec.* (1975) 96:284–7. doi: 10.1136/vr.96.13.284
87. Stevens DR, Stott J, Osburn BI, Giles R, Wieseahn GP, Barber TL. Potency and efficacy of inactivated bluetongue virus vaccines. *Prog Clin Biol Res.* (1985) 178:649–52.
88. Stott JL, Barber TL, Osburn BI. Immunologic response of sheep to inactivated and virulent bluetongue virus. *Am J Vet Res.* (1985) 46:1043–9.
89. Zientara S, Sanchez-Vizcaino JM. Control of bluetongue in Europe. *Vet Microbiol.* (2013) 165:33–7. doi: 10.1016/j.vetmic.2013.01.010
90. Breard E, Belbis G, Viarouge C, Nomikou K, Haegeman A, De Clercq K, et al. Evaluation of adaptive immune responses and heterologous protection induced by inactivated bluetongue virus vaccines. *Vaccine.* (2015) 33:512–8. doi: 10.1016/j.vaccine.2014.11.053
91. Martinelle L, Dal Pozzo F, Thys C, De Leeuw I, Van Campe W, De Clercq K, et al. Assessment of cross-protection induced by a bluetongue virus (BTV) serotype 8 vaccine towards other BTV serotypes in experimental conditions. *Vet Res.* (2018) 49:63. doi: 10.1186/s13567-018-0556-4
92. Perez de Diego AC, Sanchez-Cordon PJ, de las Heras AI, Sanchez-Vizcaino JM. Characterization of the immune response induced by a commercially available inactivated bluetongue virus serotype 1 vaccine in sheep. *Sci World J.* (2012) 2012:147158. doi: 10.1100/2012/147158
93. Moulin V, Noordegraaf CV, Makoschey B, van der Sluijs M, Veronesi E, Darpel K, et al. Clinical disease in sheep caused by bluetongue virus serotype 8, and prevention by an inactivated vaccine. *Vaccine.* (2012) 30:2228–35. doi: 10.1016/j.vaccine.2011.11.100
94. Szmaragd C, Wilson AJ, Carpenter S, Wood JL, Mellor PS, Gubbins S. The spread of bluetongue virus serotype 8 in Great Britain and its control by vaccination. *PLoS ONE.* (2010) 5:e9353. doi: 10.1371/journal.pone.0009353
95. Wackerlin R, Eschbaumer M, König P, Hoffmann B, Beer M. Evaluation of humoral response and protective efficacy of three inactivated vaccines against bluetongue virus serotype 8 one year after vaccination of sheep and cattle. *Vaccine.* (2010) 28:4348–55. doi: 10.1016/j.vaccine.2010.04.055
96. Batten CA, Edwards L, Oura CA. Evaluation of the humoral immune responses in adult cattle and sheep, 4 and 2.5 years post-vaccination with a bluetongue serotype 8 inactivated vaccine. *Vaccine.* (2013) 31:3783–5. doi: 10.1016/j.vaccine.2013.06.033
97. Breard E, Belbis G, Hamers C, Moulin V, Lilin T, Moreau F, et al. Evaluation of humoral response and protective efficacy of two inactivated vaccines

- against bluetongue virus after vaccination of goats. *Vaccine*. (2011) 29:2495–502. doi: 10.1016/j.vaccine.2010.12.105
98. Ries C, Beer M, Hoffmann B. BTV antibody longevity in cattle five to eight years post BTV-8 vaccination. *Vaccine*. (2019) 37:2656–60. doi: 10.1016/j.vaccine.2019.03.082
 99. Merial (2015) *Merial's Bluetongue Vaccine - Information for Veterinary Professionals and Farmers*, 2015. Available online at: <https://www.boehringer-ingenheim.com/animal-health/livestock-products/btv-pur>
 100. Hund A, Gollnick N, Sauter-Louis C, Neubauer-Juric A, Lahm H, Buttner M. A two year BTV-8 vaccination follow up: molecular diagnostics and assessment of humoral and cellular immune reactions. *Vet Microbiol*. (2012) 154:247–56. doi: 10.1016/j.vetmic.2011.07.019
 101. Anderson J, Mertens PP, Herniman KA. A competitive ELISA for the detection of anti-tubule antibodies using a monoclonal antibody against bluetongue virus non-structural protein NS1. *J Virol Methods*. (1993) 43:167–75. doi: 10.1016/0166-0934(93)90074-2
 102. Barros SC, Cruz B, Luis TM, Ramos F, Fagulha T, Duarte M, et al. A DIVA system based on the detection of antibodies to non-structural protein 3 (NS3) of bluetongue virus. *Vet Microbiol*. (2009) 137:252–9. doi: 10.1016/j.vetmic.2009.01.033
 103. Tacken MG, Daus FJ, Feenstra F, van Gennip RG, van Rijn PA. Development of a competitive ELISA for NS3 antibodies as DIVA test accompanying the novel Disabled Infectious Single Animal (DISA) vaccine for Bluetongue. *Vaccine*. (2015) 33:5539–45. doi: 10.1016/j.vaccine.2015.09.020
 104. Huismans H, van der Walt NT, Erasmus BJ. Immune response against the purified serotype specific antigen of bluetongue virus and initial attempts to clone the gene that codes for the synthesis of this protein. *Prog Clin Biol Res*. (1985) 178:347–53.
 105. French TJ, Inumaru S, Roy P. Expression of two related nonstructural proteins of bluetongue virus (BTV) type 10 in insect cells by a recombinant baculovirus: production of polyclonal ascitic fluid and characterization of the gene product in BTV-infected BHK cells. *J Virol*. (1989) 63:3270–8.
 106. Inumaru S, Roy P. Production and characterization of the neutralization antigen VP2 of bluetongue virus serotype 10 using a baculovirus expression vector. *Virology*. (1987) 157:472–9. doi: 10.1016/0042-6822(87)90289-3
 107. Roy P, French T, Erasmus BJ. Protective efficacy of virus-like particles for bluetongue disease. *Vaccine*. (1992) 10:28–32. doi: 10.1016/0264-410X(92)90415-G
 108. Urakawa T, French TJ, Adachi Y, Fukusho A, LeBlois H, Flamand M, et al. Synthesis of recombinant baculoviruses expressing the outer capsid protein VP2 of five BTV serotypes and the induction of neutralizing antibodies to homologous and heterologous BTV serotypes. *Virus Res*. (1994) 31:149–61. doi: 10.1016/0168-1702(94)90001-9
 109. Athmaram TN, Bali G, Kahng GG, Dwarakanath S. Heterologous expression of Bluetongue VP2 viral protein fragment in *Pichia pastoris*. *Virus Genes*. (2007) 35:265–71. doi: 10.1007/s11262-006-0061-0
 110. Fay PC, Attoui H, Batten C, Mohd Jaafar F, Lomonosoff GP, Daly JM, et al. Bluetongue virus outer-capsid protein VP2 expressed in *Nicotiana benthamiana* raises neutralising antibodies and a protective immune response in IFNAR (-/-) mice. *Vaccine*. (2019) 2:100026. doi: 10.1016/j.jvax.2019.100026
 111. Thuenemann EC, Meyers AE, Verwey J, Rybicki EP, Lomonosoff GP. A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles. *Plant Biotechnol J*. (2013) 11:839–46. doi: 10.1111/pbi.12076
 112. van Zyl AR, Meyers AE, Rybicki EP. Transient Bluetongue virus serotype 8 capsid protein expression in *Nicotiana benthamiana*. *Biotechnol Rep*. (2016) 9:15–24. doi: 10.1016/j.btre.2015.12.001
 113. Roy P. Use of baculovirus expression vectors: development of diagnostic reagents, vaccines and morphological counterparts of bluetongue virus. *FEMS Microbiol Immunol*. (1990) 2:223–34. doi: 10.1111/j.1574-6968.1990.tb03523.x
 114. Anderson J, Hagglund S, Breard E, Comtet L, Lovgren Bengtsson K, Pringle J, et al. Evaluation of the immunogenicity of an experimental subunit vaccine that allows differentiation between infected and vaccinated animals against bluetongue virus serotype 8 in Cattle. *Clin Vaccine Immunol*. (2013) 20:1115–22. doi: 10.1128/CI.00229-13
 115. Anderson J, Hagglund S, Breard E, Riou M, Zohari S, Comtet L, et al. Strong protection induced by an experimental DIVA subunit vaccine against bluetongue virus serotype 8 in cattle. *Vaccine*. (2014) 32:6614–21. doi: 10.1016/j.vaccine.2014.09.066
 116. Mohd Jaafar F, Belhouchet M, Vitour D, Adam M, Breard E, Zientara S, et al. Immunisation with bacterial expressed VP2 and VP5 of bluetongue virus (BTV) protect α/β interferon-receptor knock-out (IFNAR(-/-)) mice from homologous lethal challenge. *Vaccine*. (2014) 32:4059–67. doi: 10.1016/j.vaccine.2014.05.056
 117. Marin-Lopez A, Otero-Romero I, de la Poza F, Menaya-Vargas R, Calvo-Pinilla E, Benavente J, et al. VP2, VP7, and NS1 proteins of bluetongue virus targeted in avian reovirus muNS-Mi microspheres elicit a protective immune response in IFNAR(-/-) mice. *Antiviral Res*. (2014) 110:42–51. doi: 10.1016/j.antiviral.2014.07.008
 118. Brandariz-Nunez A, Menaya-Vargas R, Benavente J, Martinez-Costas J. A versatile molecular tagging method for targeting proteins to avian reovirus muNS inclusions. Use in protein immobilization and purification. *PLoS ONE*. (2010) 5:e13961. doi: 10.1371/journal.pone.0013961
 119. Legisa DM, Perez Aguirreburualde MS, Gonzalez FN, Marin-Lopez A, Ruiz V, Wigdorovitz A, et al. An experimental subunit vaccine based on Bluetongue virus 4 VP2 protein fused to an antigen-presenting cells single chain antibody elicits cellular and humoral immune responses in cattle, guinea pigs and IFNAR(-/-) mice. *Vaccine*. (2015) 33:2614–9. doi: 10.1016/j.vaccine.2015.03.067
 120. Belyaev AS, Roy P. Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells. *Nucleic Acids Res*. (1993) 21:1219–23. doi: 10.1093/nar/21.5.1219
 121. French TJ, Marshall JJ, Roy P. Assembly of double-shelled, viruslike particles of bluetongue virus by the simultaneous expression of four structural proteins. *J Virol*. (1990) 64:5695–700.
 122. Stewart M, Bhatia Y, Athmaram TN, Noad R, Gastaldi C, Dubois E, et al. Validation of a novel approach for the rapid production of immunogenic virus-like particles for bluetongue virus. *Vaccine*. (2010) 28:3047–54. doi: 10.1016/j.vaccine.2009.10.072
 123. Stewart M, Dovas CI, Chatzinasiou E, Athmaram TN, Papanastassopoulou M, Papadopoulos O, et al. Protective efficacy of Bluetongue virus-like and subvirus-like particles in sheep: presence of the serotype-specific VP2, independent of its geographic lineage, is essential for protection. *Vaccine*. (2012) 30:2131–9. doi: 10.1016/j.vaccine.2012.01.042
 124. Stewart M, Dubois E, Sailleau C, Breard E, Viarouge C, Desprat A, et al. Bluetongue virus serotype 8 virus-like particles protect sheep against virulent virus infection as a single or multi-serotype cocktail immunogen. *Vaccine*. (2013) 31:553–8. doi: 10.1016/j.vaccine.2012.11.016
 125. Rybicki EP. Plant-made vaccines for humans and animals. *Plant Biotechnol J*. (2010) 8:620–37. doi: 10.1111/j.1467-7652.2010.00507.x
 126. Roy P, Bishop DH, LeBlois H, Erasmus BJ. Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. *Vaccine*. (1994) 12:805–11. doi: 10.1016/0264-410X(94)90289-5
 127. Roy P. Genetically engineered structure-based vaccine for bluetongue disease. *Vet Ital*. (2004) 40:594–600. Available online at: <https://www.ncbi.nlm.nih.gov/pubmed/20422594>
 128. Nunes SF, Hamers C, Ratnien M, Shaw A, Brunet S, Hudelet P, et al. A synthetic biology approach for a vaccine platform against known and newly emerging serotypes of bluetongue virus. *J Virol*. (2014) 88:12222–32. doi: 10.1128/JVI.02183-14
 129. Boone JD, Balasuriya UB, Karaca K, Audonnet JC, Yao J, He L, et al. Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. *Vaccine*. (2007) 25:672–8. doi: 10.1016/j.vaccine.2006.08.025
 130. Perrin A, Albina E, Breard E, Sailleau C, Prome S, Grillet C, et al. Recombinant capripoxviruses expressing proteins of bluetongue virus: evaluation of immune responses and protection in small ruminants. *Vaccine*. (2007) 25:6774–83. doi: 10.1016/j.vaccine.2007.06.052
 131. Top S, Foucras G, Deplanche M, Rives G, Calvalido J, Comtet L, et al. Myxomavirus as a vector for the immunisation of sheep: protection

- study against challenge with bluetongue virus. *Vaccine*. (2012) 30:1609–16. doi: 10.1016/j.vaccine.2011.12.108
132. Franceschi V, Capocéfalo A, Calvo-Pinilla E, Redaelli M, Mucignat-Caretta C, Mertens P, et al. Immunization of knock-out alpha/beta interferon receptor mice against lethal bluetongue infection with a BoHV-4-based vector expressing BTV-8 VP2 antigen. *Vaccine*. (2011) 29:3074–82. doi: 10.1016/j.vaccine.2011.01.075
 133. Ma G, Eschbaumer M, Said A, Hoffmann B, Beer M, Osterrieder N. An equine herpesvirus type 1 (EHV-1) expressing VP2 and VP5 of serotype 8 bluetongue virus (BTV-8) induces protection in a murine infection model. *PLoS ONE*. (2012) 7:e34425. doi: 10.1371/journal.pone.0034425
 134. Marin-Lopez A, Calvo-Pinilla E, Barriales D, Lorenzo G, Brun A, Anguita J, et al. CD8T cell responses to an immunodominant epitope within the nonstructural protein NS1 provide wide immunoprotection against Bluetongue Virus in IFNAR(-/-) Mice. *J Virol*. (2018) 92:e00938-18. doi: 10.1128/JVI.00938-18
 135. Ura T, Okuda K, Shimada M. Developments in viral vector-based vaccines. *Vaccines*. (2014) 2:624–41. doi: 10.3390/vaccines2030624
 136. Grunwald T, Ulbert S. Improvement of DNA vaccination by adjuvants and sophisticated delivery devices: vaccine-platforms for the battle against infectious diseases. *Clin Exp Vaccine Res*. (2015) 4:1–10. doi: 10.7774/cevr.2015.4.1.1
 137. Jiang Y, Yu K, Zhang H, Zhang P, Li C, Tian G, et al. Enhanced protective efficacy of H5 subtype avian influenza DNA vaccine with codon optimized HA gene in a pCAGGS plasmid vector. *Antiviral Res*. (2007) 75:234–41. doi: 10.1016/j.antiviral.2007.03.009
 138. Li J, Yang T, Xu Q, Sun E, Feng Y, Lv S, et al. DNA vaccine prime and recombinant FPV vaccine boost: an important candidate immunization strategy to control bluetongue virus type 1. *Appl Microbiol Biotechnol*. (2015) 99:8643–52. doi: 10.1007/s00253-015-6697-8
 139. Calvo-Pinilla E, Rodriguez-Calvo T, Sevilla N, Ortego J. Heterologous prime boost vaccination with DNA and recombinant modified vaccinia virus Ankara protects IFNAR(-/-) mice against lethal bluetongue infection. *Vaccine*. (2009) 28:437–45. doi: 10.1016/j.vaccine.2009.10.027
 140. Calvo-Pinilla E, Navasa N, Anguita J, Ortego J. Multiserotype protection elicited by a combinatorial prime-boost vaccination strategy against bluetongue virus. *PLoS ONE*. (2012) 7:e34735. doi: 10.1371/journal.pone.0034735
 141. Jabbar TK, Calvo-Pinilla E, Mateos F, Gubbins S, Bin-Tarif A, Bachanek-Bankowska K, et al. Protection of IFNAR(-/-) mice against Bluetongue Virus serotype 8, by heterologous (DNA/rMVA) and homologous (rMVA/rMVA) vaccination, expressing outer-capsid protein VP2. *PLoS ONE*. (2013) 8:e60574. doi: 10.1371/journal.pone.0060574
 142. Matsuo E, Roy P. Minimum requirements for bluetongue virus primary replication *in vivo*. *J Virol*. (2013) 87:882–9. doi: 10.1128/JVI.02363-12
 143. Pretorius JM, Huismans H, Theron J. Establishment of an entirely plasmid-based reverse genetics system for Bluetongue virus. *Virology*. (2015) 486:71–7. doi: 10.1016/j.virol.2015.09.004
 144. van Rijn PA, van de Water SG, Feenstra F, van Gennip RG. Requirements and comparative analysis of reverse genetics for bluetongue virus (BTV) and African horse sickness virus (AHSV). *J Virol*. (2016) 13:119. doi: 10.1186/s12985-016-0574-7
 145. van Gennip RG, van de Water SG, Potgieter CA, Wright IM, Veldman D, van Rijn PA. Rescue of recent virulent and avirulent field strains of bluetongue virus by reverse genetics. *PLoS ONE*. (2012) 7:e30540. doi: 10.1371/journal.pone.0030540
 146. Shaw AE, Ratnien M, Nunes SF, Nomikou K, Caporale M, Golder M, et al. Reassortment between two serologically unrelated bluetongue virus strains is flexible and can involve any genome segment. *J Virol*. (2013) 87:543–57. doi: 10.1128/JVI.02266-12
 147. van Rijn PA, Geurts Y, van der Spek AN, Veldman D, van Gennip RG. Bluetongue virus serotype 6 in Europe in 2008-emergence and disappearance of an unexpected non-virulent BTV. *Vet Microbiol*. (2012) 158:23–32. doi: 10.1016/j.vetmic.2012.01.022
 148. van Gennip RG, van de Water SG, Maris-Veldhuis M, van Rijn PA. Bluetongue viruses based on modified-live vaccine serotype 6 with exchanged outer shell proteins confer full protection in sheep against virulent BTV8. *PLoS ONE*. (2012) 7:e44619. doi: 10.1371/journal.pone.0044619
 149. Janowicz A, Caporale M, Shaw A, Gulletta S, Di Galleonardo L, Ratnien M, et al. Multiple genome segments determine virulence of bluetongue virus serotype 8. *J Virol*. (2015) 89:5238–49. doi: 10.1128/JVI.00395-15
 150. Matsuo E, Celma CC, Boyce M, Viarouge C, Sailleau C, Dubois E, et al. Generation of replication-defective virus-based vaccines that confer full protection in sheep against virulent bluetongue virus challenge. *J Virol*. (2011) 85:10213–21. doi: 10.1128/JVI.05412-11
 151. Celma CC, Boyce M, van Rijn PA, Eschbaumer M, Wernike K, Hoffmann B, et al. Rapid generation of replication-deficient monovalent and multivalent vaccines for bluetongue virus: protection against virulent virus challenge in cattle and sheep. *J Virol*. (2013) 87:9856–64. doi: 10.1128/JVI.01514-13
 152. Celma CC, Stewart M, Wernike K, Eschbaumer M, Gonzalez-Molleda L, Breard E, et al. Replication-deficient particles: new insights into the next generation of Bluetongue virus vaccines. *J Virol*. (2017) 91:e01892-16. doi: 10.1128/JVI.01892-16
 153. Ratnien M, Shaw AE, Barry G, Gu Q, Di Galleonardo L, Janowicz A, et al. Bluetongue virus NS4 protein is an interferon antagonist and a determinant of virus virulence. *J Virol*. (2016) 90:5427–39. doi: 10.1128/JVI.00422-16
 154. Feenstra F, Drolet BS, Boonstra J, van Rijn PA. Non-structural protein NS3/NS3a is required for propagation of bluetongue virus in *Culicoides sonorensis*. *Parasit Vectors*. (2015) 8:476. doi: 10.1186/s13071-015-1063-3
 155. van Gennip RGP, Drolet BS, Rozo Lopez P, Roost AJC, Boonstra J, van Rijn PA. Vector competence is strongly affected by a small deletion or point mutations in bluetongue virus. *Parasit Vectors*. (2019) 12:470. doi: 10.1186/s13071-019-3722-2
 156. Feenstra F, van Gennip RG, Maris-Veldhuis M, Verheij E, van Rijn PA. Bluetongue virus without NS3/NS3a expression is not virulent and protects against virulent bluetongue virus challenge. *J Gen Virol*. (2014) 95(Pt 9):2019–29. doi: 10.1099/vir.0.065615-0
 157. Feenstra F, Pap JS, van Rijn PA. Application of Bluetongue disabled infectious single animal (DISA) vaccine for different serotypes by VP2 exchange or incorporation of chimeric VP2. *Vaccine*. (2015) 33:812–8. doi: 10.1016/j.vaccine.2014.12.003
 158. Feenstra F, van Gennip RG, van de Water SG, van Rijn PA. RNA elements in open reading frames of the bluetongue virus genome are essential for virus replication. *PLoS ONE*. (2014) 9:e92377. doi: 10.1371/journal.pone.0092377
 159. Maan S, Maan NS, van Rijn PA, van Gennip RG, Sanders A, Wright IM, et al. Full genome characterisation of bluetongue virus serotype 6 from the Netherlands 2008 and comparison to other field and vaccine strains. *PLoS ONE*. (2010) 5:e10323. doi: 10.1371/journal.pone.0010323
 160. Feenstra F, Maris-Veldhuis M, Daus FJ, Tacken MG, Moormann RJ, van Gennip RG, et al. VP2-serotyped live-attenuated bluetongue virus without NS3/NS3a expression provides serotype-specific protection and enables DIVA. *Vaccine*. (2014) 32:7108–14. doi: 10.1016/j.vaccine.2014.10.033
 161. van Rijn PA, Daus FJ, Maris-Veldhuis MA, Feenstra F, van Gennip RG. Bluetongue disabled infectious single animal (DISA) vaccine: studies on the optimal route and dose in sheep. *Vaccine*. (2017) 35:231–7. doi: 10.1016/j.vaccine.2016.11.081
 162. Avia M, Rojas JM, Miorin L, Pascual E, Van Rijn PA, Martin V, et al. Virus-induced autophagic degradation of STAT2 as a mechanism for interferon signaling blockade. *EMBO Rep*. (2019) 11:e48766. doi: 10.15252/embr.201948766
 163. Chauveau E, Doceul V, Lara E, Breard E, Sailleau C, Vidalain PO, et al. NS3 of bluetongue virus interferes with the induction of type I interferon. *J Virol*. (2013) 87:8241–6. doi: 10.1128/JVI.00678-13
 164. Kundlacz C, Pourcelot M, Fablet A, Amaral Da Silva Moraes R, Leger T, Morlet B, et al. Novel function of Bluetongue virus NS3 protein in regulation of the MAPK/ERK signaling pathway. *J Virol*. (2019) 93:e00336-19. doi: 10.1128/JVI.00336-19
 165. Leblanc N, Rasmussen TB, Fernandez J, Sailleau C, Rasmussen LD, Uttenthal A, et al. Development of a real-time RT-PCR assay based on primer-probe energy transfer for the detection of all serotypes of bluetongue

- virus. *J Virol Methods*. (2010) 167:165–71. doi: 10.1016/j.jviromet.2010.03.032
166. Orru G, Ferrando ML, Meloni M, Liciardi M, Savini G, De Santis P. Rapid detection and quantitation of Bluetongue virus (BTV) using a Molecular Beacon fluorescent probe assay. *J Virol Methods*. (2006) 137:34–42. doi: 10.1016/j.jviromet.2006.05.028
 167. van Rijn PA, Heutink RG, Boonstra J, Kramps HA, van Gennip RG. Sustained high-throughput polymerase chain reaction diagnostics during the European epidemic of Bluetongue virus serotype 8. *J Vet Diagn Invest*. (2012) 24:469–78. doi: 10.1177/1040638712440986
 168. van Rijn PA, van de Water SG, van Gennip HG. Bluetongue virus with mutated genome segment 10 to differentiate infected from vaccinated animals: a genetic DIVA approach. *Vaccine*. (2013) 31:5005–8. doi: 10.1016/j.vaccine.2013.08.089
 169. Potgieter AC, Wright IM, Erasmus BJ. *Live Attenuated African Horsesickness Virus* WO 2016/071850. (2014). Available online at: <https://patents.google.com/patent/EP3149158B1/en>
 170. Kramps JA, van Maanen K, Mars MH, Popma JK, van Rijn PA. Validation of a commercial ELISA for the detection of bluetongue virus (BTV)-specific antibodies in individual milk samples of Dutch dairy cows. *Vet Microbiol*. (2008) 130:80–7. doi: 10.1016/j.vetmic.2008.01.004
 171. Mars MH, van Maanen C, Vellema P, Kramps JA, van Rijn PA. Evaluation of an indirect ELISA for detection of antibodies in bulk milk against bluetongue virus infections in the Netherlands. *Vet Microbiol*. (2010) 146:209–14. doi: 10.1016/j.vetmic.2010.05.009
 172. Bumbarov V, Golender N, Erster O, Khinich Y. Detection and isolation of Bluetongue virus from commercial vaccine batches. *Vaccine*. (2016) 34:3317–23. doi: 10.1016/j.vaccine.2016.03.097

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 van Rijn. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Failure to Remove Bluetongue Serotype 8 Virus (BTV-8) From *in vitro* Produced and *in vivo* Derived Bovine Embryos and Subsequent Transmission of BTV-8 to Recipient Cows After Embryo Transfer

OPEN ACCESS

Edited by:

William C. Wilson,
United States Department of
Agriculture, United States

Reviewed by:

Bernd Hoffmann,
Friedrich Loeffler Institute, Germany
Madhuri Subbiah,
National Institute of Animal
Biotechnology, India

*Correspondence:

Andy Haegeman
andy.haegeman@sciensano.be

[†]These authors have contributed
equally to this work

*Present address:

Leen Vandaele,
Flanders Research Institute for
Agriculture, Fisheries and Food,
Animal Sciences Unit, Melle, Belgium

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 09 October 2019

Accepted: 15 November 2019

Published: 05 December 2019

Citation:

Haegeman A, Vandaele L, De Leeuw I,
Oliveira AP, Nauwynck H, Van Soom A
and De Clercq K (2019) Failure to
Remove Bluetongue Serotype 8 Virus
(BTV-8) From *in vitro* Produced and *in*
vivo Derived Bovine Embryos and
Subsequent Transmission of BTV-8 to
Recipient Cows After Embryo Transfer.
Front. Vet. Sci. 6:432.
doi: 10.3389/fvets.2019.00432

Andy Haegeman^{1*}, Leen Vandaele^{2†}, Ilse De Leeuw¹, André P. Oliveira^{2,3},
Hans Nauwynck⁴, Ann Van Soom² and Kris De Clercq¹

¹ Unit of Exotic and Particular Diseases, Sciensano, Brussels, Belgium, ² Department of Reproduction, Obstetrics and Herd Health, Ghent University, Merelbeke, Belgium, ³ EPAMIG, Escola de Veterinária da UFMG, Bolsista CAPES, Belo Horizonte, Brazil, ⁴ Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

The behavior of BTV-8 in cattle is different from most other serotypes not only with regards to clinical signs but certainly with respect to virus transmission (transplacental, contact). Therefore, the possibility of virus transmission by means of embryo transfer was examined by *in vitro* exposure of *in vitro* produced and *in vivo* derived bovine blastocysts to BTV-8 followed by different washing protocols, including longer exposure times (up to 120 s) to 0.25% trypsin at room temperature or at 37°C. None of the washing protocols used was successful in removing the viral genome completely from the *in vitro* produced and *in vivo* derived embryos as was demonstrated by real-time PCR. Moreover, BTV-8 virus was transmitted to recipient cows after embryo transfer of *in vivo* derived BTV8-exposed embryos, which had been subjected to routine decontamination as recommended by IETS, consisting of 5 washes in PBS followed by a double treatment of 0.25% trypsin for 45s at 37°C, and an additional 5 washes in PBS with 2% FCS. This study clearly demonstrates the necessity of vigorous application of the directives for screening of potential donors and the collected embryos, especially in regions with BTV-8, to prevent transmission of the disease.

Keywords: bovine embryo, IETS guidelines, Bluetongue virus, BTV-8, transmission

INTRODUCTION

Bluetongue virus (BTV) is a segmented double stranded RNA virus belonging to the genus Orbivirus, family Reoviridae (1) and is the causative agent for bluetongue disease. The disease has a significant impact on naïve populations and although BTV can infect all ruminant species, clinical signs are usually confined to sheep and white-tailed deer (2, 3). In epizootic situations the virus has the potential to cause severe socio-economic problems (4) due to loss of productivity, international movement restrictions, and lengthy and costly regulatory testing requirements of livestock and germ cell. The main transmission route for BTV is by biting midges (*Culicoides* spp.), but data have been published on contact transmission of BTV-8 (5) and BTV-26 (6). As human intervention in bovine reproduction has become common practice, with artificial insemination

and embryo transfer being routinely used in cattle breeding, other possible transmission routes need to be considered. Shedding of BTV in semen is considered to be rare in ruminants (7) and only occurs during and/or directly after the viraemic period (8). This has been mainly observed for laboratory-adapted strains (BTV-1, BTV-23) but can also occur with wild type strains (BTV-23) (8). Although not completely elucidated, the presence of BTV in the seminal plasma of bulls is thought to be caused by the infiltration of infected blood cells due to injury or inflammation of the genital tract (8). The risk of transmitting BTV by embryo transfer is considered to be negligible by the International Embryo Transfer Society (IETS) when their guidelines for embryo washing/trypsin treatment are strictly followed (9, 10). This has been substantiated by experimental findings that when these guidelines are applied, *in vitro* or *in vivo* infection of the embryos does not result in the transmission of the virus to recipient cows (11–14) or ewes (15, 16) and their offspring. However, the emergence of BTV-8 in Central and Northern Europe in 2006–2009 (4, 17) did not only challenge our understanding of the geographic distribution of BTV and its potential vectors but numerous observations and experiments clearly demonstrated the atypical behavior of this particular serotype (18, 19). There was not only a significant increase in morbidity and mortality in cattle and offspring (20, 21) but infectious virus could readily be detected and isolated from bovine semen samples in the absence of contaminating blood cells (22). The fact that BTV-8 seems to interact differently with the genital tract compared to the other serotypes is also corroborated by other observations. Just as seminal shedding, transplacental infection was considered to be associated only with vaccine or laboratory-adapted BTV strains (23–25). However during the BTV-8 epizootic in Central and Northern Europe in 2006–2009 vertical transmission could be demonstrated on numerous occasions (26, 27). This potential of BTV-8 to be vertically transmitted resulted in increased numbers of abortions/stillborns and birth abnormalities and might be related to active virus replication as was shown in *in vitro* exposed bovine hatched embryos (28–30). The underlying genetic reason for the atypical behavior of BTV-8 still has to be clarified which makes it difficult to estimate the true extent of its different behavior. In view of the apparent altered interaction of BTV-8 with the reproductive system, it was the purpose of this study to examine the possibility of BTV-8 transmission by means of embryo transfer following different washing/trypsin protocols, including the one advocated by the IETS. Both *in vitro* produced and *in vivo* derived embryos were included in this study in alignment with current bovine assisted reproductive techniques.

MATERIALS AND METHODS

Virus

The BTV-8 strain used (Bel2006/2) was isolated from an infected sheep during the 2006 epidemic through one passage on embryonated chicken eggs (ECE) and 5 passages on Baby Hamster Kidney (BHK-21) cells (ATCC-CCL10) as described by Toussaint et al. (17).

Embryo Collection

In vitro Production of Bovine Blastocysts

Bovine blastocysts ($n = 105$) were produced by the following *in vitro* methods: after collecting bovine ovaries from an abattoir, the oocytes were aspirated from follicles measuring between 4 and 8 mm in diameter and cultured for 20–24 h at 38.5°C in 5% CO₂ in air in groups of 100 in 500 µL modified bicarbonate buffered TCM-199 supplemented with 20% heat-treated fetal calf serum (FCS) (Biochrom AG, Berlin, Germany). Spermatozoa were separated from frozen-thawed bovine semen using Percoll-gradient centrifugation (Pharmacia, Uppsala, Sweden), and then washed. The mature oocytes were incubated with a sperm (sp) concentration of 1×10^6 sp/mL in an *in vitro* fertilization medium consisting of bicarbonate buffered Tyrode albumin lactate pyruvate (TALP) solution, supplemented with bovine serum albumin (BSA, fraction V, A6003, Sigma-Aldrich, Bornem, Belgium) (6 mg/mL) and heparin (25 µg/mL). After 20–24 h of incubation the presumed zygotes were vortexed to remove excess sperm and cumulus cells and subsequently cultured for a further 7 days in 50 µL droplets of synthetic oviduct fluid supplemented with amino acids and FCS (SOFaa + 5% FCS) in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂ under mineral oil (Sigma-Aldrich).

In vivo Derived Embryos

Donor cows ($n = 2$) were synchronized and super-ovulated using Stimufol® (Ulg, Liège, Belgium) and subsequently inseminated. Donor cows (blood at the start of the synchronization) and bull (blood and sperm) tested negative in the BTV real-time RT-PCR (RT-qPCR) [see Virus isolation on embryonated chicken eggs (ECE)]. At 6.5 days post insemination (dpi), embryos ($n = 14$ and $n = 3$) were non-surgically collected by uterine flushing.

Viral Exposure

At 7 days post insemination (dpi) for *in vitro* produced embryos and at 6.5 dpi for *in vivo* derived embryos, groups of 4 to 8 zona-intact embryos were placed in 800 µL of minimal essential medium (MEM), containing $10^{4.9}$ TCID₅₀/ml of BTV-8, a titer that can be found in semen from bulls naturally infected with BTV-8 (22), and incubated for 1 h at 39°C in 5% CO₂ incubator (28). In total 98 *in vitro* produced embryos and 17 *in vivo* derived embryos were exposed to BTV8. Mock-exposure of 7 zona-intact blastocysts, *in vitro* produced, was performed in 800 µL SOF or 800 µM MEM without virus to evaluate any negative effects of MEM on blastocyst viability.

Embryo Washing and Trypsin Treatment Procedures

Evaluation of Washing Procedures of BTV Exposed Embryos (*in vitro* Produced)

Preliminary evaluation of the decontamination of in vitro produced bovine embryos following the routine IETS procedure (experiment 1)

It was the purpose of this preliminary experiment to look at the efficacy of the routinely used IETS treatment/wash procedures to eliminate BTV8 from the *in vitro* produced

bovine embryos. For this purpose 8 *in vitro* produced bovine embryos were exposed to BTV8 (as described in section Viral exposure) and divided in two groups. The first group was not washed/treated and functioned as a control group, while the bovine embryos in the second group were washed and treated as follows: the embryos were washed individually in 5 consecutive petri dishes containing PBS with gentamycin (50 mg/L) and 0.4% BSA, without Ca and Mg. Subsequently, the embryos were exposed to 2 consecutive 0.25% trypsin (Invitrogen, Carlsbad, CA, 25050-014) treatments by incubation for 45s in a 5% CO₂ incubator at 37°C. Finally, another 5 consecutive washes in PBS with 2% FCS were performed. Each petri dish contained at least 2 mL of medium and was gently agitated between washes. Embryos were transferred in a maximum of 7 µL of medium and a new tip was used after every wash step. Washes 1–5 and washes 6–10 were pooled. The pooled washing fluids, trypsin liquid and the washed/treated embryos were stored at –80°C for real-time PCR evaluation.

Evaluation of increased duration of exposure of virus-exposed in vitro produced bovine embryos to trypsin at room temperature and at 37°C (experiment 2)

For the second *in vitro* experiment, three different types of trypsin treatments (T45–T120) were evaluated at 2 different incubation temperatures, namely at 37°C (G37) and at room temperature (G20), resulting in six different treatment combinations (Table 1). Per treatment the experiment was carried out in triplicate whereby each sample (replicate) consisted of 5 embryos. Each sample (E) was washed five times in PBS without BSA followed by two treatments in 0.25% trypsin-EDTA for either 45s (T45), 60s (T60), or 120s (T120) and then followed by ten washes in PBS + 0.4% BSA. For each step, the embryos were transferred in a maximum of 7 µL of medium and a new tip was used after every wash step. Only washes 11 to 15 were pooled and are indicated as W11.

All washed/treated embryos, pools and individual wash/trypsin fluids were analyzed for the presence of BTV genome using RT-qPCR and virus isolation.

TABLE 1 | Experimental set-up of the treatment procedure for the second *in vitro* experiment; E: Sample consisting of 5 embryos, Between brackets: the group assignment.

Incubation time	At 37°C	At RT (20°C)
Wash and treatment procedures		
Wash step 1–5		
Trypsin treatment 1 and 2		
45s	E1, E4, E7 (G37T45)	E10, E13, E16 (G20T45)
60s	E2, E5, E8 (G37T60)	E11, E14, E17 (G20T60)
120s	E3, E6, E9 (G37T120)	E12, E15, E18 (G20T120)

E1 was excluded due to problems during the washing/trypsin treatment as explained in section Experiment 2: Evaluation of increased duration of exposure of virus-exposed *in vitro* produced bovine embryos to trypsin at room temperature and at 37°C.

In vitro and in vivo Evaluation of the Routine IETS Wash/Treatment Procedure of BTV Exposed Embryos (in vivo Derived) (Experiment 3)

Washing and trypsin treatment

Embryos were either washed in pairs ($n = 14$; 7 pairs) or separately ($n = 3$) using identical washing and trypsin conditions as described for the embryos in the first *in vitro* experiment [see Preliminary evaluation of the decontamination of *in vitro* produced bovine embryos following the routine IETS procedure (Experiment 1)]. Washes 1–5 and washes 6–10, from the embryos washed in pairs, were pooled and analyzed for the presence of BTV-8 genome using RT-qPCR. Embryos which were not used for embryo transfer (see Embryo transfer) were similarly used for real-time PCR.

Embryo transfer

All the washed/treated *in vivo* derived embryos ($n = 17$) were examined for their suitability for embryo transfer in donor cows. In total 8 embryos, which had reached the morula or blastocyst stage were selected and washed in pairs. The embryos which were not selected for transfer, consisting of morulae, and degenerated or unfertilized oocytes, were washed following the same protocol (separately or in group). Three pairs of *in vivo* derived washed embryos were loaded in straws and transferred to three BTV negative recipient cows. The fourth pair was used for real-time PCR analysis. Two sentinel cows served as control. Cows were bled twice weekly and blood and serum samples were analyzed for the presence of BTV-8 RNA (RT-qPCR) and antibodies against BTV-8. The protocol was approved by the ethical committee of the faculty of Veterinary Medicine (Ghent, authorization number EC2011/094).

Antibody ELISA

All sera were tested for the presence of BTV-specific antibodies by means of a commercially available competitive ELISA (c-ELISA) (ID Screen® Blue Tongue Competition, ID VET, Montpellier, France) performed according to the instructions provided by the manufacturer. Results were expressed as a percentage negativity (PN) compared to the negative kit control and were classified into positive (PN ≤ 65), doubtful (PN > 65 but ≤ 75), and negative (PN > 75) results based on the optimal cut-off point for diagnostic purposes of 65 PN determined by Vandenbussche et al. (31).

RNA Extraction

RNA extractions were performed using the NucleoSpin® RNA Virus kit (Machery-Nagel, Düren, Germany) according to the manufacturer's recommendations with the exception of the addition of an external control (EC) to the RAV1 buffer (32). Hearts of chicken embryos were pre-treated as described in Garigliany et al. (33).

Real-Time RT-PCR (RT-qPCR)

The efficiency of the different washing techniques and trypsin temperature for virus removal was evaluated by using a non-serotype specific quantitative reverse-transcription PCR assay targeting BTV segment 5 (pan-BTV/S5 RT-qPCR) according to the method described by Vandenbussche et al. (32) on

embryos, washes and blood and organ samples. Test results were classified as follows: Crossing Point values (Cp-values) <40.0 were classified as positive, Cp-values ≥ 40.0 but <45.0 were classified as doubtful, and Cp-values ≥ 45.0 were classified as negative.

Virus Isolation on Embryonated Chicken Eggs (ECE)

Virus isolations from washed/treated *in vitro* produced embryos, washing fluids and trypsin residues from Evaluation of washing procedures of BTV exposed embryos (*in vitro* produced). Two were performed as described for blood samples in Toussaint et al. (17) whereby 5 ECEs were used per sample. Passages on ECEs were done by collecting blood from chicken embryos that were still alive at 7dpi. This blood was 10 times diluted in PBS supplemented with 0.2% gentamycin and one hundred micro liter of this dilution was inoculated in new ECE. A sample was considered to be negative if all 5 ECEs were still viable after 7 dpi. Samples were considered to be positive if an ECE died between 2 and 7 dpi and the presence of BTV was confirmed by real-time PCR. The latter was achieved by homogenizing the heart of a dead embryo followed by RNA extraction and subsequent real-time PCR as described in 2.6 and 2.7, respectively.

RESULTS

Experiment 1: Preliminary Evaluation of the Decontamination of *in vitro* Produced Bovine Embryos Following the Routine IETS Procedure

The four unwashed/untreated embryos of the control group were all positive for BTV with Cp-values between 31.2 and 33.9. When looking at the washed/treated group, all the pools of the first wash steps (1–5) were positive with Cp-values between 29.4 and 30. In contrast, the pools for wash step 6–10 were all negative. When the washed/treated embryos themselves were examined, two were found to be negative, one doubtful, and one was positive with a Cp value of 37.7.

Experiment 2: Evaluation of Increased Duration of Exposure of Virus-Exposed *in vitro* Produced Bovine Embryos to Trypsin at Room Temperature and at 37°C

The results of the preliminary evaluation of the routinely used IETS wash/treatment procedure with pooled wash steps (Experiment 1) asked for a more in detail evaluation of individual wash and trypsin steps. In this second experiment the washing steps were analyzed individually and different trypsin treatments were evaluated (Table 1). Due to problems during the washing/trypsin treatment of the G37T45 group in the second *in vitro* experiment, one of the triplicate repeats was excluded (E1), meaning that G37T45 consisted only of 2 replicates (E4 and E7). The initial RT-qPCR screening of the wash and trypsin fluids containing one replicate of each of the six washing/temperatures combinations (E4, E10, E5, E11, E3, and E12) showed a decreasing viral load in each subsequent

wash step (Figure 1). Five of the six samples were still positive at wash step five (W5), with Cp's between 33.2 and 38.8, while only one of the six remained positive after trypsin 45 (T45) and 60 (T60) seconds treatment. Additional doubtful results were obtained for T45 ($n = 3$) and T620 ($n = 1$). From wash step six (W6) onwards no positive results were obtained for all replicates although 2 and 1 sample were doubtful for, respectively W6 and W7. When analyzing the embryos after all the steps (washes and trypsin treatments), three replicates were found to be negative while 2 remained positive and one doubtful. Based on these results, W5–W8 of all the other replicates were tested as well as the remaining embryos. These results confirmed the initial screening with the majority of the samples being positive on W5 with rapidly decreasing Cp-values toward T45 and T60. Two differences were noted, however. One sample was positive in W6 and the majority of the embryos were positive, more precisely 12 of the 16 (Figure 2). One negative embryo was found in G37T45, G37T120, and G20T120 and one doubtful in G37T60. The average Cp-values of the washed embryos were very similar across the groups (average Cp-values between 35.5 and 36.2) with a small exception for G20T120 which had a slightly higher average Cp of 37.9. No positive RT-qPCR results were observed after W6 except one doubtful result for G37T45 at W7 from the initial screening. Although virus could be isolated on embryonated chicken eggs (ECE) from W1 and W3, no virus could be isolated from W5 onwards from the samples tested. Similarly, no virus was isolated from all the washed embryos even after 4 consecutive passages on ECE. The blood collected from the fourth passage remained negative on RT-PCR.

Experiment 3: *In vitro* and *in vivo* Evaluation of the Routine IETS Wash/Treatment Procedure of BTV Exposed Embryos (*in vivo* Derived)

When looking at the real-time PCR results of the first pooled washing step (wash 1–5) of the embryos which were transferred to recipient cows, the Cp-value ranged from 28.9 to 29.5. Consistent with the data from experiment 1 and 2, the Cp-values of the second pool (wash 6–10) were a lot higher with 2 pools being borderline positive (38.9 and 39.5), 1 doubtful (Cp = 40) and 1 negative. As these embryos themselves could not be tested as they were transferred, an embryo pair which was not transferred but washed/treated was similarly analyzed by real-time PCR. Both individual embryos were positive with Cp-values of 32.3 and 32.5. When this is compared to the Cp values of the *in vitro* produced embryos under identical washing/treatment regime (G37T45), this was found to be slightly lower. The oocytes/embryos which were unsuited for embryo transfer were washed/treated and analyzed as well with real-time PCR. The Cp value that was obtained was very similar to those for the suited embryos: (1) the pools of the first wash steps were all positive (Cp-values of 27.7 to 32.2) while only one out of three was positive for the second pool (Cp 38.13); (2) the embryos themselves ($n = 9$) were all positive (Cp-values between 31.02 and 34.11) except one.

Two embryos were transferred to each of three recipient cows (identification number: 1047, 1052, and 1082). The latter

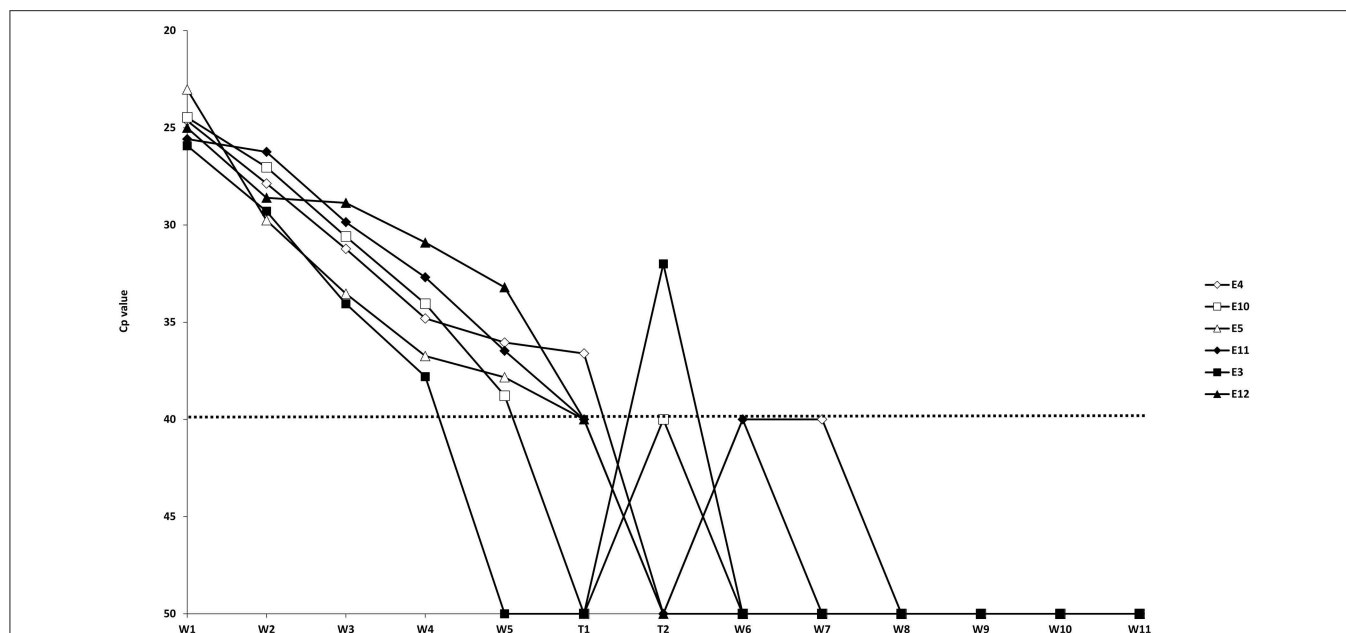


FIGURE 1 | Initial RT-qPCR-results of the wash (W)/trypsin (T) fluids. The dotted line represents the cut-off for RT-qPCR positivity. E: Sample consisting of 5 embryos.

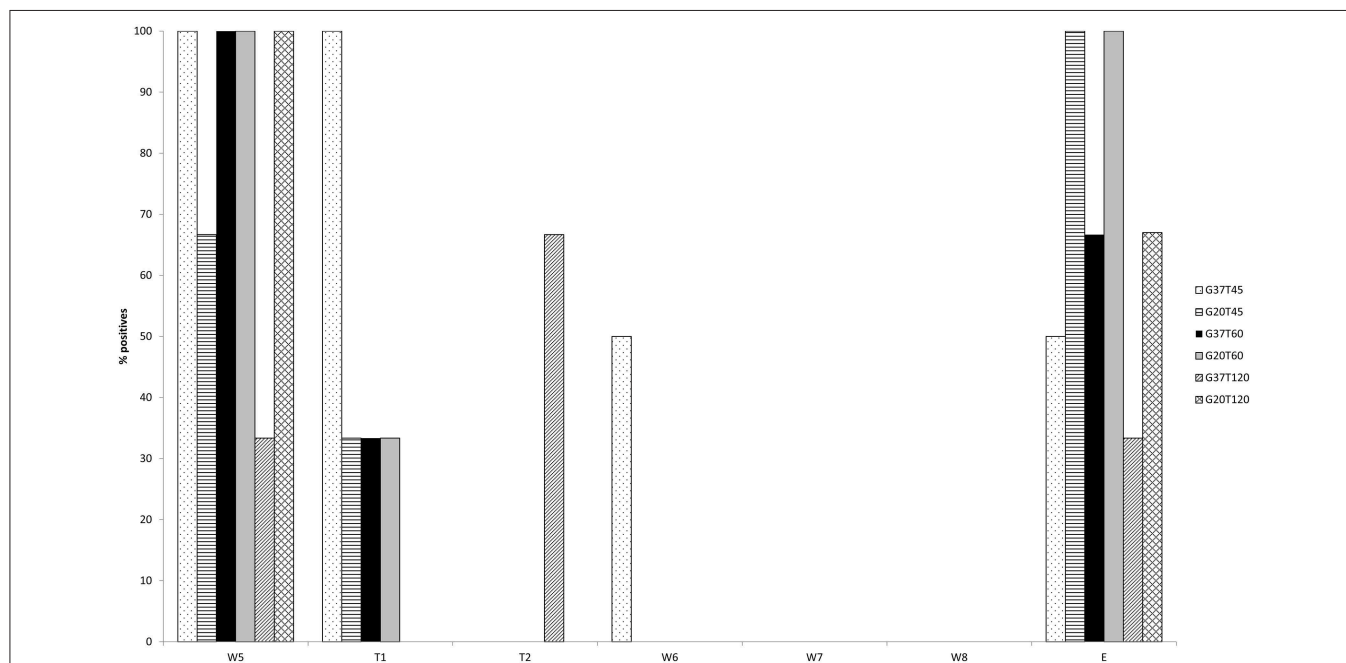


FIGURE 2 | RT-qPCR results of the wash step (W) 5–8, including both trypsin treatments and the embryos (E) after all washing steps. The percentage of positives are represented per wash/trypsin treatment group (as defined in **Table 1**).

two animals received embryos for which the second pool was negative while animal 1047 received a paired embryo sample for which the second pool scored doubtful. The two sentinel animals (identification number: 1056 and 1070) remained negative on ELISA and RT-qPCR for the complete duration of the experiment (i.e., 80 days post-transfer; dpt). The three recipient cows became viremic at the same time, namely 7 dpt and with

similar Cp-values (**Figure 3**). Recipient 1047 and 1082 displayed a similar viremic profile and remained positive until 25 and 29 dpt, respectively. Cow 1052 had a shorter viremic period and was only positive until 17 dpt. All three recipient cows also displayed a very similar serological profile as they all seroconverted at 14 dpt and remained positive until the end of the experiment (**Figure 4**).

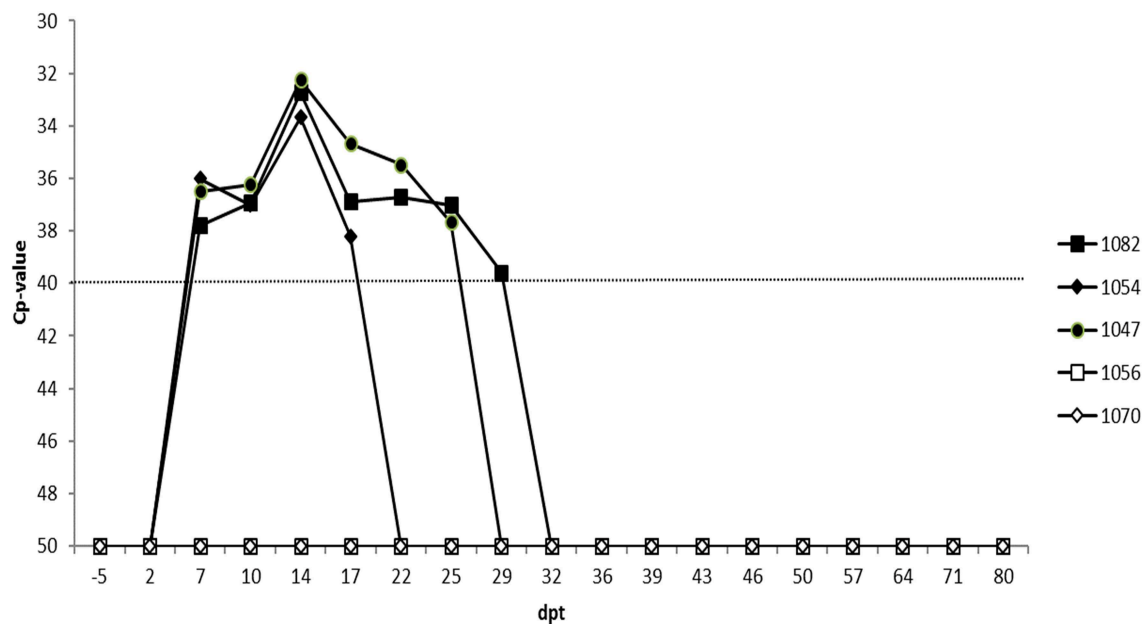


FIGURE 3 | RT-qPCR blood results of the three recipient (1082, 1054, 1047) and 2 sentinel cows (1056, 1070). The dotted line represents the cut-off for RT-qPCR positivity. Dpt, days post transfer.

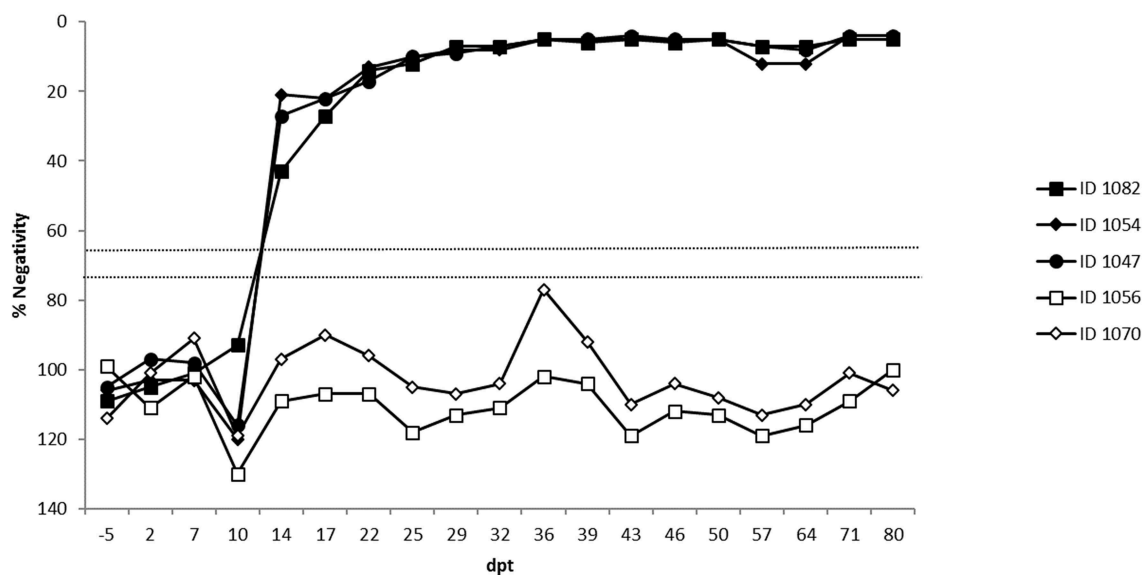


FIGURE 4 | ELISA results of the three recipient (1082, 1054, 1047) and 2 sentinel cows (1056, 1070). Percentage negativity (PN): positive PN ≤ 65 , doubtful PN > 65 but ≤ 75 and negative PN > 75 . Dpt, days post transfer.

DISCUSSION

The risk of BTV transmission by embryo transfer has been considered to be negligible, when following the prescribed guidelines of the IETs. This is largely based upon animal experiments whereby BTV transmission to the recipient cows or ewes could not be demonstrated when the appropriate washing procedures were applied. These experiments were done

using mainly BTV serotype 2, 4, 10, 11, 13, and 17 [reviewed by Wrathall et al. (10)]. Although the combined data spans several BTV serotypes, giving it more credibility, it needs to be mentioned that BTV has an important genomic diversity. This is reflected by the numerous serotypes which have been and are still being characterized (34). The serotype of BTV is defined by the structural protein VP2 whose coding sequence is the most variable of all the BTV segments. Inter-serotype

diversity of VP2 can go as high as 59% on the nucleotide level and 73% on the deduced amino acid level (35). This protein is not only the most outer capsid protein (36) but is also implemented in cell attachment and entry (37). The combination of VP2's genomic variability and its function is a potential source of different virus serotype behavior. This is exemplified by BTV-8 which seems to interact differently with the components of the genital tract compared to the other wild type serotypes (vertical transmission, seminal shedding, contact transmission, ...). Caution is therefore heeded regarding generalizations across serotypes and further investigations are warranted for serotypes displaying different behavior.

Correctly carrying out the washing procedure is an important step in the process of embryo transfer as BTV has great affinity for the zona pellucida of the embryo after *in vitro* exposure (38). This great affinity is clearly demonstrated in the Langston et al. study (39) where 12 consecutive wash steps failed to remove BTV from the bovine embryos as infectious virus could be recovered afterwards. Similar results were obtained in infected caprine and ovine embryos where 10 washes did not remove BTV completely (16, 40). Also in this study the affinity of BTV-8 for the embryos was noted as more than 80% of wash step 5 fluids were BTV-8 positive albeit with decreasing Cp-values. These data seems to be in contrast to the Venter et al. study (9) using ovine embryos where BTV (serotype 2 and 4) could only be detected in the first washing fluid and then even rarely. However, the used titers ($1 \times 10^{2.88}$ and $1 \times 10^{3.5}$, respectively) in that study were lower than in our study. Even unwashed ova were not readily detected after a first passage on cell culture in the Venter study. The benefit of implementing trypsin treatments was clearly demonstrated as only 1 out of the 17 wash/treatment fluids remained positive in the first wash step following the treatments. This is supported by the finding of Ahmad et al. (41) where the washing fluids became negative after incubation with trypsin. In contrast to infected caprine embryos, where a double 0.25% trypsin treatment of 60s removed BTV-8 completely, none of the here evaluated trypsin treatments efficiently removed all traces of the BTV-8 genome from all the bovine embryos, not even the double 60s 0.25% trypsin treatment. Only small differences were seen between temperature groups (37°C group: 62.5% positives +12.5% doubtful vs. 88.9% positives in the room temperature group) and duration groups (positives: T45 80%; T60 83% +16.7% doubtful; T120 66.7%). The small differences seen are probably due to more optimal conditions for trypsin (in regards to temperature and duration). Nevertheless, it is interesting to note that negative washing fluids did not prove that the embryos themselves were free from viral genome as was demonstrated by positive real-time PCR results. If there was still infectious virus present, however, could not be determined. Although the virus could not be isolated from the *in vitro* produced washed embryos, their Cp-values were high (> 35), meaning that the inability to isolate the virus could also be caused by a too low viral load. The instability of RNA needs also to be kept in mind specifically with the many wash and treatment steps that were carried. The continued presence, therefore, of solely genomic RNA on the embryos seems unlikely. The importance of the inability to remove BTV-8 genome from the *in vitro* infected embryos is seen

during the *in vivo* part of the study where the transfer of infected and washed, following the IETS guidelines, embryos resulted in the viremia and seroconversion in 100% of the recipients. The viremia seen in the recipient cows was in general shorter compared to naturally infected cattle (42). Although bluetongue viremia is generally perceived as prolonged (43) a short viremia (14 days) after infection with BTV-8 is no exception as shown by the extensive literature review by EFSA (44). The capacity to transmit the virus by embryo transfer to the recipients clearly demonstrates that infectious virus was present on the embryos after washing although it could not be isolated on ECE during the *in vitro* studies (Experiment 2). It needs to be stated that *in vivo* derived embryos were used for the *in vivo* part of the study while *in vitro* produced embryos were used for the *in vitro* part. This can be of importance as differences were seen in the ability to remove/inactivate BoHV-1 between *in vitro* produced and *in vivo* derived embryos using wash/trypsin treatments [reviewed by Wrathall et al. (10)]. Moreover, the zona pellucida of *in vivo* derived and *in vitro* produced bovine embryos is very different in its ability to bind virus (45). Although the washing steps were pooled for the *in vivo* trial (Experiment 3) instead of individually tested as in experiment 2, no differences were seen between both experiments in the PCR profiles and Cp-values of the washing fluids, trypsin liquids and the washed embryos. This indicated that the washing and trypsin treatments were equally ineffective in removing BTV-8 from *in vivo* derived and *in vitro* produced embryos. To our knowledge this is the first time that BTV-8 was transferred by means of embryo transfer when using the IETS guidelines for washing the embryos. Viremia and seroconversion in ewes was reported by Gilbert et al. (46) but the embryos used in this study were not washed or treated. In all other studies (9, 11, 14, 16) transmission was never reported if the IETS guidelines were followed. However, in these studies embryos were transferred from infected donors while in our study the embryos were exposed *in vitro* to the virus. The latter allows more control over the exposure of the embryo to the virus with parameters such as concentration of free virus, timeline and others. However, the question can be asked if the *in vitro* exposure is relevant for an *in vivo* situation. Firstly, the way of exposure of the embryos to the virus seems to be of importance, as more embryos had virus particles when they were exposed using an infected cell culture then when a viral suspension was used (38). Secondly, the question can be put forward if the BTV is able to come into contact with the embryo in order to infect or attach on it as harvested embryos from BTV infected donors are rarely reported to be positive (11). In many of the published studies the embryos are harvested at peak viremia in the blood under the hypothesis that this would be the time of the highest exposure of the embryos to the virus. However, data with regards to the organ distribution of BTV and its kinetics during viremia are not available to support this assumption. This could lead to a lesser or even unsuited time point for harvesting positive embryos. On the other hand, embryos can be exposed to BTV *in utero*, as the virus has been isolated from the uterus of cows infected with BTV11 (47). Furthermore, BTV circulates for a prolonged period of time in the blood of an infected cow, and the embryo can be exposed to the virus as a consequence of endometrial trauma during flushing

when collecting the embryos (13). Although in most cases this was attributed to the infiltration of BTV positive red blood cells, free BTV was also found in cell free flush fluids. The latter would be capable of infecting the embryos, although it remains difficult uncertain to which virus titer bovine embryos are exposed to *in vivo*.

In summary, this study demonstrated that although extensive washing/trypsin treatment reduces and eliminates BTV-8 viral load from the washing fluids, it cannot completely clear the virus from bovine embryos spiked with BTV8. When the latter were transferred, it can result in virus transmission to the recipient.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the ethical committee of the faculty of Veterinary Medicine; Ghent University; authorization number EC2011/094.

REFERENCES

- Mertens PPC, Maan S, Samuel A, Attoui H, Orbivirus, reoviridae. In: Fauquet CM, Mayo MA, Maniloff J, Dresselberger U, Ball LA, editors. *Virus Taxonomy*. London: Elsevier Academic Press. (2005). p. 466–83.
- Darpel KE, Batten CA, Veronesi E, Shaw AE, Anthony S, Bachanek-Bankowska K, et al. Clinical signs and pathology shown by British sheep and cattle infected with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe. *Vet Rec.* (2007) 161:253–61. doi: 10.1136/vr.161.8.253
- Howerth EW, Greene CE, Prestwood AK. Experimentally induced bluetongue virus infection in white-tailed deer—coagulation, clinical pathologic, and gross pathologic changes. *Am J Vet Res.* (1988) 49:1906–13.
- Wilson A, Mellor P. Bluetongue in Europe: vectors, epidemiology and climate change. *Parasitol Res.* (2008) 103(Suppl. 1):S69–77. doi: 10.1007/s00436-008-1053-x
- Menzies FD, McCullough SJ, McKeown IM, Forster JL, Jess S, Batten C, et al. Evidence for transplacental and contact transmission of bluetongue virus in cattle. *Vet Rec.* (2008) 163:203–9. doi: 10.1136/vr.163.7.203
- Batten C, Darpel K, Henstock M, Fay P, Veronesi E, Gubbins S, et al. Evidence for transmission of bluetongue virus serotype 26 through direct contact. *PLoS ONE.* (2014) 9:e96049. doi: 10.1371/journal.pone.0096049
- Osburn BI. The impact of bluetongue virus on reproduction. *Comp Immunol Microbiol Infect Dis.* (1994) 17:189–96. doi: 10.1016/0147-9571(94)90042-6
- Kirkland PD, Melville LF, Hunt NT, Williams CF, Davis RJ. Excretion of bluetongue virus in cattle semen: a feature of laboratory-adapted virus. *Vet Ital.* (2004) 40:497–501.
- Venter EH, Gerdes T, Wright I, Terblanche J. An investigation into the possibility of bluetongue virus transmission by transfer of infected ovine embryos. *Onderstepoort J Vet Res.* (2011) 78:17. doi: 10.4102/ojvr.v78i1.17
- Wrathall AE, Simmons HA, Van Soom A. Evaluation of risks of viral transmission to recipients of bovine embryos arising from fertilisation with virus-infected semen. *Theriogenology.* (2006) 65:247–74. doi: 10.1016/j.theriogenology.2005.05.043
- Acree JA, Echternkamp SE, Kappes SM, Luedke AJ, Holbrook FR, Pearson JE, et al. Failure of embryos from bluetongue infected cattle to transmit virus to susceptible recipients or their offspring. *Theriogenology.* (1991) 36:689–97. doi: 10.1016/0093-691X(91)90406-4
- Bowen RA, Howard TH, Elsdon RP, Seidel GE. Bluetongue virus and embryo transfer in cattle. *Prog Clin Biol Res.* (1985) 178:85–189.
- Bowen RA, Howard TH, Elsdon RP, Seidel GE Jr. Embryo transfer from cattle infected with bluetongue virus. *Am J Vet Res.* (1983) 44:1625–8. doi: 10.1016/0093-691X(83)90133-4
- Thomas FC, Singh EL, Hare WC. Embryo transfer as a means of controlling viral infections. VI Bluetongue virus-free calves from infectious semen. *Theriogenology.* (1985) 24:345–50. doi: 10.1016/0093-691X(85)90226-2
- Hare WC, Luedke AJ, Thomas FC, Bowen RA, Singh EL, Eaglesome MD, et al. Nontransmission of bluetongue virus by embryos from bluetongue virus-infected sheep. *Am J Vet Res.* (1988) 49:468–72.
- Singh EL, Dulac GC, Henderson JM. Embryo transfer as a means of controlling the transmission of viral infections. XV Failure to transmit bluetongue virus through the transfer of embryos from viremic sheep donors. *Theriogenology.* (1997) 47:1205–14. doi: 10.1016/S0093-691X(97)00100-3
- Toussaint JF, Sailleau C, Mast J, Houdart P, Czaplicki G, Demeestere L, et al. Bluetongue in Belgium, 2006. *Emerging Infect Dis.* (2007) 13:614–6. doi: 10.3201/eid1304.061136
- Elbers AR, Backx A, Mintiens K, Gerbier G, Staubach C, Hendrickx G, et al. Field observations during the Bluetongue serotype 8 epidemic in 2006. II Morbidity and mortality rate, case fatality and clinical recovery in sheep and cattle in the Netherlands. *Prev Vet Med.* (2008) 87:31–40. doi: 10.1016/j.prevetmed.2008.06.003
- Méroc E, Herr C, Verheyden B, Hooyberghs J, Houdart P, Raemaekers M, et al. Bluetongue in Belgium: episode II. *Transbound Emerg Dis.* (2009) 56:39–48. doi: 10.1111/j.1865-1682.2008.01063.x
- Dal Pozzo F, De Clercq K, Guyot H, Vandemeulebroucke E, Sarradin P, Vandenbussche F, et al. Experimental reproduction of bluetongue virus serotype 8 clinical disease in calves. *Vet Microbiol.* (2009) 136:352–8. doi: 10.1016/j.vetmic.2008.11.012
- Elbers AR, Backx A, Ekker HM, van der Spek AN, van Rijn PA. Performance of clinical signs to detect bluetongue virus serotype 8 outbreaks in cattle and sheep during the 2006-epidemic in The Netherlands. *Vet Microbiol.* (2008) 129:156–62. doi: 10.1016/j.vetmic.2007.10.034
- Vanbinst T, Vandenbussche F, Dernelle E, De Clercq K. A duplex real-time RT-PCR for the detection of bluetongue virus in bovine semen. *J Virol Methods.* (2010) 169:162–8. doi: 10.1016/j.jviromet.2010.07.019

AUTHOR CONTRIBUTIONS

AH was the author of the article and responsible of the real-time PCR evaluation of the samples Experiment 1 and 2. LV was responsible for Experiment 3. AO carried out the viral exposure, washing of the embryos in Experiment 1, 2. IL performed the real-time PCR and ELISA evaluations of Experiment 3. HN, AV, and KC were responsible for the cross-lab and cross institute coordination and critical scientific review of the data.

FUNDING

This work was supported by internal funding from Sciensano.

ACKNOWLEDGMENTS

The authors wish to thank Peter Vercauteren and Ignace Moyaert from CRV Holding for performing the donor superovulation, donor flushing and embryo transfer to the recipients. We thank Petra Van Damme and Isabel Lemahieu from RBU (Ghent University) and all technicians of Sciensano who contributed to this study for their excellent technical assistance.

23. Flanagan M, Johnson SJ. The effects of vaccination of Merino ewes with an attenuated Australian BTV serotype 23 at different stages of gestation. *Aust. Vet. J.* (1995) 72:455–7. doi: 10.1111/j.1751-0813.1995.tb03488.x
24. Kirkland PD, Hawkes RA. A comparison of laboratory and 'wild' strains of bluetongue virus—is there any difference and does it matter? *Vet Ital.* (2004) 40:448–55.
25. MacLachlan NJ, Conley AJ, Kennedy PC. Bluetongue and equine viral arteritis viruses as models of virus-induced foetal injury and abortion. *Anim Reprod Sci.* (2000) 60–1:643–51. doi: 10.1016/S0378-4320(00)00105-6
26. Darpel K, Batten CA, Veronesi E, Williamson S, Anderson P, Dennison M, et al. Transplacental transmission of bluetongue virus 8 in cattle, UK. *Emerg Infect Dis.* (2009) 15:2025–8. doi: 10.3201/eid1512.090788
27. De Clercq K, De Leeuw I, Verheyden B, Vandemeulebroucke E, Vanbinst T, Herr C, et al. Transplacental infection and apparently immunotolerance induced by a wild-type bluetongue virus serotype 8 natural infection. *Transbound Emerg Dis.* (2008) 55:352–9. doi: 10.1111/j.1865-1682.2008.01044.x
28. Vandaele L, Wesselingh W, De Clercq K, De Leeuw I, Favoreel H, Van Soom A, et al. Susceptibility of *in vitro* produced hatched bovine blastocysts to infection with bluetongue virus serotype 8. *Vet Res.* (2011) 42:14. doi: 10.1186/1297-9716-42-14
29. Vercauteren G, Miry C, Vandenbussche F, Ducatelle R, Van der Heyden S, Vandemeulebroucke E, et al. Bluetongue virus serotype 8-associated congenital hydranencephaly in calves. *Transbound Emerg Dis.* (2008) 55:29329–38. doi: 10.1111/j.1865-1682.2008.01034.x
30. Wouda W, Roumen MB, Peperkamp NH, Vos JH, van Garderen E, Muskens J. Hydranencephaly in calves following the bluetongue serotype 8 epidemic in the Netherlands. *Vet Rec.* (2008) 162:422–3. doi: 10.1136/vr.162.13.422-b
31. Vandenbussche F, Vanbinst T, Verheyden B, Van Dessel W, Demeestere L, Houdart P, et al. Evaluation of antibody-ELISA and real-time RT-PCR for the diagnosis and profiling of bluetongue virus serotype 8 during the epidemic in Belgium in 2006. *Vet Microbiol.* (2008) 129:15–27. doi: 10.1016/j.vetmic.2007.10.029
32. Vandenbussche F, Vandemeulebroucke E, De Clercq K. Simultaneous detection of bluetongue virus RNA, internal control GAPDH mRNA, and external control synthetic RNA by multiplex real-time PCR. In: King N, O'Connell J, editors. *RT-PCR Protocols (Methods in Molecular Biology)*. Totowa, NJ: Humana Press Inc. (2010). p. 97–108. doi: 10.1007/978-1-60761-629-0_7
33. Garigliany M, De Leeuw I, Kleijnen D, Vandenbussche F, Callens J, Van Loo H, et al. The presence of bluetongue virus serotype 8 RNA in Belgian cattle since 2008. *Transbound Emerg Dis.* (2011) 58:503–9. doi: 10.1111/j.1865-1682.2011.01230.x
34. Bumbarov V, Golender N, Jenckel M, Wernike K, Beer M, Khinich E, et al. Characterization of bluetongue virus serotype 28. *Transbound Emerg Dis.* (2019). doi: 10.1111/tbed.13338. [Epub ahead of print].
35. Maan S, Maan NS, Samuel AR, Rao S, Attoui H, Mertens PP. Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. *J Gen Virol.* (2007) 88:621–30. doi: 10.1099/vir.0.82456-0
36. Nason EL, Rothagel R, Mukherjee SK, Kar AK, Forzan M, Prasad BV, et al. Interactions between the inner and outer capsids of bluetongue virus. *J Virol.* (2004) 78:8059–67. doi: 10.1128/JVI.78.15.8059-8067.2004
37. Hassan SS, Roy P. Expression and functional characterization of bluetongue virus VP2 protein: role in cell entry. *J Virol.* (1990) 73:9832–42.
38. Gillespie JH, Schlafer DH, Foote RH, Quick S, Dougherty E, Schiff E, et al. Comparison of persistence of seven bovine viruses on bovine embryos following *in vitro* exposure. *Dtsch Tierarztl Wochenschr.* (1990) 97:65–8.
39. Langston NL, Stringfellow DA, Garrett GE. Failure to wash bluetongue virus from bovine IVF embryos. *Proc IETS Canada.* (1999) 51:273. doi: 10.1016/S0093-691X(99)91832-0
40. Al Ahmad MZ, Pellerin JL, Larrat M, Chatagnon G, Cécile R, Sailleau C, et al. Can bluetongue virus (BTV) be transmitted via caprine embryo transfer? *Theriogenology.* (2011) 76:126–32. doi: 10.1016/j.theriogenology.2011.01.025
41. Al Ahmad MZ, Bruyas JF, Pellerin JL, Larrat M, Chatagnon G, Roux C, et al. Evaluation of bluetongue virus (BTV) decontamination techniques for caprine embryos produced *in vivo*. *Theriogenology.* (2012) 78:1286–93. doi: 10.1016/j.theriogenology.2012.05.024
42. Zanella G, Martinelle L, Guyot H, Mauroy A, De Clercq K, Saegerman C. Clinical pattern characterization of cattle naturally infected by BTV-8. *Transbound Emerg Dis.* (2013) 60:231–7. doi: 10.1111/j.1865-1682.2012.01334.x
43. Zientara S, Ponsart C. Viral emergence and consequences for reproductive performance in ruminants: two recent examples (bluetongue and Schmallenberg viruses). *Reprod Fertil Dev.* (2015) 27:63–71. doi: 10.1071/RD14367
44. EFSA. EFSA panel on animal health and welfare (AHAW); Scientific opinion on bluetongue serotype 8. *EFSA J.* (2011) 9:2189. doi: 10.2903/j.efsa.2011.2189
45. Van Soom A, Wrathall AE, Herrler A, Nauwynck HJ. Is the zona pellucida an efficient barrier to viral infection? *Reprod Fertil Dev.* (2010) 22:21–31. doi: 10.1071/RD09230
46. Gilbert RO, Coubrough RI, Weiss KE. The transmission of bluetongue virus by embryo transfer in sheep. *Theriogenology.* (1987) 27:527–40. doi: 10.1016/0093-691X(87)90240-8
47. de la Concha-Bermejillo A, Odeon A, BonDurant RH, Osburn BI. Experimental infection of pregnant cattle with bluetongue virus serotype 11 between postbreeding days 21 and 48. *J Vet Diagn Invest.* (1993) 5:329–35. doi: 10.1177/104063879300500304

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Haegeman, Vandaele, De Leeuw, Oliveira, Nauwynck, Van Soom and De Clercq. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Emerging Trends in the Epidemiology of West Nile and Usutu Virus Infections in Southern Europe

Tatjana Vilibic-Cavlek^{1,2*}, Vladimir Savic³, Tamas Petrovic⁴, Ivan Toplak⁵, Ljubo Barbic⁶, Dusan Petric⁷, Irena Tabain¹, Ivana Hrnjakovic-Cvjetkovic^{8,9}, Maja Bogdanic¹, Ana Klobucar¹⁰, Anna Mrzljak^{2,11}, Vladimir Stevanovic⁶, Petra Dinjar-Kujundzic¹¹, Luka Radmanic⁶, Federica Monaco¹², Eddy Listes¹³ and Giovanni Savini¹²

¹ Department of Virology, Croatian Institute of Public Health, Zagreb, Croatia, ² School of Medicine, University of Zagreb, Zagreb, Croatia, ³ Poultry Center, Croatian Veterinary Institute, Zagreb, Croatia, ⁴ Department for Virology, Scientific Veterinary Institute, Novi Sad, Serbia, ⁵ Veterinary Faculty, University of Ljubljana, Ljubljana, Slovenia, ⁶ Department of Microbiology and Infectious Diseases With Clinic, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia, ⁷ Laboratory for Medical and Veterinary Entomology, Faculty of Agriculture, University of Novi Sad, Novi Sad, Serbia, ⁸ Center for Microbiology, Institute of Public Health Vojvodina, Novi Sad, Serbia, ⁹ Medical Faculty, University of Novi Sad, Novi Sad, Serbia, ¹⁰ Division of Disinfection, Disinfestation and Pest Control, Andrija Stampar Teaching Institute of Public Health, Zagreb, Croatia, ¹¹ Department of Medicine, Merkur University Hospital, Zagreb, Croatia, ¹² OIE Reference Center for West Nile Disease, Istituto Zooprofilattico Sperimentale "G. Caporale", Teramo, Italy, ¹³ Laboratory for Diagnostics, Croatian Veterinary Institute, Regional Institute Split, Split, Croatia

OPEN ACCESS

Edited by:

Armanda Bastos,
University of Pretoria, South Africa

Reviewed by:

Irit Davidson,
Kimron Veterinary Institute, Israel
Ahmed Ali,
Beni Suef University, Egypt

*Correspondence:

Tatjana Vilibic-Cavlek
tatjana.vilibic-cavlek@hzjz.hr

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 28 September 2019

Accepted: 19 November 2019

Published: 06 December 2019

Citation:

Vilibic-Cavlek T, Savic V, Petrovic T, Toplak I, Barbic L, Petric D, Tabain I, Hrnjakovic-Cvjetkovic I, Bogdanic M, Klobucar A, Mrzljak A, Stevanovic V, Dinjar-Kujundzic P, Radmanic L, Monaco F, Listes E and Savini G (2019) Emerging Trends in the Epidemiology of West Nile and Usutu Virus Infections in Southern Europe. *Front. Vet. Sci.* 6:437. doi: 10.3389/fvets.2019.00437

The epidemiology of West Nile (WNV) and Usutu virus (USUV) has changed dramatically over the past two decades. Since 1999, there have been regular reports of WNV outbreaks and the virus has expanded its area of circulation in many Southern European countries. After emerging in Italy in 1996, USUV has spread to other countries causing mortality in several bird species. In 2009, USUV seroconversion in horses was reported in Italy. Co-circulation of both viruses was detected in humans, horses and birds. The main vector of WNV and USUV in Europe is *Culex pipiens*, however, both viruses were found in native *Culex* mosquito species (*Cx. modestus*, *Cx. perexiguus*). Experimental competence to transmit the WNV was also proven for native and invasive mosquitoes of *Aedes* and *Culex* genera (*Ae. albopictus*, *Ae. detritus*, *Cx. torrentium*). Recently, *Ae. albopictus* and *Ae. japonicus* naturally-infected with USUV were reported. While neuroinvasive human WNV infections are well-documented, USUV infections are sporadically detected. However, there is increasing evidence of a role of USUV in human disease. Seroepidemiological studies showed that USUV circulation is more common than WNV in some endemic regions. Recent data showed that WNV strains detected in humans, horses, birds, and mosquitoes mainly belong to lineage 2. In addition to European USUV lineages, some reports indicate the presence of African USUV lineages as well. The trends in WNV/USUV range and vector expansion are likely to continue in future years. This mini-review provides an update on the epidemiology of WNV and USUV infections in Southern Europe within a multidisciplinary "One Health" context.

Keywords: West Nile virus, Usutu virus, epidemiology, "One Health", Southern Europe

INTRODUCTION

West Nile virus (WNV) and Usutu virus (USUV) are mosquito-borne flaviviruses characterized by similar clinical manifestations and overlapping geographic distribution, host and vector species. The epidemiology of WNV and USUV has changed dramatically over the past two decades. Since 1996, an increasing number of WNV outbreaks in humans and horses were detected, and the area of its circulation expanded in many Southern European countries (1). After USUV emergence in Austria in 2001, it subsequently spread to neighboring countries causing mortality in several wild bird species, mainly Eurasian blackbirds (2). However, a retrospective analysis of archived bird tissue samples from Italy (Tuscany region) in 1996 identified USUV, indicating a much earlier introduction of this virus into Europe (3).

Since the early 2000s, WNV circulation has been continuously monitored in some European countries with varying number of human and horse cases. USUV infections are reported in birds, while human clinical cases are rarely detected (4–6). However, there is increasing evidence of a role of USUV in human disease. Seroepidemiological studies showed that USUV circulation in humans is more common than WNV in some endemic regions where both viruses circulate (7).

Until the introduction of WNV lineage 2 in 2004 (8), WNV lineage 1 was identified as the cause of human outbreaks in Europe. In the following years, lineage 2 dispersed to the eastern part of Austria and to Southern European countries (9–11). Recent data showed that strains detected in humans, horses, birds, and mosquitoes mainly belong to WNV lineage 2 (6, 12, 13). Although the majority of USUV strains belong to European USUV lineages, some reports indicate the presence of African USUV lineages as well (5).

In 2018, WNV infections in Europe increased dramatically compared to previous transmission seasons. A total of 2,083 human cases and 285 outbreaks among equids were reported with the largest number detected in Italy, Serbia, and Greece (14). USUV was detected in asymptomatic blood donors, birds, and mosquitoes in Italy (15, 16) and in three patients with neuroinvasive disease in Croatia (6).

The Middle East represents an important transit zone for bird migration between Africa and Eurasia and provides valuable information on circulation of WNV/USUV in these regions (17–19). In Israel, serologic evidence and acute WNV infections have been reported in humans, horses, birds, and mosquitoes (20, 21). While strains sequenced from humans mainly belonged to the WNV lineage 1 (22), mosquito surveillance revealed a high genetic diversity of WNV (23). USUV strains detected in mosquitoes belonged to a putative novel lineage Europe 5 (24). WNV/USUV infections were also reported in other countries bordering the Mediterranean Sea (France, Turkey, Cyprus) (5, 25–40). This mini-review provides an update on the epidemiology/molecular epidemiology of WNV and USUV infections in Southern Europe and neighboring countries within a multidisciplinary “One Health” context.

Data on WNV/USUV infections are presented in **Table 1** and **Figure 1**.

ALBANIA

Data on WNV infections in Albania are scarce. To date, only two human cases were reported in 2011 (14). A serological study conducted among horses from 12 districts in Albania showed WNV seropositivity of 22.2%, with significant regional differences (7.7–66.7%). The highest seroprevalence rates were reported in districts near the Mediterranean Sea, whereas low or negative seroprevalence was detected in districts located further inland (41). Data on USUV infection are not available.

BOSNIA AND HERZEGOVINA

There is only one report, in 2013, of two cases of WNV neuroinvasive disease (WNND) in Bosnia and Herzegovina (detected in the Tuzla and Kladanj region). Three out of nine patients screened for WNV met clinical criteria for WNND, of which two had high serum IgM titres. RT-PCR for WNV RNA was negative. Since the neutralization test was not performed, cases were classified according to the European Union case definition as probable WNV (42). So far, there are no data on USUV infections in Bosnia and Herzegovina.

BULGARIA

In 2015, the first confirmed human case with fatal outcome reported in Bulgaria was caused by a Central/Southern-European lineage 2 WNV (43). Human cases were continuously detected in the following seasons (12). A nationwide study conducted among residents of all 28 districts in Bulgaria revealed a 1.5% WNV seroprevalence with the highest seropositivity (up to 10%) detected in districts near the Danube River (44). USUV infections were not documented in Bulgaria.

CROATIA

The first outbreak of human WNND in Croatia was detected in 2012 in eastern counties, thereafter outbreaks (2013, 2017) and sporadic cases (2014–2016) were continuously notified in continental Croatia (4, 45). The largest outbreak was recorded in 2018 with 54 cases of WNND and 7 cases of WNV fever in 10 of the 21 counties (6). During the 2013 WNV outbreak, the first three cases of neuroinvasive USUV disease were detected in Zagreb and surrounding areas (4, 46). Three additional USUV cases were confirmed in the 2018 outbreak, of which one was fatal (6). A serological study conducted from 2010 to 2011 showed WNV antibodies in 3.43% horses and 0.11% cattle with the highest seropositivity in eastern counties bordering Hungary and Serbia (47). Seropositivity and acute asymptomatic WNV infections in horses were detected continuously in subsequent years. Seroprevalence varied greatly by year and region (0–26%) with the highest seropositivity in counties with documented human cases (4, 48). Although passive monitoring of WNV in birds was established in Croatia in 2012, WNV infections were not detected until the summer of 2018, when WNV infection was confirmed serologically in one buzzard presenting with neurological symptoms, and WNV RNA was detected in two

TABLE 1 | Summary of West Nile and Usutu virus infections in Southern Europe.

Country	First human clinical cases		Humans		Horses		Birds		Mosquitoes	
	WNV	USUV	WNV	USUV	WNV	USUV	WNV	USUV	WNV	USUV
Albania	2011									
Bosnia and Herzegovina	2013									
Bulgaria	2013		Lineage 2							
Croatia	2012	2013	Lineage 2	Europe 2			Lineage 2	Europe 2		Europe 2
Cyprus	2016		Lineage 1							
France	1962	2016								Africa 2 Africa 3
Greece	2010		Lineage 2						Lineage 2	
Israel	1951		Lineage 1						Lineage 1 Lineage 2	Europe 5
Italy	2008	2009	Lineage 1 Lineage 2	Europe 2 Europe 3 Europe 4	Lineage 1 Lineage 2		Lineage 1 Lineage 2	Europe 2	Lineage 1 Lineage 2	
Macedonia	2011									
Montenegro	2012									
Portugal	2004									
Serbia	2012		Lineage 2				Lineage 2		Lineage 2	Europe 2
Slovenia	2013									
Spain	2004				Lineage 1		Lineage 1		Lineage 2	Africa 2
Turkey	2010				Lineage 1				Lineage 1	

 acute infections (clinical cases/IgM detection/seroconversion/RNA detection)

 IgG seropositivity.

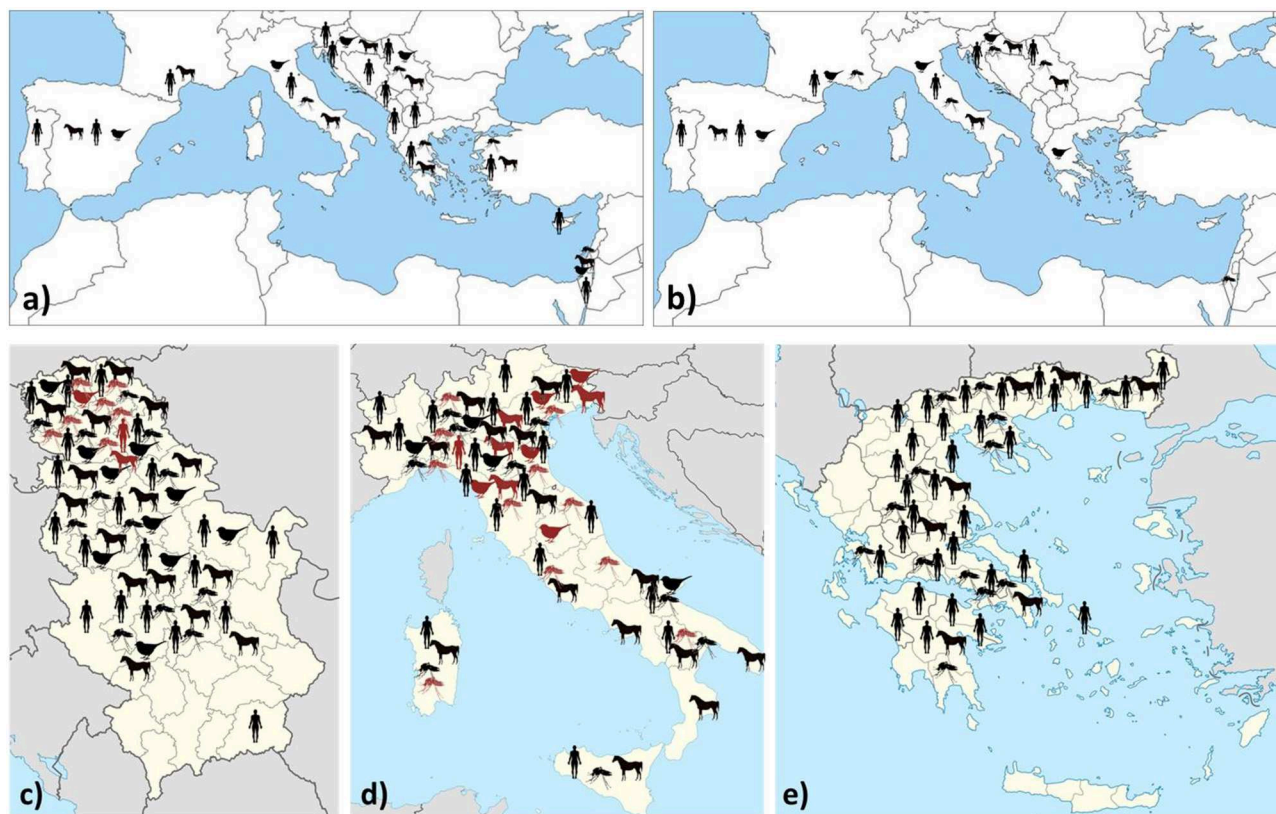


FIGURE 1 | Geographic distribution of WNV (a) and USUV (b) infections/seropositivity in southern Europe and neighboring countries. Regional distribution of WNV (black) and USUV (red) infections (clinical cases/IgM detection/seroconversion/RNA detection) in countries with a high prevalence: Serbia (c), Italy (d), and Greece (e).

dead goshawks from Northwest Croatia. Two USUV seropositive horses were documented in 2011 in Northwest Croatia (49). In 2018, USUV RNA was detected in one dead blackbird in Zagreb County (6). To date, none of the mosquito pools tested were WNV RNA positive. However, USUV-positive mosquito pools were found in 2016 (*Aedes albopictus*), 2017 (*Culex pipiens*), and 2018 (*Cx. pipiens*) in Northwestern counties (6, 50). Sequenced strains from humans, wild birds, and mosquitoes (2018) confirmed circulation of WNV lineage 2 and USUV Europe 2 lineage (6).

GREECE

No WNV clinical cases in humans or horses had been reported in Greece prior to the large WNV outbreak in 2010 when 262 human cases with extremely high fatality rates (17%) were reported mostly in Central Macedonia (51). The outbreaks continued in 2011 and 2012 in areas that had not been affected before (52, 53). Thereafter, outbreaks occurred in humans every year except 2015 and 2016 (14, 54). The Greek WNV strains detected during 2010–2018 clustered within the Central/Southern European subclade of lineage 2 (55). In 2018, a novel genetic variant was detected that belonged to the Eastern European subclade of lineage 2 (56). A nationwide

WNV seroprevalence study (2013) showed seropositivity of 1.5% in the Greek population (57), whilst WNV antibodies were detected in 4% of horse serum samples (2001–2008) (58). In 2010, WNV infection was reported in 17 horses with neurological symptoms (59). Furthermore, a high WNV seropositivity (18.6%) was reported in cattle (60). In Central Macedonia, pigeon seroprevalence was 54 and 31% at the end of the 2010 and 2011 epidemic seasons, respectively, while one serum was positive for USUV neutralizing antibodies (61). A small-scale entomological study performed in Central Macedonia, at the epicenter of the 2010 WNV outbreak found two positive *Cx. pipiens* pools. Following this large epidemic, an active mosquito surveillance system was implemented in Greece for a 3-year period (2011–2013). Positive *Cx. pipiens* pools were detected in different areas of the country and preceded the diagnosed human cases (62). WNV lineage 2 was detected in *Cx. pipiens* mosquito pools during the 2017 outbreak (63).

ITALY

A multi-species national surveillance plan was implemented by the Italian government in 2002, following the first WNV outbreak (64). In the following years, the program has been adapted according to the WNV new epidemiological scenarios and a

National Integrated Plan Monitoring USUV and WNV is in place since 2016. Human WNV outbreaks were continuously notified since 2008 (14). WNV lineage 1 was responsible for reported WNND cases in 2010. In 2011–2012, lineage 1 and 2 co-circulated with a higher proportion of lineage 1 and from 2013 to 2016 lineage 2 was most prevalent (64–66). From 2017, only lineage 2 has been detected. Since 2008, WNV infections were continuously detected also in horses and birds (65). WNV lineage 2 was confirmed in wild birds (2014) (11, 67) and horses with fatal WNV neuroinvasive infection (68). The presence of USUV in humans was serologically confirmed for the first time in four blood donors in 2009 (69). A survey conducted retrospectively on cerebrospinal fluid (CSF) and serum samples in Modena (2008–2011) found USUV RNA in 1.1% CSF samples, while WNV RNA was not detected. USUV antibody levels were significantly higher (6.57%) compared to that of WNV (2.96%) indicating that USUV infection is not a sporadic event in humans in Italy (70). A very high USUV seroprevalence was found in forestry workers (18.1%) (71). Neuroinvasive USUV infection was detected for the first time in Italy in two immunocompromised patients in 2009 (72, 73). During 2017–2018, USUV Europe 2, 3, and 4 lineages were confirmed in blood donations in the Lazio region (15). USUV neutralizing antibodies were detected in horses with a higher seropositivity in 2008 (89.2%) compared to 2009 (7.8%). In the same study, USUV RNA was found in blackbirds and magpies (Emilia Romagna and Veneto). Additionally, USUV neutralizing antibodies were detected in rock pigeon, blackbird and, magpie (74). *Culex pipiens* is the mosquito species most involved in the WNV and USUV circulation in Italy, although other species would also support the spread of both viruses during winter months (75). An entomologic investigation conducted in 2013 showed WNV RNA in 1.9% and USUV in 2.6% mosquito pools. Each virus was detected mainly in *Cx. pipiens* pools; however some pools tested positive for both viruses. The majority of the WNV strains detected in mosquitoes belonged to WNV lineage 2, while WNV lineage 1 which predominantly circulated in 2008–2012, was still detected at low levels until 2016 (64, 76). Sequence analysis of the first known isolate of USUV to cause human encephalitis indicated European USUV lineage (77). The whole genome sequences of USUV strains isolated from mosquitoes and wild birds in Northern Italy (2010–2014) showed Europe 2 and Europe 4 lineages, respectively (78).

PORTUGAL

In the summer of 2004, two human cases of WNV infection were reported in Portugal in tourists (Ria Formosa, Algarve) (79). Shortly after this report, a WNV monitoring program was established. The presence of WNV was assessed by serological surveys in horses (2004–2010), wild birds, and birds from zoological parks. Detection of WNV antibodies in horses and birds in all the years covered by the study as well as the presence of WNV IgM antibodies in horses with neurological signs supported the evidence of WNV circulation in Portugal (80). However, no human clinical cases were reported in the

country from 2005 to 2010. In 2010, another WNV human case was identified (81). Following a 5-year period of apparent absence, WNV re-emerged in southern Portugal in 2015 when WNND was diagnosed in a man from the Algarve region (82). There are no data on USUV infections in Portugal.

SERBIA

The serological surveys (2005–2010) revealed the presence of WNV IgG antibodies in 3.99% human serum samples in Vojvodina Province (northern Serbia), with varying yearly rates (1.97–6.04%) (83). The first outbreak of human WNND was detected in 2012 (Belgrade and Vojvodina) with 69 reported and 41 clinically and laboratory confirmed cases (14, 84). Each year thereafter, seasonal outbreaks were observed. In 2013, 303 cases were reported from 18/25 districts in Serbia; 202 WNND cases were confirmed with lethality of 11.6%. From 41 to 76 WNND cases were documented annually between 2014 and 2017. The largest epidemic so far was recorded in 2018 with 415 WNND cases detected in 13/25 districts (14). A serological study conducted in 2009–2010 showed for the first time WNV neutralizing antibodies in 12% of horses from Vojvodina (85). Seropositivity and asymptomatic WNV infections in horses were detected continuously in the subsequent years; 28.6% seroprevalence from 2010 to 2011 and 49.23% in 2012 in Vojvodina (86, 87). In 2014, seroconversion was detected in 2.57% horses from the whole country. In addition, in 2015, 2017, and 2018, acute WNV infections were detected in 0.53, 0.41, and 1.47% of horses tested, respectively (88–90). WNV antibodies were detected in 7.6% wild birds sampled from 2011 to 2012 in Vojvodina, of which 133 birds were found dead. Virus presence was confirmed in 9.87% of tissue samples and in the blood sample of one bird (91). From 2014 onwards, WNV was detected in wild birds, with the highest percentage of positive samples being observed in 2018 when WNV was detected in 11.61% of tissues and 6.56% of pharyngeal swabs (88–90). All WNV isolates belonged to WNV lineage 2 (88, 91). Additionally, WNV antibodies were identified in 15.4% farm pigs, 17.6% wild boars, and 18.7% roe deer sampled from 2011 to 2012 (Vojvodina) (92). WNV was detected in mosquitoes for the first time in 2010 in the city of Novi Sad. At that time, 3/841 mosquito pools were positive by WNV specific RT-PCR. The pools originated from 66 localities in 29 settlements in Vojvodina (60). More than 9% of the mosquito pools examined during 2012–2013 were positive for WNV (93). Thereafter, country-wide surveys detected WNV in 2.31, 2.09, and 2.51% of *Cx. pipiens* during 2014, 2015, and 2017, respectively, whereas 12.21% of *Cx. pipiens* tested positive in 2018. Other naturally-infected mosquito species were *Ae. vexans* and *Culiseta annulata* (83). All isolates from mosquitoes (2010–2018) were shown to belong to WNV lineage 2 (83, 88–92, 94–96). USUV neutralizing antibodies were detected in 1/349 horses (2009–2010), and 4/318 wild boars (2011–2012) in Vojvodina (85, 92). Additionally, USUV RNA was confirmed in 0.4% of *Cx. pipiens* pools from Vojvodina in 2014 (74). In 2015, USUV was detected in 0.93% of *Cx. pipiens* pools while USUV antibodies were detected in 5% of human serum samples from

South Bačka District (Vojvodina) (97). Moreover, in 2017, USUV RNA was detected in 2.75% of *Cx. pipiens* mosquitoes collected in Vojvodina. Two isolates were typed as USUV Europe 2 lineage (98). Thus, far, USUV has not yet been detected in wild birds in Serbia.

SLOVENIA

Only four human cases of WNV infections were reported in Slovenia so far. The first case was confirmed in 2013 in a patient with meningitis. Thereafter, in 2018, three cases of WND were confirmed by detection of IgM antibodies in the CSF samples (99). In 2009, 481/1912 (25.2%) clinically healthy horses were positive for WNV IgG antibodies (100). From 2003 until 2009, serum samples from 34 species of songbirds were tested with WNV seroprevalence of 4.7% (101). Furthermore, from 2010 to 2012, wild bird carcasses were collected through passive monitoring of wild bird mortality and tested for WNV RNA, however, there was no evidence of wild bird mortality due to WNV (102). As well, there was no evidence of USUV circulation in Slovenia.

SPAIN

The first human clinical case of WNV infection was diagnosed in 2004 in a patient with aseptic meningitis visiting Southwestern Spain (103). Autochthonous cases were subsequently notified in 2010, 2016, and 2018 transmission seasons (14, 104). WNV cases in horses caused by WNV lineage 1 were continuously reported since 2010 (105). A seroprevalence study conducted in equine populations of Mallorca Island (2011–2012) showed seropositivity rates to WNV and USUV of 6.4 and 1.2%, respectively (106). Another study conducted among horses from central Spain (2011–2013) found WNV seroprevalence of 1.35% (107). WNV lineage 1 was isolated from diseased and dead golden eagles in 2007 (108). In 2011, a survey conducted in waterfowl used as decoys in Andalusia showed that the frequency of seropositive decoys ranged 1.5–3.1% for WNV and 4.4–5.9% for USUV (109). Additionally, WNV and USUV antibodies were found in 23 and 10% of hunted wild red-legged partridges and pheasants from Cádiz (2011–2012) (110). In 2012, USUV was confirmed in two song thrushes (111). WNV and USUV antibodies were also detected in wild birds (2013) (112) as well as pigeons and zoo birds from Cordoba (2013–2014) (113). Moreover, USUV Africa 2 lineage in 2006 (Northeastern Spain) and 2009 (Southern Spain) as well as WNV lineage 1 in 2008 (Southern Spain) were detected in mosquitoes (114, 115).

CONCLUSIONS AND FUTURE PERSPECTIVES

An integrated “One Health” surveillance of mosquitoes and birds in several countries has proven to be useful for early detection of WNV/USUV circulation and identification of enzootic areas (48, 61, 116). Virus detection in mosquitoes and birds preceded human and horse cases (48, 62). In addition, a

high seroprevalence in sentinel animals was detected in areas with documented human cases (4, 22, 48, 61).

There are still many challenges in the epidemiology of WNV/USUV. In addition to birds, which are well-known reservoirs, WNV and USUV antibodies were found in different animal species. WNV and USUV neutralizing antibodies were documented in red deer in Spain (117). WNV seropositive dogs were detected in Italy, Spain, and Corsica Island (118), while USUV antibodies were found in hunting dogs in Italy (119). Moreover, WNV and USUV antibodies were found in gray squirrels in Italy, broadening the host range for these viruses (120). WNV neutralizing antibodies were also found in two wild rodents (*Apodemus flavicollis*) captured in forested areas of Italy (121). However, the short-term and low-level viremia makes it unlikely that these animal species play a role in the WNV transmission cycle. *Culex pipiens* mosquitoes appear to be a major vector for WNV and USUV transmission in Europe, but *Cx. modestus* and *Cx. perexiguus* play an important role in marshlands of some southern countries (115). However, detection of WNV and USUV in different field-collected native (*Ae. vexans*) and invasive (*Ae. albopictus*, *Ae. japonicus*) mosquito species indicates their possible role in promoting the overwintering of these viruses (75, 83, 122–125). Additionally, a recently published study identified *Cx. torrentium* as a highly competent vector for WNV in Central and Northern Europe (126).

While several licensed veterinary WNV vaccines are currently available, there is no WNV or USUV vaccine for humans (127, 128). Since data from Europe indicate that both WNV and USUV appear to be expanding their geographical ranges and the trends indicate that this spread is likely to continue in future years, the development of an effective vaccine is urgently needed to protect at-risk populations from neurological complications.

AUTHOR CONTRIBUTIONS

TV-C and TP made contributions to conception and design of the study, involved in data collection, and drafting the manuscript. VSa, LB, ITa, IH-C, MB, AK, AM, VSt, PD-K, LR, FM, and EL were involved in data collection and drafting the manuscript. ITa, DP, and GS revised the manuscript critically. All authors read and approved the final manuscript.

FUNDING

This work was supported by the Croatian Science Foundation, Project No. IP 2016-06-7456: Prevalence and molecular epidemiology of emerging and re-emerging neuroinvasive arboviral infections in Croatia; CRONEUROARBO (to TV-C), by bilateral project funded by Croatian Ministry of Science and Education and Serbian Ministry of Education, Science and Technological Development: Optimization of diagnosis and surveillance of emerging and re-emerging viral vector-borne zoonoses (to LB and TP), and by Project No. TR31084 funded by Serbian Ministry of Education, Science and Technological Development.

REFERENCES

- Zannoli S, Sambri V. West Nile virus and Usutu virus co-circulation in Europe: epidemiology and implications. *Microorganisms*. (2019) 7:E184. doi: 10.3390/microorganisms7070184
- Weissenböck H, Kolodziejek J, Url A, Lussy H, Rebel-Bauder B, Nowotny N. Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe. *Emerg Infect Dis*. (2002) 8:652–6. doi: 10.3201/eid0807.020094
- Weissenböck H, Bakonyi T, Rossi G, Mani P, Nowotny N. Usutu virus, Italy, 1996. *Emerg Infect Dis*. (2013) 19:274–7. doi: 10.3201/eid1902.121191
- Vilibic-Cavlek T, Kaic B, Barbic L, Pem-Novosel I, Slavic-Vrzic V, Lesnikar V, et al. First evidence of simultaneous occurrence of West Nile virus and Usutu virus neuroinvasive disease in humans in Croatia during the 2013 outbreak. *Infection*. (2014) 42:689–95. doi: 10.1007/s15010-014-0625-1
- Simonin Y, Sillam O, Carles MJ, Gutierrez S, Gil P, Constant O, et al. Human Usutu virus infection with atypical neurologic presentation, Montpellier, France, 2016. *Emerg Infect Dis*. (2018) 24:875–8. doi: 10.3201/eid2405.171122
- Vilibic-Cavlek T, Savic V, Sabadi D, Peric L, Barbic L, Klobucar A, et al. Prevalence and molecular epidemiology of West Nile and Usutu virus infections in Croatia in the “One health” context, 2018. *Transbound Emerg Dis*. (2019) 66:1946–57. doi: 10.1111/tbed.13225
- Faggioni G, De Santis R, Pomponi A, Grottola A, Serpini GF, Meacci M, et al. Prevalence of Usutu and West Nile virus antibodies in human sera, Modena, Italy, 2012. *J Med Virol*. (2018) 90:1666–8. doi: 10.1002/jmv.25230
- Bakonyi T, Ivanics E, Erdelyi K, Ursu K, Ferenczi E, Weissenböck H, et al. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. *Emerg Infect Dis*. (2006) 12:618–23. doi: 10.3201/eid1204.051379
- Hernández-Triana LM, Jeffries CL, Mansfield KL, Carnell G, Fooks AR, Johnson N. Emergence of West Nile virus lineage 2 in Europe: a review on the introduction and spread of a mosquito-borne disease. *Front Public Health*. (2014) 2:271. doi: 10.3389/fpubh.2014.00271
- Ravagnan S, Montarsi F, Cazzin S, Porcellato E, Russo F, Palei M, et al. First report outside Eastern Europe of West Nile virus lineage 2 related to the Volgograd 2007 strain, northeastern Italy, 2014. *Parasit Vectors*. (2015) 8:418. doi: 10.1186/s13071-015-1031-y
- Savini G, Capelli G, Monaco F, Polci A, Russo F, Di Gennaro A, et al. Evidence of West Nile virus lineage 2 circulation in Northern Italy. *Vet Microbiol*. (2012) 158:267–73. doi: 10.1016/j.vetmic.2012.02.018
- Kuralt IC, Krajinović V, Topić A, Kuzman I, Baršić B, Markotić A. First molecular analysis of West Nile virus during the 2013 outbreak in Croatia. *Virus Res*. (2014) 189:63–6. doi: 10.1016/j.virusres.2014.04.017
- Savini G, Puggioni G, Di Gennaro A, Di Francesco G, Rocchigiani AM, Polci A, et al. West Nile virus lineage 2 in Sardinian wild birds in 2012: a further threat to public health. *Epidemiol Infect*. (2013) 141:2313–6. doi: 10.1017/S0950268812003147
- European Centre for Disease Prevention and Control (ECDC). *Historical Data by Year - West Nile Fever Seasonal Surveillance*. (2018). Available online at: <https://ecdc.europa.eu/en/west-nile-fever/surveillance-and-disease-data/historical>
- Carletti F, Colavita F, Rovida F, Percivalle E, Baldanti F, Ricci I, et al. Expanding Usutu virus circulation in Italy: detection in the Lazio region, central Italy, 2017 to 2018. *Euro Surveill*. (2019) 24:1800649. doi: 10.2807/1560-7917.ES.2019.24.3.1800649
- IZSAM. 2018 West Nile disease in Italy. *Epidemiological Bulletin*. (2018). Available online at: <https://westnile.izs.it>
- Lustig Y, Kaufman Z, Mannasse B, Koren R, Katz-Likovnik S, Orshan L, et al. West Nile virus outbreak in Israel in 2015: phylogenetic and geographic characterization in humans and mosquitoes. *Clin Microbiol Infect*. (2017) 23:986–93. doi: 10.1016/j.cmi.2017.04.023
- Lustig Y, Kaufman Z, Mendelson E, Orshan L, Anis E, Glazer Y, et al. Spatial distribution of West Nile virus in humans and mosquitoes in Israel, 2000–2014. *Int J Infect Dis*. (2017) 64:20–6. doi: 10.1016/j.ijid.2017.08.011
- Salama M, Amitai Z, Lustig Y, Mor Z, Weiberger M, Chowders M, et al. Outbreak of West Nile Virus disease in Israel (2015): a retrospective analysis of notified cases. *Travel Med Infect Dis*. (2019) 28:41–5. doi: 10.1016/j.tmaid.2018.07.008
- Azmi K, Tirosh-Levy S, Manasrah M, Mizrahi R, Nasereddin A, Al-Jawabreh A, et al. West Nile virus: seroprevalence in animals in Palestine and Israel. *Vector Borne Zoonotic Dis*. (2017) 17:558–66. doi: 10.1089/vbz.2016.2090
- Bassal R, Shohat T, Kaufman Z, Mannasse B, Shinar E, Amichay D, et al. The seroprevalence of West Nile virus in Israel: a nationwide cross sectional study. *PLoS ONE*. (2017) 12:e0179774. doi: 10.1371/journal.pone.0179774
- Lustig Y, Gosinov R, Zuckerman N, Glazer Y, Orshan L, Sofer D, et al. Epidemiologic and phylogenetic analysis of the 2018 West Nile virus (WNV) outbreak in Israel demonstrates human infection of WNV lineage I. *Euro Surveill*. (2019) 24:1800662. doi: 10.2807/1560-7917.ES.2019.24.1.1800662
- Lustig Y, Hindiyeh M, Orshan L, Weiss L, Koren R, Katz-Likovnik S, et al. Mosquito surveillance for 15 years reveals high genetic diversity among West Nile viruses in Israel. *J Infect Dis*. (2016) 213:1107–14. doi: 10.1093/infdis/jiv556
- Mannasse B, Mendelson E, Orshan L, Mor O, Shalom U, Yeger T, et al. Usutu virus RNA in mosquitoes, Israel, 2014–2015. *Emerg Infect Dis*. (2017) 23:1699–702e. doi: 10.3201/eid2310.171017
- Del Giudice P, Schuffenecker I, Vandenbos F, Counillon E, Zellet H. Human West Nile virus, France. *Emerg Infect Dis*. (2004) 10:1885–6. doi: 10.3201/eid1010.031021
- Vittecoq M, Lecollinet S, Jourdain E, Thomas F, Blanchon T, Arnal A, et al. Recent circulation of West Nile virus and potentially other closely related flaviviruses in Southern France. *Vector Borne Zoonotic Dis*. (2013) 13:610–3. doi: 10.1089/vbz.2012.1166
- Cazeau G, Leblond A, Sala C, Froustey M, Beck C, Lecollinet S, et al. Utility of examining fallen stock data to monitor health-related events in equids: application to an outbreak of West Nile Virus in France in 2015. *Transbound Emerg Dis*. (2019) 66:1417–9. doi: 10.1111/tbed.13150
- Bahoun C, Marcillaud-Pitel C, Bournez L, Leblond A, Beck C, Hars J, et al. West Nile virus epizootics in the Camargue (France) in 2015 and reinforcement of surveillance and control networks. *Rev Sci Tech Off Int Epiz*. (2016) 35:81–6. doi: 10.20506/rst.35.3.2571
- Lecollinet S, Blanchard Y, Manson C, Lowenski S, Laloy E, Quenault H, et al. Dual Emergence of Usutu virus in common blackbirds, Eastern France, 2015. *Emerg Infect Dis*. (2016) 22:2225. doi: 10.3201/eid2212.161272
- Eiden M, Gil P, Ziegler U, Rakotoarivony I, Marie A, Frances B, et al. Emergence of two Usutu virus lineages in *Culex pipiens* mosquitoes in the Camargue, France, 2015. *Infect Genet Evol*. (2018) 61:151–4. doi: 10.1016/j.meegid.2018.03.020
- Balança G, Gaidet N, Savini G, Vollot B, Foucart A, Reiter P, et al. Low West Nile virus circulation in wild birds in an area of recurring outbreaks in Southern France. *Vector Borne Zoonotic Dis*. (2009) 9:737–41. doi: 10.1089/vbz.2008.0147
- Ozkul A, Yildirim Y, Pinar D, Akcali A, Yilmaz V, Colak D. Serological evidence of West Nile virus (WNV) in mammalian species in Turkey. *Epidemiol Infect*. (2006) 134:826–9. doi: 10.1017/S0950268805005492
- Ergunay K, Bakonyi T, Nowotny N, Ozkul A. Close relationship between West Nile virus from Turkey and lineage 1 strain from Central African Republic. *Emerg Infect Dis*. (2015) 21:352–5. doi: 10.3201/eid2102.141135
- Ergunay K, Litzba N, Brinkmann A, Günay F, Sarikaya Y, Kar S, et al. Co-circulation of West Nile virus and distinct insect-specific flaviviruses in Turkey. *Parasit Vectors*. (2017) 10:149. doi: 10.1186/s13071-017-2087-7
- Akiner MM, Öztürk M, Başer AB, Günay F, Hacıoglu S, Brinkmann A, et al. Arboviral screening of invasive *Aedes* species in northeastern Turkey: West Nile virus circulation and detection of insect-only viruses. *PLoS Negl Trop Dis*. (2019) 13:e0007334. doi: 10.1371/journal.pntd.0007334
- Yilmaz H, Barut K, Karakullukcu A, Kasapcopur O, Kocazeybek B, Altan E, et al. Serological evidence of tick-borne encephalitis and West Nile virus infections among children with arthritis in Turkey. *Vector Borne Zoonotic Dis*. (2019) 19:446–9. doi: 10.1089/vbz.2018.2349
- Öncü C, Brinkmann A, Günay F, Kar S, Öter K, Sarikaya Y, et al. West Nile virus, *Anopheles flavivirus*, a novel flavivirus as well as Merida-like rhabdovirus Turkey in field-collected mosquitoes from Thrace and Anatolia. *Infect Genet Evol*. (2018) 57:36–45. doi: 10.1016/j.meegid.2017.11.003
- Paphitou NI, Tourvas A, Floridou D, Richter J, Tryfonos C, Christodoulou C. The first human case of neuroinvasive West Nile virus infection identified in Cyprus. *J Infect Public Health*. (2017) 10:891–3. doi: 10.1016/j.jiph.2017.02.003

39. Richter J, Tryfonos C, Tourvas A, Floridou D, Paphitou NI, Christodoulou C. Complete Genome sequence of West Nile virus (WNV) from the first human case of neuroinvasive WNV infection in Cyprus. *Genome Announc.* (2017) 5:e01110-17. doi: 10.1128/genomeA.01110-17
40. Billioud G, Tryfonos C, Richter J. The prevalence of antibodies against sandfly fever viruses and West Nile virus in Cyprus. *J Arthropod Borne Dis.* (2019) 13:116–25. doi: 10.18502/jad.v13i1.938
41. Berxholi K, Ziegler U, Rexhepi A, Schmidt K, Mertens M, Korro K, et al. (2013) Indigenous West Nile virus infections in horses in Albania. *Transbound Emerg Dis.* 60(Suppl. 2):45–50. doi: 10.1111/tbed.12141
42. Ahmetagić S, Petković J, Hukić M, Smriko-Nuhanović A, Piljić D. Human West Nile virus infection in Bosnia and Herzegovina. *Med Glas.* (2015) 12:47–51.
43. Baymakova M, Trifonova I, Panayotova E, Dakova S, Pacenti M, Barzon L, et al. Fatal case of West Nile neuroinvasive disease in Bulgaria. *Emerg Infect Dis.* (2016) 22:2203–4. doi: 10.3201/eid2212.151968
44. Christova I, Panayotova E, Tchakarova S, Taseva E, Trifonova I, Gladnisha T. A nationwide seroprevalence screening for West Nile virus and Tick-borne encephalitis virus in the population of Bulgaria. *J Med Virol.* (2017) 89:1875–8. doi: 10.1002/jmv.24855
45. Pem-Novosel I, Vilibic-Cavlek T, Gjenero-Margan I, Pandak N, Peric L, Barbic L, et al. First outbreak of West Nile virus neuroinvasive disease in humans, Croatia, 2012. *Vector Borne Zoonotic Dis.* (2014) 14:82–4. doi: 10.1089/vbz.2012.1295
46. Santini M, Vilibic-Cavlek T, Barsic B, Barbic L, Savic V, Stevanovic V, et al. First cases of human Usutu virus neuroinvasive infection in Croatia, August–September 2013: clinical and laboratory features. *J Neurovirol.* (2015) 21:92–7. doi: 10.1007/s13365-014-0300-4
47. Barbić L, Listeš E, Katić S, Stevanović V, Madić J, Starešina V, et al. Spreading of West Nile virus infection in Croatia. *Vet Microbiol.* (2012) 159:504–8. doi: 10.1016/j.vetmic.2012.04.038
48. Savić V, Barbić Lj, Vilibić-Cavlek T, Balenović M, Stevanović V, Listeš E, et al. Chickens and horses as sentinels for early warning system in prevention of human West Nile virus infections in Croatia. *Slov Vet Res.* (2016) 53(Suppl 17):292–4.
49. Barbic L, Vilibic-Cavlek T, Listes E, Stevanovic V, Gjenero-Margan I, Ljubic-Sternak S, et al. Demonstration of Usutu virus antibodies in horses, Croatia. *Vector Borne Zoonotic Dis.* (2013) 13:772–4. doi: 10.1089/vbz.2012.1236
50. Klobucar A, Benic N, Krajcar D, Kosanovic-Licina ML, Tesic V, Merdic E, et al. An overview of mosquitoes and emerging arboviral infections in the Zagreb area, Croatia. *J Infect Dev Ctries.* (2016) 10:1286–93. doi: 10.3855/jidc.7988
51. Papa A, Danis K, Baka A, Bakas A, Douglas G, Lytras T, et al. Ongoing outbreak of West Nile virus infections in humans in Greece, July–August 2010. *Euro Surveill.* (2010) 15:19644. doi: 10.2807/ese.15.34.19644-en
52. Danis K, Papa A, Papanikolaou E, Douglas G, Terzaki I, Baka A, et al. Ongoing outbreak of West Nile virus infection in humans, Greece, July to August 2011. *Euro Surveill.* (2011) 16:19951.
53. Papa A. West Nile virus infections in humans-focus on Greece. *J Clin Virol.* (2013) 58:351–3. doi: 10.1016/j.jcv.2013.02.020
54. Mavrouli M, Vrioni G, Kapsimali V, Tsiamis C, Mavroulis S, Pervanidou D, et al. Reemergence of West Nile Virus Infections in Southern Greece, 2017. *Am J Trop Med Hyg.* (2019) 100:420–6. doi: 10.4269/ajtmh.18-0339
55. Barzon L, Papa A, Lavezzo E, Franchin E, Pacenti M, Sinigaglia A, et al. Phylogenetic characterization of Central/Southern European lineage 2 West Nile virus: analysis of human outbreaks in Italy and Greece, 2013–2014. *Clin Microbiol Infect.* (2015) 21:1122.e1–10. doi: 10.1016/j.cmi.2015.07.018
56. Papa A, Papadopoulou E, Chatzixanthoulou C, Glouftisios P, Pappa S, Pervanidou D, et al. Emergence of West Nile virus lineage 2 belonging to the Eastern European subclade, Greece. *Arch Virol.* (2019) 164:1673–5. doi: 10.1007/s00705-019-04243-8
57. Hadjichristodoulou C, Pournaras S, Mavrouli M, Marka A, Tserkezou P, Baka A, et al. West Nile virus seroprevalence in the Greek population in 2013: a nationwide cross-sectional survey. *PLoS ONE.* (2015) 10:e0143803. doi: 10.1371/journal.pone.0143803
58. Mangana-Vougiouka O, Boutsini S, Ntousi D, Patakakis M, Orfanou E, Zafiropoulou K, et al. Epizootiological investigation of the most important infectious equine diseases in Greece. *Rev Sci Tech.* (2013) 32:755–87. doi: 10.20506/rst.32.2.2217
59. Bouzalas IG, Diakakis N, Chaintoutis SC, Brellou GD, Papanastassopoulou M, Danis K, et al. Emergence of equine West Nile encephalitis in Central Macedonia, Greece, 2010. *Transbound Emerg Dis.* (2016) 63:e219–27. doi: 10.1111/tbed.12334
60. Giadinis N, Katsoulos P, Chochlakakis D, Tselentis Y, Ntais P, Lafi S, et al. Serological investigation for West Nile virus, *Anaplasma ovis* and *Leishmania infantum* in Greek cattle. *Vet Ital.* (2015) 51:205–9. doi: 10.12834/VetIt.174.523.4
61. Chaintoutis SC, Dovas CI, Papanastassopoulou M, Gewehr S, Danis K, Beck C, et al. Evaluation of a West Nile virus surveillance and early warning system in Greece, based on domestic pigeons. *Comp Immunol Microbiol Infect Dis.* (2014) 37:131–41. doi: 10.1016/j.cimid.2014.01.004
62. Patsoula E, Vakali A, Balatsos G, Pervanidou D, Beleri S, Tegos N et al. West Nile virus circulation in mosquitoes in Greece (2010–2013). *Biomed Res Int.* (2016) 2016:2450682. doi: 10.1155/2016/2450682
63. Mavridis K, Fotakis EA, Kioulos I, Mpellou S, Konstantas S, Varela E, et al. Detection of West Nile virus - lineage 2 in *Culex pipiens* mosquitoes, associated with disease outbreak in Greece, 2017. *Acta Trop.* (2018) 182:64–8. doi: 10.1016/j.actatropica.2018.02.024
64. *West Nile Disease.* (2018). Available online at: http://sorveglianza.izs.it/emergenze/west_nile/emergenze_en.html
65. Rizzo C, Napoli C, Venturi G, Pupella S, Lombardini L, Calistri P, et al. West Nile virus transmission: results from the integrated surveillance system in Italy, 2008 to 2015. *Euro Surveill.* (2016) 21:30340. doi: 10.2807/1560-7917.ES.2016.21.37.30340
66. Magurano F, Remoli ME, Baggieri M, Fortuna C, Marchi A, Fiorentini C, et al. Circulation of West Nile virus lineage 1 and 2 during an outbreak in Italy. *Clin Microbiol Infect.* (2012) 18:E545–7. doi: 10.1111/1469-0691.12018
67. Chiari M, Prosperi A, Faccin F, Avisani D, Cerioli M, Zanoni M, et al. West Nile virus surveillance in the Lombardy Region, Northern Italy. *Transbound Emerg Dis.* (2015) 62:343–9. doi: 10.1111/tbed.12375
68. Calistri P, Monaco F, Savini G, Guercio A, Purpari G, Vicari D, et al. Further spread of West Nile virus in Italy. *Vet Ital.* (2010) 46:467–74.
69. Gaibani P, Piero A, Alicino R, Rossini G, Cavrini F, Landini MP, et al. Detection of Usutu-virus-specific IgG in blood donors from northern Italy. *Vector-Borne Zoonotic Dis.* (2012) 12:431–3. doi: 10.1089/vbz.2011.0813
70. Grottola A, Marcacci M, Tagliazucchi S, Gennari W, Di Gennaro A, Orsini M, et al. Usutu virus infections in humans: a retrospective analysis in the municipality of Modena, Italy. *Clin Microbiol Infect.* (2017) 23:33–7. doi: 10.1016/j.cmi.2016.09.019
71. Percivalle E, Sasseria D, Rovida F, Isernia P, Fabbi M, Baldanti F, et al. Usutu virus antibodies in blood donors and healthy forestry workers in the Lombardy region, Northern Italy. *Vector Borne Zoonotic Dis.* (2017) 17:658–61. doi: 10.1089/vbz.2017.2126
72. Pecorari M, Longo G, Gennari W, Grottola A, Sabbatini A, Tagliazucchi S, et al. First human case of Usutu virus neuroinvasive infection, Italy, August–September 2009. *Euro Surveill.* (2009) 14:19446. doi: 10.2807/ese.14.50.19446-en
73. Cavrini F, Gaibani P, Longo G, Piero AM, Rossini G, Bonilauri P, et al. Usutu virus infection in a patient who underwent orthotopic liver transplantation, Italy, August–September 2009. *Euro Surveill.* (2009) 14:19448. doi: 10.2807/ese.14.50.19448-en
74. Savini G, Monaco F, Terregino C, Di Gennaro A, Bano L, Pinoni C, et al. Usutu virus in Italy: an emergence or a silent infection? *Vet Microbiol.* (2011) 151:264–74. doi: 10.1016/j.vetmic.2011.03.036
75. Mancini G, Montarsi F, Calzolari M, Capelli G, Dottori M, Ravagnan S, et al. Mosquito species involved in the circulation of West Nile and Usutu viruses in Italy. *Vet Ital.* (2017) 53:97–110. doi: 10.12834/VetIt.114.933.4764.2
76. Calzolari M, Pautasso A, Montarsi F, Albieri A, Bellini R, Bonilauri P, et al. West Nile virus surveillance in 2013 via mosquito screening in Northern Italy and the influence of weather on virus circulation. *PLoS ONE.* (2015) 10:e0140915. doi: 10.1371/journal.pone.0140915
77. Gaibani P, Cavrini F, Gould EA, Rossini G, Piero A, Landini MP, et al. Comparative genomic and phylogenetic analysis of the first Usutu virus isolate from a human patient presenting with neurological symptoms. *PLoS ONE.* (2013) 8:e64761. doi: 10.1371/journal.pone.0064761

78. Calzolari M, Chiapponi C, Bonilauri P, Lelli D, Baioni L, Barbieri I, et al. Co-circulation of two Usutu virus strains in Northern Italy between 2009 and 2014. *Infect Genet Evol.* (2017) 51:255–62. doi: 10.1016/j.meegid.2017.03.022
79. Almeida AP, Freitas F, Novo MT, Sousa CA, Rodrigues JC, Alves R, et al. Mosquito surveys and West Nile virus screening in two different areas of Southern Portugal, 2004–2007. *Vector Borne Zoonotic Dis.* (2010) 10:673–680. doi: 10.1089/vbz.2009.0245
80. Barros SC, Ramos F, Fagulha T, Duarte M, Henriques M, Luís T, et al. Serological evidence of West Nile virus circulation in Portugal. *Vet Microbiol.* (2011) 152:407–10. doi: 10.1016/j.vetmic.2011.05.013
81. Alves MJ, Poças JM, Luz T, Amaro F, Zé-Zé L, Osório HC. West Nile virus infection in Portugal: considerations about a clinical case with febrile syndrome and rash. *Rev. Port Doenças Infec.* (2012) 8:46–51.
82. Barros SC, Ramos F, Fagulha T, Duarte M, Henriques AM, Waap H, et al. West Nile virus in horses during the summer and autumn seasons of 2015 and 2016: Portugal. *Vet Microbiol.* (2017) 212:75–9. doi: 10.1016/j.vetmic.2017.11.008
83. Petrić D, Hrnjaković Cvjetković I, Radovanov J, Cvjetković D, Jerant Patić V, Milošević V, et al. West Nile virus surveillance in humans and mosquitoes and detection of cell fusing agent virus in Vojvodina province (Serbia). *HealthMED.* (2012) 6:462–8.
84. Popović N, Milošević B, Urošević A, Poluga J, Popović N, Stevanović G, et al. Clinical characteristics and functional outcome of patients with West Nile neuroinvasive disease in Serbia. *J Neurol.* (2014) 261:1104–11. doi: 10.1007/s00415-014-7318-7
85. Lupulović D, Martín-Acebes M, Lazić S, Alonso-Padilla J, Blázquez AB, Escribano-Romero E, et al. First serological evidence of West Nile virus activity in horses in Serbia. *Vector Borne Zoonotic Dis.* (2011) 11:1303–5. doi: 10.1089/vbz.2010.0249
86. Medić S, van den Hoven R, Petrović T, Lupulović D, Nowotny N. Serological evidence of West Nile virus infection in the horse population of northern Serbia. *J Infect Dev Ctries.* (2014) 8:914–8. doi: 10.3855/jidc.3885
87. Petrović T, Lazić S, Lupulović D, Lazić G, Bugarski D, Vidanović D, et al. Serological study on WNV presence in horses in Vojvodina after the human outbreak in Serbia in 2012. *Arch Biol Sci Belgrade.* (2014) 66:473–81. doi: 10.2298/ABS1402473P
88. Petrović T, Šekler M, Petrić D, Lazić S, Debeljak Z, Vidanović D, et al. Methodology and results of integrated WNV surveillance programmes in Serbia. *PLoS ONE.* (2018) 13:e0195439. doi: 10.1371/journal.pone.0195439
89. Petrović T, Šekler M, Petrić D, Debeljak Z, Vidanović D, Lazić G, et al. Results of WNV monitoring program in Serbia in 2017. In: *Book of Abstracts, XX Symposium of Epizootiologists and Epidemiologists.* Vrnjačka Banja (2018). p. 92–5.
90. Petrović T, Šekler M, Debeljak Z, Petrić D, Labus T, Vidanović D et al. Results of the WNV monitoring program in Serbia in 2018. In: *Book of Abstracts, XXI Symposium of Epizootiologists and Epidemiologists.* Novi Sad (2019). p. 42–5.
91. Petrović T, Blázquez AB, Lupulović D, Lazić G, Escribano-Romero E, Fabijan D, et al. Monitoring West Nile virus (WNV) infection in wild birds in Serbia during 2012: first isolation and characterisation of WNV strains from Serbia. *Euro Surveill.* (2013) 18:20622. doi: 10.2807/1560-7917.ES2013.18.44.20622
92. Escribano-Romero E, Lupulović D, Merino-Ramos T, Blázquez AB, Lazić G, Lazić S, et al. West Nile virus serosurveillance in pigs, wild boars, and roe deer in Serbia. *Vet Microbiol.* (2015) 176:365–9. doi: 10.1016/j.vetmic.2015.02.005
93. Petrović T, Šekler M, Petrić D, Vidanović D, Potkonjak A, Hrnjaković Cvjetković I, et al. Flaviviruses at the territory of Serbia - present situation and challenges. *Arch Vet Med.* (2018) 11:53–70.
94. Petrić D, Petrović T, Hrnjaković Cvjetković I, Zgomba M, Milošević V, Lazić G, et al. West Nile virus 'circulation' in Vojvodina, Serbia: Mosquito, bird, horse and human surveillance. *Mol Cell Probes.* (2017) 31:28–36. doi: 10.1016/j.mcp.2016.10.011
95. Kemenesi G, Krtinić B, Milankov V, Kutas A, Dallos B, Oldal M, et al. West Nile virus surveillance in mosquitoes, April to October 2013: Vojvodina province, Serbia: implications for the 2014 season. *Euro Surveill.* (2014) 19:20779. doi: 10.2807/1560-7917.ES2014.19.16.20779
96. Zana B, Kemenesi G, Herczeg R, Dallos B, Oldal M, Marton S, et al. Genomic characterization of West Nile virus strains derived from mosquito samples obtained during 2013 Serbian outbreak. *J Vector Borne Dis.* (2016) 53:379–83.
97. Kemenesi G, Buzás D, Zana B, Kurucz K, Krtinić B, Kepner A, et al. First genetic characterization of Usutu virus from *Culex pipiens* mosquitoes Serbia, 2014. *Infect Genet Evol.* (2018) 63:58–61. doi: 10.1016/j.meegid.2018.05.012
98. Hrnjaković Cvjetković I, Petrović T, Petric D, Milosevic U, Radovanov J, Kovacevic G, et al. Usutu virus: an emerging flavivirus in Europe. *Arch Vet Med.* (2017) 10:25–35.
99. Knap N, Korva M, Ivović V, Kalan K, Jelovšek M, Zakotnik S, et al. West Nile infections in Slovenia. In: Vilibić-Cavlek T, Barbić Lj, Savić V, Kaić B, editors. *Book of Abstracts. Symposium Diagnosis and Surveillance of West Nile Virus Infections in the "One Health" Context.* Zagreb (2019). p. 1.
100. Malovrh T, Krt B. Serological monitoring of West Nile fever in Slovenia in horses. *Slov Vet Res.* (2011) 48(Suppl 13):300–2.
101. Račnik J, Trilar T, Jelovšek M, Zadravec M, Slavec B, Zorman Rojs O, et al. West Nile virus in birds in Slovenia. In: Madić J, editor. *Book of Abstracts. Symposium Epidemiological and Clinical Features of West Nile virus in Croatia and Neighboring Countries.* Zagreb (2012). p. 9–10.
102. Račnik J, Slavec B, Zadravec M, Zorman Rojs O. West Nile virus monitoring in wild birds in Slovenia. *Rad HAZU Med Sci.* (2013) 39:89–93.
103. Kaptoul D, Viladrich PF, Domingo C, Niubo J, Martinez-Yelamos S, De Ory F, et al. West Nile virus in Spain: report of the first diagnosed case (in Spain) in a human with aseptic meningitis. *Scand J Infect Dis.* (2007) 39:70–1. doi: 10.1080/00365540600740553
104. López-Ruiz N, Montaña-Remacha MDC, Durán-Pla E, Pérez-Ruiz M, Navarro-Marí JM, Salamanca-Rivera C, et al. West Nile virus outbreak in humans and epidemiological surveillance, west Andalusia, Spain, 2016. *Euro Surveill.* (2018) 23:17-00261. doi: 10.2807/1560-7917.ES2018.23.14.17-00261
105. García-Bocanegra I, Belkhiria J, Napp S, Cano-Terriza D, Jiménez-Ruiz S, Martínez-López B. Epidemiology and spatio-temporal analysis of West Nile virus in horses in Spain between 2010 and 2016. *Transbound Emerg Dis.* (2018) 65:567–77. doi: 10.1111/tbed.12742
106. Vanhomwegen J, Beck C, Desprès P, Figuerola A, García R, Lecollinet S, et al. Circulation of zoonotic arboviruses in equine populations of Mallorca Island (Spain). *Vector Borne Zoonotic Dis.* (2017) 17:340–6. doi: 10.1089/vbz.2016.2042
107. Abad-Cobo A, Llorente F, Barbero MDC, Cruz-López F, Forés P, Jiménez-Clavero MÁ. Serosurvey reveals exposure to West Nile virus in asymptomatic horse populations in central Spain prior to recent disease foci. *Transbound Emerg Dis.* (2017) 64:1387–92. doi: 10.1111/tbed.12510
108. Jiménez-Clavero MA, Sotelo E, Fernandez-Pinero J, Llorente F, Blanco JM, Rodriguez-Ramos J, et al. West Nile virus in golden eagles, Spain, 2007. *Emerg Infect Dis.* (2008) 14:1489–91. doi: 10.3201/eid1409.080190
109. Jurado-Tarifa E, Napp S, Lecollinet S, Arenas A, Beck C, Cerdà-Cuellar M, et al. Monitoring of West Nile virus, Usutu virus and Meaban virus in waterfowl used as decoys and wild raptors in southern Spain. *Comp Immunol Microbiol Infect Dis.* (2016) 49:58–64. doi: 10.1016/j.cimid.2016.10.001
110. Llorente F, Pérez-Ramírez E, Fernández-Pinero J, Soriguer R, Figuerola J, Jiménez-Clavero MA. Flaviviruses in game birds, southern Spain, 2011–2012. *Emerg Infect Dis.* (2013) 19:1023–5. doi: 10.3201/eid1906.130122
111. Höfle U, Gamino V, de Mera IG, Mangold AJ, Ortiz JA, de la Fuente J. Usutu virus in migratory song thrushes, Spain. *Emerg Infect Dis.* (2013) 19:1173–5. doi: 10.3201/eid1907.130199
112. Ferraguti M, LA Puente JM, Soriguer R, Llorente F, Jiménez-Clavero MÁ, Figuerola J. West Nile virus-neutralizing antibodies in wild birds from southern Spain. *Epidemiol Infect.* (2016) 144:1907–11. doi: 10.1017/S0950268816000133
113. Cano-Terriza D, Guerra R, Lecollinet S, Cerdà-Cuellar M, Cabezon O, Almería S, et al. Epidemiological survey of zoonotic pathogens in feral pigeons (*Columba livia* var. domestica) and sympatric zoo species in Southern Spain. *Comp Immunol Microbiol Infect Dis.* (2015) 43:22–7. doi: 10.1016/j.cimid.2015.10.003
114. Busquets N, Alba A, Allepuz A, Aranda C, Ignacio Nuñez J. Usutu virus sequences in *Culex pipiens* (Diptera: Culicidae), Spain. *Emerg Infect Dis.* (2008) 14:861–3. doi: 10.3201/eid1405.071577
115. Vázquez A, Ruiz S, Herrero L, Moreno J, Molero F, Magallanes A, et al. West Nile and Usutu viruses in mosquitoes in Spain, 2008–2009. *Am J Trop Med Hyg.* (2011) 85:178–81. doi: 10.4269/ajtmh.2011.11-0042

116. Bellini R, Calzolari M, Mattivi A, Tamba M, Angelini P, Bonilauri P, et al. The experience of West Nile virus integrated surveillance system in the Emilia-Romagna region: five years of implementation, Italy, 2009 to 2013. *Euro Surveill.* (2014) 19:20953. doi: 10.2807/1560-7917.ES2014.19.44.20953
117. García-Bocanegra I, Paniagua J, Gutiérrez-Guzmán AV, Lecollinet S, Boadella M, Arenas-Montes A, et al. Spatio-temporal trends and risk factors affecting West Nile virus and related flavivirus exposure in Spanish wild ruminants. *BMC Vet Res.* (2016) 12:249. doi: 10.1186/s12917-016-0876-4
118. Maquart M, Dahmani M, Marié JL, Gravier P, Leparc-Goffart I, Davoust B. First serological evidence of West Nile virus in horses and dogs from Corsica Island, France. *Vector Borne Zoonotic Dis.* (2017) 17:275–7. doi: 10.1089/vbz.2016.2024
119. Montagnaro S, Piantedosi D, Ciarcia R, Loponte R, Veneziano V, Fusco G, et al. Serological evidence of mosquito-borne flaviviruses circulation in hunting dogs in Campania region, Italy. *Vector Borne Zoonotic Dis.* (2019) 19:142–7. doi: 10.1089/vbz.2018.2337
120. Romeo C, Lecollinet S, Caballero J, Isla J, Luzzago C, Ferrari N, et al. Are tree squirrels involved in the circulation of flaviviruses in Italy? *Transbound Emerg Dis.* (2018) 65:1372–6. doi: 10.1111/tbed.12874
121. Cosseddu GM, Sozio G, Valleriani F, Di Gennaro A, Pascucci I, Gavaudan S, et al. Serological survey of hantavirus and flavivirus among wild rodents in central Italy. *Vector Borne Zoonotic Dis.* (2017) 17:777–9. doi: 10.1089/vbz.2017.2143
122. Fortuna C, Remoli ME, Severini F, Di Luca M, Toma L, Fois F, et al. Evaluation of vector competence for West Nile virus in Italian *Stegomyia albopicta* (= *Aedes albopictus*) mosquitoes. *Med Vet Entomol.* (2015) 29:430–3. doi: 10.1111/mve.12133
123. Blagrove MSC, Sherlock K, Chapman GE, Impoinvil DE, McCall PJ, Medlock J, et al. Evaluation of the vector competence of a native UK mosquito *Ochlerotatus detritus Aedes detritus* for dengue, chikungunya and West Nile viruses. *Parasit Vectors.* (2016) 9:1–6. doi: 10.1186/s13071-016-1739-3
124. Puggioli A, Bonilauri P, Calzolari M, Lelli D, Carrieri M, Urbanelli S, et al. Does *Aedes albopictus* (Diptera: Culicidae) play any role in Usutu virus transmission in Northern Italy? Experimental oral infection and field evidences. *Acta Trop.* (2017) 172:192–6. doi: 10.1016/j.actatropica.2017.05.006
125. Camp J, Kolodziejek J, Nowotny N. Targeted surveillance reveals native and invasive mosquito species infected with Usutu virus. *Parasites Vectors.* (2019) 12:46. doi: 10.1186/s13071-019-3316-z
126. Jansen S, Heitmann A, Lühken R, Leggewie M, Helms M, Badusche M, et al. *Culex torrentium*: a potent vector for the transmission of West Nile virus in Central Europe. *Viruses.* (2019) 11:E492. doi: 10.3390/v11060492
127. Amanna IJ, Slifka MK. Current trends in West Nile virus vaccine development. *Expert Rev Vaccines.* (2014) 13:589–608. doi: 10.1586/14760584.2014.906309
128. Brandler S, Tangy F. Vaccines in development against West Nile virus. *Viruses.* (2013) 5:2384–409. doi: 10.3390/v5102384

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Vilibic-Cavlek, Savic, Petrovic, Toplak, Barbic, Petric, Tabain, Hrnjakovic-Cvjetkovic, Bogdanic, Klobucar, Mrzljak, Stevanovic, Dinjar-Kujundzic, Radmanic, Monaco, Listes and Savini. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Detection of Neutralizing Antibodies to Tembusu Virus: Implications for Infection and Immunity

Junfeng Lv, Lixin Yang, Shenghua Qu, Runze Meng, Qingxiangzi Li, Huicong Liu, Xiaoyan Wang* and Dabing Zhang*

Key Laboratory of Animal Epidemiology and Zoonosis of Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, Beijing, China

OPEN ACCESS

Edited by:

William C. Wilson,
United States Department of
Agriculture, United States

Reviewed by:

Qingtao Liu,
Jiangsu Academy of Agricultural
Sciences, China
Faten Abdelal Okda,
St. Jude Children's Research
Hospital, United States

*Correspondence:

Xiaoyan Wang
wxy320@cau.edu.cn
Dabing Zhang
zdb@cau.edu.cn

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 26 September 2019

Accepted: 26 November 2019

Published: 10 December 2019

Citation:

Lv J, Yang L, Qu S, Meng R, Li Q,
Liu H, Wang X and Zhang D (2019)
Detection of Neutralizing Antibodies to
Tembusu Virus: Implications for
Infection and Immunity.
Front. Vet. Sci. 6:442.
doi: 10.3389/fvets.2019.00442

Neutralizing antibodies are the key mediators of protective immune response to flaviviruses after both infection and vaccination. Plaque reduction neutralization test (PRNT) is considered the “gold standard” for measurement of the immunity. To date, little is known regarding neutralizing antibody response to Tembusu virus (TMUV), a novel flavivirus emerging in ducks in 2010. Here, we developed a PRNT for detection of TMUV neutralizing antibodies. Following optimization and validation, the PRNT was applied to test serum samples from different flocks of ducks. Using sera prepared in experimental conditions, the levels of 50% end point titer (neutralizing dose, ND₅₀) generated from positive sera (5,012–79,433) were significantly higher than those from mock-infected sera (10 to 126), indicating that the test can be used in the detection of TMUV-specific neutralizing antibodies. Dose-dependent efficacy test of a cell-derived 180th passage of a plaque-purified virus of the PS TMUV isolate (PS180) in combined with immunization-challenge experiments revealed that ND₅₀ titer of ~1,258 is the minimum capable of providing adequate protection against challenge with virulent TMUV. In the investigation of serum samples collected from three flocks infected by TMUV and four flocks vaccinated with a licensed attenuated vaccine (the 120th passage virus), ND₅₀ titers peaked at 1 week after both disease onset (7,943–125,893) and vaccination (3,612–79,432), and high levels of ND₅₀ titer were detected in sera collected at 15 weeks after disease onset (5,012–63,095) and 17 weeks after vaccination (3,981–25,119). Together these findings demonstrated that spontaneous and experimental infections by TMUV and vaccination with the licensed TMUV attenuated vaccine elicit high, long-lasting neutralizing antibodies. The highest ND₅₀ titer of neutralizing antibodies elicited by PS180 was determined to be 3,162, suggesting that attenuation of TMUV by more passages has a dramatic impact on the neutralizing antibody response of the virus.

Keywords: duck, Tembusu virus, TMUV infection, TMUV attenuated vaccine, humoral immune response, neutralizing antibody, plaque reduction neutralization test

INTRODUCTION

Humoral immune response plays a significant role in protection of the host from flavivirus infections (1). Generally, neutralizing antibodies are thought to be the key mediators of protection against flaviviruses following both infection and vaccination (2, 3). Under some circumstances, however, antibodies may enhance flavivirus infection, a phenomenon called antibody-dependent

enhancement (ADE) of infection (4, 5). In the case of dengue virus (DENV), the ADE phenomenon most frequently occurs in secondary infections with different DENV serotypes and in children with maternal antibodies declined to sub-neutralizing concentrations (6, 7). In fact, any antibody that neutralizes at sufficiently high concentrations can enhance flavivirus infectivity at sub-neutralizing concentrations (3). Therefore, high levels of neutralizing antibody are crucial to exerting a protective effect, especially in the context of vaccine-mediated humoral immunity.

For assessing the host's specific immune response to flaviviruses, many useful serological tests have been developed, such as hemagglutination inhibition, complement fixation, enzyme-linked immunosorbent assay (ELISA), and plaque reduction neutralization test (PRNT) (8). However, flavivirus serological tests may present a classical challenge in differential diagnosis owing to strong cross-reactivity between antibodies and heterologous viral antigens (9, 10). PRNT measures neutralizing antibodies, and is the most virus-specific serological test. Thus, this test is considered the "gold standard" for the differentiation of flavivirus infections and the measurement of immunity to flaviviruses although it is time-consuming to perform (2, 8, 11–18).

Tembusu virus (TMUV) is currently classified within the genus *Flavivirus* of the family *Flaviviridae* (<https://talk.ictvonline.org/taxonomy>). Based on the mode of transmission and serological cross-reactivity, TMUV is also classified within the Ntaya group of the mosquito-borne flavivirus group, along with Bagaza virus (BAGV), Ntaya virus (NTAV), and Zika virus (ZIKV) (8). TMUV-related disease in ducks emerged in 2010, which affects mainly ducks during egg-laying periods. The disease is characterized by sudden onset, rapid spread, severe drops in egg production, and degenerate ovaries with hemorrhagic lesions (19–22). In affected flocks, the egg production rate may reduce to 10% or less within ~1 week after disease onset (23). To control the disease, several vaccine candidates have been developed, including live-attenuated (24), inactivated (25, 26), and subunit-based (27–31) vaccine candidates. Live-attenuated and inactivated vaccines have been licensed to use in ducks in China (32, 33).

It has been shown previously that humoral immune response to TMUV can be developed in ducks following vaccination with various vaccine candidates as described above. Most data regarding to antibody response were generated by using ELISA-based assays, such as indirect ELISA (34), competition ELISA (35), and blocking ELISA (36). In the study by Chen et al. (27), a neutralization test was applied to detect serum antibodies of ducks vaccinated with a vectored duck enteritis virus expressing the TMUV envelope. That investigation showed that the vaccine candidate elicits neutralizing antibodies, with titers of $1:2^8$ at 7 days after immunization and $1:2^4$ at 15 weeks after immunization. A serological investigation performed on 60 serum samples collected from six farms by using a blocking ELISA revealed a high prevalence of 56.7% (36). To date, data relating to TMUV neutralizing antibody response elicited by infection and vaccine is still limited.

In this study, we describe the development of a PRNT for the detection of TMUV neutralizing antibodies. We also describe the

application of the test to field serum samples from different flocks of diseased and immunized ducks.

MATERIALS AND METHODS

Cells

BHK-21 cells were maintained in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM; Gibco, NY, USA) supplemented with 10% fetal calf serum (FCS; Gibco, NY, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin.

Viruses

The PS and Y isolates were originally recovered in China from outbreaks of TMUV-related disease in a flock of egg-laying ducks in 2011 and in a flock of 74-day-old ducks, respectively. The fourth passage (strain PS4) in BHK-21 cells of the PS isolate was applied to produce the working virus in PRNT. The fourth passage (strain Y4) in BHK-21 cells of the Y isolate was applied to produce TMUV antibody-positive sera. The 180th passage (strain PS180) of a plaque-purified virus of the PS isolate was used in immunization-challenge experiments. The PS180 virus was prepared in our laboratory, which underwent five passages in 9-days-old specific pathogen free chicken embryos, three passages in BHK-21 cells, three-rounds of plaque purification, and 180 passages in BHK-21 cells (37, 38).

Virus Propagation

BHK-21 cells were prepared in T25 flasks, which were seeded at 8×10^6 cells/flask. When confluent, the cells were washed three times with phosphate buffered solution (PBS), and inoculated with PS4 at a multiplicity of infection (MOI) of 0.01 plaque forming unit (PFU)/cell. Following 1-h adsorption at 37°C, the inoculum was removed, and 5 ml of maintenance medium consisting of DMEM supplemented with 2% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin was added to each flask. The cells were incubated at 37°C in a 5% CO₂ atmosphere. At about 60 h post inoculation (pi), when a remarkable cytopathic effect (CPE) was developed, the cell culture was subjected to a cycle of freeze and thawing, followed by centrifugation at $10,000 \times g$ for 1 min. Cell-free supernatant was harvested for additional three passages, generating the PS5, PS6, PS7, and PS8 strains. The viruses were stored at –80°C until use.

Serum Samples

TMUV antibody-negative and positive sera were prepared in Pekin ducklings (*Anas platyrhynchos domestica*) and used for the development of PRNT. The newly hatched ducklings were obtained from a local duck farm, where TMUV infection had never been observed and their parents had never been immunized with TMUV vaccine. Antisera ($n = 10$) against Y4 were taken from 14-days-old survived ducklings that were inoculated intramuscularly (i.m.) with strain Y4 at 7 days of age at the dose of 10^4 PFU per duckling. The TMUV antibody-negative sera were collected from 1-day-old ducklings ($n = 10$), and also prepared from 14-days-old ducklings ($n = 10$) which were inoculated i.m. with 0.2 ml of DMEM at 7 days of age.

Three groups of serum samples collected from ducks showing drops in egg production were used for investigation of the levels of neutralizing antibody induced by TMUV infection. The first group consisted of 20 samples collected in Inner Mongolia autonomous region from 29-weeks-old Pekin ducks in which the egg drop disease had been observed at 28 weeks of age. The egg laying rate decreased by 20–30%, lower than those caused by TMUV infection (23). Using previously reported TMUV-specific reverse transcription (RT)-PCR assays (19, 20), no positives were found in theca folliculi of diseased ducks. The second group consisted of 50 samples collected at 1 week after disease onset, including 20 samples taken in Hubei province from 28-weeks-old Shaoxing duck (*Anas platyrhynchos domestica*) and 30 samples taken in Shandong province from 31-weeks-old Cherry Valley Pekin ducks (a strain of Pekin duck; *Anas platyrhynchos domestica*). The presence of TMUV infection in the flocks were confirmed based on clinical features and detection of TMUV in theca folliculus samples by using the RT-PCR assays (19, 20). The third group comprised 75 samples collected in Liaoning province from a flock of Jinding ducks (*Anas platyrhynchos domestica*). In the flock, a severe drop in egg production was observed at 25-weeks-old. TMUV was detected in theca folliculus samples. The serum samples were taken at 1, 3, 7, 11, and 15 weeks after disease onset. At each sampling time point, 15 samples were collected.

Two groups of serum samples collected from immunized ducks were used for investigation of the levels of neutralizing antibody induced by immunization with a licensed TMUV live-attenuated vaccine, the 120th passage of the WF TMUV isolate (32). The first group consisted of 110 samples collected at 1 week after immunization, including 30 samples collected in Anhui province from 19-weeks-old Cherry Valley Pekin ducks, 30 samples collected in Shandong province from 21-weeks-old Cherry Valley Pekin ducks, and 50 samples collected in Liaoning province from 21-weeks-old Jinding ducks. The second group comprised 70 serum samples, which were collected in Shandong province from a flock of Cherry Valley Pekin ducks. The ducks were vaccinated at 20 weeks of age. The samples were taken at 1 week before immunization, and at 1, 3, 5, 9, 13, and 17 weeks after immunization. At each sampling time point, 10 samples were collected. No clinical signs were observed during the course of sampling.

Plaque Assay

The virus was prepared in serial 10-fold dilutions (10^{-1} – 10^{-6}) with DMEM. BHK-21 cells were cultured on 24-well plates (Corning, NY, USA), which were seeded at 4×10^5 cells/well. When confluent, the cells were washed three times with PBS, and inoculated with 0.1 ml of diluted virus, three cultures per dilution. Following 1 h adsorption at 37°C, the inoculum was removed and the cells were washed three times with PBS. The plates received 0.5 ml of overlay medium consisting of DMEM containing 2% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% low melting-point agarose (Macgene, Beijing, China), and placed at 4°C for 15 min for agarose coagulation. After 3 days further incubation at 37°C in a 5% CO₂, the cells were fixed with 0.5 ml of 4% paraformaldehyde at room temperature for 90 min. The paraformaldehyde and agarose were removed and the cells were

stained with 0.5 ml of 0.2% (w/v) crystal violet. Fifteen minutes later, crystal violet was removed and plaques were read. Virus titer was calculated as described previously (39).

Growth Kinetics

Confluent monolayers of BHK-21 cells cultured in T25 flasks were inoculated with TMUV at a MOI of 0.01 PFU/cell as described above. For assessing virus growth, medium was sampled every 12 h between 12 and 84 hpi. At each sampling point, 0.2 ml sample was collected. Virus titers were determined using plaque assay as described above.

Development of PRNT

The PS7 strain was used as a working virus to develop PRNT for the detection of TMUV-specific neutralizing antibodies in sera of ducks. Ten antisera against the Y4 isolate and 10 sera from 1-day-old ducklings were tested in a preliminary protocol. Six antisera with 50% end point titers (neutralizing dose, ND₅₀) ranging from 10,000 to 125,893 and four sera with ND₅₀ titers ranging from 10 to 125 were selected for optimization of the PRNT. To determine the optimal dilution of working virus, four different dilutions ($1:5 \times 10^3$, $1:7 \times 10^3$, $1:10^4$, and $1:2 \times 10^4$) of the PS7 virus were tested against the 10 sera. Subsequently, varying incubation times around the following steps of PRNT were tested: (i) virus-serum neutralization (30, 45, 60, 75, and 90 min); (ii) adsorption of virus-serum inoculum to the cell surface (30, 40, 50, and 60 min); (iii) incubation after the plates received overlay medium (60, 66, 72, 78, and 84 h). For each condition, each serum sample was tested three times, generating three ND₅₀ titers (the observed results) and one median titer. The optimal condition was confirmed if all serum samples tested had the observed results within one 3-fold difference of the median titer. For determination of the optimal dilution of working virus and the optimal incubation time after the plates received overlay medium, the conditions to get readily discernable plaques and minimize plaque overlap were considered to be acceptable.

PRNT Protocol

Serum was inactivated at 56°C for 30 min and diluted in a 10-fold step to 10^{-5} with DMEM. Each dilution of the sera was mixed with an equal volume of diluted virus, and incubated at 37°C for 1 h. BHK-21 cell cultures were prepared in 24-well plates as described above. When confluent, the cells were washed and inoculated with 0.2 ml of the virus-serum mixtures, and plaque assay was conducted as described above. Controls were included in the experiment, including three virus control groups, in which 0.1 ml of virus was used to infect BHK-21 cells, and an uninfected BHK-21 cell control group, in which 0.1 ml of DMEM was used as inoculum. The PRNT titer was calculated using the Kärber method and expressed as ND₅₀. The formula is: $\log_{10} \text{ND}_{50} = m - \Delta (\sum p - 0.5)$, where m indicates $\log_{10}$ of the highest dilution, Δ indicates $\log_{10}$ of dilution coefficient, and $\sum p$ indicates the sum of number of plaques produced by virus-serum inoculum/average number of plaques produced by virus controls (40, 41).

Validation of PRNT

The optimized PRNT assay was validated as described previously (42). The precision of the assay was assessed by using 20 TMUV antibody-negative sera and 10 antisera against Y4. The intra-assay precision was determined by testing a single sample in three independent assays by the same operator, and the inter-assay precision was determined by testing a single sample in three independent assays by three operators. The sensitivity of the test was evaluated using 10 antisera against Y4. The serum samples were individually diluted in DMEM at a 10-fold step to 10^{-3} . Each of undiluted and diluted samples was tested three times by using the optimized PRNT.

Detection of TMUV Neutralizing Antibodies in Serum Samples of Ducks

The PRNT was applied to test serum samples of ducks collected in the field. To investigate the neutralizing antibody response induced by PS180, vaccination experiments were conducted. Sixty 1-day-old Pekin ducklings, which were derived from non-immune breeders that had no antibody to TMUV, were randomly divided into six groups (10 birds/group). Four groups were vaccinated i.m. with PS180 at doses of 10^4 (designated PS180- 10^4), 10^3 (PS180- 10^3), 10^2 (PS180- 10^2), and 10 (PS180-10) PFU per ducklings, respectively. Two groups (mock and control) were inoculated with 0.2 ml of DMEM. The birds were separately reared in different isolators. Serum samples were taken from all ducklings at 7 days p.i. and tested for neutralizing antibodies by using PRNT.

Vaccination and Challenge Experiments

To assess the immunogenicity of TMUV PS180, the vaccinated birds were challenged i.m. with TMUV Y4 (10^5 PFU per duckling) at 7 days p.i. after serum collection. The groups were designated PS180- 10^4 /Y4, PS180- 10^3 /Y4, PS180- 10^2 /Y4, and PS180-10/Y4, respectively. Of the two unvaccinated groups, one (mock/Y4) was challenged as described above, and another (control) was inoculated i.m. with 0.2 ml of DMEM. Ducklings were monitored daily for 7 days. Living ducks were weighed at the end of the experiment.

Statistical Analysis

The data derived from growth kinetic analysis, PRNT, and immunization-challenge experiments were calculated at mean $\pm$ standard deviation (SD). Difference between groups were compared using the analysis of variance method implemented in the GraphPad Prism Software (Version 5.0; GraphPad Software Inc., San Diego, CA, USA). A $P < 0.05$ value was considered statistically significant.

RESULTS

The Seventh Passage of TMUV PS in BHK-21 Cells Was Selected as Working Virus

It has been shown previously that the fifth to tenth passages of cell-derived virus are generally used as working virus in the PRNT assay (13). To produce the working virus for TMUV

PRNT, the PS4 strain was passed for four times in BHK-21 cells and the growth properties of the resulting four strains (PS5–PS8) were compared.

All the four viruses grew well in BHK-21 cells and exhibited similar growth kinetics (Figure 1). The titers of the four viruses showed increase since 12 hpi, and peaked at 60 hpi. Since then, the titers of the viruses tended downwards. During the observation period, the titers of PS7 and PS8 were slightly higher than those of PS5 and PS6, whereas no significant differences were observed between the viruses ($P > 0.05$). All the viruses produced clear plaques in BHK-21 cells. Differences were detected in plaque sizes, with 1–2 mm in diameter in the PS7- and PS8-infected cells and <1 mm in diameter in the PS5- and PS6-infected cells (Figure 2).

Based on above observations, the PS7 isolate was used as working virus in TMUV PRNT. Thus, the working virus was produced by infecting BHK-21 cells with the PS6 strain. The PS7 virus was harvested at 60 hpi, dispensed into small aliquots, and stored at -80°C . The titer was determined to be 4.2×10^6 PFU/ml.

The TMUV Antibody-Positive Sera Can Be Clearly Distinguished From the TMUV Antibody-Negative Sera by the PRNT

The $1:5 \times 10^3$ dilution of the working virus produced ~ 80 plaques/well, which had an impact on the accuracy of counting. The $1:7 \times 10^3$, $1:10^4$, and $1:2 \times 10^4$ dilutions of the working virus produced ~ 60 , 40, and 20 plaques/well, respectively, all of which assured a good precision of counting. In PRNT using $1:7 \times 10^3$ and $1:10^4$ dilutions of the working virus, all serum samples tested showed the three observed values within one 3-fold difference of the median ND_{50} titer (Supplementary Table S1). $1:10^4$ was selected as the optimal dilution of the working virus. On this basis, the 45, 60, and 75 min neutralization times and the 40, 50, and 60 min adsorption times were all shown to assure the accuracy of PRNT (Supplementary Tables S2, S3). Sixty minutes, which was generally employed in the routine neutralization test, was regarded as the optimal incubation time for both neutralization and adsorption. After the plates received

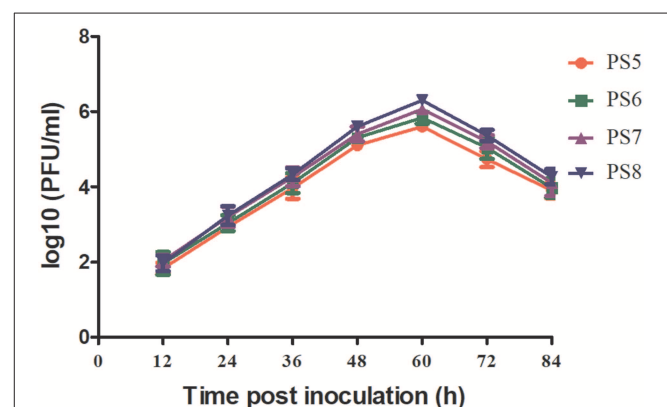


FIGURE 1 | TMUV growth kinetics in BHK-21 cells. Virus titers in plaque-forming units (PFU)/ml were measured between 12 and 84 hpi with a 12 h interval.

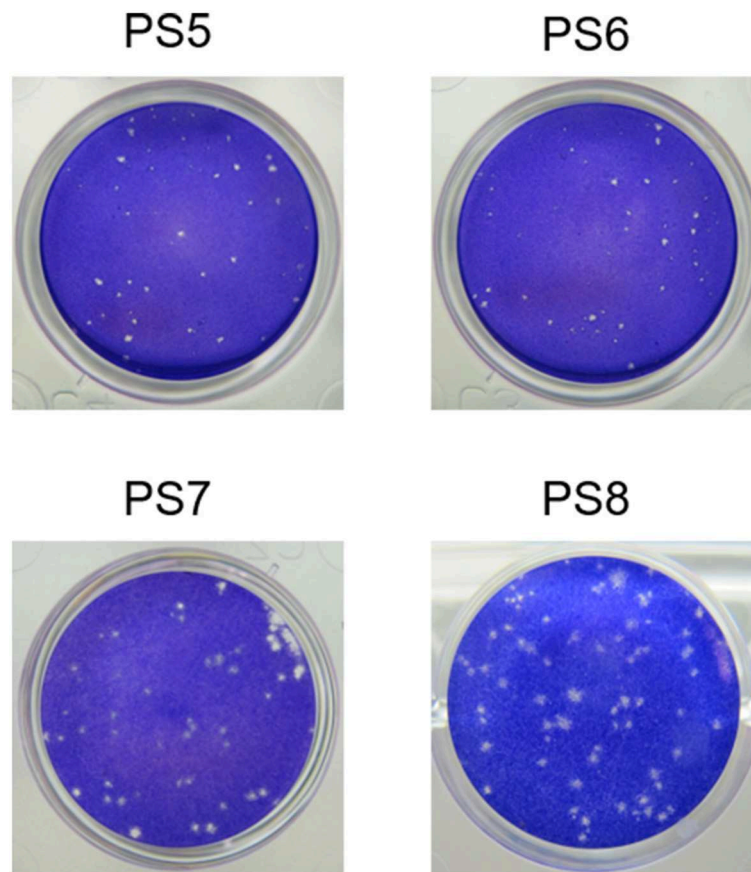


FIGURE 2 | TMUV plaques in BHK-21 cells at 72 h post inoculation.

overlay medium, the 72, 78, and 84 h incubation times were all considered to be acceptable as plaques with 1–2 mm in diameter formed at these time points were easily recognized and counted (**Supplementary Figure S1**). Seventy-two hours was selected as the optimal incubation time.

As shown in **Tables 1, 2**, there were only three and two samples exhibited results that were outside ± 3 -fold of the median ND_{50} titer of the three results in the intra- and inter-assay precision analyses, respectively. In the sensitivity analysis, all the samples were shown to have an observed and expected ND_{50} titer within 3-fold of each other. For all serum samples tested, antibodies were detectable at a 1:100 dilution, but not at a 1:1,000 dilution (**Table 3**). In the light of criteria previously defined for DENV (42), the precision of intra- and inter-assays and the sensitivity of the TMUV PRNT were considered acceptable.

The 10 antisera against Y4 produced high levels of ND_{50} titer, ranging from 5,012 to 79,433 (**Tables 1, 2**). In sharp contrast to the antisera, the 10 sera collected from 1-day-old ducklings and the 10 sera prepared from 14-days-old ducklings that had been inoculated with DMEM produced significantly lower ND_{50} values, ranging from 10 to 126 (**Tables 1, 2**). These data indicated that the TMUV antibody-positive sera can be distinguished clearly from the TMUV antibody-negative sera by the optimized PRNT.

The Cell-Derived PS180 Virus Exhibits Satisfied Immunogenicity

To assess the immunogenicity of the cell-derived 180th passage of PS virus (PS180), groups of one-day-old Pekin ducklings were vaccinated with PS180 at different doses and challenged with virulent TMUV (Y4) seven days later. No clinical signs were observed in control and ducks that had been vaccinated with 10^4 and 10^3 PFU of PS180. In contrast, ducks in other groups developed signs of illness at 3 days after challenge. Mild signs, such as mild depression and a slight decrease in feed intake, were observed in ducklings vaccinated with 10^2 PFU of PS180. More serious signs, including dramatic decrease in feed intake, paralysis, and encephalitis, were seen in ducklings vaccinated with 10 PFU of PS180 and unvaccinated ducklings (**Table 4**).

One duckling vaccinated with 10^2 PFU of PS180 died within 6–7 days post challenge (p.c.); four ducklings vaccinated with 10 PFU of PS180 died within 6–7 days p.c.; five unvaccinated ducklings died within 5–7 days p.c. In contrast, no deaths occurred in control and ducklings that had been vaccinated with 10^4 or 10^3 PFU of PS180. These data indicated that one-day-old ducklings immunized i.m. with PS180 at higher doses (10^4 and 10^3 PFU) withstood an i.m. challenge of 10^5 PFU of virulent TMUV (Y) 7 days later, whereas 10^2 and 10 PFU of PS180 did not offered adequate protection (**Table 4**).

TABLE 1 | Determination of intra-assay precision for TMUV PRNT.

Sample no.	ND ₅₀ for antibody-negative sera [†]			Sample no.	ND ₅₀ for antibody-negative sera [†]			Sample no.	ND ₅₀ for anti-Y4 sera		
	Test 1	Test 2	Test 3		Test 1	Test 2	Test 3		Test 1	Test 2	Test 3
1	10	16	10	1	13	10	10	1	12,589	15,848	7,943
2	13	20	20	2	25	16	20	2	6,309	10,000	7,943
3	50	63	40	3	25	13	32	3	50,118	31,622	39,811
4	13	16	12	4	32	40	40	4	7,943	10,000	12,589
5	63	79	50	5	126	100	63	5	31,622*	10,000	10,000
6	32	25	40	6	100	79	79	6	5,012	6,309	10,000
7	126	79	63	7	100	79	25*	7	15,849	19,953	15,849
8	100	126	79	8	63	79	50	8	25,119	25,119	39,811
9	32	50	63	9	100	100	126	9	63,096	79,433	19,953*
10	13	16	10	10	40	32	32	10	10,000	12,589	15,849

[†] Sera collected from 1-day-old ducklings.[‡] Sera prepared from 14-days-old ducklings that had been inoculated with DMEM at 7 days of age.* Outside ± 3 -fold of the median titer of three results obtained in triplicates.**TABLE 2** | Determination of inter-assay precision for TMUV PRNT.

Sample no.	ND ₅₀ for antibody-negative sera [†]			Sample no.	ND ₅₀ for antibody-negative sera [†]			Sample no.	ND ₅₀ for anti-Y4 sera		
	Test 1	Test 2	Test 3		Test 1	Test 2	Test 3		Test 1	Test 2	Test 3
1	10	20	25	1	13	20	13	1	12,589	10,000	10,000
2	13	16	20	2	25	20	20	2	6,309	7,943	31,623*
3	50	64	50	3	20	10	20	3	50,118	39,811	63,096
4	13	25	16	4	32	25	32	4	7,943	10,000	15,849
5	63	79	63	5	100	79	50	5	31,622	39,811	15,849
6	32	40	50	6	126	32*	63	6	5,012	12,589	7,943
7	126	100	79	7	63	79	79	7	15,849	19,953	19,953
8	100	50	63	8	100	50	63	8	25,119	31,622	19,953
9	32	40	63	9	79	50	50	9	63,096	50,119	79,433
10	13	20	10	10	40	50	25	10	10,000	19,953	15,849

[†] Sera collected from 1-day-old ducklings.[‡] Sera prepared from 14-days-old ducklings that had been inoculated with DMEM at 7 days of age.* Outside ± 3 -fold of median titer of three results obtained in triplicates.

The body weights of living ducks in vaccinated and unvaccinated groups at 7 days after challenge were measured to investigate further the immunogenicity of PS180 in terms of weight gain. Challenge had no significant impact on weight gain in ducks vaccinated with 10^4 and 10^3 PFU of PS180 when compared with control ($P > 0.05$). In contrast, mean body weights of ducks immunized with 10^2 and 10 PFU of PS180 and unvaccinated ducks were all significantly lower than that of control ($P < 0.05$) (Table 4). Taken together, it is concluded that PS180 exhibits satisfied immunogenicity.

Neutralizing Antibodies With a ND₅₀ Titer of Higher Than 1,258 Provides Adequate Protection Against Infection With Virulent TMUV

To define the minimal value of TMUV neutralizing antibodies protecting ducks against TMUV infections, serum samples

were collected before challenge in above trials for vaccination-challenge experiments and tested for TMUV neutralizing antibodies by using PRNT (Table 4). Higher ND₅₀ values, ranging from 1,584 to 3,162 and 1,258 to 2,511, were derived from sera of ducklings vaccinated with 10^4 and 10^3 PFU/bird PS180, respectively. Relatively low ND₅₀ values, ranging from 631 to 1,548 and 251 to 398, were detected in sera of ducks after immunization with 10^2 and 10 PFU of PS180, respectively. The levels of ND₅₀ titer detected in sera taken from unvaccinated ducks were very low, ranging from 25 to 158, which were considered TMUV antibody-negative according to the criterion defined above. These data, in conjunction with results derived in the immunization-challenge experiments, suggested that ND₅₀ titer of $\sim 1,258$ can be considered the minimum capable of providing adequate protection against challenge with virulent TMUV.

TABLE 3 | Evaluation of the sensitivity for TMUV PRNT.

Sample no.	ND ₅₀ titer of neutralizing antibody in serum sample with dilution indicated					
	Undiluted	1:10		1:100		1:1,000
	Median [†]	Median	Expected [‡]	Median	Expected	Median
1	39,811	5,012	3,981	794	398	20
2	7,943	1,000	794	50	79	16
3	15,849	3,162	1,585	79	159	13
4	15,849	1,995	1,585	100	159	50
5	50,119	6,310	5,012	316	501	63
6	15,848	1,000	1,585	200	159	20
7	12,589	1,000	1,259	79	126	32
8	31,622	5,012	3,162	200	316	63
9	12,589	2,511	1,259	126	126	25
10	79,433	7,943	7,943	501	794	125

[†] Median titer, mean value of three titers obtained in triplicates from each of diluted serum samples.

[‡] Expected titer, median titer of the undiluted sample divided by the dilution factor.

TABLE 4 | Correlation between levels of neutralizing antibody and protective efficacies conferred by TMUV PS180.

Group [†]	ND ₅₀ titer	Mortality (%)	Mean body weight (g) [§]
PS180-10 ⁴ /Y4	1,584–3,162	0 (0/10) [‡]	572 ± 20
PS180-10 ³ /Y4	1,258–2,511	0 (0/10)	560 ± 30
PS180-10 ² /Y4	631–1,548	10 (1/10)	450 ± 26*
PS180-10/Y4	251–398	40 (4/10)	414 ± 50*
Mock/Y4	25–158	50 (5/10)	400 ± 39*
Control	32–126	0 (0/10)	580 ± 18

[†] Pekin ducks were inoculated with 10⁴ to 10⁵ PFU of PS180 and DMEM at 1 day of age. Ducks in five groups were challenged with 10⁵ PFU of TMUV Y4 at 7 days of age. Serum was sampled from 7-days-old ducks before challenge.

[‡] No. of death/no. of ducks challenged.

[§] The ducks were weighed at 7 days after challenge.

*Significant difference compared with control ($P < 0.05$).

TMUV Natural Infection Elicits High-Level, Long-Term Neutralizing Antibody Response in Ducks

To investigate the humoral immune response induced by TMUV natural infections, the PRNT was applied to test serum samples collected at 1 week after disease onset from two flocks in which TMUV infection had been confirmed and a flock in which TMUV infection had been excluded. High levels of ND₅₀ titer were generated from the serum samples of Shaoxing ducks in Hubei province and of Cherry Valley Pekin ducks in Shandong province, ranging from 7,943 to 79,433 and from 10,000 to 125,893 respectively. In contrast, the ND₅₀ values produced from the serum samples of Pekin ducks in Inner Mongolia autonomous region ranged from 25 to 200, significantly lower than those obtained in Hubei and Shandong provinces ($P < 0.001$) (Figure 3).

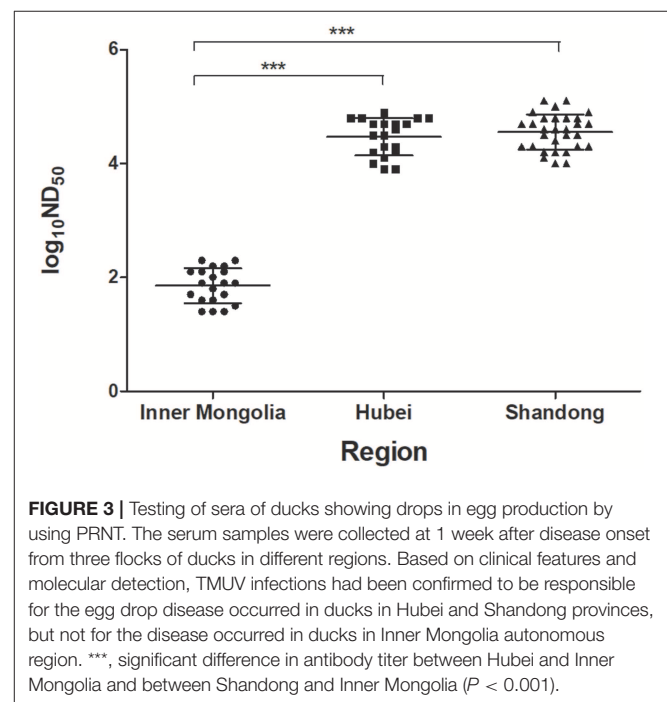
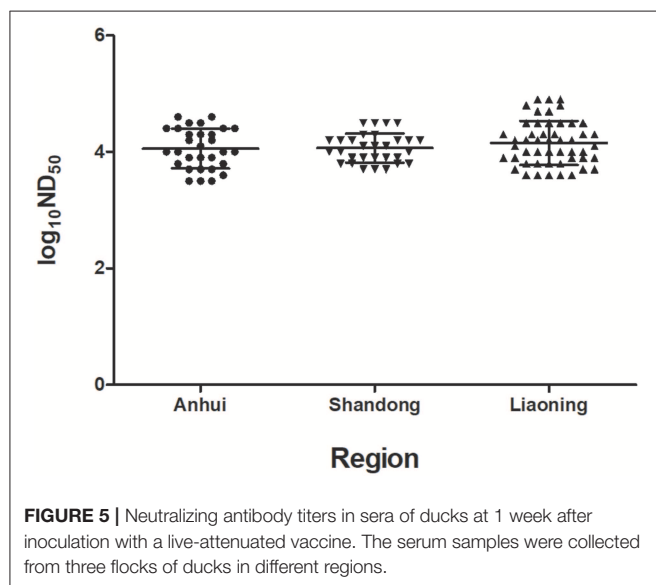
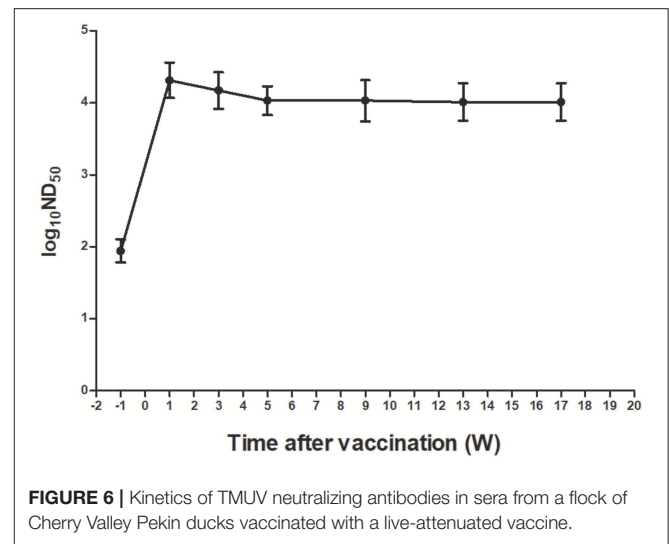
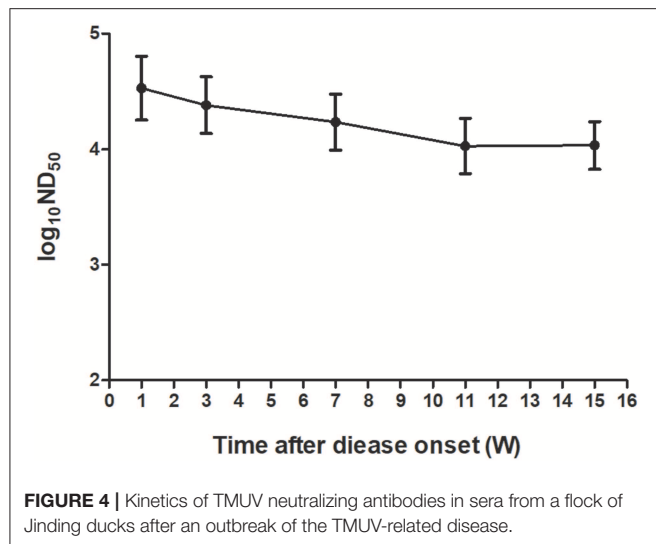


FIGURE 3 | Testing of sera of ducks showing drops in egg production by using PRNT. The serum samples were collected at 1 week after disease onset from three flocks of ducks in different regions. Based on clinical features and molecular detection, TMUV infections had been confirmed to be responsible for the egg drop disease occurred in ducks in Hubei and Shandong provinces, but not for the disease occurred in ducks in Inner Mongolia autonomous region. ***, significant difference in antibody titer between Hubei and Inner Mongolia and between Shandong and Inner Mongolia ($P < 0.001$).

To investigate the kinetics of neutralizing antibodies in sera of ducks after TMUV natural infections, the PRNT was performed on serum samples collected at different time points from a flock of Jinding ducks in which TMUV infection had been confirmed. High neutralizing antibody titers ranging from 12,589 to 100,000 appeared in sera at 1 week after disease onset. Although the ND₅₀ titers showed tendency to decline since then, high ND₅₀ values ranging from 5,012 to 63,095 were detected in sera collected at 15 weeks after disease onset (Figure 4).



Immunization With TMUV Attenuated Vaccine Elicits High-Level, Long-Term Neutralizing Antibody Response in Ducks

To evaluate the humoral immune response induced by TMUV attenuated vaccine, the PRNT assay was applied to test serum samples collected from three different flocks at 1 week after immunization. The ND_{50} values were generally high, ranging from 3,612 to 37,811 in sera of Cherry Valley Pekin ducks in Anhui province, from 5,012 to 31,623 in sera of Cherry Valley Pekin ducks in Shandong province, and from 3,981 to 79,432 in sera of Jinding ducks in Liaoning province (**Figure 5**).

To investigate further the kinetics of neutralizing antibodies induced by TMUV attenuated vaccine, the PRNT was employed to test serum samples from a flock of Cherry Valley Pekin ducks at 1 week before immunization and at different time points after immunization. ND_{50} values detected in sera of ducks before immunization were generally lower, ranging from 50 to 158. Following vaccination, the neutralizing antibodies increased

rapidly and reached 7,943–50,119 at 1 week after vaccination. On subsequent 16 weeks the ND_{50} titers declined gradually. Nevertheless, higher ND_{50} values ranging from 3,981 to 25,119 were detected at 17 weeks after vaccination (**Figure 6**).

DISCUSSION

The present study describes the development of a PRNT for the detection of neutralizing antibodies against TMUV by using a seventh cell-derived working virus. The PRNT was optimized by comparative testing of different conditions and validated by determination of intra- and inter-assay variations. The detection limit was determined to be about 1:100 to 1:1,000 of serum dilution, suggesting that the sensitivity of the test is acceptable. The specificity of the test was proven by detection of neutralizing antibodies induced by experimental and natural infections and vaccination with two TMUV attenuated strains. We were unable to carry out the differential detection of antibodies against BAGV, NTAV, and ZIKV which have been classified within the same antigenic complex group with TMUV (8, 43) since the reference antisera or strains for these flaviviruses are unavailable. However, we reasoned that the test might not exhibit cross-reactions with other flaviviruses based on the following points. First, it has been shown previously that PRNT is the most virus-specific serological test (2, 8, 11–18). Second, there have been no reports describing infections in ducks caused by other flaviviruses (e.g., BAGV, NTAV, and ZIKV) so far. We showed that the ND_{50} values obtained from negative sera can be easily distinguished from those generated from positive sera. Using TMUV antibody negative serum samples prepared in laboratory and collected from flocks uninfected by TMUV in the field, the ND_{50} values were determined to be <200 , which can be regarded as a cut-off ND_{50} value. The availability of a TMUV PRNT provides a useful diagnostic tool with which investigations into TMUV antibody-mediated protection can be undertaken.

Based on clinical features and molecular detection of tissue samples collected from affected ducks, TMUV infections were confirmed to be responsible for the egg drop disease occurred

in Shaoxing ducks in Hubei province, Cherry Valley Pekin ducks in Shandong province, and Jinding ducks in Liaoning province, but not for the disease occurred in Pekin ducks in Inner Mongolia autonomous region. Using the serum samples from diseased ducks, we focused on understanding the duck neutralizing antibody response to TMUV infection. We showed that high levels of neutralizing antibody (7,943–125,893) were detected in sera of ducks in Hubei and Shandong provinces, indicating that ducks exposed to TMUV infections develop strong neutralizing antibody response as early as 1 week after disease onset. The detection of low ND₅₀ values in sera collected from Inner Mongolia autonomous region, indicating that these samples could be considered negative for TMUV antibodies. Thus, application of PRNT in serum samples is of help to the definitive diagnosis of the TMUV-related disease. Further detection of the kinetics of TMUV antibodies in a flock of Jinding ducks demonstrated that higher levels of TMUV neutralizing antibody induced by natural infection can persist in sera of ducks for at least 15 weeks. These findings indicate that natural infection can elicit high-level, long-lasting immunity to TMUV.

To date, a TMUV attenuated vaccine (32) has been widely used in ducks in China. In this study, therefore, we also focused on understanding humoral immunity elicited by the vaccine, which is directly relevant to control of the TMUV-related disease. We showed that neutralizing antibodies with ND₅₀ titers higher than 1,258 offer adequate protection against infection of virulent TMUV. The detection of ND₅₀ titers ranging from 3,612 to 79,432 in sera collected at 1 week after vaccination from three different flocks located in Anhui, Shandong, and Liaoning provinces supported the view that the licensed TMUV attenuated vaccine elicits high levels of neutralizing antibody rapidly. Further detection of the kinetics of neutralizing antibodies in sera of an immunized flock of Cherry Valley Pekin ducks in Shandong province further confirmed that TMUV attenuated vaccine leads to a rapid seroresponse from seronegative before immunization to seropositive status after vaccination and a rapid rise of TMUV-specific serum neutralizing antibody titers. Moreover, higher levels of neutralizing antibody persist for at least 17 weeks. Together these findings indicate that vaccination with the licensed live-attenuated vaccine can confer high-level, long-lasting immunity to TMUV.

Compared with those of the licensed live-attenuated vaccine (32), the PS180 strain elicited noticeably lower levels of neutralizing antibody, which might be attributed to more passages of PS180. This investigation may be of help for further studies on molecular mechanism for TMUV attenuation and antibody-mediated protection.

Taken together, we have developed a PRNT for the detection of TMUV-specific neutralizing antibodies in sera of ducks. From the investigations of TMUV antibody-positive and negative sera we conclude that the test described here is a useful diagnostic tool to serological diagnosis of TMUV infection and measurement of neutralizing antibody-mediated immunity to TMUV. Application of the test in sera from infected and immunized ducks provided evidence that neutralizing antibodies is a critical component of immunity to TMUV following both infection and vaccination, which contributes to the understanding of neutralizing antibody response to TMUV. The data obtained from detection of the kinetics of neutralizing antibodies in naturally infected and vaccinated flocks provides a scientific basis for rational use of TMUV live-attenuated vaccine.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Welfare and Ethics Censor Committee of China Agricultural University (license number CAU20171011-2). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

DZ and JL designed the study. JL performed the experiments. LY and SQ participated in the data analysis. RM, QL, and HL participated in the animal experiments. JL, XW, and DZ wrote the manuscript.

FUNDING

This work was supported by the National Key Research and Development Program of China (2016YFD0500107), and the China Agriculture Research System (CARS-42).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2019.00442/full#supplementary-material>

REFERENCES

- Pierson TC, Diamond MS. A game of numbers: the stoichiometry of antibody-mediated neutralization of flavivirus infection. *Prog Mol Biol Transl Sci*. (2015) 129:141–66. doi: 10.1016/bs.pmbts.2014.10.005
- Putnak JR, de la Barrera R, Burgess T, Pardo J, Dessy F, Gheysen D, et al. Comparative evaluation of three assays for measurement of dengue virus neutralizing antibodies. *Am J Trop Med Hyg*. (2008) 79:115–22. doi: 10.4269/ajtmh.2008.79.115
- Pierson TC, Xu Q, Nelson S, Oliphant T, Nybakken GE, Fremont DH, et al. The stoichiometry of antibody-mediated neutralization and enhancement of West Nile virus infection. *Cell Host Microbe*. (2007) 1:135–45. doi: 10.1016/j.chom.2007.03.002
- Halstead SB, O'Rourke EJ. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. *J Exp Med*. (1977) 146:201–17. doi: 10.1084/jem.146.1.201
- Peiris JS, Porterfield JS. Antibody-mediated enhancement of Flavivirus replication in macrophagelike cell

- lines. *Nature*. (1979) 282:509–11. doi: 10.1038/282509a0
6. Halstead SB. Neutralization and antibody-dependent enhancement of dengue viruses. *Adv Virus Res*. (2003) 60:421–67. doi: 10.1016/S0065-3527(03)60011-4
7. Flipse J, Wilschut J, Smit JM. Molecular mechanisms involved in antibody-dependent enhancement of dengue virus infection in humans. *Traffic*. (2013) 14:25–35. doi: 10.1111/tra.12012
8. Maeda A, Maeda J. Review of diagnostic plaque reduction neutralization tests for flavivirus infection. *Vet J*. (2013) 195:33–40. doi: 10.1016/j.tvjl.2012.08.019
9. Mansfield KL, Horton DL, Johnson N, Li L, Barrett ADT, Smith DJ, et al. Flavivirus-induced antibody cross-reactivity. *J Gen Virol*. (2011) 92:2821–9. doi: 10.1099/vir.0.031641-0
10. Ledermann JP, Loroño-Pino MA, Ellis C, Saxton-Shaw KD, Blitvich BJ, Beaty BJ, et al. Evaluation of widely used diagnostic tests to detect West Nile virus infections in horses previously infected with St. Louis encephalitis virus or dengue virus type 2. *Clin Vaccine Immunol*. (2011) 18:580–7. doi: 10.1128/CVI.00201-10
11. Russell PK, Nisalak A, Sukhavachana P, Vivona S. A plaque reduction test for dengue virus neutralizing antibodies. *J Immunol*. (1967) 99:285–90.
12. Hombach J, Solomon T, Kurane I, Jacobson J, Wood D. Report on a WHO consultation on immunological endpoints for evaluation of new Japanese encephalitis vaccines, WHO, Geneva, 2–3 September, 2005. *Vaccine*. (2005) 23:5205–11. doi: 10.1016/j.vaccine.2005.07.002
13. Roehrig JT, Hombach J, Barrett AD. Guidelines for plaque-reduction neutralization testing of human antibodies to dengue viruses. *Viral Immunol*. (2008) 21:123–32. doi: 10.1089/vim.2008.0007
14. Johnson BW, Kosoy O, Hunsperger E, Beltran M, Delorey M, Guirakhoo F, et al. Evaluation of chimeric Japanese encephalitis and Dengue viruses for use in diagnostic plaque reduction neutralization tests. *Clin Vaccine Immunol*. (2009) 16:1052–9. doi: 10.1128/CVI.00095-09
15. Rainwater-Lovett K, Rodriguez-Barraquer, Cummings DA, Lessler J. Variation in dengue virus plaque reduction neutralization testing: systematic review and pooled analysis. *BMC Infect Dis*. (2012) 12:233. doi: 10.1186/1471-2334-12-233
16. Simões M, Camacho LA, Yamamura AM, Miranda EH, Cajaraville AC, da Silva Freire M. Evaluation of accuracy and reliability of the plaque reduction neutralization test (micro-PRNT) in detection of yellow fever virus antibodies. *Biologicals*. (2012) 40:399–404. doi: 10.1016/j.biologicals.2012.09.005
17. Suthar MS, Diamond MS, Gale M Jr. West Nile virus infection and immunity. *Nat Rev Microbiol*. (2013) 11:115–28. doi: 10.1038/nrmicro2950
18. Lukman N, Salim G, Kosasih H, Susanto NH, Parwati I, Fitri S, et al. Comparison of the hemagglutination inhibition test and IgG ELISA in categorizing primary and secondary dengue infections based on the plaque reduction neutralization test. *Biomed Res Int*. (2016) 2016:5253842. doi: 10.1155/2016/5253842
19. Cao ZZ, Zhang C, Liu Y, Liu YH, Ye WC, Han JW, et al. Tembusu virus in ducks, china. *Emerg Infect Dis*. (2011) 17:1873–5. doi: 10.3201/eid1710.101890
20. Yan PX, Zhao YS, Zhang X, Xu DW, Dai XG, Teng QY, et al. An infectious disease of ducks caused by a newly emerged Tembusu virus strain in mainland China. *Virology*. (2011) 417:1–8. doi: 10.1016/j.virol.2011.06.003
21. Su JL, Li S, Hu XD, Yu XL, Wang YY, Liu PP, et al. Duck egg-drop syndrome caused by BYD virus, a new Tembusu-related flavivirus. *PLoS ONE*. (2011) 6:e18106. doi: 10.1371/journal.pone.0018106
22. Thontiravong A, Ninvilai P, Tunterak W, Nonthabenjawan N, Chaiyavong S, Angkabkingkaew K, et al. Tembusu-related flavivirus in ducks, Thailand. *Emerg Infect Dis*. (2015) 21:2164–7. doi: 10.3201/eid2112.150600
23. Zhang D. Tembusu virus infection of ducks. In: Brugere-Picoux J, Vaillancourt JP, Shivaprasad HL, Venne D, Bouzouaia M, editors. *Manual of Poultry Diseases*. Paris: AFAS (2015). p. 612–5.
24. Li GX, Gao XY, Xiao YL, Liu SQ, Peng S, Li XS, et al. Development of a live attenuated vaccine candidate against duck Tembusu viral disease. *Virology*. (2014) 450–451:233–42. doi: 10.1016/j.virol.2013.12.028
25. Lin J, Liu YH, Wang XQ, Yang BS, He PY, Yang ZY, et al. efficacy evaluation of an inactivated duck Tembusu virus vaccine. *Avian Dis*. (2015) 59:244–8. doi: 10.1637/10960-101514-Reg
26. Zhang LJ, Li ZH, Zhang QS, Sun MX, Li S, Su WL, et al. Efficacy assessment of an inactivated Tembusu virus vaccine candidate in ducks. *Res Vet Sci*. (2017) 110:72–8. doi: 10.1016/j.rvsc.2016.11.002
27. Chen PC, Liu JX, Jiang YP, Zhao YH, Li QM, Wu L, et al. The vaccine efficacy of recombinant duck enteritis virus expressing secreted E with or without PrM proteins of duck Tembusu virus. *Vaccine*. (2014) 32:5271–7. doi: 10.1016/j.vaccine.2014.07.082
28. Zhao D, Huang X, Han K, Liu Y, Yang J, Liu Q, et al. Protective immune response against newly emerging goose tembusu virus infection induced by immunization with a recombinant envelope protein. *Lett Appl Microbiol*. (2015) 61:318–24. doi: 10.1111/lam.12459
29. Zou Z, Liu ZG, Jin ML. Efficient strategy to generate a vectored duck enteritis virus delivering envelope of duck Tembusu virus. *Viruses*. (2014) 6:2428–43. doi: 10.3390/v6062428
30. Ma TF, Liu YX, Cheng J, Liu YH, Fan WT, Cheng ZQ, et al. Liposomes containing recombinant E protein vaccine against duck Tembusu virus in ducks. *Vaccine*. (2016) 34:2157–63. doi: 10.1016/j.vaccine.2016.03.030
31. Wang HJ, Liu L, Li XF, Ye Q, Deng YQ, Qin ED, et al. *In vitro* and *in vivo* characterization of chimeric duck Tembusu virus based on Japanese encephalitis live vaccine strain SA14-14-2. *J Gen Virol*. (2016) 97:1551–6. doi: 10.1099/jgv.0.000486
32. Wang L, Zhang QC, Peng JY, Zhang ZB, Gong ZH, Wu H, et al. *An Attenuated Duck TMUV Vaccine Strain and Its Application*. Chinese patent CN105274064A (2015).
33. Liu YH, Luo S, Lin J, Zhang YX, Yang ZY, Wang JQ et al. *A Method for Preparing TMUV Inactivated Vaccine and a TMUV Inactivated Vaccine*. Chinese patent CN108721615B (2018).
34. Yin XC, Lv R, Chen XD, Liu M, Hua RH, Zhang Y. Detection of specific antibodies against Tembusu virus in ducks by use of an E protein-based enzyme-linked immunosorbent assay. *J Clin Microbiol*. (2013) 51:2400–2. doi: 10.1128/JCM.00361-13
35. Fu YG, Ji YH, Liu B, Dafallah RM, Zhu QY. Development of a solid-phase competition ELISA to detect antibodies against newly emerged Duck Tembusu virus. *J Virol Methods*. (2015) 224:73–6. doi: 10.1016/j.jviromet.2015.08.017
36. Li XS, Li GX, Teng QY, Yu L, Wu XG, Li ZJ. Development of a blocking ELISA for detection of serum neutralizing antibodies against newly emerged duck Tembusu virus. *PLoS ONE*. (2012) 7:e53026. doi: 10.1371/journal.pone.0053026
37. Liu XX. *Isolation and identification of a virulent Tembusu virus and development of a cell-adapted Tembusu virus (in Chinese)* (MA thesis). China Agricultural University, Beijing, China (2015).
38. Liang T. *Molecular basis for different virulence of Tembusu viruses (in Chinese)* (MA thesis). China Agricultural University, Beijing, China (2016).
39. Roberts EA, Coote GG. The estimation of concentration of viruses and bacteria from dilution counts. *Biometrics*. (1965) 21:600–15. doi: 10.2307/2528544
40. Grist NR, Ross CAC, Bell EJ, Stott EJ. *Diagnostic Methods in Clinical Virology*. Oxford: Blackwell Scientific Publications (1996).
41. Cohen BJ, Audet S, Andrews N, Beeler J, WHO working group on measles plaque reduction neutralization test. Plaque reduction neutralization test for measles antibodies: description of a standardised laboratory method for use in immunogenicity studies of aerosol vaccination. *Vaccine*. (2007) 26:59–66. doi: 10.1016/j.vaccine.2007.10.046
42. Timiryasova TM, Bonaparte MI, Luo P, Zedar R, Hu BT, Hildreth SW. Optimization and validation of a plaque reduction neutralization test for the detection of neutralizing antibodies to four serotypes of dengue virus used in support of dengue vaccine development. *Am J Trop Med Hyg*. (2013) 88:962–70. doi: 10.4269/ajtmh.12-0461
43. Calisher CH, Gould EA. Taxonomy of the virus family Flaviviridae. *Adv Virus Res*. (2003) 59:1–19. doi: 10.1016/S0065-3527(03)59001-7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Lv, Yang, Qu, Meng, Li, Liu, Wang and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Rift Valley Fever Virus, Japanese Encephalitis Virus, and African Swine Fever Virus: Three Transboundary, Vector-Borne, Veterinary Biothreats With Diverse Surveillance, and Response Capacity Needs

Rebekah C. Kading^{1*}, Edward O. Abworo² and Gabriel L. Hamer³

¹ Arthropod-Borne Infectious Disease Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO, United States, ² International Livestock Research Institute, Nairobi, Kenya, ³ Department of Entomology, College of Agriculture and Life Sciences, Texas A&M University, College Station, TX, United States

OPEN ACCESS

Edited by:

Juergen A. Richt,
Kansas State University, United States

Reviewed by:

Artur Summerfield,
Institute of Virology and Immunology
(IVI), Switzerland

Ahmed Ali,
Beni Suef University, Egypt

*Correspondence:

Rebekah C. Kading
rebekah.kading@colostate.edu

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 27 September 2019

Accepted: 28 November 2019

Published: 13 December 2019

Citation:

Kading RC, Abworo EO and
Hamer GL (2019) Rift Valley Fever
Virus, Japanese Encephalitis Virus,
and African Swine Fever Virus: Three
Transboundary, Vector-Borne,
Veterinary Biothreats With Diverse
Surveillance, and Response Capacity
Needs. *Front. Vet. Sci.* 6:458.
doi: 10.3389/fvets.2019.00458

Early detection of emerging foreign animal diseases is critical to pathogen surveillance and control programs. Rift valley fever virus (RVFV), Japanese encephalitis virus (JEV), and African swine fever virus (ASFV) represent three taxonomically and ecologically diverse vector-borne viruses with the potential to be introduced to the United States. To promote preparedness for such an event, we reviewed the current surveillance strategies and diagnostic tools in practice around the world for these emerging viruses, and summarized key points pertaining to the availability of existing guidelines and strategic approaches for early detection, surveillance, and disease management activities. We compare and contrast the surveillance and management approaches of these three diverse agents of disease as case studies to emphasize the importance of the ecological context and biology of vectors and vertebrate hosts. The information presented in this review will inform stakeholders of the current state of surveillance approaches against these transboundary foreign animal disease which threaten the United States.

Keywords: emerging arboviruses, ticks, mosquitoes, biosurveillance, introduction, guidelines

INTRODUCTION

Rift Valley fever virus (RVFV), African swine fever virus (ASFV), and Japanese encephalitis virus (JEV), are three vector-borne veterinary pathogens with the potential to invade the United States (US). All of these viruses are notifiable animal diseases (1), and Rift Valley fever (RVF) is also included on the “World Health Organization (WHO) Blueprint of priority pathogens” (2). While these viruses would all have devastating health and economic impacts, the ecological context and potential transmission dynamics of each are quite different and would require unique preparedness and mitigation efforts. All of these agents are capable of vector-borne as well as direct transmission (Table 1). JEV and RVFV are also zoonotic and capable of causing disease in humans, and the potential vector, domestic, and wild animal species involved in enzootic and spillover transmission are variable. Routes of potential entry into the US span importation of infected vectors (all three agents), viremic travelers (RVFV), infected animal products (ASFV), or contaminated fomites

TABLE 1 | Similarities and differences among the invasion and establishment of Rift Valley fever virus, Japanese encephalitis virus, and African swine fever virus into the United States.

	Rift Valley fever	Japanese encephalitis	African swine fever
Human pathogen	Yes	Yes	No
Vaccine available	No*	Yes	No
Vector-borne potential	Mosquitoes	Mosquitoes	Ticks
Direct transmission	Yes	Yes	Yes
Likely wildlife reservoir	Deer	Birds	Feral hogs
Affected domestic hosts	Goats, sheep, cattle	Pigs	Pigs
Possible route of entry	People, mosquitoes	Mosquitoes	Swine products, fomites

*Currently there is no vaccine licensed in the U.S., but multiple vaccines have or are being developed.

(ASFV), hence requiring very different preparedness efforts. This complexity in disease ecology and introduction potential presents a challenge to our health infrastructure to prepare for one or multiple such events.

The risk of introduction and establishment for RVFV, JEV, and ASFV to the US have all been investigated. The emergence of RVFV on the Arabian Peninsula (3) and recent reports of RVFV-infected travelers returning to France and China from areas of active RVFV transmission (4, 5) demonstrate the feasibility of pathogen importation via air travel. Considering five potential pathways of entry, Golnar et al. predicted the most likely introduction would be through an infected human traveler, but invasion of infected mosquitoes by plane or ship also carried quantifiable risk (6). In a separate qualitative analysis, Kasari et al. also concluded that RVFV could feasibly enter the US through several pathways including importation of infected animals, people, vectors, or live virus (7). If introduced, many competent mosquito vectors exist in the US (8, 9), and maintenance of the virus in mosquito populations by vertical transmission is possible (10–12). Deer have recently been shown to be a susceptible amplifying host (13) with viremias capable of infecting blood-feeding mosquitoes, which significantly increases the probability of enzootic establishment (14). Intensive livestock production in the US also creates a vulnerable environment for virus transmission among human and livestock communities by opportunistic mosquito vectors adapted to agricultural environments (15). Barker et al. modeled the potential for RVFV transmission in California considering local vectors, hosts and environmental conditions, but understanding the long-term persistence of RVFV depended on the parameterization of the models with data that were not available at the time this model was developed (16).

Japanese encephalitis virus strains have a history of invasion and establishment among new areas in Asia. Molecular epidemiological evidence suggests that JEV strains are introduced from continental south-east Asia to Japan regularly (17–19). Introductions of JEV within Asia and between Asia and Australia are believed to be through either infected windblown mosquitoes or birds, where the virus then circulates enzootically among native birds and *Culex* spp. mosquitoes (20–22). Considering seven possible pathways of JEV entry into the US, entry of infected adult mosquitoes through air travel was the most likely

scenario, however, the risk of establishment of JEV in the US following this type of introduction was variable (23). Several parameters complicated the establishment in the US, including a relatively short host viremia, unpredictable contact rates between hosts and vectors, particularly near airports, virus strain and genotype differences, and the cross-protection of JEV with other flaviviruses endemic to the US (23). North American *Culex* mosquitoes have been shown to be competent for infection with JEV under experimental conditions (24, 25). The predictable and stochastic factors influencing an introduction are complex, which makes risk assessment efforts challenging.

African swine fever virus has demonstrated its ability to cross national borders and establish in diverse geographical areas around the globe (26–29). A unique risk for introduction of ASFV is that this DNA virus is exceptionally stable in the environment, and remains viable in animal products. This property opens a potential route of entry not available to RVFV and JEV. If ASFV invades the US, competent vertebrate amplifying hosts as well as transmission-competent soft ticks are known to be present, although many questions remain (30). California, Texas, and Florida were among the states with the highest predicted risk for ASFV introduction through imported swine or swine products (31, 32). Further, these states plus much of the southwestern US were predicted to have increased risk for ASFV establishment should this virus be introduced. Additionally, operational procedures that characterize small-scale and organic pig producers throughout the southern US made these enterprises particularly vulnerable to ASFV circulation given the potential exposure to feral swine or *Ornithodoros* spp. soft ticks (32).

Preventing establishment of any or all of these viral pathogens would require biosecurity and health infrastructure to have a broad focus able to evaluate the risk of introduction and establishment of these disease agents. For example, monitoring passengers with febrile illness entering via airplane from RVFV-endemic countries, inspecting aircraft entering the US from JEV and RVFV-endemic countries for mosquitoes, testing swine products for ASFV, educating agricultural, wildlife, and veterinary professionals to recognize signs and symptoms of infection with any of these agents, conducting surveillance on a diversity of wildlife species spanning birds, deer, and feral swine, and being prepared to mitigate local vector-borne transmission

through mosquito and tick vector control. As an example of extreme caution, the 2019 World Pork Expo to be held in June in Des Moines, Iowa was canceled by the National Pork Producers Council due to the concern for the risk of ASFV infected products or fomites being introduced from infected countries (33). Executing the scope and complexity of these activities effectively with limited resources will make identification and early response to any of these agents particularly challenging. Diagnostic labs in the US testing samples from animals or arthropod vectors do not routinely test for these three agents specifically. Still, an extensive national network of veterinary diagnostic laboratories, and valuable knowledge, guidelines, and diagnostic tools exist for each of these viral pathogens to help prepare for such events. The following sections compile information on the current status of surveillance practices and diagnostic tools in use for RVFV, JEV, and ASFV.

RIFT VALLEY FEVER VIRUS

Rift Valley fever is a notifiable disease to the World Organization for Animal Health (OIE) and is additionally cross-listed as a select agent with the USDA and US Department of Health and Human Services (HHS). Due to the RVF disease severity, sizeable outbreaks, and the significant health and economic impacts, national and international guidelines for RVF surveillance and response activities have been developed.

Surveillance

The ability to predict likely times when an outbreak may occur would provide incredible power toward the timely mobilization of intervention and control measures. For RVF outbreaks in East Africa, a close association between epizootic activity and the occurrence of El Niño has been documented (34), providing an opportunity for monitoring weather patterns as an early indicator of conditions consistent with historic outbreak conditions. In particular, NASA has developed an outbreak risk mapping system for RVFV that inputs a variety of satellite measurements to produce a dynamic map of areas at potential risk of virus transmission. This “*Rift Valley Fever Monitor*” (35), records monthly RVF risk for Africa and the Middle East based on interpretation of satellite vegetation indices, and is maintained by the USDA and NASA (11, 36). In 2008, the WHO convened a joint meeting of experts with the United Nations Food and Agriculture Organization (FAO) to share experiences and discuss the predictability of forecasting models for RVE, and develop guidelines for improving prediction and response capabilities to RVF outbreaks (37). How these predictive models might translate to environmental risk in the US has been approached (16) but needs further study. Still, the precedent has been set for a potential contribution of climate monitoring and predictive modeling into RVFV outbreak prevention.

In 2014, the African Union-InterAfrican Bureau for Animal Resources (AU-IBAR) published a framework to support consistency and coordination of disease control activities, referred to as the “Standard Methods and Procedures (SMPs) for control of RVF in the Greater Horn of Africa” (38). The overall approach concerning the development of these SMPs relies on

the coordination of disease prevention and control activities in each participating region (i.e., Greater Horn of Africa) to a set of regional minimum standards and procedures that align with the OIE standards (38). This SMP document provides valuable information on surveillance and epidemiology, diagnosis and laboratory detection, disease control, disease reporting and information management, RVF and trade, and risk analysis and risk mapping, and is supported by specific Standard Operating Procedures (SOPs) for each subject area in accordance with the structure and capabilities of each nation.

Of particular relevance to a potential introduction of RVFV to the US, the AU-IBAR SMP document lists recommendations for surveillance approaches in areas which have not yet reported RVFV activity, in order to promote early detection and rapid response to a virus introduction. Among these recommendations are a combination of passive (continuous) and active surveillance (as needed, according to perceived risk) (38) (Table 2). Passive surveillance would involve the distribution of educational materials to veterinary personnel, and syndromic surveillance conducted by veterinarians, livestock handlers, and wildlife agencies. A specific reporting network is outlined for when suspicious cases are identified. Active surveillance would include serosurveillance, syndromic surveillance, and the regular monitoring of RVFV antibody levels in sentinel herds or flocks. Enhanced vector surveillance is recommended during interepidemic activities, to monitor for increased virus infection rates in mosquito populations. Supporting these surveillance activities should be administrative preparations, such as capacity building and training, and development of a policy framework to support surveillance activities (38).

The rapid deployment of available vaccines may aid considerably in curtailing virus circulation. RVF vaccines currently being deployed under emergency circumstances in Africa include the formalin-inactivated attenuated Smithburn strain. The Smithburn strain vaccine is “recommended for use in pregnant animals and in RVF-free countries experiencing outbreaks” (47); even though multiple boosters may be required. At the time of this writing, there is no RVF vaccine approved for human or veterinary use in the US, however RVF vaccine research and development is being pursued (48–51). If the decision to vaccinate is made, the vaccine must meet the standards in the “OIE Manual of Standards for Diagnostic Tests and Vaccines.” The final decision to vaccinate will be made by the US Department of Agriculture (USDA) Animal Plant Health Inspection service (APHIS) VS Deputy Administrator (US Chief Veterinary Officer) (40). Once vaccination is implemented, the ability to differentiate among infected and vaccinated animals (DIVA) will be essential in order to assess vaccine coverage as compared with virus exposure levels (48, 52).

The USDA APHIS has published “The Foreign Animal Disease Preparedness and Response Plan (FAD PReP)—Disease Response Strategy: Rift Valley Fever” (2013) (40) (Table 2). This document is intended to provide responders with the critical information necessary to mount an effective response effort against RVF in domestic livestock in the US (40). This document recommends approaches to disease control and eradication in the event of an introduction, including the roles and responsibilities

TABLE 2 | Guidelines and control recommendations for Rift Valley fever virus, Japanese encephalitis virus, and African swine fever virus.

Pathogen	Available Guidelines and Resources	Disease control recommendations	References
Rift Valley Fever virus	Standard Methods and Procedures (SMPs) for control of Rift Valley Fever (RVF) in the Greater Horn of Africa	• Mass vaccination—Smithburn vaccine or Clone 13 • Vector control • Animal quarantine and movement control • Public education	(38)
	Rift Valley fever surveillance: FAO Animal Health and Production Manual No. 21	• Mass vaccination • Vector control • Communication and public outreach	(39)
	The Foreign Animal Disease Preparedness and Response Plan (FAD PreP)—Disease Response Strategy: Rift Valley Fever	• Animal quarantine and movement control (state level) • Possible restrictions on interstate commerce • Vaccination, if vaccine is available • Possible euthanasia and mass depopulation of affected animals • Wildlife management • Vector control	(40)
Japanese Encephalitis Virus	"Japanese encephalitis virus infection, diagnosis and control in domestic animals"	• Vaccination of animals • Vector control	(41)
	WHO-recommended standards for surveillance of selected vaccine-preventable diseases	• Vaccination of people, • Assessment of the impact of vaccination • Syndromic surveillance for acute encephalitis syndrome • Standardized, aggregate, data reporting	(42)
	Manual for the Laboratory Diagnosis of Japanese Encephalitis Virus Infection	• Detailed specimen handling, laboratory testing and data management recommendations	(43)
African Swine Fever Virus	The Foreign Animal Disease Preparedness and Response Plan (FAD PReP)—Disease Response Strategy: Japanese encephalitis virus	• Quarantine and movement control • Stamping out—swine depopulation on infected premises within 24 h • Vaccination, if vaccine is available • Public outreach campaigns • Wildlife management • Vector control	(40)
	African swine fever. In OIE terrestrial manual 2012.	• Detailed information provided on specific diagnostic procedures	(44)
	The Foreign Animal Disease Preparedness and Response Plan (FAD PReP)—Disease Response Strategy: Rift Valley Fever (2013)	• Biocontainment and stamping out on infected premises • Public outreach campaigns • Prevent contact between feral and commercial swine • Possible feral swine depopulation in affected areas • Quarantine and movement control, particularly for fomites • Possible complete movement standstill for live swine	(45)
	USDA: Swine Hemorrhagic Fevers: African and Classical Swine Fever Integrated Surveillance Plan	• Detailed sampling and testing recommendations, case definitions, and reporting for commercial, backyard, and feral swine	(46)

of US government agencies (40). These control and eradication strategies are based on four epidemiological principles: "1. Prevent contact between RVFV and susceptible animals (i.e., quarantine and movement control), 2. Stop the production of RVFV by infected or exposed animals, 3. Stop the production of RVFV by insect vectors (i.e., vector surveillance and control), and 4. Increase the disease resistance of susceptible animals to RVFV (i.e., vaccination)" (40). Vector surveillance is only considered in the context of an outbreak and rapid response situation in these guidelines, and not as a potential strategy for early detection. Because of the diverse infrastructure for how vector surveillance and control is managed, these decisions would be left to the agency responsible for conducting vector

surveillance activities at the county or municipal government level (40). While these guidelines take some important first steps in providing a framework for priority activities by responsible parties, additional planning is needed to determine mechanisms for inter-agency collaboration during surveillance and response efforts, and identify capacity gaps for implementation of this strategic plan on the ground in an emergency situation. Further, no plan is outlined for conducting pre-introduction surveillance activities, either active or passive, as is detailed in the USAID plan (38). These gaps should be addressed for diseases, such as RVFV which are considered serious risks for introduction.

In December of 2006, the "APHIS Centers for Epidemiology and Animal Health in Fort Collins, Colorado," hosted a

working group meeting comprised of representatives from several academic institutions and government agencies to discuss the state of research and preparedness for RVF (53). Participants presented and discussed key research areas to improve the national capacity to respond to an introduction of RVFV, including more research regarding the relative importance of North American mosquitoes to transmission of RVFV, and the role of wildlife species to enzootic maintenance of RVFV circulation. Since the time of this publication, additional research has been conducted on the vector competence of North American mosquito vectors for RVFV (8, 9, 15), and the susceptibility of deer (13). Discussion was held on interagency cooperation and collaboration during outbreak events. On the response side, organized vector control in the US is principally concentrated in larger cities with high human densities. Rural landscapes where the enzootic cycle of RVFV is likely to amplify and spill-over into human and animals generally lacks organized vector control. Vector control in large rural landscapes often relies on partnerships with military capability (54). Similarly, there is a deficit in veterinary surveillance, and the tools to perform this type of surveillance safely. Veterinary surveillance is and will be very important for early detection of invasive vectors and pathogens. As an example, observations of Myiasis in Florida key deer populations in 2016 led to the discovery of an invasion of the screw worm fly, *Cochliomyia hominivorax* (Coquerel), which had been eradicated from the US since 1959 (55). Re-eradication of this fly was declared in March of 2017, after 35 releases of sterile flies (55). Participants of the RVF Working Group recommended training relevant professionals “to recognize the early signs of an RVF epizootic or epidemic,” and identified a shortage in high containment biosafety laboratories to safely conduct the necessary research (53).

Diagnostic Tools

The diagnostic tests for identification of RVFV listed in the “OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2012)” include virus isolation, reverse transcriptase polymerase chain reaction (RT-PCR)-based assays, an agar gel immunodiffusion (AGID) assay, an enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry (40, 44). Mansfield et al. comprehensively reviewed clinical diagnostic methods for RVFV infection as well as advancements in vaccine development (56). Therefore, only some representative assays for these widely-used techniques are highlighted below:

Serological Diagnosis

VanVuren and Paweska developed an ELISA based on the capture of the nucleocapsid protein (NP) (57). Alternatively, a monoclonal antibody-based competitive ELISA developed by Kim et al. for the detected antibodies against RVFV in goats and cattle with a sensitivity/specificity of 94.7/99.7% between 9 and 11 days post-inoculation (58). A multiplex fluorescence microsphere immunoassay (FMIA) also detected cattle and sheep IgM and IgG antibodies to the RVFV glycoprotein, non-structural proteins, and nucleoprotein (59). They also reported that the “N protein was the best target for early detection of infection” (59). A novel lateral flow strip test has recently been

developed based on detection of the N protein, for use as a pen side diagnostic (60). Importantly, McElroy et al. developed an ELISA that can be used to differentiate infected from those that had been vaccinated a Δ NSm/ Δ NSs double-mutant vaccine (48, 52).

The MAGPIX[®] and the Luminex xMAP platforms have also been used to simultaneously detect IgG antibodies against a multitude of key emerging viral species and families including RVFV (61, 62). Therefore, many options exist for pathogen detection, depending on the goals of the surveillance program and institutional capabilities.

Molecular Detection

qRT-PCR

Bird et al. developed a robust pan-RVF virus quantitative RT-PCR assay (63). Primers and a probe were designed from the genome alignments of 40 virus isolates derived from representative vector and vertebrate hosts over time. This assay was successfully validated on human RVF clinical samples, and has clinical diagnostic capabilities out to at least 10 days post-symptom-onset. The assay developed by Wilson et al. amplifies the L and M segments as confirmatory targets, and includes a third target gene, NSs, which is deleted in multiple vaccine candidates (64). This approach will be tremendously advantageous for simultaneously monitoring vaccine coverage vs. virus spread.

Isothermal Amplification

Recently, isothermal nucleic acid amplification methods have gained popularity for their ability to more rapidly detect pathogen nucleic acids directly in a clinical or field sample, and not necessarily require RNA extraction. Euler et al. developed an “isothermal ‘recombinase polymerase amplification (RPA)’ assay” on an “ESEquant tubescanner device.” While lacking in sensitivity compared to qRT-PCR, the portability of this system could be a potential tool for field, pen side or bedside diagnostics (65).

Nanotrap Particles

One innovative new approach to sample preparation, which also addresses sensitivity issues that might arise during surveillance, is the use of nanotrap particles to improve the detection of RVFV in low-titer samples by ~100-fold (66). Inactivation of RVFV by detergents or heat treatments did not affect the efficacy of virus binding to nanotrap particles, demonstrating that field samples can be safely inactivated and processed with nanotrap enrichment method to maximize sensitivity and maintain safety precautions (66).

Multiplex Pathogen Detection

Rather than focus on specific detection of RVFV, there are several multiplex assays that incorporate RVFV into the testing panel. Fajfr et al. developed multiplex assays targeting hemorrhagic filoviruses, arenaviruses, and bunyaviruses including RVFV, using previously-published primers (67). These assays are optimized for several types of real-time PCR instruments, including a portable “Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.)” (Idaho Technology,

Inc.). Liu et al. developed a real-time PCR-based TaqMan array card (TAC) that can screen up to eight samples within 3 h for up to 26 agents simultaneously (68). The comprehensive panel includes 15 viruses, eight bacteria, and three protozoa (68). Another multiplex platform is the Lawrence Livermore Microbial Detection Array which “detects 10,261 species of microbes including 4,219 viruses, 5,367 bacteria, 293 archaeobacteria, 265 fungi, and 117 protozoa”; RVFV spiked into *Culex* mosquitoes were successfully detected by the platform (69).

BSL-2 Reagent Production

The select agent status of RVFV limits the tests and control material that can be handled by veterinary diagnostic laboratories that operate at BSL-2. Therefore, Drolet et al. “modified an existing one-step real-time RT-PCR (rRT-PCR) assay for quick virus inactivation and use in BSL-2 laboratories” (70). Further, an immunohistochemical (IHC) assay was developed for use by BSL-2 laboratories, using antiserum against recombinant RVFV-nucleocapsid (N) (70). These advancements provide necessary diagnostic tools to public health and veterinary labs to safely test samples for RVFV and thereby enhance capacity for rapid disease detection and response activities for this high containment pathogen.

Diagnostic Considerations

When considering a diagnosis of RVF in animals in the US, the symptoms observed tend to be non-specific. Therefore, the below diseases are recommended to comprise the testing panel for diagnosis: anthrax, bacterial septicemias, bluetongue, brucellosis, heartwater, ephemeral fever, enterotoxemia of sheep, Nairobi sheep disease, ovine enzootic abortion, peste des petits ruminants, toxic plants, trichomonosis, vibriosis, and Wesselsbron disease (40). Due to biosafety and biosecurity concerns, aerosolization potential, and select agent status, diagnostic testing for suspected RVF cases would be performed at the Foreign Animal Disease Diagnostic Laboratory (FADDL), part of the National Veterinary Services Laboratories (NVSL) (40).

JAPANESE ENCEPHALITIS

Globally, Japanese encephalitis virus (JEV) is one of the most prevalent encephalitic arboviruses, mainly found in eastern and southern regions of Asia (71). Japanese encephalitis virus is primarily recognized as a human pathogen, but affects animal health as well. This virus is transmitted enzootically among mosquitoes (i.e., *Culex tritaeniorhynchus*), swine and wading birds, which serve as amplification hosts (72). Adult pigs are typically asymptomatic to JEV infection, however the virus is known to cause severe reproductive complications and fetal abnormalities among infected swine (41). Infection of horses with JEV has also been documented (73). People are exposed to JEV through the bite of an infectious mosquito but are considered dead-end hosts, as there is no human-to-human transmission. Japanese encephalitis virus is comprised of five genotypes (GI–GV) which are classified according to the phylogeny of the envelope

(E) gene (74), with emergence and circulation of different genotypes alerting public health professionals throughout areas of endemicity. Commercial vaccines are available for humans (75), and pigs and horses (76), however veterinary vaccines for JEV are relatively less available than human vaccines (41).

Surveillance

Japanese encephalitis virus (JEV), West Nile virus (WNV), and St. Louis encephalitis virus (SLE) share the same serocomplex (77). There is existing field and laboratory surveillance infrastructure for these other flaviviruses in the US. Operationally, collection and testing of *Culex* mosquitoes and sentinel surveillance programs in place for WNV could simultaneously also support vector surveillance for JEV through expansion of the testing panel, and funding should allow for supplementary surveillance activities.

The WHO publication, the “Manual for the Laboratory Diagnosis of Japanese Encephalitis Virus Infection” (43), covers JEV epidemiology, the role of the laboratory in JEV surveillance, prevention, and control, coordination of laboratory activities at local and national scales, specimen collection and handling, laboratory diagnosis procedures, testing algorithms/workflow, and data management (Table 2). Similarly, the WHO publication, “WHO-recommended standards for surveillance of selected vaccine-preventable diseases” (42) provides surveillance guidelines for a number of diseases including JE. Information included in this report include the accepted case definition, laboratory criteria for confirmation, and recommended types of surveillance (Table 2). Comprehensive syndromic surveillance for acute encephalitis syndrome (AES) is recommended for JEV (42). These resources will provide some initial guidance in establishing a public health laboratory surveillance network and testing algorithm for JEV in the US that is consistent with global recommendations.

Surveillance for JEV in the US would comprise passive and active surveillance of mosquito vectors, wildlife hosts, and domestic animals as well as people. Considering the genotype diversity of JEV, phylogenetic models have been developed to study JEV host changes and patterns of dispersal for different JEV genotypes using data collected through active and passive surveillance programs. Through this study, the authors concluded that active surveillance of mosquitoes was important in characterizing the circulating strain diversity of JEV, epidemiology, and transmission patterns, which informed virus spatial and evolutionary patterns (78). Yoshikawa et al. studied the serological and molecular epidemiology of JEV on an island of Japan, by conducting active surveillance of pigs as well as mosquitoes. Pigs were sampled at slaughterhouses and pig farms, and mosquito collections were coordinated near pig farms. The resulting data demonstrate seasonality of virus-positive mosquitoes and seroconversions and virus isolation from pigs over a 7-years period (19). This study presents the type of information that is useful in identifying not only spatial foci of transmission, but also seasonal patterns, which will direct evidence-based surveillance and control programs and help

conserve resources. Vector surveillance would play a key role in monitoring JEV circulation after a virus introduction.

The practice of using of sentinel animals to detect active transmission through seroconversion is widespread in arbovirus surveillance programs. Cohorts of sentinel pigs in addition to mosquito collections have been used to determine the rate of JEV transmission to pigs in Cambodia (79). While virus infection rates in mosquitoes were low, intense transmission was detected in pigs through the seroconversion of almost all of the pigs over the course of the study. This finding may be attributed to the potential for transmission of JEV directly between pigs through oral-nasal secretions (80), providing further support for an integrated surveillance program involving vector and vertebrate sampling. Chickens have also served as sentinel animals for detecting virus circulation in serological surveillance programs for WNV, JEV (81), and other flaviviruses (82).

Diagnostic Tools

A variety of serological and molecular diagnostics, using both single- and multiplex platforms, have been developed for detection of JEV (43).

Serological Diagnosis

Antibody detection (IgM and IgG) by capture ELISA is the recognized standard for JE diagnosis (83, 84). However, an important consideration for JE serological diagnosis is the extraordinary cross-reactivity among flaviviruses due to shared viral epitopes (85). This cross-reactivity challenges the ability of diagnosticians to verify the identity of the infecting flavivirus, particularly in endemic areas where multiple flaviviruses are co-circulating. Diagnostic approaches to overcome these challenges with flavivirus serology include the use of specific IgM ELISAs during the early stages of infection, and plaque reduction neutralization test (PRNT) (43). While PRNT may provide serological differentiation among closely-related and/or co-circulating flaviviruses, the technique is labor-intensive and involves working with live virus, which may not be feasible for many laboratories.

Currently, the recommended method for JE diagnosis of human infections is the JEV-specific IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) in CSF and serum (86). Several commercial ELISA kits have been developed for the diagnosis of JEV and other flaviviruses. These kits offer a standardized testing method for widespread use, however sensitivity and specificity issues complicate interpretation of results and may mis-inform stakeholders due to false negatives or antigen-reactivity. The “JEV MAC-ELISA” has been used since 2006 for laboratory-based surveillance of JE by the WHO Japanese encephalitis (JE) laboratory network (86). Of the available MAC ELISA kits available, comparative testing determined that the “Panbio JE-Dengue IgM combo ELISA” (Inverness Medical Innovations Inc., Queensland, Australia) had a higher specificity than either the “InBios JE Detect™ MAC-ELISA (JE Detect)” (InBios International Inc., Seattle, WA) or the JEV CheX (XCyton Diagnostics Ltd., Bangalore, India) kits, however the PanBio kit has not been commercially produced since 2013 (86). Johnson et al. screened a panel of

JEV+ and DENV+ control serum and CSF with JE Detect and DENV Detect InBios kits and developed a testing algorithm for differential diagnosis of these two viral infections (86).

Recognizing the problem of serological cross-reactivity among flaviviruses and its impact on surveillance and diagnosis, Cleton et al. used sera from naturally and experimentally-infected horses to develop a protein microarray to differentiate flaviviral infections in horses by NS1-antigen (87). Differentiation among flaviviral infections in horses using this method was possible, however some cross-reactivity was still noted, and differentiation between vaccinated and infected horses was not possible. Still, this type of platform could be a tool for screening serum samples of symptomatic individuals for multiple flaviviruses at once (87). Microseroneutralization tests have also been used in serosurveillance of JEV (88).

Molecular Detection

JEV viral RNA can be detected from viremic vertebrate samples and mosquitoes by many different available assays spanning traditional single-plex RT-PCR (89, 90), to more complex multiplex assays to screen samples for multiple arboviruses simultaneously (91). The multiplex assay developed by Wang et al. targets; “WNV, Saint Louis encephalitis virus, Venezuelan equine encephalomyelitis virus, Western equine encephalomyelitis virus, Eastern equine encephalomyelitis virus, Highlands J virus and JEV” (91).

AFRICAN SWINE FEVER

African swine fever virus (ASFV) (*Asfviridae: Asfavirus*), is transmitted among wild vertebrate reservoir species by soft ticks in the genus *Ornithodoros*. Infection of wild and domestic suids with this virus is associated with high mortality, and hemorrhagic fever, however infections in African suid species are asymptomatic (92). Until the 1980s ASF remained confined to Africa, with the exception of Sardinia. In 2007 ASF was reported from the country of Georgia (93). From Georgia, ASFV expanded its range throughout the Caucasus and the Russian Federation, and was reported from the European Union (EU) in 2014. Incursions of ASFV into Caribbean and Brazil were met with aggressive and costly responses that successfully eradicated the virus (94). ASFV strains belonging to the vp72 genotype II currently circulate endemically among wild boar populations in Eastern Europe and cause epidemics in domestic pig and wild boar populations, while genotype I circulates in Sardinia. From this history, many lessons have been learned from curtailing ASFV circulation in resource-poor endemic areas in Africa, as well as combatting outbreak activity in areas of recent introduction throughout Europe.

Surveillance

While ASFV is known to be transmitted by ticks in the genus *Ornithodoros*, most surveillance efforts are focused on disease detection and diagnosis in domestic and wild suids, perhaps due to the inconsistent involvement of ticks in outbreak situations. In eastern and southern African countries, ASFV circulates enzootically between ticks of the *O. moubata* species complex

and common warthogs. In contrast, transmission in West Africa, appears to occur among domestic pigs in the absence of ticks (95). All available experimental data to date on which vertebrate species become viremic upon infection with ASFV is restricted to animals in the family Suidae, and was reviewed by Golnar et al. Of these hosts, domestic pigs produced the highest circulating virus titers, with bush pigs and warthogs producing lesser but detectable viremias (30). *Ornithodoros erraticus* was determined to be responsible maintaining ASFV transmission in outdoor pig production facilities on the Iberian Peninsula. This is in contrast to many other locations throughout Europe in which the distribution and role of *Ornithodoros* ticks in the transmission of ASF is poorly known (95). A recombinant rTSGP1 ELISA kit can detect antibodies to *O. moubata* salivary gland proteins with 100% sensitivity and 99.4% specificity and directly determines the exposure history of pigs to blood feeding ticks (96). This and similar tests can therefore be used in addition to conventional tests for surveillance to determine potential exposure that could portend risk to ASF through ticks. While it will be important to understand the competence and distribution of potential tick vectors of ASFV in the US and areas at risk of ASFV establishment (30, 32), efforts to monitor for an introduction event should likely focus on evidence for infection among wild and domestic suids. Once ASFV establishes itself in a new area, the role of ticks will become increasingly more important to the environmental maintenance of this virus.

Passive surveillance of deceased wild boar and symptomatic animals could serve as first indicators of ASFV activity in new area of introduction (97, 98). Petrov et al. demonstrated that molecular detection of ASFV as well as classical swine fever virus were also detected reliably by quantitative PCR from dry and semi-dry blood swabs collected from carcasses (98). Similarly, Carlson et al. evaluated the reliability of detecting ASFV antibody from blood swabs taken from deceased animals in the field using commercially-available antibody-based detection kits (97). Sensitivity and specificity of these kits on dried blood swabs well-exceeded 90%. These studies demonstrate that molecular and serological diagnostics are easily applied to a passive surveillance strategy targeting carcasses, and a practical approach for early detection of ASFV circulation.

Mur et al. tested oral fluids as a non-invasive alternative to serum for ASF diagnosis (99). Oral fluid samples were collected by allowing experimentally-infected pigs to chew on ropes, and collecting fluid from the ropes. Paired samples of oral secretions and serum were tested for antibody against ASFV by both ELISA and the immunoperoxidase test (IPT). ASFV antibodies were detected in oral secretions for the 65-days duration of the experiment by both methods, introducing the possibility of saliva-based surveillance and diagnostics for ASF.

Fernandez-Carrion et al. developed a motion-based video surveillance system, based on the concept of monitoring animal behavior for signs of illness characteristic ASFV disease (100). Among experimentally-infected pigs, changes in mobility patterns preceded the onset of other disease symptoms (100). Further, motion-based video surveillance can be combined with temperature monitoring via biosensors, and linked to a smart

system to inform care takers when animals become feverish and/or lethargic (101). This system offers a non-invasive early-warning system for commercial animal facilities.

One challenge to surveillance is the potential for some suids to serve as asymptomatic carriers of ASFV and drive epizootic activity. Pigs surviving infection can contribute to virus spread within the population as persistently-infected carriers (102). Abworo et al. studied infection of pigs with ASFV genotype IX in small farming operations along the border of Kenya and Uganda (103). Approximately 16% of clinically-healthy pigs sampled had ASFV detected in their tissues, suggesting a role for persistently-infected pigs maintaining the virus in these production systems (103). Bellini et al. reviewed precautionary measures focused on minimizing the risk of spreading ASFV in and among pig farms in the epidemiological context of European systems (104).

Several gaps and priorities have been identified relative to ASFV control and surveillance: “(1) raise awareness among hunters, farmers and veterinarians, (2) ensure farm locations are far from suitable wild boar areas, and in affected areas, promote confinement, (3) prepare early warning systems, contingency plans, and control measures, (4) implementation of surveillance activities based on the risk of potential exposure, introduction, and spread” (105). Strict sanitation and early detection of ASFV in new areas are paramount, because no vaccine is currently available (102). Educational campaigns will be instrumental in raising awareness among key stakeholders to ensure rapid detection and response (106). Rapid disease recognition in the field and on farms is critical, followed by laboratory confirmation. Awareness of hunters, farmers, and veterinarians to the signs and symptoms of ASF, further understanding of ASFV epidemiology in areas of introduction, and preparedness with appropriate diagnostic capabilities are all critical next steps to the detection and mitigation of an introduction of ASFV into the US. The USDA-APHIS Veterinary Services (VS) recently proposed an integrated active surveillance plan for ASF and classical swine fever targeting higher-risk populations, sick pigs, and mortality events. Currently, detection of ASF will rely on passive disease reporting. The approval of tonsil and spleen tissues as additional valid tissue types for diagnostic testing in the National Animal Health Laboratory Network (NAHLN) presents an opportunity to integrate ASF surveillance with current CSF surveillance efforts as an active surveillance plan for swine diseases (46). USDA APHIS has also posted a disease response strategy for ASF (45). A subset of NAHLN laboratories has trained and proficiency tested analysts ready to participate in foreign animal disease investigations or surge capacity testing as needed (107).

Diagnostic Tools

Multiple molecular and serological detection platforms exist for ASFV, and are reviewed in detail by Gallardo et al. (102) and Arias et al. (105). Importantly, Gallardo et al. also provide valuable information on the interpretation of ASF diagnostic results in the context of differing clinical presentation and strain diversity (108).

Serological Detection

Currently, there is no approved vaccine for ASFV. Therefore, all antibody detections in field samples are indicative of natural exposure to virus circulation. Though caution must be taken in endemic epidemiological situations zero antibody positivity has been observed PCR ASF positive animal (109). Average animal positivity for ASFV by PCR in Southwestern Kenya was 28%, despite these animals being asymptomatic and sero-negative by ELISA (109). Such results demand for parallel confirmatory test to rule out infection or exposure, e.g., combination of molecular tools and serology. Gallardo et al. compared three commercial ELISAs with varying antigenic targets, the OIE-ELISA test, and the confirmatory immunoperoxidase test (IPT) (102). While the ELISA-based tests were rapid, high-throughput, and could be automated, sensitivity was generally poor compared to that of the Universal Probe Library (UPL-PCR) molecular test (see below) (102). The IPT was advantageous in detection of ASF antibodies earlier during the course of infection when titers were low (102). More recently, an indirect ELISA can be used to detect ASFV antibodies in either serum or oral secretions, and has been validated on field samples (110). Serological assays optimized for use on both oral and serum samples will be useful in endemic areas where virulence is low (110).

Molecular Detection

Recommended PCR assays for ASFV have become standard diagnostic tool in reference laboratories (111, 112). A side-by-side proficiency test of the UPL-PCR (113), and the recommended OIE conventional (44, 111) and real-time PCR assays (44, 111) assays revealed almost perfect agreement between these methods, however the UPL-PCR had greater diagnostic sensitivity for early detection of the disease. Due to some potential primer mis-matches between the primers in the OIE PCR assay (111). Luo et al. designed novel conventional PCR primers to address these sensitivity issues observed with the OIE assay for detection of European strains of ASFV (114). This updated PCR assay demonstrated improved diagnostic sensitivity than the two OIE over comparable PCR assays (111, 112) when detecting diverse virus strains (114). Wozniakowski et al. recently developed a polymerase cross-linking spiral reaction (PCLSR). This sensitive new test does not cross-react with other porcine pathogens, and has been validated on multiple sample types derived from ASFV-infected wild boars and pigs (115).

Because ASF is only one porcine pathogen of veterinary significance, some diagnostic efforts have included ASF in multiplex assays targeting multiple agents. Grau et al. developed a multiplex real-time PCR (mRT-qPCR) for concurrent detection of ASFV, classical swine fever virus, and foot and mouth disease virus, and evaluated use of this assay on swine oral secretions (116). This mRT-qPCR consisted of previously published singleplex RT-qPCR or qPCR assays (117–119) in use at the USDA-NVSL-FADDL and within the NAHLN, that for this study were combined into the multiplex format (116). A multi-plexed real time PCR assay has also been developed for ASFV and classical swine fever virus (120).

DISCUSSION

While all three of these pathogens are arthropod-borne viruses, the unique biology of these three systems demonstrates that there is no single approach for the surveillance and diagnostics. Vector, animal, and public health infrastructure needs to sustain broad capacity to rapidly detect and respond to these three viruses and more. The NVSL system, including the over 60 NAHLN labs and the FADDL will play a critical role in the diagnostic testing and confirmation of these disease agents. Currently, many NAHLN laboratories are already approved for ASFV testing (107). While vector collection and vector-based surveillance approaches are an integral component to post-introductory surveillance and management (Table 2), the first indicators of an introduction event of any of these diseases would likely be through recognition of sick wildlife or domestic animals or pathogen detection from carcasses. The USDA foreign animal diseases preparedness plans for each of these transboundary viruses includes a component of animal culling for biocontainment purposes, together with vector control and vaccination, if possible (40, 45) (Table 2). With this in mind, capacity for passive surveillance focused on the recognition of disease symptoms in domestic and wild animals by appropriate professionals (i.e., producers, veterinarians, wildlife professionals), particularly those near high risk ports of entry and in first-responding agencies, should be prioritized in order to detect and diagnose these cases early. Rapid response and containment will minimize the spread of the disease, and broader impacts on producers. Aspects of these diseases that have been exploited as predictors of virus transmission in other parts of the world may also be effective surveillance tools in the US, including outbreak forecasting using climate models for RVF. Vector surveillance activities will likely be most informative and important immediately after initial pathogen detection in animals or humans. Vector surveillance will help to incriminate particular vector species, determine vector abundance and infection rates, genotype circulating virus strains, and evaluate the efficacy of control strategies on vector populations and pathogen infection rates. National or international guidelines on surveillance and diagnosis also exist for each of these three agents (Table 2). These documents comprise a valuable starting place for critical discussions and development of action plans for surveillance and response activities in the US.

AUTHOR CONTRIBUTIONS

GH and RK: conceptualization. GH: funding acquisition. RK: original draft. RK, GH, and EA: review and editing.

FUNDING

This work was supported by the Department of Homeland Security (DHS) Science and Technology Directorate (S&T) under agreement # HSHQDC-17-C-B0003.

REFERENCES

- OIE. *OIE-Listed Diseases 2019*. OIE–World Organisation for Animal Health (2019). Available online at: <https://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2019/> (accessed August 16, 2019).
- WHO. *WHO|List of Blueprint Priority Diseases*. WHO (2019). Available online at: <http://www.who.int/blueprint/priority-diseases/en/> (accessed August 16, 2019).
- Al-Afaeq AI, Hussein MF. The status of Rift Valley fever in animals in Saudi Arabia: a mini review. *Vector Borne Zoonotic Dis.* (2011) 11:1513–20. doi: 10.1089/vbz.2010.0245
- Haneche F, Leparac-Goffart I, Simon F, Hentzien M, Martinez-Pourcher V, Caumes E, et al. Rift Valley fever in kidney transplant recipient returning from Mali with viral RNA detected in semen up to four months from symptom onset, France, autumn 2015. *Euro Surveill.* (2016) 21:30222. doi: 10.2807/1560-7917.ES.2016.21.18.30222
- Liu J, Sun Y, Shi W, Tan S, Pan Y, Cui S, et al. The first imported case of Rift Valley fever in China reveals a genetic reassortment of different viral lineages. *Emerg Microbes Infect.* (2017) 6:e4. doi: 10.1038/emi.2016.136
- Golnar AJ, Kading RC, Hamer GL. Quantifying the potential pathways and locations of Rift Valley fever virus entry into the United States. *Transbound Emerg Dis.* (2018) 65:85–95. doi: 10.1111/tbed.12608
- Kasari TR, Carr DA, Lynn TV, Weaver JT. Evaluation of pathways for release of Rift Valley fever virus into domestic ruminant livestock, ruminant wildlife, and human populations in the continental United States. *J Am Vet Med Assoc.* (2008) 232:514–29. doi: 10.2460/javma.232.4.514
- Golnar AJ, Turell MJ, LaBeaud AD, Kading RC, Hamer GL. Predicting the mosquito species and vertebrate species involved in the theoretical transmission of Rift Valley fever virus in the United States. *PLoS Negl Trop Dis.* (2014) 8:e3163. doi: 10.1371/journal.pntd.0003163
- Turell MJ, Wilson WC, Bennett KE. Potential for North American mosquitoes (Diptera: Culicidae) to transmit rift valley fever virus. *J Med Entomol.* (2010) 47:884–9. doi: 10.1093/jmedent/47.5.884
- Bergren NA, Kading RC. The ecological significance and implications of transovarial transmission among the vector-borne bunyaviruses: a review. *Insects.* (2018) 9:E173. doi: 10.3390/insects9040173
- Linthicum KJ, Britch SC, Anyamba A. Rift Valley Fever: an emerging mosquito-borne disease. *Annu Rev Entomol.* (2016) 61:395–415. doi: 10.1146/annurev-ento-010715-023819
- Linthicum KJ, Davies FG, Kairo A, Bailey CL. Rift Valley fever virus (family Bunyaviridae, genus Phlebovirus). Isolations from Diptera collected during an inter-epizootic period in Kenya. *J Hyg (Lond).* (1985) 95:197–209. doi: 10.1017/S0022172400062434
- Wilson WC, Kim IJ, Trujillo JD, Sunwoo SY, Noronha LE, Urbaniak K, et al. Susceptibility of white-tailed deer to Rift Valley fever virus. *Emerging Infect Dis.* (2018) 24:1717–9. doi: 10.3201/eid2409.180265
- Kakani S, LaBeaud AD, King CH. Planning for Rift Valley fever virus: use of geographical information systems to estimate the human health threat of white-tailed deer (*Odocoileus virginianus*)-related transmission. *Geospat Health.* (2010) 5:33–43. doi: 10.4081/gh.2010.185
- Hartman DA, Rice LM, DeMaria J, Borland EM, Bergren NA, Fagre AC, et al. Entomological risk factors for potential transmission of Rift Valley fever virus around concentrations of livestock in Colorado. *Transbound Emerg Dis.* (2019) 66:1709–17. doi: 10.1111/tbed.13206
- Barker CM, Niu T, Reisen WK, Hartley DM. Data-driven modeling to assess receptivity for Rift Valley fever virus. *PLoS Negl Trop Dis.* (2013) 7:e2515. doi: 10.1371/journal.pntd.0002515
- Nabeshima T, Loan HTK, Inoue S, Sumiyoshi M, Haruta Y, Nga PT, et al. Evidence of frequent introductions of Japanese encephalitis virus from south-east Asia and continental east Asia to Japan. *J Gen Virol.* (2009) 90:827–32. doi: 10.1099/vir.0.007617-0
- Nga PT, del Carmen Parquet M, Cuong VD, Ma SP, Hasebe F, Inoue S, et al. Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from Southeast Asia to East Asia. *J Gen Virol.* (2004) 85:1625–31. doi: 10.1099/vir.0.79797-0
- Yoshikawa A, Nabeshima T, Inoue S, Agoh M, Morita K. Molecular and serological epidemiology of Japanese encephalitis virus (JEV) in a remote island of western Japan: an implication of JEV migration over the East China Sea. *Trop Med Health.* (2016) 44:8. doi: 10.1186/s41182-016-0010-0
- Ritchie SA, Rochester W. Wind-blown mosquitoes and introduction of Japanese encephalitis into Australia. *Emerging Infect Dis.* (2001) 7:900–3. doi: 10.3201/eid0705.017524
- Solomon T, Thao TT, Lewthwaite P, Ooi MH, Kneen R, Dung NM, et al. A cohort study to assess the new WHO Japanese encephalitis surveillance standards. *Bull World Health Organ.* (2008) 86:178–86. doi: 10.2471/BLT.07.043307
- Takashima I, Watanabe T, Ouchi N, Hashimoto N. Ecological studies of Japanese encephalitis virus in Hokkaido: interepidemic outbreaks of swine abortion and evidence for the virus to overwinter locally. *Am J Trop Med Hyg.* (1988) 38:420–7. doi: 10.4269/ajtmh.1988.38.420
- Oliveira ARS, Piaggio J, Cohnstaedt LW, McVey DS, Cernicchiaro N. Introduction of the Japanese encephalitis virus (JEV) in the United States—a qualitative risk assessment. *Transbound Emerg Dis.* (2019) 66:1558–74. doi: 10.1111/tbed.13181
- Huang Y-JS, Hettenbach SM, Park SL, Higgs S, Barrett ADT, Hsu W-W, et al. Differential infectivities among different Japanese encephalitis virus genotypes in culex quinquefasciatus mosquitoes. *PLoS Negl Trop Dis.* (2016) 10:e0005038. doi: 10.1371/journal.pntd.0005038
- Huang Y-JS, Harbin JN, Hettenbach SM, Maki E, Cohnstaedt LW, Barrett ADT, et al. Susceptibility of a North American culex quinquefasciatus to Japanese encephalitis virus. *Vector Borne Zoonotic Dis.* (2015) 15:709–11. doi: 10.1089/vbz.2015.1821
- Li X, Tian K. African swine fever in China. *Vet Rec.* (2018) 183:300–1. doi: 10.1136/vr.k3774
- Oura C. African swine fever virus: on the move and dangerous. *Vet Rec.* (2013) 173:243–5. doi: 10.1136/vr.f5327
- Zhao D, Liu R, Zhang X, Li F, Wang J, Zhang J, et al. Replication and virulence in pigs of the first African swine fever virus isolated in China. *Emerg Microbes Infect.* (2019) 8:438–47. doi: 10.1080/22221751.2019.1590128
- Zhou X, Li N, Luo Y, Liu Y, Miao F, Chen T, et al. Emergence of African swine fever in China, 2018. *Transbound Emerg Dis.* (2018) 65:1482–4. doi: 10.1111/tbed.12989
- Golnar ME, Wormington JD, Kading RC, Teel PD, Hamer SA, Hamer GL. Reviewing the potential vectors and hosts of African swine fever virus transmission in the United States. *Vector Borne Zoonotic Dis.* (2019) 19:512–24. doi: 10.1089/vbz.2018.2387
- Herrera-Ibatá DM, Martínez-López B, Quijada D, Burton K, Mur L. Quantitative approach for the risk assessment of African swine fever and Classical swine fever introduction into the United States through legal imports of pigs and swine products. *PLoS ONE.* (2017) 12:e0182850. doi: 10.1371/journal.pone.0182850
- Wormington JD, Golnar A, Poh KC, Kading RC, Martin E, Hamer SA, et al. Risk of African swine fever virus sylvatic establishment and spillover to domestic swine in the United States. *Vector Borne Zoonotic Dis.* (2019) 19:506–11. doi: 10.1089/vbz.2018.2386
- NPPC. *National Pork Producers Council Cancels 2019 World Pork Expo as Precaution*. (2019). Available online at: <https://nppc.org/nppc-cancels-2019-world-pork-expo-as-precaution/> (accessed November 13, 2019).
- Anyamba A, Linthicum KJ, Tucker CJ. Climate-disease connections: Rift Valley fever in Kenya. *Cad Saude Publ.* (2001) 17:133–40. doi: 10.1590/S0102-311X2001000700022
- USDA. *The Rift Valley Fever Monitor*. (2015). Available online at: <https://www.ars.usda.gov/southeast-area/gainesville-fl/center-for-medical-agricultural-and-veterinary-entomology/docs/rvf-monthlyupdates/> (accessed August 8, 2019).
- Linthicum KJ, Anyamba A, Tucker CJ, Kelley PW, Myers MF, Peters CJ. Climate and satellite indicators to forecast Rift Valley fever epidemics in Kenya. *Science.* (1999) 285:397–400. doi: 10.1126/science.285.5426.397
- Joint FAO-WHO Experts Consultation. *Rift Valley Fever Outbreaks Forecasting Models*. Rome: World Health Organization (2008).
- AU-IBAR. *Standard Methods and Procedures (SMPs) for Control of Rift Valley Fever (RVF) in the Greater Horn of Africa*. Nairobi: AU-IBAR (2014).
- Mariner J. *Rift Valley Fever Surveillance*. FAO Animal Production and Health Manual No. 21. Rome: Food and Agriculture Organization of the United Nations (FAO) (2018).

40. USDA. *Disease Response Strategy: Rift Valley Fever. Foreign Animal Disease Preparedness and Response Plan*. Riverdale, MD: United States Department of Agriculture; Animal Plant Health Inspection Service, Veterinary Services (2013).
41. Mansfield KL, Hernández-Triana LM, Banyard AC, Fooks AR, Johnson N. Japanese encephalitis virus infection, diagnosis and control in domestic animals. *Vet Microbiol.* (2017) 201:85–92. doi: 10.1016/j.vetmic.2017.01.014
42. WHO. *WHO-Recommended Standards for Surveillance of Selected Vaccine-Preventable Diseases*. WHO/V&B/03.01. Geneva: World Health Organization (2003).
43. WHO. *WHO/Japanese Encephalitis*. WHO (2007). Available online at: http://www.who.int/immunization/diseases/japanese_encephalitis/en/ (accessed July 16, 2019).
44. OIE. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. (2012). Available online at: <http://www.oie.int/standard-setting/terrestrial-manual/access-online/> (accessed July 16, 2019).
45. USDA. *Disease Response Strategy: African Swine Fever*. Riverdale, MD: United States Department of Agriculture; Animal Plant Health Inspection Service, Veterinary Services (2019).
46. Rotolo M, Beemer O, Antognolio C, Hsi D. *Swine Hemorrhagic Fevers: African and Classical Swine Fever. An Integrated Surveillance Plan*. Fort Collins, CO: United States Department of Agriculture; Animal Plant Health Inspection Service, Veterinary Services - Center for Epidemiology and Animal Health-Surveillance Design and Analysis Unit (2019).
47. Ikegami T, Makino S. Rift valley fever vaccines. *Vaccine*. (2009) 27:D69–72. doi: 10.1016/j.vaccine.2009.07.046
48. Bird BH, Maartens LH, Campbell S, Erasmus BJ, Erickson BR, Dodd KA, et al. Rift Valley fever virus vaccine lacking the NSs and NSm genes is safe, nonteratogenic, and confers protection from viremia, pyrexia, and abortion following challenge in adult and pregnant sheep. *J Virol.* (2011) 85:12901–9. doi: 10.1128/JVI.06046-11
49. Bird BH, Albariño CG, Hartman AL, Erickson BR, Ksiazek TG, Nichol ST. Rift valley fever virus lacking the NSs and NSm genes is highly attenuated, confers protective immunity from virulent virus challenge, and allows for differential identification of infected and vaccinated animals. *J Virol.* (2008) 82:2681–91. doi: 10.1128/JVI.02501-07
50. CEPI. *CEPI Awards Funding Agreement Worth Up to US\$9.5 Million to Colorado State University to Develop a Human Vaccine Against Rift Valley Fever*. CEPI (2019). Available online at: https://cepi.net/news_cepi/cepi-awards-funding-agreement-worth-up-to-us9-5-million-to-colorado-state-university-to-develop-a-human-vaccine-against-rift-valley-fever/ (accessed November 13, 2019).
51. Wilson WC, Drolet BS. *Project: USDA ARS*. (2013). Available online at: <https://www.ars.usda.gov/research/project/?accnNo=418710&showpars=true&fy=2011> (accessed July 16, 2019).
52. McElroy AK, Albariño CG, Nichol ST. Development of a RVFV ELISA that can distinguish infected from vaccinated animals. *Virol J.* (2009) 6:125. doi: 10.1186/1743-422X-6-125
53. Britch SC, Linthicum KJ. Developing a research agenda and a comprehensive national prevention and response plan for Rift Valley fever in the United States. *Emerg Infect Dis.* (2007) 13:e1. doi: 10.3201/eid1308.070551
54. MacPherson J. *Air Force Reserve Bombs Mosquitoes in North Dakota Posted on July 4*. missoulain.com (2009). Available online at: https://missoulain.com/news/state-and-regional/air-force-reserve-bombs-mosquitoes-in-north-dakotaposted-on-july/article_185bff1d-5283-5bee-bd15-673fa5ef67cc.html (accessed August 16, 2019).
55. Skoda SR, Phillips PL, Welch JB. Screwworm (Diptera: Calliphoridae) in the United States: response to and elimination of the 2016–2017 outbreak in Florida. *J Med Entomol.* (2018) 55:777–86. doi: 10.1093/jme/tjy049
56. Mansfield KL, Banyard AC, McElhinney L, Johnson N, Horton DL, Hernández-Triana LM, et al. Rift Valley fever virus: a review of diagnosis and vaccination, and implications for emergence in Europe. *Vaccine*. (2015) 33:5520–31. doi: 10.1016/j.vaccine.2015.08.020
57. van Vuren PJ, Paweska JT. Comparison of enzyme-linked immunosorbent assay-based techniques for the detection of antibody to Rift Valley fever virus in thermochemically inactivated sheep sera. *Vector Borne Zoonotic Dis.* (2010) 10:697–9. doi: 10.1089/vbz.2009.0213
58. Kim H-J, Nah J-J, Moon J-S, Ko Y-J, Yoo H-S, Kweon C-H. Competitive ELISA for the detection of antibodies to Rift Valley fever virus in goats and cattle. *J Vet Med Sci.* (2012) 74:321–7. doi: 10.1292/jvms.11-0259
59. Hossain MM, Wilson WC, Faburay B, Richt J, McVey DS, Rowland RR. Multiplex detection of IgG and IgM to Rift Valley fever virus nucleoprotein, nonstructural proteins, and glycoprotein in ovine and bovine. *Vector Borne Zoonotic Dis.* (2016) 16:550–7. doi: 10.1089/vbz.2014.1721
60. Cêtre-Sossah C, Pédarrieu A, Juremalm M, Vuren PJV, Brun A, Mamy ABOE, et al. Development and validation of a pen side test for Rift Valley fever. *PLoS Negl Trop Dis.* (2019) 13:e0007700. doi: 10.1371/journal.pntd.0007700
61. O'Hearn AE, Voorhees MA, Fetterer DP, Wauquier N, Coomber MR, Bangura J, et al. Serosurveillance of viral pathogens circulating in West Africa. *Virol J.* (2016) 13:163. doi: 10.1186/s12985-016-0621-4
62. Wu W, Zhang S, Qu J, Zhang Q, Li C, Li J, et al. Simultaneous detection of IgG antibodies associated with viral hemorrhagic fever by a multiplexed Luminex-based immunoassay. *Virus Res.* (2014) 187:84–90. doi: 10.1016/j.virusres.2013.12.037
63. Bird BH, Bawiec DA, Ksiazek TG, Shoemaker TR, Nichol ST. Highly sensitive and broadly reactive quantitative reverse transcription-PCR assay for high-throughput detection of Rift Valley fever virus. *J Clin Microbiol.* (2007) 45:3506–13. doi: 10.1128/JCM.00936-07
64. Wilson WC, Romito M, Jaspersen DC, Weingartl H, Binopal YS, Maluleke MR, et al. Development of a Rift Valley fever real-time RT-PCR assay that can detect all three genome segments. *J Virol Methods.* (2013) 193:426–31. doi: 10.1016/j.jviromet.2013.07.006
65. Euler M, Wang Y, Nentwich O, Piepenburg O, Hufert FT, Weidmann M. Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus. *J Clin Virol.* (2012) 54:308–12. doi: 10.1016/j.jcv.2012.05.006
66. Shafagati N, Narayanan A, Baer A, Fite K, Pinkham C, Bailey C, et al. The use of NanoTrap particles as a sample enrichment method to enhance the detection of Rift Valley Fever Virus. *PLoS Negl Trop Dis.* (2013) 7:e2296. doi: 10.1371/journal.pntd.0002296
67. Fajfr M, Neubauerová V, Pajer P, Kubičková P, Ružek D. Detection panel for identification of twelve hemorrhagic viruses using real-time RT-PCR. *Epidemiol Mikrobiol Immunol.* (2014) 63:238–44.
68. Liu J, Ochieng C, Wiersma S, Ströher U, Towner JS, Whitmer S, et al. Development of a TaqMan array card for acute-febrile-illness outbreak investigation and surveillance of emerging pathogens, including ebola virus. *J Clin Microbiol.* (2016) 54:49–58. doi: 10.1128/JCM.02257-15
69. Martin E, Borucki MK, Thissen J, Garcia-Luna S, Hwang M, Wise de Valdez M, et al. Adaptation of a microbial detection array as a monitoring tool revealed the presence of mosquito-borne viruses and insect-specific viruses in field-collected mosquitoes. *Appl Environ Microbiol.* (2019) 85:e01202–19. doi: 10.1128/AEM.01202-19
70. Drolet BS, Weingartl HM, Jiang J, Neufeld J, Marszal P, Lindsay R, et al. Development and evaluation of one-step rRT-PCR and immunohistochemical methods for detection of Rift Valley fever virus in biosafety level 2 diagnostic laboratories. *J Virol Methods.* (2012) 179:373–82. doi: 10.1016/j.jviromet.2011.11.025
71. Tiwari S, Singh RK, Tiwari R, Dhole TN. Japanese encephalitis: a review of the Indian perspective. *Braz J Infect Dis.* (2012) 16:564–73. doi: 10.1016/j.bjid.2012.10.004
72. Scherer WF, Buescher EL. Ecologic studies of Japanese encephalitis virus in Japan. I Introduction. *Am J Trop Med Hyg.* (1959) 8:644–50. doi: 10.4269/ajtmh.1959.8.644
73. Sugiura T, Shimada K. Seroepizootiological survey of Japanese encephalitis virus and Getah virus in regional horse race tracks from 1991 to 1997 in Japan. *J Vet Med Sci.* (1999) 61:877–81. doi: 10.1292/jvms.61.877
74. Chen WR, Tesh RB, Rico-Hesse R. Genetic variation of Japanese encephalitis virus in nature. *J Gen Virol.* (1990) 71:2915–22. doi: 10.1099/0022-1317-71-12-2915
75. Zanin MP, Webster DE, Martin JL, Wesselingh SL. Japanese encephalitis vaccines: moving away from the mouse brain. *Expert Rev Vaccines.* (2003) 2:407–16. doi: 10.1586/14760584.2.3.407

76. Nah J-J, Yang D-K, Kim H-H, Song J-Y. The present and future of veterinary vaccines for Japanese encephalitis in Korea. *Clin Exp Vaccine Res.* (2015) 4:130–6. doi: 10.7774/cevr.2015.4.2.130
77. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB. Phylogeny of the genus *Flavivirus*. *J Virol.* (1998) 72:73–83.
78. Pham TT, Meng S, Sun Y, Lv W, Bahl J. Inference of Japanese encephalitis virus ecological and evolutionary dynamics from passive and active virus surveillance. *Virus Evol.* (2016) 2:vev009. doi: 10.1093/ve/vev009
79. Cappelle J, Duong V, Pring L, Kong L, Yakovlev M, Prasetyo DB, et al. Intensive circulation of Japanese encephalitis virus in peri-urban sentinel pigs near Phnom Penh, Cambodia. *PLoS Negl Trop Dis.* (2016) 10:e0005149. doi: 10.1371/journal.pntd.0005149
80. Ricklin ME, García-Nicolás O, Brechbühl D, Python S, Zumkehr B, Nougairé A, et al. Vector-free transmission and persistence of Japanese encephalitis virus in pigs. *Nat Commun.* (2016) 7:10832. doi: 10.1038/ncomms10832
81. Yap G, Lim XF, Chan S, How CB, Humaidi M, Yeo G, et al. Serological evidence of continued Japanese encephalitis virus transmission in Singapore nearly three decades after end of pig farming. *Parasit Vectors.* (2019) 12:244. doi: 10.1186/s13071-019-3501-0
82. Buckley A, Dawson A, Gould EA. Detection of seroconversion to West Nile virus, Usutu virus and Sindbis virus in UK sentinel chickens. *Virol J.* (2006) 3:71. doi: 10.1186/1743-422X-3-71
83. Bundo K, Igarashi A. Antibody-capture ELISA for detection of immunoglobulin M antibodies in sera from Japanese encephalitis and dengue hemorrhagic fever patients. *J Virol Methods.* (1985) 11:15–22. doi: 10.1016/0166-0934(85)90120-X
84. Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, Chongswasdi V, Suntayakorn S, et al. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. *Am J Trop Med Hyg.* (1989) 40:418–27. doi: 10.4269/ajtmh.1989.40.418
85. Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, et al. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol.* (1989) 70:37–43. doi: 10.1099/0022-1317-70-1-37
86. Johnson BW, Goodman CH, Jee Y, Featherstone DA. Differential diagnosis of Japanese encephalitis virus infections with the inbios JE Detect™ and DEN Detect™ MAC-ELISA Kits. *Am J Trop Med Hyg.* (2016) 94:820–8. doi: 10.4269/ajtmh.15-0631
87. Cleton NB, van Maanen K, Bergervoet SA, Bon N, Beck C, Godeke G-J, et al. A serological protein microarray for detection of multiple cross-reactive flavivirus infections in horses for veterinary and public health surveillance. *Transbound Emerg Dis.* (2017) 64:1801–12. doi: 10.1111/tbed.12569
88. Pan J, Yan J, Zhou J, Tang X, He H, Xie R, et al. Sero-molecular epidemiology of Japanese encephalitis in Zhejiang, an Eastern Province of China. *PLoS Negl Trop Dis.* (2016) 10:e0004936. doi: 10.1371/journal.pntd.0004936
89. Chu H, Wu Z, Chen H, Li C, Guo X, Liu R, et al. Japanese encephalitis virus infection rate and detection of genotype I from *Culex tritaeniorhynchus* collected from Jiangsu, China. *Vector Borne Zoonotic Dis.* (2017) 17:503–9. doi: 10.1089/vbz.2016.2086
90. Tanaka M. Rapid identification of flavivirus using the polymerase chain reaction. *J Virol Methods.* (1993) 41:311–22. doi: 10.1016/0166-0934(93)90020-R
91. Wang Y, Ostlund EN, Jun Y, Nie F-P, Li Y-G, Johnson DJ, et al. Combining reverse-transcription multiplex PCR and microfluidic electrophoresis to simultaneously detect seven mosquito-transmitted zoonotic encephalomyelitis viruses. *Vet J.* (2016) 212:27–35. doi: 10.1016/j.tvjl.2015.10.040
92. Chenais E, Depner K, Guberti V, Dietze K, Viltrop A, Ståhl K. Epidemiological considerations on African swine fever in Europe 2014–2018. *Porcine Health Manag.* (2019) 5:6. doi: 10.1186/s40813-018-0109-2
93. Rowlands RJ, Michaud V, Heath L, Hutchings G, Oura C, Vosloo W, et al. African swine fever virus isolate, Georgia, 2007. *Emerging Infect Dis.* (2008) 14:1870–4. doi: 10.3201/eid1412.080591
94. Costard S, Wieland B, de Glanville W, Jori F, Rowlands R, Vosloo W, et al. African swine fever: how can global spread be prevented? *Philos Trans R Soc Lond B Biol Sci.* (2009) 364:2683–96. doi: 10.1098/rstb.2009.0098
95. Sánchez-Vizcaino JM, Mur L, Bastos ADS, Penrith ML. New insights into the role of ticks in African swine fever epidemiology. *Rev Off Int Epizoot.* (2015) 34:503–11. doi: 10.20506/rst.34.2.2375
96. Díaz-Martín V, Manzano-Román R, Siles-Lucas M, Oleaga A, Pérez-Sánchez R. Cloning, characterization and diagnostic performance of the salivary lipocalin protein TSGP1 from *Ornithodoros moubata*. *Vet Parasitol.* (2011) 178:163–72. doi: 10.1016/j.vetpar.2010.12.014
97. Carlson J, Zani L, Schwaiger T, Nurmoja I, Viltrop A, Vilem A, et al. Simplifying sampling for African swine fever surveillance: assessment of antibody and pathogen detection from blood swabs. *Transbound Emerg Dis.* (2018) 65:e165–e172. doi: 10.1111/tbed.12706
98. Petrov A, Schotte U, Pietschmann J, Dräger C, Beer M, Anheyer-Behnenburg H, et al. Alternative sampling strategies for passive classical and African swine fever surveillance in wild boar. *Vet Microbiol.* (2014) 173:360–5. doi: 10.1016/j.vetmic.2014.07.030
99. Mur L, Gallardo C, Soler A, Zimmermann J, Pelayo V, Nieto R, et al. Potential use of oral fluid samples for serological diagnosis of African swine fever. *Vet Microbiol.* (2013) 165:135–9. doi: 10.1016/j.vetmic.2012.12.034
100. Fernández-Carrión E, Martínez-Avilés M, Ivorra B, Martínez-López B, Ramos AM, Sánchez-Vizcaino JM. Motion-based video monitoring for early detection of livestock diseases: The case of African swine fever. *PLoS ONE.* (2017) 12:e0183793. doi: 10.1371/journal.pone.0183793
101. Martínez-Avilés M, Fernández-Carrión E, López García-Baones JM, Sánchez-Vizcaino JM. Early detection of infection in pigs through an online monitoring system. *Transbound Emerg Dis.* (2017) 64:364–73. doi: 10.1111/tbed.12372
102. Gallardo C, Nieto R, Soler A, Pelayo V, Fernández-Pinero J, Markowska-Daniel I, et al. Assessment of African swine fever diagnostic techniques as a response to the epidemic outbreaks in Eastern European Union countries: how to improve surveillance and control programs. *J Clin Microbiol.* (2015) 53:2555–65. doi: 10.1128/JCM.00857-15
103. Abworo EO, Onzere C, Oluoch Amimo J, Riitho V, Mwangi W, Davies J, et al. Detection of African swine fever virus in the tissues of asymptomatic pigs in smallholder farming systems along the Kenya-Uganda border: implications for transmission in endemic areas and ASF surveillance in East Africa. *J Gen Virol.* (2017) 98:1806–14. doi: 10.1099/jgv.0.000848
104. Bellini S, Rutili D, Guberti V. Preventive measures aimed at minimizing the risk of African swine fever virus spread in pig farming systems. *Acta Vet Scand.* (2016) 58:82. doi: 10.1186/s13028-016-0264-x
105. Arias M, Jurado C, Gallardo C, Fernández-Pinero J, Sánchez-Vizcaino JM. Gaps in African swine fever: analysis and priorities. *Transbound Emerg Dis.* (2018) 65:235–47. doi: 10.1111/tbed.12695
106. Guinat C, Wall B, Dixon L, Pfeiffer DU. English pig farmers' knowledge and behaviour towards African swine fever suspicion and reporting. *PLoS ONE.* (2016) 11:e0161431. doi: 10.1371/journal.pone.0161431
107. USDA. USDA APHIS/NAHLN Surveillance and Preparedness. (2016). Available online at: https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/lab-info-services/nahln/ct_surveillance_efforts (accessed September 15, 2019).
108. Gallardo C, Fernández-Pinero J, Arias M. African swine fever (ASF) diagnosis, an essential tool in the epidemiological investigation. *Virus Res.* (2019) 271:197676. doi: 10.1016/j.virusres.2019.197676
109. Okoth E, Gallardo C, Macharia JM, Omere A, Pelayo V, Bulimo DW, et al. Comparison of African swine fever virus prevalence and risk in two contrasting pig-farming systems in South-west and Central Kenya. *Prev Vet Med.* (2013) 110:198–205. doi: 10.1016/j.prevetmed.2012.11.012
110. Giménez-Lirola LG, Mur L, Rivera B, Mogler M, Sun Y, Lizano S, et al. Detection of African swine fever virus antibodies in serum and oral fluid specimens using a recombinant protein 30 (p30) dual matrix indirect ELISA. *PLoS ONE.* (2016) 11:e0161230. doi: 10.1371/journal.pone.0161230
111. Agüero M, Fernández J, Romero L, Sánchez Mascaraque C, Arias M, Sánchez-Vizcaino JM. Highly sensitive PCR assay for routine diagnosis of African swine fever virus in clinical samples. *J Clin Microbiol.* (2003) 41:4431–4. doi: 10.1128/JCM.41.9.4431-4434.2003

112. King DP, Reid SM, Hutchings GH, Grierson SS, Wilkinson PJ, Dixon LK, et al. Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus. *J Virol Methods*. (2003) 107:53–61. doi: 10.1016/S0166-0934(02)00189-1
113. Fernández-Pinero J, Gallardo C, Elizalde M, Robles A, Gómez C, Bishop R, et al. Molecular diagnosis of African swine fever by a new real-time PCR using universal probe library. *Transbound Emerg Dis*. (2013) 60:48–58. doi: 10.1111/j.1865-1682.2012.01317.x
114. Luo Y, Atim SA, Shao L, Ayebazibwe C, Sun Y, Liu Y, et al. Development of an updated PCR assay for detection of African swine fever virus. *Arch Virol*. (2017) 162:191–9. doi: 10.1007/s00705-016-3069-3
115. Wozniakowski G, Fraczyk M, Kowalczyk A, Pomorska-Mól M, Niemczuk K, Pejsak Z. Polymerase cross-linking spiral reaction (PCLSR) for detection of African swine fever virus (ASFV) in pigs and wild boars. *Sci Rep*. (2017) 7:42903. doi: 10.1038/srep42903
116. Grau FR, Schroeder ME, Mulhern EL, McIntosh MT, Bounpheng MA. Detection of African swine fever, classical swine fever, and foot-and-mouth disease viruses in swine oral fluids by multiplex reverse transcription real-time polymerase chain reaction. *J Vet Diagn Invest*. (2015) 27:140–9. doi: 10.1177/1040638715574768
117. Callahan JD, Brown F, Osorio FA, Sur JH, Kramer E, Long GW, et al. Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *J Am Vet Med Assoc*. (2002) 220:1636–42. doi: 10.2460/javma.2002.220.1636
118. Eberling AJ, Bieker-Stefanelli J, Reising MM, Siev D, Martin BM, McIntosh MT, et al. Development, optimization, and validation of a Classical swine fever virus real-time reverse transcription polymerase chain reaction assay. *J Vet Diagn Investig*. (2011) 23:994–8. doi: 10.1177/1040638711416970
119. Zsak L, Borca MV, Risatti GR, Zsak A, French RA, Lu Z, et al. Preclinical diagnosis of African swine fever in contact-exposed swine by a real-time PCR assay. *J Clin Microbiol*. (2005) 43:112–9. doi: 10.1128/JCM.43.1.112-119.2005
120. Haines FJ, Hofmann MA, King DP, Drew TW, Crooke HR. Development and validation of a multiplex, real-time RT PCR assay for the simultaneous detection of classical and African swine fever viruses. *PLoS ONE*. (2013) 8:e71019. doi: 10.1371/journal.pone.0071019

Disclaimer: The viewpoints described herein represent those of the authors and not necessarily their institutions or funding agency.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Kading, Abworo and Hamer. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Potential of Using Capripoxvirus Vectored Vaccines Against Arboviruses in Sheep, Goats, and Cattle

Mahder Teffera^{1,2} and Shawn Babiuk^{1,2*}

¹ Canadian Food Inspection Agency, National Centre for Foreign Animal Disease, Winnipeg, MB, Canada, ² Department of Immunology, University of Manitoba, Winnipeg, MB, Canada

OPEN ACCESS

Edited by:

Dana Mitzel,
United States Department of
Agriculture, United States

Reviewed by:

Santhamani Ramasamy,
Albert Einstein College of Medicine,
United States

Paulo Henrique Verardi,
University of Connecticut,
United States

*Correspondence:

Shawn Babiuk
shawn.babiuk@canada.ca

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 30 September 2019

Accepted: 27 November 2019

Published: 20 December 2019

Citation:

Teffera M and Babiuk S (2019)
Potential of Using Capripoxvirus
Vectored Vaccines Against
Arboviruses in Sheep, Goats, and
Cattle. *Front. Vet. Sci.* 6:450.
doi: 10.3389/fvets.2019.00450

The genus capripoxvirus consists of sheeppox virus, goatpox virus, and lumpy skin disease virus, which affect sheep, goats, and cattle, respectively. Together capripoxviruses cause significant economic losses to the sheep, goat, and cattle industry where these diseases are present. These diseases have spread into previously free bordering regions most recently demonstrated with the spread of lumpy skin disease virus into the Middle East, some Eastern European countries, and Russia. This recent spread has highlighted the transboundary nature of these diseases. To control lumpy skin disease virus, live attenuated viral vaccines are used in endemic countries as well as in response to an outbreak. For sheeppox and goatpox, live attenuated viral vaccines are used in endemic countries; these diseases can also be contained through slaughter of infected animals to stamp out the disease. The thermostability, narrow host range, and ability of capripoxviruses to express a wide variety of antigens make capripoxviruses ideal vectors. The ability to immunize animals against multiple diseases simultaneously increases vaccination efficiency by decreasing the number of vaccinations required. Additionally, the use of capripoxvirus vectored vaccines allows the possibility of differentiating infected from vaccinated animals. Arboviruses such as bluetongue virus and Rift Valley fever viruses are also responsible for significant economic losses in endemic countries. In the case of Rift Valley fever virus, vaccination is not routinely practiced unless there is an outbreak making vaccination not as effective, therefore, incorporating Rift Valley fever vaccination into routine capripoxvirus vaccination would be highly beneficial. This review will discuss the potential of using capripoxvirus as a vector expressing protective arboviral antigens.

Keywords: capripoxviruses, Rift Valley fever, bluetongue, vaccine, arboviruses, vector

CAPRIPOXVIRUSES

Capripoxviruses represent a genus of the poxviridae family under the subfamily chordopoxvirinae; the genus includes three animal virus species that have a devastating impact on sheep, goats, and cattle in Africa, Asia, and most recently Eastern Europe (1–4). The viruses in the genus are sheeppox virus (SPPV), goatpox virus (GTPV), and lumpy skin disease virus (LSDV) which affect sheep, goats, and cattle, respectively (1, 4). Capripoxviruses share 98% sequence similarity between

all three species; 147 putative genes are shared between goatpox and sheepox while lumpy skin disease virus has nine additional genes which are not functional in SPPV and GTPV (2).

It is believed that SPPV was first reported in the second century in central Asia before spreading to surrounding countries and Europe (5, 6). SPPV/GTPV are endemic in a large portion of the world [North and central Africa, the Middle East, Indian subcontinent, Southwest and central Asia (7)]. Outbreaks of sheep and goatpox can occur in new regions bordering endemic regions as illustrated by outbreaks in Mongolia and Vietnam (8). The transmission of sheep and goatpox can occur via aerosol, contact with contaminated material such as bedding, direct contact between infected animals (1, 9). Historically LSDV is a relatively new disease first described in 1929 originating in sub-Saharan Africa, where it has spread into most regions of Africa (10) and was historically thought of a disease affecting only Africa. Unfortunately, lumpy skin disease has expanded its geographic range out of Africa into the Middle East to Eastern Europe and Asia (7, 11). Most recently, LSDV spread in the Balkans including, Greece, Bulgaria, Serbia, Kosovo, Albania, Montenegro, the Caucasus Region including Russia, and Asian countries of Kazakhstan and recently China (11, 12). The rapid spread of LSDV into previously free regions is cause for concern, since if not effectively dealt with through using mass vaccination with an effective vaccine, LSDV will spread into bordering regions through either animal movement or dispersion of insect vectors (11). Therefore, there are numerous at risk countries for LSDV outbreaks in Asia with more countries becoming endemic with sheepox, goatpox, and LSDV.

Although LSDV is not an arbovirus, insect and arthropod vectors spread the disease through mechanical transmission. The most likely vectors involved in transmission of LSDV are stable flies, mosquitos and hard ticks (13, 14). Since capripoxviruses have a tissue tropism for epithelial tissue, this allows transmission of the virus by insect or arthropod vectors to be efficient in the absence of replication in the vector (15). Capripoxviruses cause severe production losses and are world organization for animal health (OIE) listed diseases (2, 5, 16–19). LSDV also has an additional effect on lactation causing decreased milk production as well as temporary and permanent infertility (7, 19). SPPV and GTPV are associated with a relatively high morbidity and mortality (16), while LSDV is usually associated with a high morbidity and low mortality rates ranging between 1 and 5% (17, 19). The damage and loss caused by capripoxvirus on small ruminants and cattle causes substantial economic loss due to trade restrictions, limitations on movement of animals, and co-ordination and implementation of vaccination campaigns (19). This not only affects countries which rely on export of small ruminants and cattle and by products but it also impacts small scale farmers and pastoral societies whose livelihood is directly affected by the survival of their herds (5, 19). Control of sheep and goatpox can be achieved through slaughter of infected animals. Unfortunately with LSDV slaughter is not effective and can only be achieved using live attenuated vaccines (1, 2, 17); illustrated by mass vaccination of cattle in Eastern Europe where vaccination has eliminated clinical disease (6, 12).

CURRENTLY USED VACCINES FOR CONTROL OF CAPRIPOXVIRUSES

The most effective and widely used vaccines against capripoxviruses are live attenuated vaccines (19). These live attenuated vaccines are generated by passaging field isolated viruses serially in tissue culture and/or eggs until attenuation is achieved (9, 20). An example of a commonly used vaccine is one developed in 1997 by Precausta et al. (20) which is a Romanian SPPV vaccine developed through passaging in lamb kidney cells 30 times until attenuated. This vaccine demonstrated protection against disease and generation of neutralizing serum antibodies (9, 20). The vaccine is a freeze-dried vaccine without an adjuvant and can be stored for 2 years at 6 degrees allowing for flexibility in storage and production (9). There are numerous live attenuated capripoxvirus vaccines which are used in the field reviewed by Tuppurainen et al. (21). The close antigenic relation between sheepox, goatpox, and lumpy skin disease in theory allows a single vaccine to protect against all members. However, sheepox virus based vaccines do not seem to protect cattle against lumpy skin disease virus. There have also been reported cases where vaccination with the RM65 strain of sheepox virus did not elicit complete protection against LSDV (22). For this reason, capripoxvirus vaccines require evaluation in all animal species to ensure they are efficacious.

Due to regulatory issues related to trade, preventative vaccinations against capripoxviruses are not in use in disease free countries (2, 19). In South Africa, sheepox, and goatpox vaccines are not used, instead licensed attenuated LSDV vaccines such as the OBP LSDV vaccine have been demonstrated to be safe for use and elicit long-term immunity in immunized animals (23). In other regions of Africa that are affected by all three capripoxviruses, several different capripoxvirus vaccines are used (7).

ARBOVIRUSES

Arboviruses are a diverse group of arthropod-borne viruses that are able to replicate in arthropods and vertebrate hosts (24–26). Arboviruses are classified based on their transmission cycle and consist of a variety of RNA and DNA viruses (25). The transmission of arboviruses through arthropods occurs by an injection of an infected blood meal followed by replication of the virus in the arthropod. Viral replication occurs specifically in the salivary glands, allowing transmission to a vertebrate host; after which the infected host will most likely become viremic, a period that can last from 2 days to over a week (24–26). The ability of Arboviruses to remain in circulation is due to the maintenance of a reservoir cycle by both types of hosts (arthropod and vertebrate), which are equally necessary (24, 26). Horizontal transmission of arboviruses occurs through bites and vertical transmission through eggs (24). The main arboviral viruses affecting trade in sheep, goats, and cattle are Rift Valley fever virus and bluetongue virus.

BLUETONGUE VIRUS

Bluetongue virus (BTV) is a virus in the family reoviridae under the genus orbivirus that causes bluetongue disease, an OIE listed hemorrhagic disease, in wild and domestic ruminants (27–30). BTV is a non-enveloped segmented double stranded RNA virus with five core proteins surrounded by a triple layered icosahedral capsid made up of two major proteins (28, 29). BTV is responsible for a significant damage of ruminant populations and the associated economic loss in countries where it is endemic (30–32). *Culicoides* midges exclusively transmit BTV to ruminants (33, 34). In sheep, clinical signs of disease are fever, nasal discharge, drooling, facial edema, and muscle weakness, accompanied by viremia (27, 35, 36). Animals surviving acute infection still remain at risk for long-term effects such as chronic dermatitis and the presence of lesions at mucosal and inter-digital surfaces (37). Mortality rates of BTV vary significantly between outbreaks; these outbreaks occur due to integration of susceptible sheep breeds into BTV endemic areas or through the spread of virus to BTV free sheep from infected sheep in areas between endemic and non-endemic areas (34). All ruminants are susceptible to BTV; however, European breeds of sheep are usually more severely affected (34). While disease is generally associated with sheep, BTV is also able to infect cattle asymptotically; despite the disease's asymptomatic nature, IgE mediated hypersensitivity can occur in cattle (27, 38). In fact, it has been observed that insect vectors of BTV prefer to feed on cattle leading to a hypothesis that the virus reservoir is maintained by a cycle of infection going from vector to cattle (27, 39, 40).

There are 29 BTV serotypes that have been characterized to date, with different serotypes distributed among different continents, including Africa, Asia, Europe, the Americas; BTV was most recently detected in Australia in 2017 (30). There is also diversity observed within the same serotype in which viruses of a single serotype undergo genetic drift as a result of mutations and re-assortment of gene segments (30, 41, 42). Since 1988, there have been numerous BTV outbreaks in Europe which resulted in widespread vaccination campaigns to stop the spread of the disease; prior to 1988 there were only sporadic outbreaks in Mediterranean countries (41, 43, 44). Climate change is likely responsible for the rapid spread of BTV globally due to increasing vectoral capability of *Culicoides* midges (45). The rapid spread of BTV and the emergence of new strains throughout the years is cause for concern and greatly impacts approaches to vaccination and surveillance. Low levels of cross-protection have been observed between different serotypes making vaccination strategies even more difficult (36).

VACCINES USED AGAINST BLUETONGUE VIRUS

Two types of vaccines against BTV in use are modified-live virus (MLV) vaccines or inactivated vaccines, neither of which is available for all serotypes of BTV (39, 46). MLV BTV vaccines are attenuated by passage in embryonated chicken eggs and/or

tissue culture (28, 47, 48). MLV vaccines developed in South Africa are widely used in the control of BTV and its spread in Africa (38). After the re-introduction of BTV in Europe, MLV vaccines were used to vaccinate sheep despite the risks involved with re-assortment (43). Modified-live virus vaccines generally provide a good protection and are relatively inexpensive to manufacture, however, they can result in clinical signs and side effects along with the possibility of re-assortment with genes of wild type virus (38, 41). The negative effects associated with MLV vaccines include but are not limited to viremia, teratogenic effects, abortion, and reduced milk production (39). The possible unwanted effects of MLV vaccines along with trade restrictions due to the lack of differentiation between vaccinated and infected animals has highlighted the need for new vaccine strategies to control the spread of BTV (31, 39).

Inactivated or killed vaccines have also been commercially available to immunize against BTV. They are inactivated chemically, using heat, or through exposure to UV or gamma radiation (39, 49, 50). Inactivated virulent BTV strains have demonstrated long-term protective immunity (49). These vaccines have been used in Europe, namely in France and Italy (41). A downside to using inactivated virus vaccines is the decreased immunity generated due to lack of replication in these vaccines, requiring multiple injections to confer protective immunity (38). Inactivated vaccines are more expensive than using MLV vaccines, however, inactivated vaccines can prevent clinical disease, lower economic loss due to outbreaks, and allow for the safe trade of animals (36). Inactivated vaccines used against BTV serotype 8 were proven effective in Europe in 2006 during the emergence of the highly pathogenic virus by significantly reducing the potential economic impact of an outbreak (43); however they are still not considered ideal because of cost (39). Due to the obvious downsides of vaccines currently in use against BTV, it is important to consider novel vaccination strategies to account for the presence of numerous serotypes of the virus that show diverse antigenicity; a secondary issue that has yet to be addressed is the ability to distinguish vaccinated animals from infected ones.

Next generation BTV vaccines include recombinant vaccines (sub-unit, vectored, virus-like particles) and disabled infectious single cycle vaccines. Recombinant subunit vaccines use a specific protein expressed *in vitro* (28). Notably, immunization with purified VP2 resulted in the production of neutralizing antibodies and was able to protect experimentally infected sheep, demonstrating the utility of VP2 as a vaccine antigen (28, 51). VP5 can also induce neutralizing antibodies and the inclusion of both VP2 and VP5 in vaccination strategies has resulted in better protection of experimentally infected animals (52). Virus like particles (VLPs) for bluetongue have been generated by expression of VP2, VP3, VP5, and VP7 using baculovirus (53, 54). VLPs have the structural antigenicity of the virus without the genetic information, allowing these vaccines to have a high safety profile. A multi-serotype cocktail VLPs vaccine can protect against several serotypes (55).

Disabled infectious single cycle/animal (DISC/A) vaccines have also been developed against BTV. These vaccines generally lack an essential gene, which results in an inability to replicate in

the host cells for more than one cycle. A DISC vaccine lacking VP6, a structural protein has been successfully produced and has been experimentally shown to provide immunity against challenge in sheep (56–58). Although a much higher dose of vaccine is required to elicit protective immunity, they are a safer alternative to using MLV vaccines.

Recombinant vectored vaccines are live attenuated virus vaccines modified to express genes encoding antigens to elicit protective immunity. Many viral vectors have a limited capacity to express foreign antigens. Therefore, it is important to select the best antigen(s) to elicit protective immunity following vaccination. For BTV, it has been shown from the use of subunit vaccines that structural proteins VP2, VP5, and VP7 confer protective immunity, VP2 being the most effective (28). Multiple different viral vectors have been generated to express different BTV proteins (VP2, VP5, VP7, NS1, and NS3) including poxviruses such as vaccinia virus (52, 59), canarypox virus (60), capripox virus (31, 61), herpes virus vectors including equine herpesvirus 1 (62), bovine herpesvirus type 4 (63), adenoviruses including canine adenovirus 2 (64), and human adenovirus 5 (65) as well as vesicular stomatitis virus (43, 66). These viral vectors elicited different levels of protection against bluetongue challenge and given the different antigens expressed as well as the different BTV challenge models it is difficult to directly compare the results. Nevertheless, there is room for improvement for vectored BTV vaccines. No viral vector to date has expressed VLPs using VP2, VP5, VP3, and VP7 proteins as previously demonstrated using baculovirus or plant expression systems (67). In addition, there is no vaccine currently available to differentiate vaccinated and infected animals (DIVA). There are currently available diagnostics for BTV serology using a competitive ELISA against VP7 (68). It may be possible to identify the specific epitope interacting with the monoclonal antibody used in the test though epitope mapping and then modify the VP7 antigen to allow for a DIVA vaccine.

RIFT VALLEY FEVER VIRUS

Rift Valley fever virus (RVFV) is an enveloped segmented negative stranded RNA virus of the family Bunyaviridae, genus Phlebovirus (69, 70). It causes Rift valley fever (RVF) in livestock and humans (69, 71). Despite the presence of several lineages of RVFV, there is low genetic diversity observed with up to 99% similarity at the protein level (72).

RVFV has been responsible for devastating outbreaks throughout the African continent and has most recently been reported in the Arabian Peninsula (71, 73, 74). RVF was first described in 1931 after the infection of sheep in Kenya where close to 5,000 animals died within a month (71, 75). RVFV was endemic only in Africa and Madagascar until 2000, after when outbreaks were reported in Saudi Arabia and Yemen (73, 74). Suitable habitats for maintenance RVFV are known to be shallow depressions with the presence of wet soil or flood plains of rivers; this might explain why RVFV has only been detected in the Afrotropical region (72). Artificial interference such as irrigation and direct intervention of natural ecosystems, which modify

water flow, have also been associated with increased RVFV (76). RVFV outbreaks are generally associated with increased abortion of neonates reaching 100% and mortality rates averaging 10–20% in adult livestock (71, 75). The varying mortality rate in adult ruminants is thought to be because of differences in host genetic background. Severity of RVFV can also differ within the same breed of sheep (75). Due to the zoonotic nature of RVFV, it is a threat not only to the veterinary medical communities but the overall public health of a community (71, 73, 77).

RVFV is transmitted through an infected insect bite or direct contact of infected animal tissues and body fluids (78–80). RVFV is transmitted by mosquitoes with *Aedes* spp. being the primary vectors (77, 81, 82). RVFV can also be transmitted transovarially to offspring in mosquito vectors (73, 83), allowing maintenance of the pathogen between outbreaks (83). RVFV vectors are generally divided into maintenance or amplifying, which refer to *Aedes* spp. mosquitoes found in fresh flood and semi-permanent fresh-water or *Culex* spp. found in more permanent fresh-water (72, 77). Natural events such as rainfall and flooding increase freshwater species of mosquitoes which in turn increases the risk of RVFV outbreaks in a given area (73, 84). RVFV can infect a number of vectors and vertebrate hosts including: sheep, goats, cattle, rodents, and humans (72, 84). Although mosquitoes are considered primary vectors, other vectors such as ticks (85), midges (86), and sandflies (87) have also been reported (80). Following transmission of RVFV to a host, there is an incubation period, ranging from 24 to 36 h depending on variables such as dose, strain, route of infection, and age of animal (88). The incubation period is followed by the appearance of clinical signs which can last up to 5 days usually characterized by a high fever of over 42°C and viremia (88, 89). Based on experimental infections, RVFV infections result in severe acute lethal infection, mild infection, or delayed onset complications of infection (89–91). The liver is the primary site of lesions in RVFV infections and hepatic damage is associated with severe RVF disease (73, 89, 92) although RVFV also replicates in the spleen, kidney, lung, and skin (82, 93).

Neutralizing antibodies against the RVFV proteins can protect against disease (94). Due to the damage and economic loss associated with an RVFV outbreak, successful vaccination campaigns are necessary to prevent and lower the amount of virulent RVFV circulating in endemic countries (73, 84). Unfortunately, the cyclical nature of RVFV outbreaks leads to reduced annual vaccination as the disease is out of mind.

VACCINES USED AGAINST RIFT VALLEY FEVER VIRUS

The first vaccine developed against RVFV was a live attenuated vaccine generated from the Entebbe RVFV isolate that was attenuated by serially inoculating mice intracerebrally (75, 95, 96). This vaccine, known as the Smithburn vaccine, is partially attenuated and can cause abortions and teratogenesis following vaccination. Despite this, the Smithburn vaccine and its modified live virus variants are still in use during outbreaks in non-pregnant animals (70, 72, 75, 96). Following an outbreak in Egypt

in 1977, the United States army medical research institute of infectious diseases developed another RVFV vaccine known as MP12. This vaccine was generated using random mutagenesis of a virulent Egyptian strain (ZH548) of RVFV using 5-fluorouracil over twelve passages of the virus (46, 69, 94). The MP12 vaccine was more attenuated than the Smithburn vaccine as it had mutations in all its segments and showed no virulence when tested in mice. It also induced full protection in ruminants during experimental infection with a virulent RVFV strain (46, 94, 97, 98). MP12 evaluation trials in South Africa resulted in abortions and teratogenesis in pregnant ewes; despite this, MP12 is still under development to be used against RVFV in animals and humans (72, 99). The third live attenuated vaccine known as Clone 13 was obtained through a large deletion in the non-structural S protein (100, 101). Clone 13 is an ideal vaccine because of its ability to grow to very high titres in cell culture while reversion to the original strain is prevented by the large deletion in the genome. Additionally, Clone 13 was demonstrated to elicit full protective immunity in immunized animals with the lack of any negative effects in ruminants, including pregnant ewes (75, 100, 101). Despite the associated risks, live attenuated vaccines are the most effective vaccines used in the field. While most of the focus has been on the development of modified live vaccines, formalin inactivated RVFV vaccines have been used to immunize laboratory workers and veterinary staff (101). The associated high cost, difficulty in production, and low yield makes inactivated RVFV vaccines not ideal in controlling the spread of RVFV in livestock (99).

Current vaccine candidates in development include recombinant RVFV vaccines, vectored subunit vaccines, subunit vaccines and virus-like particle vaccines. A recombinant MP12 vaccine has been developed where there is a mutation in the S segment similar to the clone 13 vaccine (72). Additional MP12 vaccines have been generated by deletion of the non-structural S protein completely and through a dual mutation of the non-structural S and M proteins. These vaccines were able to elicit protective immunity in trials while remaining non-virulent upon immunization (72). The other types of vaccines that have been developed are based on the expression of RVFV glycoproteins in recombinant vectors (72). The vectors that have been utilized include lumpy skin disease virus (70, 102), an alphavirus (103, 104), an adenovirus (105, 106), and the new castle disease virus (107, 108). A subunit vaccine based on Gn and Gc glycoproteins expressed using baculovirus was demonstrated to protect sheep following two vaccinations (109). Baculoviruses and tissue culture have also been utilized to express RVFV glycoproteins that then assemble into VLPs (110, 111).

CAPRIPOXVIRUS AS A VECTOR

It has been demonstrated that other poxviruses have been used as successfully as vectors, including vaccinia virus to control rabies in wildlife (112) and fowl pox to protect chickens against Newcastle disease (113). The genomic stability, thermostability, relatively large genomic size of capripoxviruses allowing large genes to be inserted, and ability to be administered at a relatively

low dose make them good candidates for use as recombinant vaccines (19, 114, 115). The tissue tropism of capripoxviruses to epithelial cells in the skin and nasal turbinate (15, 17) allows for intradermal as well as potential intranasal administration of vaccines. One of the most important features of capripoxvirus is the ability of this vector to elicit protective immunity consisting of both antibody and cellular immunity following a single immunization. This is especially important in regions that do not have high levels of veterinary services available. The major advantage of using capripoxvirus as a vector over vaccinia virus is its limited host range and being non-pathogenic to humans (23, 114, 116). This has led to the use of capripoxviruses as a suitable recombinant vector to protect cattle from diseases like rinderpest (114, 117). The thymidine kinase gene is a common gene insertion site in vectored vaccines (23). The idea of bi/multivalent vaccines is very important because it allows protective immune responses against two or more antigens of interest using a single dose of vaccine (118, 119). Due to the many advantages, capripoxviruses are increasingly being utilized as vectors to make recombinant vaccines (119, 120). Though the exact method of immunity elicited by the recombinant vaccines is not clearly defined, it is assumed to be cell mediated and humoral (117). The North African KS-1 vaccine which is a LSDV and the South African Neethling LSDV vaccine have been the most commonly used capripoxvirus vaccine strains to generate recombinant vectors (23).

The first recombinant capripoxvirus vaccine developed conferred dual protection against Rinderpest Virus (RPV) and LSDV in cattle. The recombinant vaccine was generated in lamb testicular cells using LSDV. The cells were then transfected with plasmid DNA containing the fusion (F) protein of RPV and a selectable marker (gpt) to replace the TK gene of LSDV; recombinant virus was then isolated through rounds of plaque purification (114). This vaccine was able to protect cattle completely against challenge with a virulent strain of RPV and LSDV (114, 121). The success of the first recombinant capripoxvirus experimental vaccine led to the development of numerous recombinant capripoxvirus vectored vaccines against an array of diseases afflicting small ruminants and cattle. Following the development of the first dual capripoxvirus vaccine, recombinant KS-1 capripoxvirus vaccine strains expressing either the F or hemagglutinin (H) genes of RPV were developed followed by their subsequent evaluation as possible dual vaccines against peste des petits ruminants [PPR (122)]. Both vaccines were found to be protective in experimental settings in goats against lethal challenge with PPR due to the similarity of the H and F proteins of PPR and RPV (122). In 1996, it was reported that expression of the outer capsid protein VP7 of BTV on the KS-1 strain of SPPV was able to provide partial protection of sheep against a virulent BTV challenge (31). The use of recombinant capripoxvirus to protect sheep against BTV and capripoxvirus began because of previous experiments that showed vaccination with structural proteins could elicit protective immunity in experimental animals (31). Recombinant capripoxvirus generation was also done by Ngichabe et al. (117, 118) where they generated LSDV expressing RPV H and F proteins followed by immunization. They reported full

protection against challenge with both diseases; protection was also observed several years after initial vaccination in some animals (117). An attenuated LSDV vaccine strain (Neethling) was similarly utilized to successfully express a rabies virus glycoprotein in cattle where there was an antibody response from the cattle upon inoculation with the recombinant virus (115).

Wallace and Viljoen (23) generated recombinant LSDV (SA-Neethling) expressing the glycoproteins of RVFV and Bovine ephemeral fever virus (BEFV). These bivalent vaccines were constructed by inserting the foreign genes into the LSDV TK gene, conferred protective immunity against challenge with both viruses, respectively. The recombinant BEFV vaccine challenges resulted in the production of neutralizing antibodies similar to that elicited by commercial vaccines in cattle; this however, did not result in full protection in cattle while the RVFV recombinant vaccine did (23). In 2006, Diallo et al., were also able to make a recombinant capripoxvirus (KS-1 strain) expressing the H protein from PPR, they reported that at their suggested dose, it was able to protect goats against virulent PPR. This was contrary to observations where a 100X lower dose expressing the F protein of PPR showed complete protective immunity (123). The use of capripoxviruses as recombinant vectors has continued with proteins from numerous infectious viruses being expressed to provide full or partial protection against virulent challenge (124–126).

GENERATION OF RECOMBINANT CAPRIPPOXVIRUS VECTORS

Homologous Recombination

Homologous recombination is a commonly used method of editing genomes and has been used to successfully delete or add antigen-encoding genes into capripoxviruses. Recombinant capripoxvirus vaccine generation using homologous recombination is achieved by infection of permissive cells with a capripoxvirus vector followed by a transfection with a transfer plasmid. The transfer plasmid contains selectable markers and the gene of interest with flanking regions for a non-essential capripoxvirus gene, often thymidine kinase (TK) (114–116, 120). Other insertion sites such as the IL-10 homolog gene (127) and interferon-gamma receptor-like gene have also been used as insertion sites (128). Deletion of the TK as well as open reading frames 8–18 was demonstrated to further attenuate the AV41 sheeppox vaccine (129). Deletion of the sheeppox-019 kelch like protein gene from a virulent Kazakhstan sheeppox isolate was able to attenuate the virus (130). These studies demonstrate that there are many different sites available to insert genes. There are likely many more non-essential gene targets for use as insertion sites, which have not been demonstrated to date.

Wallace et al. (120) evaluated different selection methods in order to determine the most appropriate markers. The selectable markers evaluated were *Escherichia coli* (*E. coli*) β -galactosidase gene, use of green fluorescent protein (GFP) genes and/or the use of *E. coli* xanthine phosphoribosyltransferase (gpt) gene (23, 120). Efficiency wise, it is logical to use a dual selectable marker to allow for a visual confirmation and an additional marker that

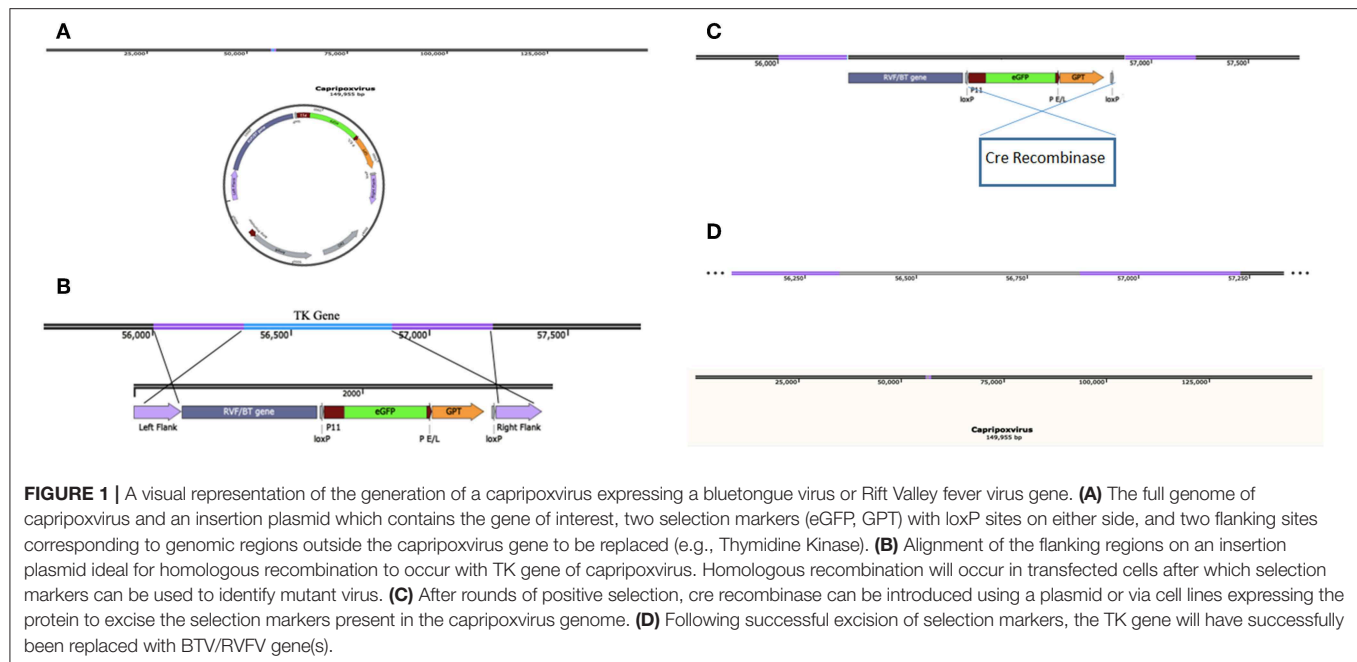
allows for growth in a selective media. LacZ and GFP act as visual markers where expression of these genes demonstrates homologous recombination has occurred without any further process than infection and transfection (120). Gpt is a dominant selectable marker and an added advantage as it allows for the selective growth of virus expressing the gene of interest on gpt selective media (120). Selectable markers are not acceptable to use in a licensed vaccine and can be removed in one of two ways. The first method would be to insert a P11 promoter oriented in the same direction placed before and after the selectable markers (131). The promoter is able to drive the expression of the selectable markers while also allowing for a recombinant excision of the markers once the selective pressure is removed from the growth media of the virus during negative selection (131). The second method to remove selection markers is the cre-loxP system. Similar to the presence of the P11 promoter, it involves the incorporation of a loxP sequence on either side of the selectable markers. Then following positive selection, once a pure recombinant virus is present it would be passaged in cells expressing cre recombinase which will recombine the two loxP sites and excise the selectable markers (**Figure 1**) (126, 132).

CRISPR/Cas

CRISPR refers to clustered regularly interspaced short palindromic repeats. CRISPR is found in prokaryotes where it functions as a defense system to attack invading foreign DNA where the foreign DNA is inserted following the CRISPR sequence and CRISPR associated (Cas) genes to produce guide RNAs that then target the sequence of foreign DNA for destruction should it ever be re-introduced into the prokaryote (133, 134). CRISPR/Cas is a system that can be utilized in place of or in parallel with homologous recombination for the generation of recombinant vaccines. Although CRISPR/Cas has yet to be reported in the generation of recombinant capripoxvirus, it has been reported in the modification of vaccinia virus (134) and African swine fever virus (135) to improve the efficiency of genetic engineering. The similarity between pox and vaccinia opens the door for the use of CRISPR/Cas system as a gene-editing tool in the process of recombinant vaccine generation (133, 134).

Synthetic Generation of Capripoxviruses

A novel method of recombinant poxvirus generation has recently been demonstrated involving large scale gene synthesis (136). The process involves the synthetic generation of large fragments of DNA up to 30 kb containing overlapping sequences of at least 1 kb. The fragments are synthesized in a plasmid then restricted and ligated in optimized cells with the presence of a helper virus to generate functional poxvirus (136). Using the molecular methods stated, horsepox virus was generated from 10 fragments of synthetic DNA using Shope Fibroma virus as a helper virus (136). The potential of this research is limitless in terms of new capripoxvirus vaccine generation. The ability to synthetically make capripoxvirus would allow for the modification of multiple genes at once reducing the laborious process of plaque purification and selection. In addition, using



synthetic biology will allow for tailoring of the vector to enhance safety and immunogenicity.

Recombinant Capripoxvirus Vaccines as DIVA Vaccines

Differentiating infected from vaccinated individual (DIVA) vaccines are possibly the most promising means to control and monitor the spread of rapidly spreading infectious diseases in small ruminants and cattle. Previously known as marker vaccines, DIVA vaccines refer to genetically altered conventional vaccines, which have at least one antigenic region missing (137). This results in quantifiably different antibody response from a vaccinated animal where there is a lack of antibodies against the missing antigen, allowing for the development of a test to differentiate the antibody response (16, 137, 138). This not only allows for the differentiation of vaccinated and unvaccinated animals but it also will likely decrease the amount of wildtype virus circulating in animal populations aiding in the possible eradication of a given virus (137, 138). Previously, the advantage of DIVA vaccines and accompanying serological diagnostic tests has been experimentally shown to be effective against Aujeszky's Disease virus (139) and herpes virus (138, 140). The expression of foreign proteins on capripoxvirus vectors allows for the application a DIVA companion diagnostic test allowing differentiation of vaccinated animals based on the absence of antibodies for proteins not expressed by the vectored vaccine. For example, a capripoxvirus vectored vaccine expressing the GnGc glycoproteins would generate antibodies against GnGc but would not generate antibodies against RVFV NP. The expression of foreign antigens also allows for the development of a test to detect the presence of antibodies specific to the foreign proteins expressed with the absence of other antibodies that would be present during a natural infection (141). Additionally,

the simultaneous removal of a non-essential but antigenically relevant gene on the capripoxvirus vector would allow for DIVA capability for capripoxvirus vaccination with the development of an accompanying serological assay.

Currently there is no DIVA vaccine and companion diagnostic test for capripoxviruses, although there are molecular based methods available to discriminate between vaccine and wild type viruses (142–144). The development of a DIVA capripoxvirus vaccine and companion diagnostic test is theoretically feasible and technically possible. However, to do this, first a validated diagnostic ELISA is required and the antigen target used in the test must be a non-essential protein for the capripoxvirus. These two requirements are prerequisites for the development of a DIVA vaccine and companion diagnostic test.

Future Directions to Improve Capripoxvirus Vaccine Vectors

The continuing spread of lumpy skin disease into previously free regions is leading to more countries where all capripoxvirus members are present. Since these viruses cannot be differentiated using serology, the only method to identify the specific virus is PCR and/or sequencing. The historical method used to identify the virus used the ruminant host that the virus was isolated from to characterize the virus. This worked generally well; however, there is an exception where this method did not identify the virus properly (7). The sequencing and analysis done by Tulman et al. (2, 145) has allowed for the study of capripoxvirus genes leading to studies where specific genes have been used to differentiate between sheeppox virus, goatpox virus, and lumpy skin disease virus. For example, the RPO30 and GPCR homolog genes in capripoxvirus have been used to develop real time and classical PCR tests to differentiate sheeppox from the other two capripoxviruses and between all three viruses, respectively

(146–148). Although the above mentioned genes have been used to determine the species of capripoxvirus, unfortunately, the understanding of what specific genes/mutations and or gene combinations are involved in determining whether a capripoxvirus is a sheepox, goatpox or LSDV is unknown. Analyzing the sequence information obtained from several capripoxviruses including virulent wild type and attenuated vaccines offers insight for future recombinant vaccine design (149). Understanding gene deletions found in capripoxvirus vaccines will allow strategic attenuation to target ideal virulence genes without compromising the vaccine integrity. It is likely that there are many possible gene deletion combinations available to generate a live attenuated vaccine. This information can be used to develop improved capripoxvirus vectors based on sheepox, goatpox, and lumpy skin disease for different regions. To alleviate this issue, a universal capripoxvirus vector generated through gene synthesis, with specific gene markers for the different capripoxviruses deleted could be developed, with the inclusion of a DIVA capability with a companion diagnostic test to alleviate political issues and potentially allow the vector used in non-endemic regions. This universal capripoxvirus vaccine would be able to protect against all capripoxviruses in sheep, goats and cattle.

Capripoxvirus vectors can be tailored to include antigens for specific disease agents in the region. This is especially important in the case of BTV where there are 29 serotypes present, designing vectored vaccines based on geographically prevalent and cross-reactive serotypes is crucial to maximize the protective capability of a multivalent vaccine. The limit of the number of foreign antigens expressed simultaneously in a capripoxvirus vector is currently unknown; however, it is likely more than two antigens. In addition, it is possible that VLPs can be expressed using a

capripoxvirus vector, however, this has not been demonstrated to date. It is possible to develop a capripoxvirus vector encoding the protective antigen GnGc from Rift Valley fever virus along with protective VLPs from bluetongue to generate a multivalent vaccine to protect sheep, goats and cattle from these diseases.

CONCLUSIONS

Capripoxvirus vectors have tremendous potential for use as multivalent vaccines to protect sheep, goats and cattle from arboviruses, capripoxviruses and other devastating diseases such as peste des petits ruminants. The difficulty in vaccinating animals against arboviruses such as Rift Valley fever virus is the cyclical nature of the disease where producers do not have the resources to vaccinate for a disease that may or may not occur. Using a multivalent capripoxvirus vaccine can alleviate these issues by having a vaccine that can protect against Rift Valley fever together with endemic capripoxvirus diseases that occur much more frequently (150). The use of multivalent recombinant vaccines can provide a cost efficient strategy compared to the use of multiple conventional vaccines.

AUTHOR CONTRIBUTIONS

MT wrote the first draft of the manuscript. SB wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

This work was funded by the Canadian Food Inspection Agency.

REFERENCES

- Babiuk S, Bowden T, Boyle D, Wallace D, Kitching R. Capripoxviruses: an emerging worldwide threat to sheep, goats and cattle. *Transbound Emerg Dis.* (2008) 55:263–72. doi: 10.1111/j.1865-1682.2008.01043.x
- Tulman E, Alfonso C, Lu Z, Zsak L, Sur J, Sandybaev N, et al. The genomes of sheepox and goatpox viruses. *J Virol.* (2002) 76:6054–61. doi: 10.1128/JVI.76.12.6054-6061.2002
- Kitching R, Bhat P, Black D. The characterization of African strains of capripoxvirus. *Epidemiol Infect.* (1989) 102:335–43. doi: 10.1017/S0950268800030016
- Kitching R. Vaccines for lumpy skin disease, sheep pox and goat pox. *Dev Biol.* (2003) 114:161–7.
- Bhanuprakash V, Indrani B, Hosamani M, Sign R. The current status of sheep pox disease. *Comp Immunol Microbiol Infect Dis.* (2006) 29:27–60. doi: 10.1016/j.cimid.2005.12.001
- Ylmaz E, Arayici P, Maharramov A, Mustafaeva Z. Approaches to designing of new generation vaccines against the sheep pox disease. *Biotechnol Acta.* (2016) 9:7. doi: 10.15407/biotech.9.06.007
- Tuppurainen E, Pearson C, Bachanek-Bankowska K, Knowles N, Amareen S, Frost L, et al. Characterization of sheep pox virus vaccine for cattle against lumpy skin disease virus. *Antiviral Res.* (2014) 109:1–6. doi: 10.1016/j.antiviral.2014.06.009
- Beard P, Sugar S, Bazarragchaa E, Gerelmaa U, Tserendorj S, Tuppurainen E, et al. A description of two outbreaks of capripoxvirus disease in Mongolia. *Vet Microbiol.* (2010) 142:427–31. doi: 10.1016/j.vetmic.2009.10.018
- Kitching P. Progress towards sheep and goat pox vaccines. *Vaccine.* (1983) 1:4–9. doi: 10.1016/0264-410X(83)90004-X
- Woods J. Lumpy skin disease – a review. *Trop Anim Health Prod.* (1988) 20:11–7. doi: 10.1007/BF02239636
- Mercier A, Arsevska E, Bournez L, Bronner A, Calavas D, Cauchard J, et al. Speed rate of lumpy skin disease in the Balkans, 2015–2016. *Transbound Emerg Dis.* (2018) 65:240–3. doi: 10.1111/tbed.12624
- EFSA, Calistri P, DeClercq K, Gubbins S, Klement E, Stegeman A, et al. Lumpy skin disease. III. Data collection and analysis. *EFSA J.* (2019) 17:5638. doi: 10.2903/j.efsa.2019.5638
- Chihota C, Rennie L, Kitching R, Mellor P. Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae). *Epidemiol Infect.* (2001) 126:317–21. doi: 10.1017/S0950268801005179
- Sprygin A, Pestova Y, Wallace D, Tuppurainen E, Kononov A. Transmission of lumpy skin disease virus: a short review. *Virus Res.* (2019) 269:197637. doi: 10.1016/j.virusres.2019.05.015
- Bowden T, Babiuk S, Parkyn G, Copps J, Boyle D. Capripoxvirus tissue tropism and shedding: A quantitative study in experimentally infected sheep and goats. *Virology.* (2008) 371:380–93. doi: 10.1016/j.virol.2007.10.002
- Madhavan A, Venkatesan G, Kumar A. Capripoxviruses of small ruminants: current updates and future perspectives. *Asian J Animal Vet Adv.* (2016) 11:757–70. doi: 10.3923/ajava.2016.757.770
- Babiuk S, Bowden T, Parkyn G, Dalman B, Manning L, Neufeld J, et al. Quantification of lumpy skin disease virus following experimental

- infection in cattle. *Transbound Emerg Dis.* (2008) 55:299–307. doi: 10.1111/j.1865-1682.2008.01024.x
18. Tuppurainen E, Venter E, Coetzer J. The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques. *Onderstepoort J Vet Res.* (2005) 72:153–64. doi: 10.4102/ojvr.v72i2.213
 19. Tuppurainen E, Oura C. Review: lumpy skin disease: an emerging threat to Europe, the middle east and Asia. *Transboundary Emerg Dis.* (2011) 59:40–8. doi: 10.1111/j.1865-1682.2011.01242.x
 20. Precasta P, Kato F, Vellut G. A new freeze-dried living virus vaccine against sheep-pox. *Comp Immunol Microbiol Infect Dis.* (1979) 1:305–19. doi: 10.1016/0147-9571(79)90032-8
 21. Tuppurainen E, Venter E, Shisler J, Gari G, Mekonnen G, Juleff N, et al. Review: capripoxvirus diseases: current status and opportunities for control. *Transbound Emerg Dis.* (2015) 64:729–45. doi: 10.1111/tbed.12444
 22. Brenner J, Bellaiche M, Gross E, Elad D, Oved Z, Haimovitz M, et al. Appearance of skin lesions in cattle populations vaccinated against lumpy skin disease: statutory challenge. *Vaccine.* (2009) 27:1500–3. doi: 10.1016/j.vaccine.2009.01.020
 23. Wallace D, Viljoen G. Immune responses to recombinants of the South African vaccine strain of lumpy skin disease virus generated by using thymidine kinase gene insertion. *Vaccine.* (2005) 23:3061–7. doi: 10.1016/j.vaccine.2004.10.006
 24. Shope R, Meegan J. Arboviruses. In: Evans AS, Kaslow RA, editors. *Viral Infections of Humans.* Boston, MA: Springer (1997). p. 151–83. doi: 10.1007/978-1-4899-0036-4_6
 25. Chamberlain R. Arboviruses, the arthropod-borne animal viruses. In: Maramorosch K, editor. *Insect Viruses. Current Topics in Microbiology and Immunology.* Vol. 42. Berlin: Springer (1968). p. 38–58. doi: 10.1007/978-3-642-46115-6_3
 26. Huang Y, Higgs S, Vanlandingham D. Emergence and re-emergence of mosquito-borne arboviruses. *Curr Opin Virol.* (2019) 34:104–9. doi: 10.1016/j.coviro.2019.01.001
 27. MacLachlan J. The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comp Immunol Microbiol Infect Dis.* (1994) 17:197–206. doi: 10.1016/0147-9571(94)90043-4
 28. Roy P, Urakawa T, Van Dijk A, Erasmus B. Recombinant virus vaccine for bluetongue disease in sheep. *J Virol.* (1990) 64:1998–2003.
 29. Grimes J, Burroughs J, Gouet P, Diprose J, Malby R, Zientara S, et al. The atomic structure of the bluetongue virus core. *Nature.* (1998) 395:470–8. doi: 10.1038/26694
 30. MacLachlan N, Zientara S, Wilson W, Richt J, Savini G. Bluetongue and epizootic hemorrhagic disease viruses: recent developments with these globally re-emerging arboviral infections of ruminants. *Curr Opin Virol.* (2019) 34:56–62. doi: 10.1016/j.coviro.2018.12.005
 31. Wade-evans A, Romero C, Mellor P, Takaatsu H, Anderson J, Thevasagayam J, et al. Expression of the major core structural protein (vp7) of bluetongue virus, by a recombinant capripox virus, provides partial protection of sheep against a virulent heterotypic bluetongue virus challenge. *Virology.* (1996) 220:227–31. doi: 10.1006/viro.1996.0306
 32. MacLachlan N, Osburn B. Impact of bluetongue virus infection on the international movement and trade of ruminants. *J Am Vet Med Assoc.* (2006) 228:1346–9. doi: 10.2460/javma.228.9.1346
 33. Mellor P, Boorman J, Baylis M. Culicoides biting midges: their role as arbovirus vectors. *Annu Rev Entomol.* (2000) 45:307–40. doi: 10.1146/annurev.ento.45.1.307
 34. MacLachlan N, Drew K, Darpel K, Worwa G. The pathology and pathogenesis of Bluetongue. *J Compar Path.* (2009) 141:1–16. doi: 10.1016/j.jcpa.2009.04.003
 35. Erasmus B. Bluetongue in sheep and goats. *Aust Vet J.* (1975) 51:165–70. doi: 10.1111/j.1751-0813.1975.tb00048.x
 36. Mayo C, Lee J, Kopanke J, MacLachlan N. A review of potential bluetongue virus vaccine strategies. *Vet Microbiol.* (2017) 206:84–90. doi: 10.1016/j.vetmic.2017.03.015
 37. Brodie S, Wilson W, O'Hearn P, Muthui D, Diem K, Pearson L. The effects of pharmacological and lentivirus-induced immune suppression on orbivirus pathogenesis: assessment of virus burden in blood monocytes and tissues by reverse transcription-*in situ* PCR. *J Virol.* (1998) 72:5599–609.
 38. Schwartz-Cornil I, Mertens P, Contreras V, Hemati B, Pascale F, Breard E, et al. Bluetongue virus: virology, pathogenesis and immunity. *Vet Res.* (2008) 39:46. doi: 10.1051/vetres:2008023
 39. Noad R, Roy P. Bluetongue vaccines. *Vaccine.* (2009) 27:D86–9. doi: 10.1016/j.vaccine.2009.08.037
 40. Hardy A, Stewart M, Shaw A, Varela M, Wilson S, Randall R, et al. Species-specific restriction of Bluetongue virus replication correlates to host resilience. *Access Microbiol.* (2019) 1. doi: 10.1099/acmi.ac2019.po0130
 41. Breard E, Belbis G, Hamers C, Moulin V, Lilin T, Moreau F, et al. Evaluation of humoral response and protective efficacy of two inactivated vaccines against bluetongue virus after vaccination of goats. *Vaccine.* (2011) 29:2495–502. doi: 10.1016/j.vaccine.2010.12.105
 42. Nomikou K, Hughes J, Wash R, Kellam P, Breard E, Zientara S, et al. Widespread reassortment shapes the evolution and epidemiology of bluetongue virus following european invasion. *PLoS Pathog.* (2015) 11:e1005056. doi: 10.1371/journal.ppat.1005056
 43. Savini G, MacLachlan N, Sanchez-Vizcaino J-M, Zientara S. Vaccines against bluetongue in Europe. *Comp Immunol Microbiol Infect Dis.* (2008) 31:101–20. doi: 10.1016/j.cimid.2007.07.006
 44. Roy P, Boyce M, Noad R. Prospects for improved bluetongue vaccines. *Nat Rev Microbiol.* (2009) 7:120–8. doi: 10.1038/nrmicro2052
 45. Purse B, Mellor P, Rogers D, Samuel A, Mertens P, Baylis M. Climate change and the recent emergence of bluetongue in Europe. *Nat Rev Microbiol.* (2005) 3:171–81. doi: 10.1038/nrmicro1090
 46. Saluzzo J, Smith J. Use of reassortant viruses to map attenuating and temperature-sensitive mutations of the Rift Valley fever virus MP-12 vaccine. *Vaccine.* (1990) 8:369–75. doi: 10.1016/0264-410X(90)90096-5
 47. Alexander R, Haig D. The use of egg attenuated bluetongue virus in the production of a polyvalent vaccine for sheep: a propagation of the virus in sheep. *Onderstepoort J Vet Sci Anim Ind.* (1951) 25:3–15.
 48. Hunter P, Modumo J. A monovalent attenuated serotype 2 bluetongue virus vaccine confers homologous protection in sheep. *Onderstepoort J Vet Res.* (2001) 68:331–3.
 49. Parker J, Herniman K, Gibbs E, Sellers R. An experimental inactivated vaccine against bluetongue. *Vet Rec.* (1975) 96:284–7. doi: 10.1136/vr.96.13.284
 50. Campbell C. Immunogenicity of bluetongue virus inactivated by gamma irradiation. *Vaccine.* (1985) 3:401–6. doi: 10.1016/0264-410X(85)90131-8
 51. Huismans H, van der Walt N, Erasmus B. Immune response against the purified serotype specific antigen of bluetongue virus and initial attempts to clone the gene that codes for the synthesis of this protein. *Prog Clin Biol Res.* (1985) 178:347–53.
 52. Lobato Z, Coupar B, Gray C, Lunt R, Andrew M. Antibody responses and protective immunity to recombinant vaccinia virus-expressed bluetongue virus antigens. *Vet Immunol Immunopathol.* (1997) 59:293–309. doi: 10.1016/S0165-2427(97)00084-6
 53. Roy P, Bishop D, LeBlois H, Erasmus B. Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. *Vaccine.* (1994) 12:805–11. doi: 10.1016/0264-410X(94)90289-5
 54. Stewart M, Bhatia Y, Athmaran T, Noad R, Gastaldi C, Dubois E, et al. Validation of a novel approach for the rapid production of immunogenic virus-like particles for bluetongue virus. *Vaccine.* (2010) 28:3047–54. doi: 10.1016/j.vaccine.2009.10.072
 55. Stewart M, Dubois E, Sailleau C, Breard E, Viarouge C, Desprat A, et al. Bluetongue virus serotype 8 virus-like particles protect sheep against virulent virus infection as a single or multi-serotype cocktail immunogen. *Vaccine.* (2013) 31:553–8. doi: 10.1016/j.vaccine.2012.11.016
 56. Matsuo E, Celma C, Boyce M, Viarouge C, Sailleau C, Dubois E, et al. Generation of replication-defective virus-based vaccines that confer full protection in sheep against virulent bluetongue virus challenge. *J Virol.* (2011) 85:10213–21. doi: 10.1128/JVI.05412-11
 57. Celma C, Boyce M, van Rijn P, Eschbaumer M, Wernike K, Hoffmann B, et al. Rapid generation of replication-deficient monovalent and multivalent vaccines for Bluetongue virus: protection against virulent virus challenge in cattle and sheep. *J Virol.* (2013) 87:9856–64. doi: 10.1128/JVI.01514-13
 58. van Rijn P, Daus F, Maris-Veldhuis M, Feenstra F, van Gennip R. Bluetongue Disabled Infectious Single Animal (DISA) vaccine: Studies

- on the optimal route and dose in sheep. *Vaccine*. (2017) 35:231–7. doi: 10.1016/j.vaccine.2016.11.081
59. Marin-Lopez A, Ortego J. Generation of recombinant modified vaccinia virus Ankara encoding VP2, NS1, and VP7 proteins of Bluetongue virus. *Methods Mol Biol*. (2016) 1349:137–50. doi: 10.1007/978-1-4939-3008-1_9
 60. Boone J, Balasuriya U, Karaca K, Audonnet J, Yao J, He L, et al. Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. *Vaccine*. (2007) 25:672–8. doi: 10.1016/j.vaccine.2006.08.025
 61. Perrin A, Albina E, Breard E, Sailleau C, Prome S, Grillet C, et al. Recombinant capripoxviruses expressing proteins of Bluetongue virus: evaluation of immune responses and protection in small ruminants. *Vaccine*. (2007) 25:6774–83. doi: 10.1016/j.vaccine.2007.06.052
 62. Ma G, Eschbaumer M, Said A, Hoffmann B, Beer M, Osterrieder N. An equine herpesvirus type 1 (EHV-1) expressing VP2 and VP5 of serotype 8 bluetongue virus (BTV-8) induces protection in a murine infection model. *PLoS ONE*. (2012) 7:e34425. doi: 10.1371/journal.pone.0034425
 63. Franceschi V, Capocéfalo A, Calvo-Pinilla E, Redaelli M, Mucignat-Caretta C, Mertens P, et al. Immunization of knock-out α/β interferon receptor mice against lethal bluetongue infection with a BoHV-4-based vector expressing BTV-8 VP2 antigen. *Vaccine*. (2011) 29:3074–82. doi: 10.1016/j.vaccine.2011.01.075
 64. Bouet-Cararo C, Contreras V, Caruso A, Top S, Szelechowski M, Bergeron C, et al. Expression of VP7, a Bluetongue virus group specific antigen by viral vectors: analysis of the induced immune responses and evaluation of protective potential in sheep. *PLoS ONE*. (2014) 9:e11605. doi: 10.1371/journal.pone.0111605
 65. Martin V, Pascual E, Avia M, Pena L, Valcarcel F, Sevilla N. Protective efficacy in sheep of adenovirus-vectored vaccines against bluetongue virus is associated with specific T cell responses. *PLoS ONE*. (2015) 10:e0143273. doi: 10.1371/journal.pone.0143273
 66. Kochinger S, Renevey N, Hofmann M, Zimmer G. Vesicular stomatitis virus replicon expressing the VP2 outer capsid protein of bluetongue virus serotype 8 induces complete protection of sheep against challenge infection. *Vet Res*. (2014) 45:64. doi: 10.1186/1297-9716-45-64
 67. Thuenemann E, Meyers A, Verwey J, Rybicki E, Lomonosoff G. A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles. *Plant Biotechnol J*. (2013) 11:839–46. doi: 10.1111/pbi.12076
 68. Afshar A, Eaton B, Wright P, Pearson J, Anderson J, Jeggo M, et al. Competitive ELISA for serodiagnosis of bluetongue: evaluation of group-specific monoclonal antibodies and expressed VP7 antigen. *J Vet Diagn Invest*. (1992) 4:231–7. doi: 10.1177/104063879200400301
 69. Caplen H, Peters C, Bishop D. Mutagen-directed attenuation of Rift Valley fever virus as a method for vaccine development. *J Gen Virol*. (1985) 66:2271–7. doi: 10.1099/0022-1317-66-10-2271
 70. Ayari-Fakhfakh E, Ghram A, Albina E, Cetre-Sossah C. Expression of cytokines following vaccination of goats with a recombinant capripoxvirus vaccine expressing Rift Valley fever virus proteins. *Vet Immunol Immunopathol*. (2018) 197:15–20. doi: 10.1016/j.vetimm.2018.01.001
 71. Bird B, Ksiazek T, Nichol S, MacLachlan N. Rift Valley fever virus. *J Am Vet Med Assoc*. (2009) 234:883–93. doi: 10.2460/javma.234.7.883
 72. Pepin M, Bouloy M, Bird B, Kemp A, Paweska J. Rift Valley fever virus (Bunyaviridae: Phelovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. *Vet Res*. (2010) 4:61. doi: 10.1051/vetres/2010033
 73. Ikegami T, Makino S. The pathogenesis of Rift Valley fever. *Viruses*. (2011) 3:493–519. doi: 10.3390/v3050493
 74. Zakham F, Alaoui A, Vapalahti O. Rift Valley fever in the middle East North Africa (MENA) region. *Curr Trop Med Rep*. (2018) 5:257–63. doi: 10.1007/s40475-018-0165-3
 75. Bishop D, Calisher C, Chumakov M, Gaidamovich S, Hannoun C, Lvov D, et al. Bunyaviridae. *Intervirology*. (1980) 14:125–43. doi: 10.1159/000149174
 76. Thonnon J, Picquet M, Thiongane Y, Lo M, Sylla R, Vercruysse J. Rift valley fever surveillance in the lower Senegal river basin: update 10 years after the epidemic. *Trop Med Int Health*. (1999) 4:580–5. doi: 10.1046/j.1365-3156.1999.00437.x
 77. Himeidan Y. Rift Valley fever: current challenges and future prospects. *Res Rep Trop Med*. (2016) 4:1–9. doi: 10.2147/RRTM.S63520
 78. Turell M, Gargan T, Bailey C. Replication and dissemination of Rift Valley fever virus in culex pipiens. *Am J Trop Med Hyg*. (1984) 33:176–81. doi: 10.4269/ajtmh.1984.33.176
 79. Anyangu A, Gould L, Sharif S, Nguku P, Omolo J, Mutonga D, et al. Risk factors for severe Rift Valley fever infection in Kenya. *Am J Trop Med Hyg*. (2010) 83:14–21. doi: 10.4269/ajtmh.2010.09-0293
 80. Lumley S, Horton D, Hernandez-Triana L, Johnson N, Fooks A, Hewson R. Rift Valley fever virus: strategies for maintenance, survival and vertical transmission in mosquitoes. *J Gen Virol*. (2017) 98:875–87. doi: 10.1099/jgv.0.000765
 81. Gargan T, Clark G, Dohm D, Turell M, Bailey C. Vector potential of selected north american mosquito species for Rift Valley fever virus. *Am J Trop Med Hyg*. (1988) 38:440–6. doi: 10.4269/ajtmh.1988.38.440
 82. Kroeker A, Smid V, Embury-Hyatt C, Moffat E, Collignon B, Lung O, et al. RVFV infection in goats by different routes of inoculation. *Viruses*. (2018) 10:709. doi: 10.3390/v10120709
 83. Linthicum K, Davies F, Kairo A, Bailey C. Rift Valley fever virus (family Bunyaviridae, genus Phlebovirus). Isolations from Diptera collected during an inter-epizootic period in Kenya. *J Hyg*. (1985) 95:197–209. doi: 10.1017/S0022172400062434
 84. Wright D, Kortekaas J, Bowden T, Warimwe G. Rift Valley fever: biology and epidemiology. *J Gen Virol*. (2019) 100:1187–99. doi: 10.1099/jgv.0.001296
 85. Linthicum K, Logan T, Bailey C, Dohm D, Moulton J. Transstadial and horizontal transmission of Rift Valley fever virus in *Hyalomma truncatum*. *Am J Trop Med Hyg*. (1989) 41:491–6. doi: 10.4269/ajtmh.1989.41.491
 86. Lee V. Isolation of viruses from field populations of culicoides (Diptera: Ceratopogonidae) in Nigeria. *J Med Entomol*. (1979) 16:76–9. doi: 10.1093/jmedent/16.1.76
 87. Turell M, Perkins P. Transmission of Rift Valley fever virus by the sand fly, *Phlebotomus duboscqi* (Diptera: Psychodidae). *Am J Trop Med Hyg*. (1990) 42:185–8. doi: 10.4269/ajtmh.1990.42.185
 88. Nfon C, Marszal P, Zhang S, Weingartl H. Innate immune response to Rift Valley Fever virus in goats. *PLoS Negl Trop Dis*. (2012) 6:e1623. doi: 10.1371/journal.pntd.0001623
 89. Terasaki K, Makino S. Interplay between the Virus and Host in Rift Valley Fever Pathogenesis. *J Innate Immun*. (2015) 7:450–8. doi: 10.1159/000373924
 90. Coackley W, Pini A, Gosden D. Experimental infection of cattle with pantropic Rift Valley Fever Virus. *Res in Vet Sci*. (1967) 8:399–405. doi: 10.1016/S0034-5288(18)34598-3
 91. Tomori O. Clinical, virological and serological response of the West African dwarf sheep to experimental infection with different strains of Rift Valley fever virus. *Res Vet Sci*. (1979). 26:152–9. doi: 10.1016/S0034-5288(18)32908-4
 92. Coetzer J, Ishak K. Sequential development of the liver lesions in new-born lambs infected with Rift Valley fever virus. I Macroscopic and microscopic pathology. *Onderstepoort J Vet Res*. (1982) 49:103–8.
 93. Odendaal L, Clift S, Fosgate G, Davis A. Lesions and cellular tropism of natural Rift Valley Fever Virus Infection in Adult Sheep. *Vet Pathol*. (2019) 56:61–77. doi: 10.1177/0300985818806049
 94. Hubbard K, Baskerville A, Stephenson J. Ability of a mutagenized virus variant to protect young lambs from Rift Valley fever. *Am J Vet Res*. (1991) 52:50–5.
 95. Smithburn K. Rift Valley fever: the neurotropic adaptation of the virus and the experimental use of this modified virus as a vaccine. *Br J Exp Pathol*. (1949) 30:1–16.
 96. Botros B, Omar A, Elian K, Mohamed G, Soliman A, Salib A, et al. Adverse response of non-indigenous cattle of European breeds to live attenuated Smithburn Rift Valley fever vaccine. *J Med Virol*. (2006) 78:787–91. doi: 10.1002/jmv.20624
 97. Morrill J, Jennings G, Caplen H, Turell M, Johnson A, Peters C. Pathogenicity and immunogenicity of a mutagen-attenuated Rift Valley fever virus immunogen in pregnant ewes. *Am J Vet Res*. (1987) 48:1042–9.
 98. Morrill J, Mebus C, Peters C. Safety and efficacy of a mutagen-attenuated Rift Valley fever virus vaccine in cattle. *Am J Vet Res*. (1997) 58:1104–9.

99. Mirrill J, Peters C. Pathogenicity and neurovirulence of a mutagen-attenuated Rift Valley fever vaccine in rhesus monkeys. *Vaccine*. (2003) 21:2994–3002. doi: 10.1016/S0264-410X(03)00131-2
100. Muller R, Saluzzo J, Lopez N, Drier T, Turell M, Smith J, et al. Characterization of clone 13, a naturally attenuated avirulent isolate of Rift Valley fever virus, which is altered in the small segment. *Am J Trop Med Hyg*. (1995) 53:405–11. doi: 10.4269/ajtmh.1995.53.405
101. Ikegami T, Won S, Peters C, Makino S. Rescue of infectious Rift Valley fever virus entirely from cDNA, analysis of virus lacking the NSs gene, and expression of a foreign gene. *J Virol*. (2006) 80:2933–40. doi: 10.1128/JVI.80.6.2933-2940.2006
102. Wallace D, Ellis C, Espach A, Smith S, Greyling R, Viljoen G. Protective immune responses induced by different recombinant vaccine regimes to Rift Valley fever. *Vaccine*. (2006) 24:7181–9. doi: 10.1016/j.vaccine.2006.06.041
103. Heise M, Whitmore A, Thompson J, Parsons M, Grobbelaar A, Kemp A, et al. An alphavirus replicon-derived candidate vaccine against Rift Valley fever virus. *Epidemiol Infect*. (2009) 137:1309–18. doi: 10.1017/S0950268808001696
104. Lundstrom K. Alphavirus-based vaccines. *Viruses*. (2014) 6:2392–415. doi: 10.3390/v6062392
105. Holman D, Penn-Nicholson A, Wang D, Woraratanadharm J, Harr M-K, Luo M, et al. A complex adenovirus-vectored vaccine against Rift Valley fever virus protects mice against lethal infection in the presence of preexisting vector immunity. *Clin Vaccine Immunol*. (2009) 16:1624–32. doi: 10.1128/CVI.00182-09
106. Warimwe G, Gesharisha J, Carr B, Otieno S, Otingah K, Wright D, et al. Chimpanzee adenovirus vaccine provides multispecies protection against Rift Valley Fever. *Sci Rep*. (2016) 6:20617. doi: 10.1038/srep20617
107. Kortekaas J, de Boer S, Kant J, Vloet R, Antonis A, Moormann R. Rift Valley fever virus immunity provided by a paramyxovirus vaccine vector. *Vaccine*. (2010) 28:4394–401. doi: 10.1016/j.vaccine.2010.04.048
108. Kim S-H, Samal S. Newcastle disease virus as a vaccine vector for development of human and veterinary vaccines. *Viruses*. (2016) 8:183. doi: 10.3390/v8070183
109. Fabruary B, Wilson W, Gaudreault N, Davis A, Shivanna V, Bawa B, et al. A recombinant Rift Valley fever virus glycoprotein subunit vaccine confers full protection against Rift Valley Fever Challenge in Sheep. *Sci Rep*. (2016) 6:27719. doi: 10.1038/srep27719
110. Liu L, Celma C, Roy P. Rift Valley fever virus structural proteins: expression, characterization and assembly of recombinant proteins. *Virol J*. (2008) 5:82. doi: 10.1186/1743-422X-5-82
111. Naslund J, Lagerqvist N, Hagan M, Lundkvist A, Evander M, Ahlm C, et al. Vaccination with virus-like particles protects mice from lethal infection of Rift Valley fever virus. *Virology*. (2009) 385:409–15. doi: 10.1016/j.virol.2008.12.012
112. Brochier B, Languet B, Blancou J, Kieny M, Lecocq J, Costy F, et al. Use of recombinant vaccinia-rabies virus for oral vaccination of fox cubs (*Vulpes vulpes*) against rabies. *Vet Microbiol*. (1988) 18:103–6. doi: 10.1016/0378-1135(88)90055-7
113. Bournsnel M, Green P, Campbell J, Deuter A, Peters R, Tomley F, et al. A fowlpox virus vaccine vector with insertion sites in the terminal repeats: demonstration of its efficacy using the fusion gene of Newcastle disease virus. *Vet Microbiol*. (1990) 23:305–16. doi: 10.1016/0378-1135(90)90161-N
114. Romero C, Barrett T, Evans S, Kitching P, Greshon P, Bostock C, et al. Single capripoxvirus recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease. *Vaccine*. (1993) 11:737–42. doi: 10.1016/0264-410X(93)90258-Y
115. Aspden K, van Dijk A, Bingham J, Cox D, Passmore J, Williamson A. Immunogenicity of a recombinant lumpy skin disease virus (neethling vaccine strain) expressing the rabies virus glycoprotein in cattle. *Vaccine*. (2002) 20:2693–701. doi: 10.1016/S0264-410X(02)00203-7
116. Liu F, Fan X, Li L, Ren W, Han X, Wu X, et al. Development of recombinant goatpox virus expressing *Echinococcus granulosus* EG95 vaccine antigen. *J Virol Methods*. (2018) 261:28–33. doi: 10.1016/j.jviromet.2018.08.002
117. Ngichabe C, Wamwayi H, Ndungu E, Mirangi P, Bostock C, Black D, et al. Long term immunity in african cattle vaccinated with a recombinant capripox-rinderpest virus vaccine. *Epidemiol Infect*. (2002) 128:343–9. doi: 10.1017/S0950268801006513
118. Ngichabe C, Wamwayi H, Barrett T, Ndungu E, Black D, Bostock C. Trial of a capripoxvirus-rinderpest recombinant vaccine in African cattle. *Epidemiol Infect*. (1997) 118:63–70. doi: 10.1017/S0950268896007200
119. Yokoyama N, Maeda K, Mikami T. Recombinant viral vector vaccines for the veterinary use. *J Vet Med Sci*. (1997) 59:311–22. doi: 10.1292/jvms.59.311
120. Wallace D, Weyer J, Nel L, Viljoen G. Improved method for the generation and selection of homogenous lumpy skin disease virus (SA-Neethling) recombinants. *J Virol Methods*. (2007) 146:52–60. doi: 10.1016/j.jviromet.2007.06.004
121. Romero C, Barrett T, Kitching R, Carn V, Black D. Protection of cattle against rinderpest and lumpy skin disease with a recombinant capripoxvirus expressing the fusion protein gene of rinderpest virus. *Vet Record*. (1994) 135:152–4. doi: 10.1136/vr.135.7.152
122. Romero C, Barrett T, Kitching R, Bostock C, Black D. Protection of goats against peste des petits ruminants with recombinant capripoxviruses expressing the fusion and haemagglutinin protein genes of rinderpest virus. *Vaccine*. (1995) 13:36–40. doi: 10.1016/0264-410X(95)80008-2
123. Diallo A, Minet C, Berhe G, Le Goff C, Black D, Fleming M, et al. Goat immune response to capripox vaccine expressing the hemagglutinin protein of peste des petits ruminants. *Ann N Y Acad Sci*. (2006) 969:88–91. doi: 10.1111/j.1749-6632.2002.tb04356.x
124. Kyriakis C. Tomorrow's vector vaccines for small ruminants. *Vet Microbiol*. (2015) 181:47–52. doi: 10.1016/j.vetmic.2015.08.003
125. Chervyakova O, Zaitsev V, Isakov B, Tailakova E, Storchkov V, Sultankulova K, et al. Recombinant sheep pox virus proteins elicit neutralizing antibodies. *Viruses*. (2016) 8:159. doi: 10.3390/v8060159
126. Liu F, Li L, Wang Z. An improved system for constructing marker-free recombinant goatpox viruses to express foreign proteins. *Res Vet Sci*. (2019) 126:124–6. doi: 10.1016/j.rvsc.2019.08.017
127. Boshra H, Truong T, Nfon C, Bowden T, Gerds V, Tikoo S, et al. A lumpy skin disease virus deficient of an IL-10 gene homologue provides protective immunity against virulent capripoxvirus challenge in sheep and goats. *Antiviral Res*. (2015) 123:39–49. doi: 10.1016/j.antiviral.2015.08.016
128. Kara P, Mather A, Pretorius A, Chetty T, Babiuk S, Wallace D. Characterisation of putative immunomodulatory gene knockouts of lumpy skin disease virus in cattle towards an improved vaccine. *Vaccine*. (2018) 36:4708–15. doi: 10.1016/j.vaccine.2018.06.017
129. Zhu Y, Li Y, Bai B, Fang J, Zhang K, Yin X, et al. Construction of an attenuated goat pox virus AV41 strain by deleting the TK gene and ORF 8-18. *Antiviral Res*. (2018) 157:111–9. doi: 10.1016/j.antiviral.2018.07.008
130. Balinsky C, Delhon G, Afonso C, Risatti G, Borca M, French R, et al. Sheeppox virus kelch-like gene SPPV-019 affects virus virulence. *J Virol*. (2007) 81:11392–401. doi: 10.1128/JVI.01093-07
131. Boshra H, Cao J, Babiuk S. Generation of recombinant capripoxvirus vectors for vaccines and gene knockout function studies. In: Brun A, editor. *Vaccine Technologies for Veterinary Viral Diseases*. Methods in Molecular Biology. New York, NY: Humana Press (2016). p. 151–61. doi: 10.1007/978-1-4939-3008-1_10
132. Liu F, Zhang H, Liu W. Construction of recombinant capripoxviruses as vaccine vectors for delivering foreign antigens: Methodology and application. *Comp Immunol Microbiol Infect Dis*. (2019) 65:181–8. doi: 10.1016/j.cimid.2019.05.013
133. Yuan M, Zhang W, Wang J, Al Yaghchi C, Ahmed J, Chard L, et al. Efficiently editing the vaccinia virus genome by using the CRISPR-Cas9 system. *J Virol*. (2015) 89:5176–9. doi: 10.1128/JVI.00339-15
134. Okoli A, Okeke M, Tryland M, Moens U. CRISPR/Cas9—advancing orthopoxvirus genome editing for vaccine and vector development. *Viruses*. (2018) 10:50. doi: 10.3390/v10010050
135. Borca M, Holinka L, Berggren K, Gladue D. CRISPR-Cas9, a tool to efficiently increase the development of recombinant African swine fever viruses. *Sci Rep*. (2018) 8:3154. doi: 10.1038/s41598-018-21575-8
136. Noyce R, Lederman S, Evans D. Construction of an infectious horsepox virus vaccine from chemically synthesized DNA fragments. *PLoS ONE*. (2018) 13:e0188453. doi: 10.1371/journal.pone.0188453
137. Van Oisshot J. Diva vaccines that reduce virus transmission. *J Biotechnol*. (1999) 73:195–205. doi: 10.1016/S0168-1656(99)00121-2

138. Van Oirschot J, Kaashoek M, Rijsewijk F, Stegeman J. The use of marker vaccines in eradication of herpesviruses. *J Biotechnol.* (1996) 44:75–81. doi: 10.1016/0168-1656(95)00129-8
139. Van oirschot J, Rziha H, Moonen P, Pol J, Van Zaane D. Differentiation of serum antibodies from pigs vaccinated or infected with Aujeszky's Disease virus by a competitive enzyme immunoassay. *J Gen Virol.* (1986) 67:1179–82. doi: 10.1099/0022-1317-67-6-1179
140. Van Oirschot J, Kaashoek M, Maris-Veldhuis M, Weerdmeester K, Rijsewijk F. An enzyme-linked immunosorbent assay to detect antibodies against glycoprotein gE of bovine herpesvirus 1 allows differentiation. *J Virol Methods.* (1997) 67:23–34. doi: 10.1016/S0166-0934(97)00073-6
141. Diallo A, Minet C, Le Goff C, Berhe G, Albina E, Libeau G, et al. The threat of peste des petits ruminants: progress in vaccine development for disease control. *Vaccine.* (2007) 25:5591–7. doi: 10.1016/j.vaccine.2007.02.013
142. Chibssa T, Grabherr R, Litsch A, Settypalli T, Tuppurainen E, Nwankpa N, et al. A gel-based PCR method to differentiate sheeppox virus field isolates from vaccine strains. *Virol J.* (2018) 15:59. doi: 10.1186/s12985-018-0969-8
143. Orlova E, Shcherbakov A, Diev V, Zakharov V. Differentiation of capripoxvirus species and strains by polymerase chain reaction. *Mol Biol.* (2006) 40:139–45. doi: 10.1134/S0026893306010183
144. Pestova Y, Byadovskaya O, Kononov A, Sprygin A. A real time high-resolution melting PCR assay for detection and differentiation among sheep pox virus, goat pox virus, field and vaccine strains of lumpy skin disease virus. *Mol Cell Probes.* (2018) 41:57–60. doi: 10.1016/j.mcp.2018.08.003
145. Tulman E, Alfonso C, Lu Z, Zsak L, Kutish G, Rock D. Genome of lumpy skin disease virus. *J Virol.* (2001) 75:7122–30. doi: 10.1128/JVI.75.15.7122-7130.2001
146. Le Goff C, Lamien C, Fakhfakh E, Chadeyras A, Aba-Adulugba E, Libeau G, et al. Capripoxvirus G-protein-coupled chemokine receptor: a host-range gene suitable for virus animal origin discrimination. *J Gen Virol.* (2009) 90:1967–77. doi: 10.1099/vir.0.010686-0
147. Lamien C, Le Goff C, Silber R, Wallace D, Gulyaz V, Tuppurainen E, et al. Use of the Capripoxvirus homologue of Vaccinia virus 30 kDa RNA polymerase subunit (RPO30) gene as a novel diagnostic and genotyping target: development of a classical PCR method to differentiate Goat poxvirus from Sheep poxvirus. *Vet Microbiol.* (2011) 149:30–9. doi: 10.1016/j.vetmic.2010.09.038
148. Lamien C, Leleuta M, Goger W, Silber R, Tuppurainen E, Matijevic M, et al. Real time PCR method for simultaneous detection, quantitation and differentiation of capripoxviruses. *J Virol Methods.* (2011) 171:134–40. doi: 10.1016/j.jviromet.2010.10.014
149. Biswas S, Noyce R, Babiuk L, Lung O, Bulach D, Bowden T, et al. Extended sequencing of vaccine and wild-type capripoxvirus isolates provides insights into genes modulating virulence and host range. *Transbound and Emerg Dis.* (2019). doi: 10.1111/tbed.13322. [Epub ahead of print].
150. Boshra H, Truong T, Nfon C, Gerdt V, Tikoo S, Babiuk L, et al. Capripoxvirus-vectored vaccines against livestock diseases in Africa. *Antiviral Res.* (2013) 98:217–27. doi: 10.1016/j.antiviral.2013.02.016

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Teffera and Babiuk. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Magnetic Nanotrap Particles Preserve the Stability of Venezuelan Equine Encephalitis Virus in Blood for Laboratory Detection

Ivan Akhrymuk^{1†}, Shih-Chao Lin^{1†}, Mei Sun², Anurag Patnaik³, Caitlin Lehman¹, Louis Altamura², Timothy Minogue², Ben Lepene³, Monique L. van Hoek¹ and Kylene Kehn-Hall^{1*}

¹ National Center for Biodefense and Infectious Diseases, School of Systems Biology, George Mason University, Manassas, VA, United States, ² United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, United States, ³ Ceres Nanosciences, Inc., Manassas, VA, United States

OPEN ACCESS

Edited by:

Stéphane Zientara,
Agence Nationale de Sécurité
Sanitaire de l'Alimentation, de
l'Environnement et du Travail
(ANSES), France

Reviewed by:

Suresh H. Basagoudanavar,
Indian Veterinary
Research Institute, India
Daniel Marc,
INRA Centre Val de Loire, France

*Correspondence:

Kylene Kehn-Hall
kkehnhal@gmu.edu

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 27 September 2019

Accepted: 23 December 2019

Published: 28 January 2020

Citation:

Akhrymuk I, Lin S-C, Sun M,
Patnaik A, Lehman C, Altamura L,
Minogue T, Lepene B, van Hoek ML
and Kehn-Hall K (2020) Magnetic
Nanotrap Particles Preserve the
Stability of Venezuelan Equine
Encephalitis Virus in Blood for
Laboratory Detection.
Front. Vet. Sci. 6:509.
doi: 10.3389/fvets.2019.00509

Most of the modern techniques used for identification of viral-induced disease are based on identification of viral antigens and/or nucleic acids in patient's blood. Diagnosis in the field or in remote locations can be challenging and alternatively samples are shipped to diagnostic labs for testing. Shipments must occur under controlled temperature conditions to prevent loss of sample integrity. We have tested the ability of magnetic Nanotrap[®] (NT) particles to improve stability and detection of Venezuelan equine encephalitis virus (VEEV), viral capsid protein, and viral genomic RNA in whole human blood at elevated temperature and prolonged storage conditions. NT particles have previously been shown to capture and enrich multiple pathogens including respiratory syncytial virus, influenza virus, coronavirus, and Rift Valley fever virus. Our study indicates that samples incubated with NT particles had detectable levels of infectious VEEV in blood equal to or greater than samples without NT treatment across all temperatures. Viral RNA detection was increased in the presence of NT particles at later time points (72 h) and higher temperature (40°C) conditions. Likewise, detection of VEEV capsid protein was enhanced in the presence of NT particles up to 72 h at 40°C. Finally, we intranasally infected C3H mice with TC-83, the live attenuated vaccine strain of VEEV, and demonstrated that NT particles could substantially increase the detection of VEEV capsid in infected blood incubated up to 72 h at 40°C. Samples without NT particles had undetectable capsid protein levels. Taken together, our data demonstrate the ability of NT particles to preserve and enable detection of VEEV in human and mouse blood samples over time and at elevated temperatures.

Keywords: alphavirus, diagnostics, surveillance, Venezuelan equine encephalitis virus, nanotrap particles, nanopartcles

INTRODUCTION

Venezuelan equine encephalitis virus (VEEV) is one of the most neglected viruses among biowarfare agents. It is classified as a Category B biothreat pathogen by National Institute of Allergy and Infectious Diseases (NIAID), USA, due to its high dissemination rate, minimal infectious dose to induce disease in human, and the requirement for specific and enhanced diagnostic capacities.

VEEV causes disease with symptoms ranging from influenza-like illness to more severe illnesses including myalgia, arthralgia, and neurological disorders that can lead to lethal encephalitis in susceptible hosts. All these characteristics made VEEV an attractive candidate for weaponization (1). VEEV was first isolated from an infected horse brain during a VEEV outbreak in Venezuela in 1938 followed by major outbreaks in Venezuela and Columbia in 1960s (2), infecting thousands of people and animals. Despite its high contagiousness, VEEV has drawn little public attention due to only sporadic outbreaks occurring in Central and South America since 1995. Other reasons for being neglected may be a low mortality rate in humans (<1%) while as high as 90% in horses, as well as no reports of VEEV outbreaks in the US since the only epidemic outbreak in Texas in 1971 (3). However, the spillover of VEEV infection from infected horses to humans during epidemics remains a concern.

VEEV is an arthropod-borne epizootic RNA virus, belonging to the *Togaviridae* family, genus *Alphavirus*, and is maintained within lower animals (rodents) and mosquitos (4). The transmission of VEEV is primarily mediated by mosquitoes, where it replicates in salivary glands and is passed to hosts, such as human and horses with overt symptoms (5, 6). Moreover, aerosolized VEEV can be directly disseminated and can infect humans or susceptible experimental animal models, causing encephalitis, and possibly limb paralysis (7–9). Historically, since the late 1930s the former Soviet Union regarded VEEV as an operational biological weapon to incapacitate the rear services and reinforcement behind the front line, leading to the spread of the disease among infected individuals with flu-like symptoms that are difficult to distinguish from epidemic influenza outbreaks. Weaponized VEEV was not expected to kill the soldiers, but cause panic and ultimately maim the military targets (10). Notably, there are no effective antiviral agents available and approved by the US Food and Drug Administration (FDA) and only supportive treatment is available for humans. As a result, it is important to establish a preventive surveillance system based on prompt diagnosis methods.

Viral stability is an essential factor for diagnosis of VEEV to confirm the presence of virions or viral RNA in a clinical sample. The current diagnostic approaches to confirm VEEV infection in humans or horses rely on direct detection of viral nucleic acids in serum or spinal fluid samples during the acute-phase of infection using reverse-transcription PCR (RT-PCR) (11) and ELISA for VEEV-specific IgM (12). However, despite high sensitivity of the above-mentioned methods, false negative results can be obtained if samples have been collected during the initial asymptomatic phase of infection where the viral load is low (13–15). Moreover, the necessity of extra steps of plasma or serum preparation complicates fast virus identification and diagnostic. Therefore, virus stabilization directly in collected sample without extra preparation steps is highly desirable. Ideally, supplementation of the blood collection device with some type of stabilization agent that can minimize pathogen loss during sample transportation and storage at ambient temperatures is appealing. In view of these challenges, Nanotrap® (NT) particles were evaluated for their ability to stabilize VEEV. NT particles are hydrogel polymer particles

comprised of N-isopropylacrylamide (NIPAm), allylamine (AA), and crosslinked with N,N'-methylenebisacrylamide (BIS). These particles are functionalized with various dye affinity baits that facilitate capture and retention of analytes from complex biological matrixes (such as blood, saliva, nasal swabs and urine) and concentrate them into a smaller volume (16–20). Previous work with NT particles has shown the benefit of their use in the enrichment of infectious virus and viral genomic material of Rift Valley fever virus (RVFV), coronavirus, influenza virus, and respiratory syncytia virus (19, 21).

In this study, we sought to apply new magnetic NT particles that consist of NIPAm copolymers functionalized with reactive red 120 to evaluate the efficacy of preservation of infectious VEEV, viral RNA, and VEEV capsid protein in whole blood samples at ambient and elevated temperature as well as at low and high humidity conditions. Our results indicate that: (i) magnetic NT particles enhance preservation of infectious VEEV in whole human blood at 40°C; (ii) NT maintain significantly higher levels of VEEV RNA in whole human blood at 40°C; (iii) NT retain their functional activity at both normal and elevated humidity conditions and significantly preserve VEEV infectivity in such an environment; and (iv) blood samples from VEEV TC-83 infected animals are better protected from capsid protein degradation if they are incubated with NT. Our results demonstrate for the first time the capability of NT particles to stabilize virus in blood at elevated temperatures, the direct interaction of NT particles with VEEV (via transmission electron microscopy), and the utility of NT particles with viral clinical samples.

MATERIALS AND METHODS

Viruses, Body Fluids, and NT Particles

VEEV-TC83 viral stocks were produced from electroporation of *in vitro* transcribed viral RNA generated from the pTC83 plasmid [a kind gift from Ilya Frolov, The University of Texas Medical Branch at Galveston] as described (22). All experiments were performed under BSL-2 conditions. Whole human blood and plasma was purchased from BioIVT (www.bioivt.com). All of the NT particles were provided by Ceres Nanoscience, Manassas, VA (ceresnano.com).

NT Particle Screening

VEEV TC83 was diluted to 1×10^6 PFU/mL in whole human blood followed by incubation with NT particle suspension (0.5 mg of NT/ml of sample or 1.25 mg of NT/ml of sample) or without NT particles, rotating for 30 min at room temperature. Viral RNA was purified using RNeasy kit (Qiagen). The amount of the viral RNA was determined by RT-qPCR using SuperScript™ III Platinum™ SYBR™ Green One-Step qPCR Kit w/ROX (Thermo Fisher Scientific) with the following set of primers (Integrated DNA Technologies): 5'-TCTGACAAG ACGTTCCTCAATCA-3' and 5'-GAATAACTTCCCTCCG ACCACA-3'. The Taq-Man probe (5'-6-carboxyfluorescein-TGTTGGAAGGGAAGATAAACGGCTACGC-6-carboxy-N,N,N',N'-tetramethylrhodamine-3') was designed against the RNA packaging signal as described previously (23). RT-qPCR was performed using a StepOne Plus Real Time PCR System

instrument from ABI. Fold enrichment was calculated based on the enriched viral genomic copy number divided by those in the “without NT particle” group.

Virus Enrichment Experiment

VEEV TC83 was diluted to 1×10^6 PFU/mL in whole human blood or plasma followed by incubation with or without NT particles at the indicated humidity and temperature conditions for various time points. Viral RNA was purified using a combination of a TriZol[®] LS from Ambion and RNeasy mini kit (Qiagen). Briefly, whole human blood containing viral virions was mixed in 1:3 ratio with TriZol LS. Hundred microliter of PBS were added to the sample in order to increase the amount of the aqueous fraction. Samples were vortexed and 200 μ L of chloroform were added to the blood TriZol LS mixture. After intensive vortexing and spinning down the upper aqueous fraction containing nucleic acids was collected and used for RNA purification by RNeasy kit (Qiagen) according to the manufacturer’s protocol. Purified RNA was used for cDNA synthesis followed by PCR reaction (30 cycles) using a One Step RT-PCR kit (ThermoFisher Scientific). For this purpose, the following set of primers was used: 5'-CTG CTC GCC AAT GTG ACG TTC-3'; 5'-AGC CTG CTC TGT TGA CTA TAG TGT TAT ACG-3'. To visualize the quantity of viral cDNA 10 μ l of the PCR product were loaded on 1% agarose gel supplemented with ethidium bromide, followed by gel electrophoresis. Samples were visualized on a ChemiDoc instrument from Bio-Rad and densitometrically analyzed using Image Lab Software from Bio-Rad.

Plaque Assay

1×10^6 PFU of the VEEV TC-83 were spiked into whole human blood supplemented (1.25 mg of NT/ml of sample) or not supplemented with NT particles. Samples were incubated at the indicated temperature and humidity conditions for the designated amount of time. At the end of the incubation, NT particles containing samples were placed on a magnet rack to separate NT particles from the blood. Particles were resuspended in 100 μ l of PBS and used for standard plaque assay as described elsewhere (24). Samples that were incubated without NT particles were processed using the same method.

Western Blotting

VEEV TC83 was diluted to 1×10^6 PFU/mL in plasma followed by incubation with or without NT particles (1.25 mg of NT/ml of sample) at indicated humidity and temperature conditions for various time points. Blue lysis buffer (2 $\times$ Novex Tris-Glycine Sample Loading Buffer SDS, T-PER Tissue Protein Extraction Reagent, 0.5 M EDTA, complete protease cocktail tablets, 0.1 M Na₃VO₄, 0.1 M NaF, and 1 M DTT) was added to the samples in 1:1 volume ratio, followed by boiling at 95°C for 10 min. Protein lysates were then electrophoresed in a 4–12% of Bis-Tris SDS-PAGE gel and transferred to a PVDF membrane. A 1:2,000 dilution of primary anti-VEEV capsid antibody (Cat#NR-9403, BEI, Manassas, VA USA) in 5% non-fat milk blocking buffer was applied on the membrane at 4°C for overnight incubation followed by washing with PBS +

0.05% Tween 20 three times. Horseradish peroxidase (HRP)-conjugated anti-goat IgG secondary antibody (ThermoFisher Scientific) at a 1:2,000 dilution was incubated with the membrane for 1 h at room temperature. The membrane was washed an additional three times with PBS + 0.05% Tween 20 before adding SuperSignal[™] West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) to develop the membrane and image the chemiluminescent signal. Samples were visualized on ChemiDoc instrument from Bio-Rad and densitometrically analyzed by the Image Lab Software from Bio-Rad.

Animal Experiment

A total of 27 C3H/HeN mice (6–8-week-old) were randomly divided into three groups ($n = 9$) and VEEV-TC83 was diluted to 2×10^7 PFU/mL with PBS followed by intranasally administrated to anesthetized mice in the amount of 20 μ L/mouse. By 2, 3, and 4 days post-infection, 9 mice were sacrificed at each time point and the blood was collected in EDTA-treated tubes to prevent coagulation. RT-qPCR was performed to quantify the level of viremia and the 6 samples with the highest viremia were pooled together and then incubated with or without NT particles to compare the capsid stability by western blot analysis. Experiments were performed in animal biosafety level 2 (ABSL-2) laboratories in accordance with the National Research Council’s *Guide for the Care and Use of Laboratory Animals* (25) and under George Mason University IACUC protocol number 0384.

Protein Docking Prediction

The VEEV structural protein (E1, E2, E3, and capsid) was retrieved from PDB website (ID: 3J0C) and the chemical structure of reactive red 120 was downloaded from Chemspider website. The heterodimer of E1 and E2 were extracted from the total protein structure with Molsoft ICM-Browser followed by submitting it to Swissdock for prediction of possible docking pockets (26). The docking models were displayed in ball-and-stick style of reactive red 120 and residue surface style of each amino acid on the heterodimer and represented images were selected based on two of the lowest free energy values (ΔG) on each subunit.

Negative Staining and Electron Microscopy (EM) Imaging

Inside the laboratory biosafety cabinet (BSC) the NT particle—VEEV suspension was mixed well with the same volume of 4% Glutaraldehyde to achieve a final concentration of 2% Glutaraldehyde. Virus samples were inactivated with 2% Glutaraldehyde inside a BSC for 24 h according to industry standard practice (27), prior to removal and transfer to the BSL-2 EM facility. A drop (8 μ l) of the glutaraldehyde treated sample was placed onto a formvar/carbon coated transmission electron microscopy (TEM) grid for 10 min in a moist chamber to reduce evaporation. This step ensures that the grid did not dry. Using fine forceps to hold the grid, the liquid was wicked away from the grid surface from the side with filter paper.

The grid was then washed three times by touching the grid to the surface of drops of deionized (dI) water. Remaining water was wicked away by touching filter paper to the side of the grid. A small drop of 1% phosphotungstic acid (PTA) was applied to the grid and allowed to remain from 10 s to 1 min depending on the sample. The stain was wicked away by touching the edge of the grid to a piece of filter paper. The grid was air dried at room temperature and stored for subsequent TEM imaging.

TEM grids were evaluated on a JEOL 1011 transmission electron microscope at 80 kV and all digital images were acquired using an Advanced Microscopy Techniques (AMT) camera system.

Statistical Analysis

Kruskal-Wallis test and unpaired *t*-test were performed throughout this study to calculate the statistical significance among groups with and without NT particles unless indicated elsewhere.

RESULTS

Screening of NT Particle Types for Enriching VEEV-TC83

NT particles contain affinity baits that enable interactions with analytes through poorly defined characteristics. For example, triazine dyes such as cibacron blue and reactive red 120 bind to proteins through hydrophobic and electrostatic interactions (28). Therefore, the initial step of our study was to screen the panel of magnetic NT particles shown in **Table 1**, to evaluate their efficacies in enriching VEEV-TC83. NT particles were mixed with blood-spiked VEEV-TC83 (0.5 mg of NT/ml of sample) and incubated for 30 min prior to determination of viral RNA levels by RT-qPCR. We screened 9 different types of NT particles and found that all the NT particles were capable of enriching VEEV, but to different levels (**Figures 1A,B**). To further confirm and test the limit of capture efficiency of the NT particles, we selected three batches of NT particles shown with the best binding efficiencies and enrichment performance, CN3170, CN3160, and CN3080, and mixed NT particles with the blood-spiked VEEV-TC83 (1.25 mg of NT/ml of sample). All NT particles were capable of enriching VEEV when used at this concentration, but CN3080 showed the most significant enrichment (bound/unbound = ~ 10 , **Figure 2A**), suggesting that the NT particle conjugated with reactive red 120 chemical bait could be the most suitable for the following experiments. We also compared the ability of CN3080 to a non-magnetic version (CN1030) and found that CN3080 was more effective at capturing VEEV (**Supplemental Figure 1**).

To confirm virion capturing by CN3080, we conducted western blot analysis toward the VEEV-TC83 capsid protein. The capsid protein is only detected in samples that have been incubated with CN3080, but not in samples without NT particles regardless of whether virus was spiked into blood or plasma (**Figure 2B**). These data demonstrate the documented ability of NT particles to enrich viral analytes that are below the limit of detection (20). To characterize the performance of CN3080 we

TABLE 1 | Nanotrap particles tested.

NT#	Description
CN3150	Nanotrap ice blue
CN3170	Nanotrap neutral red
CN3140	Nanotrap blue—custom magnetic
CN3160	Nanotrap basic fuchsin
CN3080	Nanotrap reactive red 120
CN3000	Nanotrap purple
CN3130	Nanotrap yellow
CN3120	Nanotrap orange
CN3110	Nanotrap blue

utilized various concentrations of CN3080 ranging from samples without NT particles to a 5 mg of NT/ml of sample to determine the optimal concentration for VEEV capsid enrichment and detection. To our surprise, the lower concentrations (up to 1.25 mg of NT/ml of sample) seemed to exhibit the most abundant enrichment, but not higher concentrations like 5 and 2.5 mg of NT/ml of sample (**Figure 2C**). Next, we tested for the optimal incubation time required for NT particle capture of the majority of virions in a sample. Thus, we measured the viral capsid concentration at different incubation time-points. As early as after 5 min of incubation, VEEV capsid protein was detected and by 15–30 min, the capture ability started to reach a saturation level (**Figure 2D**). These data show that CN3080 magnetic NT particles provide efficient capture of VEEV TC-83 virions over short time periods and the binding efficiency of CN3080 to viral samples can be optimized by adjusting both the incubation time and the NT particle concentration. Based on these data, we chose an incubation time of 30 min and a NT particle concentration of 1.25 mg/ml of sample for the remainder of our experiments to ensure maximal VEEV capture.

VEEV Binds to the Outside of NT Particles

It has been shown in our previous studies that NT particles capture and enrich various viruses, such as influenza virus and RVFV (19, 21, 29), but it has never been shown if NT particles can directly bind to viruses. Using transmission electronic microscopy (TEM) we visualized the direct interaction between NT particles and virions. The photo in **Figure 3** reveals that a single NT particle is capable of capturing multiple virions.

Our TEM data clearly showed that NT particles directly interact with viral particles. Therefore, we sought to elucidate the sites of the interaction between the NT particles and VEEV-TC83. We applied docking simulation via the SwissDock website and determined the free energy of binding (ΔG) toward the affinity bait, reactive red 120, and viral envelope proteins, E1 and E2 (PDB ID: 3J0C) (30). As shown in **Figure 4**, the docking predictions against the external domains of E1-E2 heterodimer protein reveal that the affinity bait overall interacted with E1 and E2 subunits where the strongest binding was predicted in the groove between E1 and E2 (panel

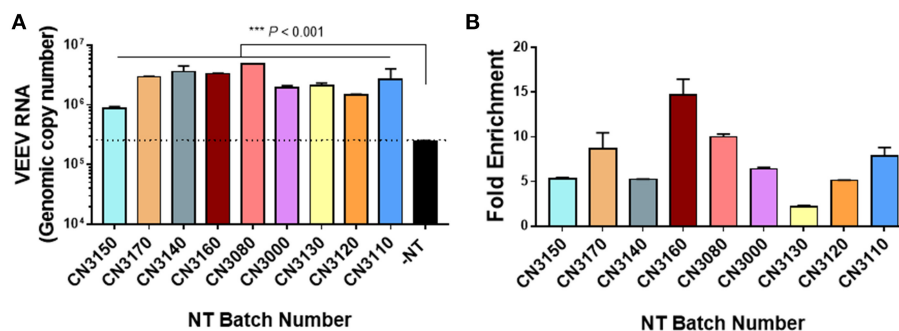


FIGURE 1 | Screening of magnetic NT particles for the ability to enrich VEEV TC-83. VEEV at 1×10^6 PFU/mL was spiked into human blood followed by incubation with nine different magnetic NT particles (0.5 mg of NT/ml of sample). **(A)** The genomic copies of VEEV were determined by RT-qPCR after incubation for 30 min in the presence of NT particles. The dashed line indicates the minimal threshold for identification of enrichment. **(B)** Fold enrichment was calculated based on the following formula: NT-captured RNA/total input viral RNA. Data were presented as mean $\pm$ SEM from results of two independent experiments ($n = 2$). Statistical significance was determined by Kruskal-Wallis test where P -value under 0.05 was considered significant.

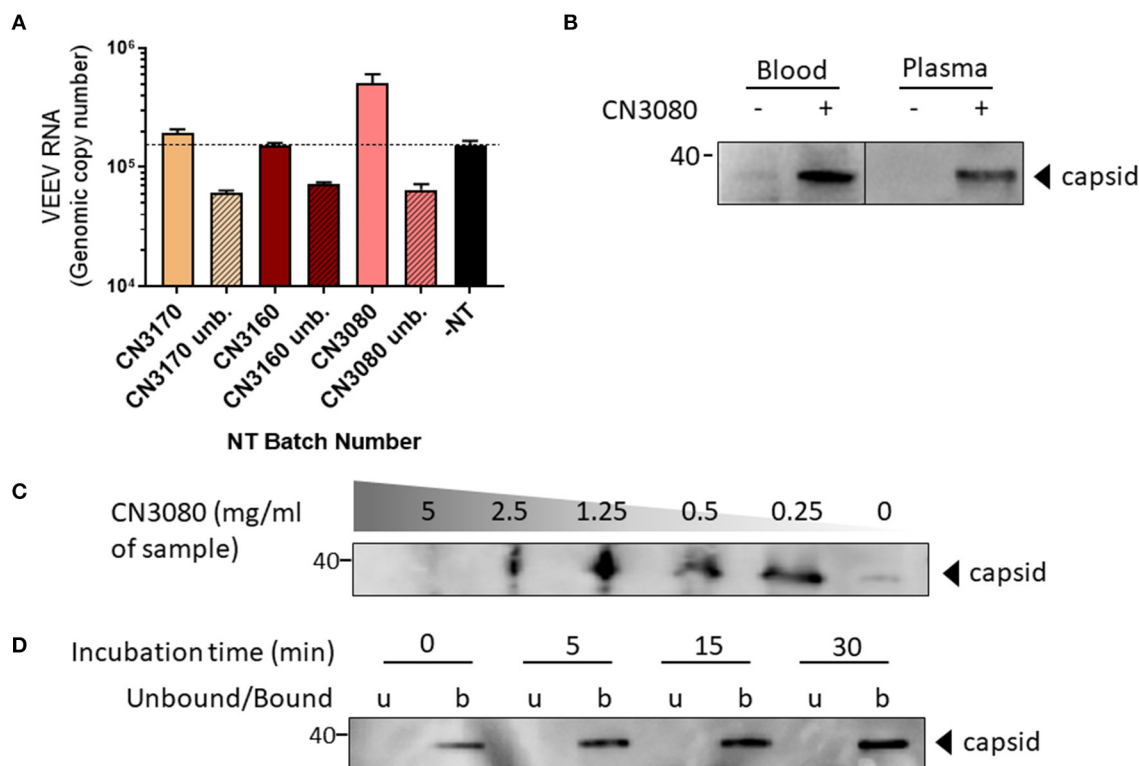
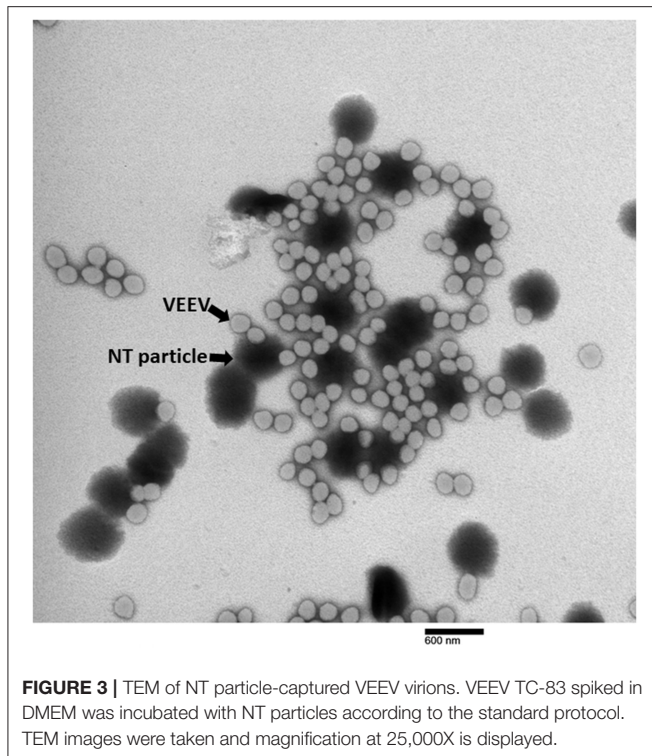


FIGURE 2 | Validation of CN3080 for enrichment of VEEV. **(A)** VEEV at 1×10^6 PFU/mL was spiked into human blood followed by incubation with CN3170, CN3160, or CN3080 magnetic NT particles (1.25 mg of NT/ml of sample). Unbound (Unb) = virus remaining in solution following NT particle incubation, -NT = samples processed without NT particles. The dashed line emphasizes the minimal threshold of enrichment. **(B)** VEEV TC-83 was spiked into blood or plasma at a final concentration 1×10^6 PFU/mL and incubated with CN3080 (1.25 mg of NT/ml of sample). The level of VEEV capsid protein was determined via western blot analysis. **(C)** The concentration-dependent efficiency of CN3080 to preserve VEEV-TC83 was established using different concentration of NT beads to human blood (as indicated). The efficiency of VEEV capture was judged based on the capsid levels visualized by western blot analysis. **(D)** Time-dependent incubation of VEEV TC-83 in presence of CN3080 at room temperature. Data were evaluated based on western blot analysis of the capsid levels captured from a 1:20 blood:PBS mixture spiked with VEEV.

2a). The free energies of NT binding to each E1 and E2 subunits is summarized in **Supplemental Table 1**. In general, the prediction results suggest that there may be strong interactions

between NT particles and VEEV envelope proteins which may potentially contribute to the viral enrichment exerted by CN3080.



Stability of VEEV-TC83 Virions With or Without Nt Particles

Previous studies have shown that NT particles are efficient in the preservation of RVFV and nucleoproteins in biologically relevant matrices (19, 29). However, the effect of NT in the stabilization of virions in a complex matrix such as whole human blood has never been tested before. Thus, after determination of the best NT composition that is most efficient in capturing VEEV TC-83, our next step was to test stability of the virus in whole blood at ambient and elevated temperatures for extended time periods. For this purpose, we spiked 10^6 PFU of VEEV-TC83 in 0.5 mL of human whole blood and incubated samples at temperatures of 22, 37, 40, and 54°C for 0, 24, 48, and 72 h followed by measurement of viral infectivity by plaque assays. We observed a gradual drop of virus titers at 37 and 40°C, whereas viral infectivity remained relatively consistent at 22°C (**Figure 5A**). However, our several attempts to incubate virus at 54°C for 24 h repeatedly resulted in complete virus inactivation. It should be noted that at 54°C after 24 h of incubation the blood samples became solidified which made it impossible to completely resuspend samples in buffer for quantitation via plaque assay (**Figure 5A**). Therefore, we cannot exclude the possibility that some viral particles were captured by coagulated blood and still remained in the sample. As a result of these findings, for most of the following experiments we focus primarily on 37 and 40°C temperature conditions.

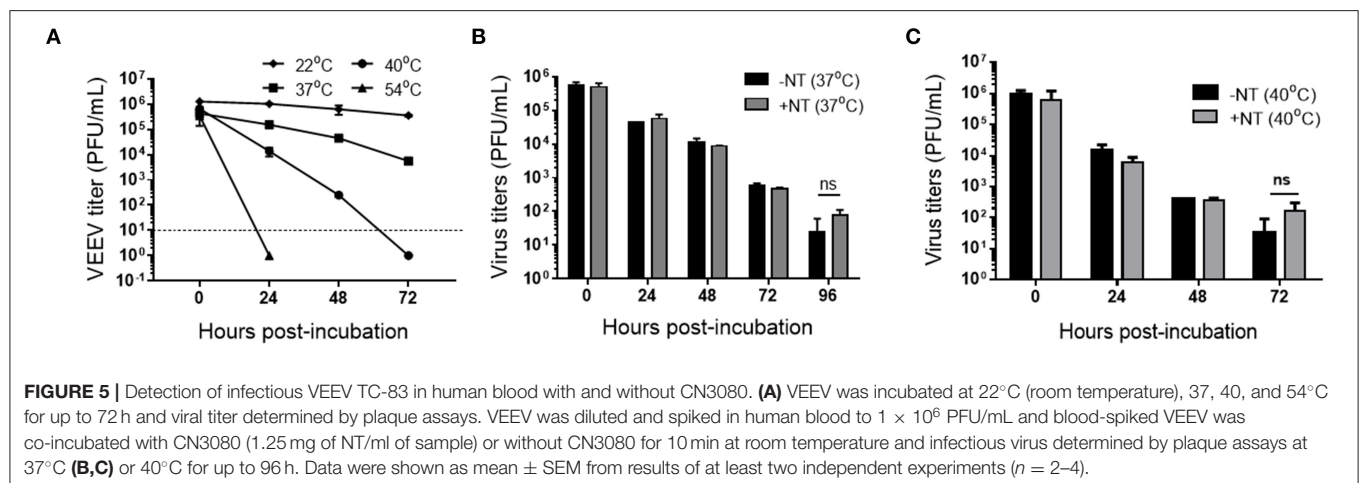
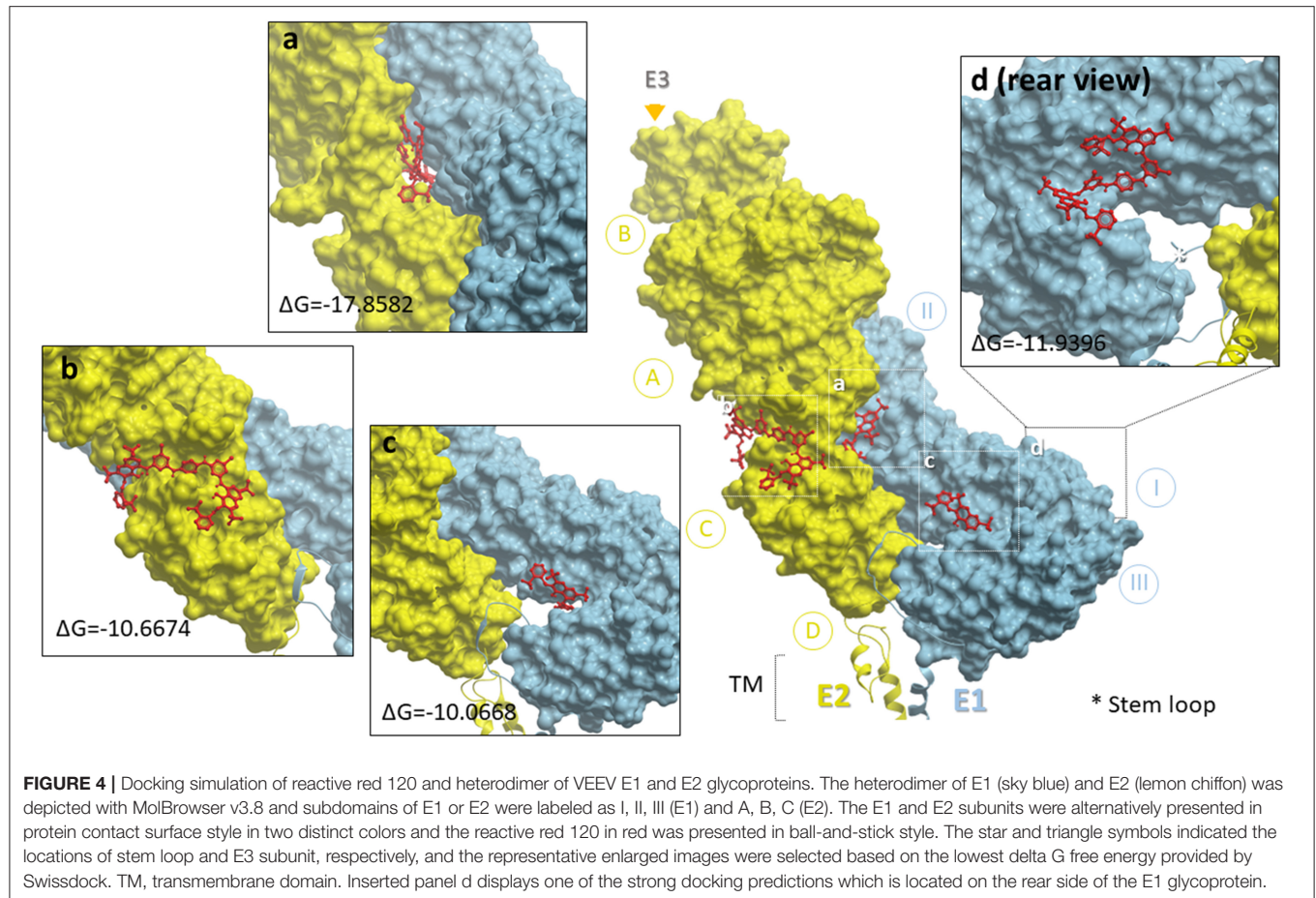
We next investigated whether CN3080 can prolong the stability of VEEV-TC83 in human whole blood. We found that the stability of VEEV at 37°C over the time points we

tested was not substantially sustained in the presence or absence of CN3080 (**Figure 5B**) whereas the infectivity of VEEV TC-83 after 72 h of incubation at 40°C appeared to be better preserved despite the lack of statistical significance (**Figure 5C**). However, it should be noted that only once in four repeats did we observe two plaques developed by virus after 72 h of incubation without NT. In contrast, in all four repeats we were able to detect virus-produced plaques (ranging from 2 to 6 plaques per well) if samples were incubated in presence of CN3080. This indicates that the addition of CN3080 in samples may stabilize the VEEV virions and preserve infectivity for diagnostic purposes.

NT Particles Help to Preserve Viral RNA and Capsid Protein Detection at Elevated Temperatures

The primary approach for confirming VEEV infection in suspected clinical cases is based on detection of the virus specific IgM and neutralizing antibodies. Reactions based on amplification of the nucleic acid is often used in fatal cases to confirm pathogen presence (5, 19, 31). RT-qPCR is a very reliable method and highly sensitive for the detection of minimal amounts of viral nucleic acids in the patient samples (11). However, this method is based on the detection of relatively short fragments of the viral genome and analysis of a longer stretch of viral RNA (vRNA) would provide a more stringent measure of RNA stability. Thus, we investigated the stability of both long viral RNA (1.5 kb product) by RT-PCR and short vRNA (75 bp) by RT-qPCR. VEEV was spiked in blood and incubated in the presence or absence of CN3080 for the indicated amount of time, followed by RNA purification, cDNA synthesis and PCR or qPCR reactions. Minimal differences in long vRNA levels were observed in samples incubated at 37°C (**Figure 6A**). CN3080 overall stabilized long vRNA and increased its detection by RT-PCR especially at higher temperature (40°C) conditions compared to samples that were incubated without NT particles (**Figure 6B**). Densitometric analysis of the PCR products indicated preservation of long vRNA in the presence of CN3080 after 72 h of incubation at 40°C. Likewise, RT-qPCR results indicated preservation of short vRNA after 72 h at 40°C (**Figure 6C**).

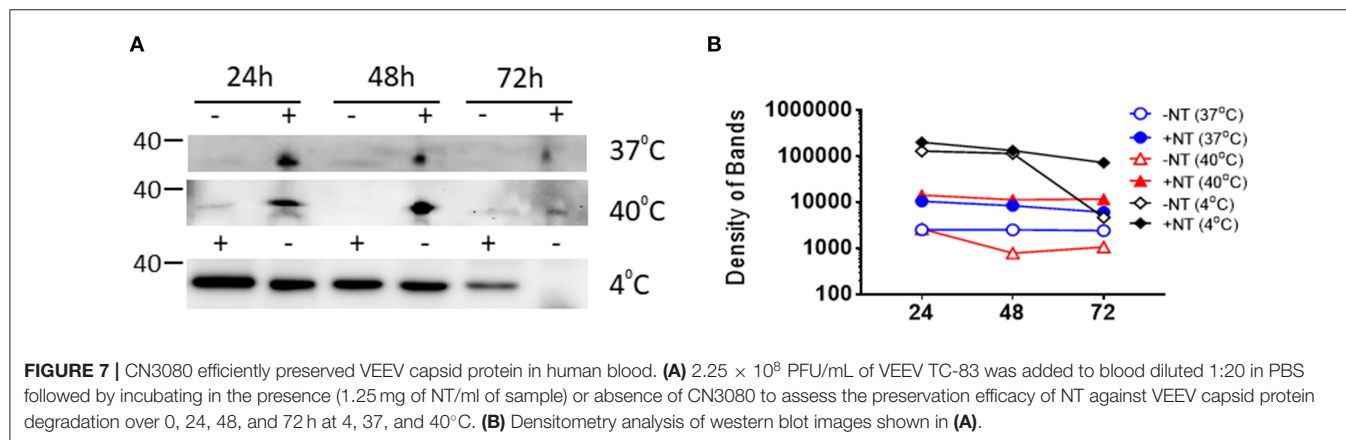
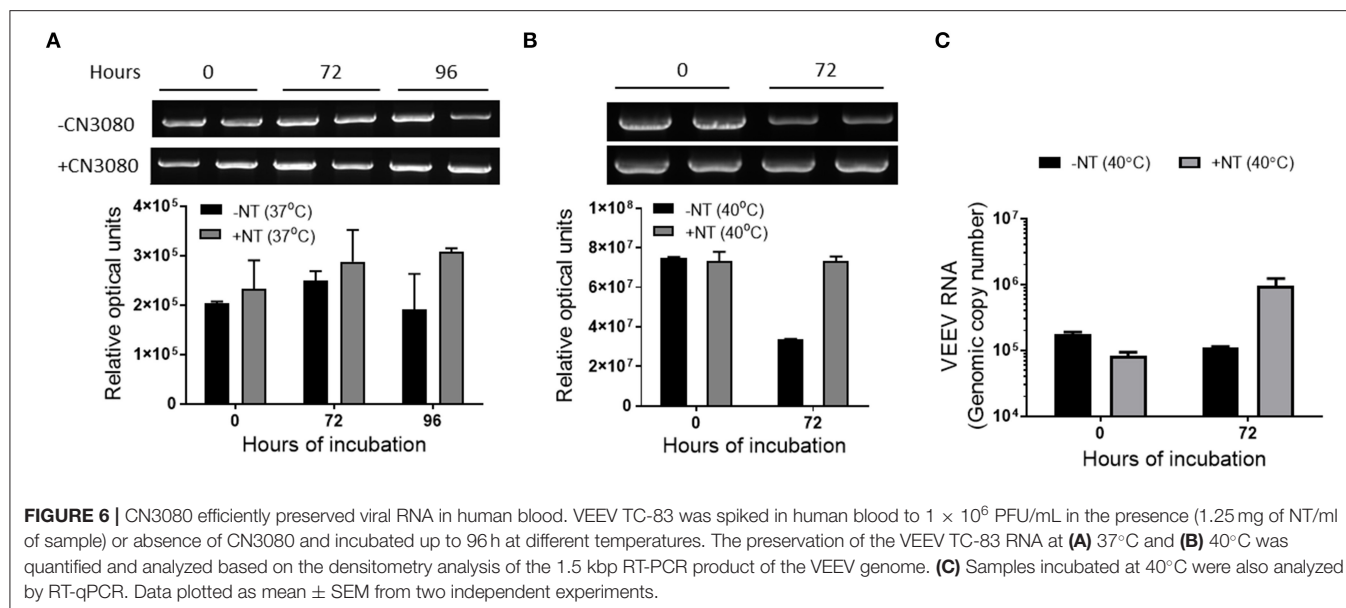
For comparison, we also monitored the degree of VEEV capsid protein decay in the presence or absence of CN3080. Given the difficulty in analysis of blood samples via western blot, these experiments were performed in blood diluted 1:20 with PBS. As a starting point we tested the ability of CN3080 to prevent capsid protein degradation at standard blood storage conditions. For this purpose, we incubated virus with and without CN3080 at 4°C and assessed by western-blot analysis the amount of capsid protein in the incubated samples. Even though VEEV capsid was overall more stable at 4°C, we observed a decrease in capsid protein detection by 72 h, which was rescued by incubation with CN3080 (**Figure 7A**, bottom panel). Western blot analysis of the samples incubated at 37 and 40°C also demonstrated that only when incubating with CN3080, capsid can be preserved and more available for detection (**Figures 7A,B**). Thus, NT particles not



only profoundly enriched capsid protein in all samples at all temperature conditions but in their presence the capsid protein degradation was significantly reduced at high temperature. Results from the above RT-PCR and protein analyses suggest that CN3080 could efficiently enrich and preserve VEEV RNA and capsid protein against the harsh temperature storage conditions.

Stability of Viral Titer of VEEV-TC83 With or Without NT Particles at Various Humidity Conditions

Some studies have shown that relative humidity can affect the stability of airborne viruses including aerosolized VEEV (32–34). Therefore, we investigated whether CN3080 could contribute to



the blood-spiked VEEV stability across environmental humidity ranges. We placed the viral samples with or without CN3080 in two incubators with relative humidity at 16 or 98% at 37°C and incubated them for up to 96 h followed by determination of viral titers. Our plaque assay results indicated that viral titers of the blood-spiked VEEV-TC83 were preserved at both 16% and 98% humidity when incubating with CN3080 for 72 and 96 h post-incubation (Figure 8). Samples incubated in higher humidity conditions were generally more stable, but the addition of CN3080 further enhanced the stability regardless of the level of humidity. Our data support the conclusion that NT particles efficiently stabilize infectious virus at elevated and dry humidity conditions for an extended amount of time.

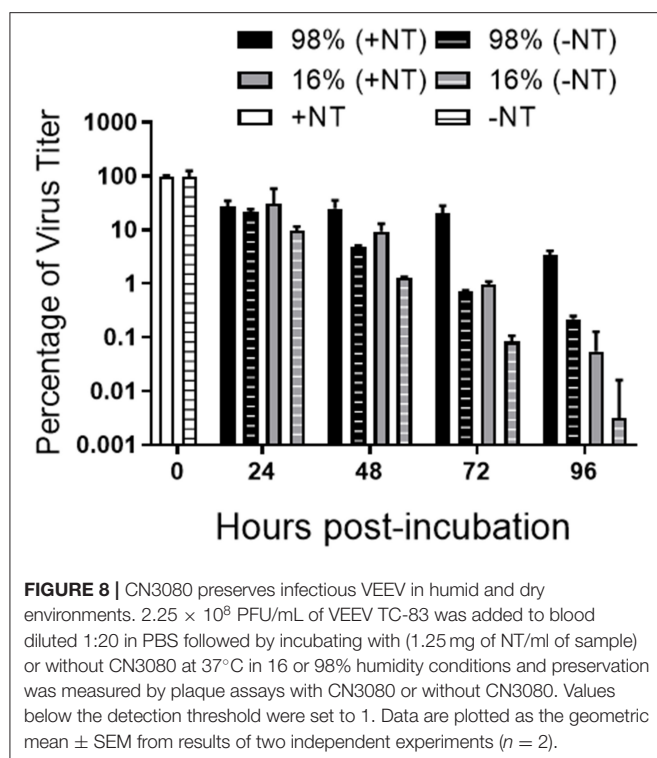
NT Particles Stabilized VEEV Capsid Protein in Blood Samples Collected From VEEV-TC83 Infected Mice

To better illustrate the application of CN3080 in clinical samples, we infected C3H/HeN mice via intranasal infection of VEEV-TC83 and measured the viral load in mouse blood at 2, 3,

and 4 days post-infection (d.p.i.) followed by supplying CN3080 to demonstrate the preservation capability of NT particles *ex vivo*. Since the mouse blood samples from day 2 showed the highest viral RNA concentration among all tested time points (Figure 9A), we used blood harvested from mice at 2 d.p.i., added CN3080, and compared the capsid protein stability with or without NT particles at 40°C for the indicated amount of time. This experiment was performed with whole blood. In the absence of NT particles, VEEV capsid was undetectable at all time points (Figure 9B). Incubation of mouse blood with CN3080 enable capsid detection at each time point, highlighting the potential use of NT particles in clinical samples.

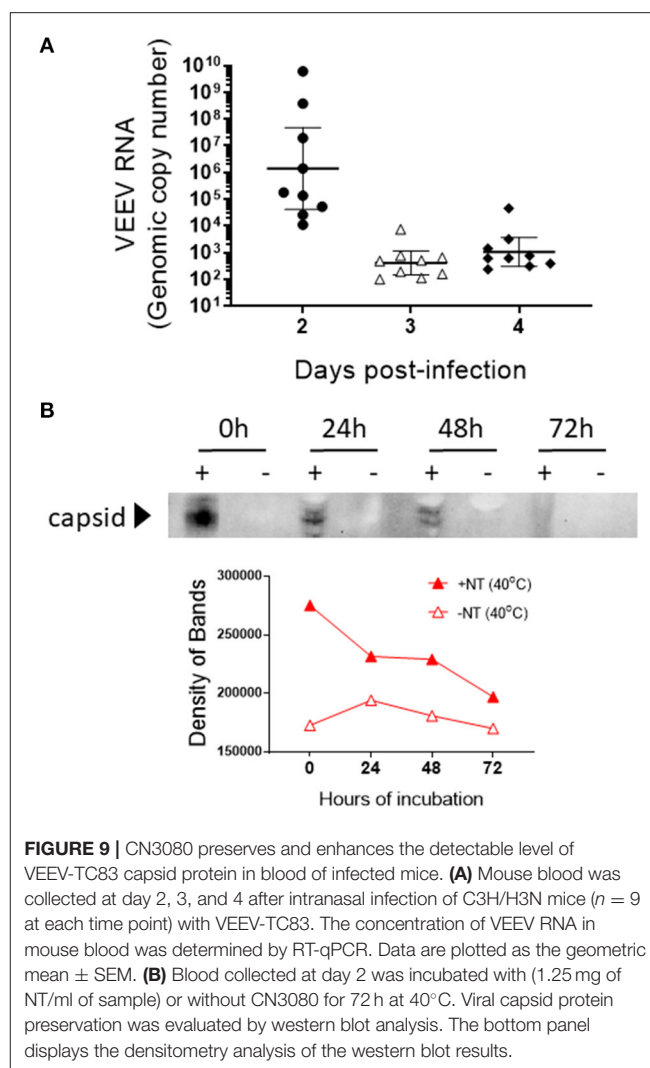
DISCUSSION

NT particles are made of a hydrogel matrix and can be customized with various affinity baits such as acrylic acid, Cibacron blue dye, and reactive red dye by polymerization (35). As such, they can bind a range of biomolecules. In our previous studies, we have found that NT particles containing a reactive



red 120 affinity bait allows NT particles to capture a wide range of RNA viruses (19, 29). Using the newly developed magnetic NT particles could simplify workflows allowing the isolation of NT particles in 3–5 s instead of long centrifugation steps. This processing allows the virions present in clinical samples and bound to NT particles to be easily and quickly separated from body fluids using either manual or automated methods. However, one of the obstacles in pathogen identification in whole blood is the natural abundance of host proteins and red blood cells that prevent direct identification of the pathogen from blood and often require additional preparation steps. Another inconvenience related to working with whole blood or other body fluid sample is a necessity to keep it at low temperatures during transportation. Such obstacles make blood a very difficult matrix for sample preparation and diagnostic analysis. However, despite the existence of the above-mentioned obstacles, we observed efficient separation of VEEV viral particles from blood by NT particles at various temperature and humidity conditions. Moreover, NT particles efficiently preserved infectious VEEV TC-83 at conditions where it was otherwise undetectable.

As much as our NT particle work has shown a significant benefit in preservation and enhancing detection of VEEV virions confirmed by stabilization of viral capsid protein, viral RNA, as well as infectious virions, there are limitations in the use of NT particles. When the environmental conditions are too harsh, the NT particles are only capable of partially alleviating the negative effects. Despite the trend showing more preservative via NT particles, not all viral titers at the later time points



exhibited statistical differences, suggesting the preservation of NT particles may not be sufficient to prevent the ultimate decay of virions under the most extreme environmental conditions. Another limitation for the use of NT particles is the difficulty in separating virions from NT particles. We have previously shown very strong binding of RVFV virions to NT particles (19). Working with RVFV we were able to elute only 5.5% from the total virions bound to NT. For this purpose we used sodium chloride in the highest concentration that does not induce virus degradation (2 M), but even this concentration was not enough to completely elute virions from NT. Although strong lysis buffers, such as Trizol reagent and RIPA buffer, can be utilized for precipitating RNA or extracting viral proteins for diagnosis purposes, we have not found a method to elute the virions while retaining the viral infectivity. As shown in our electronic micrograph (Figure 3), a NT particle is potentially bound with dozens of virions, indicating the efficient capturing ability of NT particles. However, the principle of plaque assay is to dilute viral samples until a single virion can form a

plaque, so called plaque-forming unit, visualized, and countable by investigators. Overwhelming capture affinity of NT particles to virions is an obstacle for the accuracy of the plaque assay method, which might lead to an underestimation of initial viral titers (Figure 5). Therefore, a better way to elute virions needs to be developed to enable more accurate viral titer determination. One possibility for future testing is to competitively elute VEEV via addition of increasing concentration of the reactive red affinity bait.

Despite the limitations discussed above, our data indicate that NT particles are a useful innovation to researchers. The NT particles can be customized with various affinity baits to bind different microorganisms or biomolecules. In addition to the viruses, NT particles have been shown to be capable of binding the outer surface protein of *Borrelia*, the bacteria that causes Lyme disease, and increasing the specificity of diagnosis for early stage of Lyme disease (18). Also, biomarkers in clinical fluid samples or secreted extracellular organelles like exosomes can be captured and the sensitivity of their detection enhanced via NT particles (36–38). The ability of the NT particles to preserve VEEV and its components including capsid protein and genomic RNA in such complex matrix such as whole blood highlight an importance to further study the capabilities of the NT particles.

Overall, the magnetic NT particle presented in this study demonstrated efficient binding affinity to capture one of the biological warfare agents, VEEV, allowing laboratory technicians to detect this particular pathogen from clinical samples of patients or soldiers in the front line. Moreover, the high efficiency in preservation of live pathogen and its proteins and nucleic acids make NT particles an attractive candidate to be used as a stabilization agent in a new blood collection medical device that are currently undergoing development and testing. Hence, utilization of the NT particles can be an alternative method to enhance the surveillance system to monitor and prevent the outbreaks. Our study provides mechanistic insights for broad use of NT particles.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

REFERENCES

1. Ryan JR. *Biosecurity and Bioterrorism: Containing and Preventing Biological Threats*. Amsterdam: Elsevier/BH, Butterworth-Heinemann is an imprint of Elsevier (2016).
2. Beck CE, Wyckoff RW. Venezuelan equine encephalomyelitis. *Science*. (1938) 88:530. doi: 10.1126/science.88.2292.530
3. Zehmer RB, Dean PB, Sudia WD, Calisher CH, Sather GE, Parker RL. Venezuelan equine encephalitis epidemic in Texas, 1971. *Health Serv Rep*. (1974) 89:278–82. doi: 10.2307/4595031
4. Knipe DM, Howley PM. *Fields Virology*. Philadelphia, PA: Wolters Kluwer/Lippincott Williams & Wilkins Health (2013).
5. Zacks MA, Paessler S. Encephalitic alphaviruses. *Vet Microbiol*. (2010) 140:281–6. doi: 10.1016/j.vetmic.2009.08.023

ETHICS STATEMENT

The animal study was reviewed and approved by George Mason University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

IA, S-CL, AP, LA, TM, BL, MH, and KK-H designed experiments. IA, S-CL, and CL performed the experiments and analyzed the data. MS provided TEM data and analysis. IA and S-CL wrote the manuscript. AP, MS, LA, BL, MH and KK-H edited the manuscript.

ACKNOWLEDGMENTS

The authors thank Kathleen Kuehl and Rahul Raychaudhuri, United States Army Medical Research Institute for Infectious Diseases, for assistance with the TEM sample preparation and imaging, respectively. The authors thank Erwin Berthier and Emily Welch, Tasso Inc, for helpful discussions. The following reagent was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: Polyclonal Anti-Venezuelan Equine Encephalitis Virus, TC-83 (Subtype IA/B) Capsid Protein (antiserum, Goat), NR-9403. This work was supported by a grant from Defense Threat Reduction Agency (DTRA) grant, HDTRA1-17-C-0069, to Tasso, Inc with a subcontract to KK-H and MH. DTRA does not have any role in the design of the study and collection, analysis, and interpretation of data and nor in writing the manuscript.

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army, the U.S. Department of Defense, or the U.S. Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2019.00509/full#supplementary-material>

6. Sharma A, Knollmann-Ritschel B. Current understanding of the molecular basis of venezuelan equine encephalitis virus pathogenesis and vaccine development. *Viruses*. (2019) 11:E164. doi: 10.3390/v11020164
7. Steele KE, Davis KJ, Stephan K, Kell W, Vogel P, Hart MK. Comparative neurovirulence and tissue tropism of wild-type and attenuated strains of Venezuelan equine encephalitis virus administered by aerosol in C3H/HeN and BALB/c mice. *Vet Pathol*. (1998) 35:386–97. doi: 10.1177/030098589803500508
8. Julander JG, Skirpstunas R, Siddharthan V, Shafer K, Hoopes JD, Smee DF, et al. C3H/HeN mouse model for the evaluation of antiviral agents for the treatment of Venezuelan equine encephalitis virus infection. *Antiviral Res*. (2008) 78:230–41. doi: 10.1016/j.antiviral.2008.01.007
9. Rusnak JM, Dupuy LC, Niemuth NA, Glenn AM, Ward LA. Comparison of aerosol- and percutaneous-acquired venezuelan equine encephalitis

- in humans and nonhuman primates for suitability in predicting clinical efficacy under the animal rule. *Comp Med*. (2018) 68:380–95. doi: 10.30802/AALAS-CM-18-000027
10. Leitenberg M, Zilinskas RA, Kuhn JH. *The Soviet Biological Weapons Program: A History*. Cambridge, MA: Harvard University Press (2012).
 11. Linssen B, Kinney RM, Aguilar P, Russell KL, Watts DM, Kaaden OR, et al. Development of reverse transcription-PCR assays specific for detection of equine encephalitis viruses. *J Clin Microbiol*. (2000) 38:1527–35.
 12. Martin DA, Muth DA, Brown T, Johnson AJ, Karabatsos N, Roehrig JT. Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays for routine diagnosis of arboviral infections. *J Clin Microbiol*. (2000) 38:1823–6.
 13. de Moraes Bronzoni RV, Baleotti FG, Ribeiro Nogueira RM, Nunes M, Moraes Figueiredo LT. Duplex reverse transcription-PCR followed by nested PCR assays for detection and identification of Brazilian alphaviruses and flaviviruses. *J Clin Microbiol*. (2005) 43:696–702. doi: 10.1128/JCM.43.2.696-702.2005
 14. Wang E, Paessler S, Aguilar PV, Carrara AS, Ni H, Greene IP, et al. Reverse transcription-PCR-enzyme-linked immunosorbent assay for rapid detection and differentiation of alphavirus infections. *J Clin Microbiol*. (2006) 44:4000–8. doi: 10.1128/JCM.00175-06
 15. Laurent P, Le Roux K, Grivard P, Bertil G, Naze F, Picard M, et al. Development of a sensitive real-time reverse transcriptase PCR assay with an internal control to detect and quantify chikungunya virus. *Clin Chem*. (2007) 53:1408–14. doi: 10.1373/clinchem.2007.086595
 16. Luchini A, Geho DH, Bishop B, Tran D, Xia C, Dufour RL, et al. Smart hydrogel particles: biomarker harvesting: one-step affinity purification, size exclusion, and protection against degradation. *Nano Lett*. (2008) 8:350–61. doi: 10.1021/nl072174l
 17. Luchini A, Longo C, Espina V, Petricoin EF 3rd, Liotta LA. Nanoparticle technology: Addressing the fundamental roadblocks to protein biomarker discovery. *J Mater Chem*. (2009) 19:5071–7. doi: 10.1039/b822264a
 18. Douglas TA, Tamburro D, Fredolini C, Espina BH, Lepene BS, Ilag L, et al. The use of hydrogel microparticles to sequester and concentrate bacterial antigens in a urine test for Lyme disease. *Biomaterials*. (2011) 32:1157–66. doi: 10.1016/j.biomaterials.2010.10.004
 19. Shafagati N, Narayanan A, Baer A, Fite K, Pinkham C, Bailey C, et al. The use of NanoTrap particles as a sample enrichment method to enhance the detection of Rift Valley Fever Virus. *PLoS Negl Trop Dis*. (2013) 7:e2296. doi: 10.1371/journal.pntd.0002296
 20. Shafagati N, Patanarut A, Luchini A, Lundberg L, Bailey C, Petricoin E 3rd, et al. The use of Nanotrap particles for biodefense and emerging infectious disease diagnostics. *Pathog Dis*. (2014) 71:164–76. doi: 10.1111/2049-632X.12136
 21. Shafagati N, Fite K, Patanarut A, Baer A, Pinkham C, An S, et al. Enhanced detection of respiratory pathogens with nanotrap particles. *Virulence*. (2016) 7:756–69. doi: 10.1080/21505594.2016.1185585
 22. Lundberg L, Pinkham C, de la Fuente C, Brahms A, Shafagati N, Wagstaff KM, et al. Selective inhibitor of nuclear export (SINE) compounds alter new world alphavirus capsid localization and reduce viral replication in mammalian cells. *PLoS Negl Trop Dis*. (2016) 10:e0005122. doi: 10.1371/journal.pntd.0005122
 23. Kim DY, Firth AE, Atasheva S, Frolova EI, Frolov I. Conservation of a packaging signal and the viral genome RNA packaging mechanism in alphavirus evolution. *J Virol*. (2011) 85:8022–36. doi: 10.1128/JVI.00644-11
 24. Baer A, Kehn-Hall K. Viral concentration determination through plaque assays: using traditional and novel overlay systems. *J Vis Exp*. (2014) e52065. doi: 10.3791/52065
 25. Council NR. *Guide for the Care and Use of Laboratory Animals*. 8th ed. Washington, DC: The National Academies Press (2011).
 26. Grosdidier A, Zoete V, Michielin O. SwissDock, a protein-small molecule docking web service based on EADock DSS. *Nucleic Acids Res*. (2011) 39:W270–7. doi: 10.1093/nar/gkr366
 27. Moller L, Schunadel L, Nitsche A, Schwebke I, Hanisch M, Laue M. Evaluation of virus inactivation by formaldehyde to enhance biosafety of diagnostic electron microscopy. *Viruses*. (2015) 7:666–79. doi: 10.3390/v7020666
 28. Gianazza E, Arnaud P. Chromatography of plasma proteins on immobilized Cibacron Blue F3-GA. *Mech Mol Interact Biochem J*. (1982) 203:637–41. doi: 10.1042/bj2030637
 29. Shafagati N, Lundberg L, Baer A, Patanarut A, Fite K, Lepene B, et al. The use of Nanotrap particles in the enhanced detection of Rift Valley fever virus nucleoprotein. *PLoS ONE*. (2015) 10:e0128215. doi: 10.1371/journal.pone.0128215
 30. Zhang R, Hryc CF, Cong Y, Liu X, Jakana J, Gorchakov R, et al. 4.4 Å cryo-EM structure of an enveloped alphavirus Venezuelan equine encephalitis virus. *EMBO J*. (2011) 30:3854–63. doi: 10.1038/emboj.2011.261
 31. Jaworski E, Saifuddin M, Sampey G, Shafagati N, Van Duynne R, Iordanskiy S, et al. The use of Nanotrap particles technology in capturing HIV-1 virions and viral proteins from infected cells. *PLoS ONE*. (2014) 9:e96778. doi: 10.1371/journal.pone.0096778
 32. Ehrlich R, Miller S. Effect of relative humidity and temperature on airborne Venezuelan equine encephalitis virus. *Appl Microbiol*. (1971) 22:194–9.
 33. Zhao Y, Aarnink AJ, Dijkman R, Fabri T, de Jong MC, Groot Koerkamp PW. Effects of temperature, relative humidity, absolute humidity, and evaporation potential on survival of airborne Gumboro vaccine virus. *Appl Environ Microbiol*. (2012) 78:1048–54. doi: 10.1128/AEM.06477-11
 34. Kudo E, Song E, Yockey LJ, Rakib T, Wong PW, Homer RJ, et al. Low ambient humidity impairs barrier function and innate resistance against influenza infection. *Proc Natl Acad Sci USA*. (2019) 116:10905–10. doi: 10.1073/pnas.1902840116
 35. Patanarut A, Luchini A, Botterell PJ, Mohan A, Longo C, Vorster P, et al. Synthesis and characterization of hydrogel particles containing Cibacron Blue F3G-A. *Colloids Surf A Physicochem Eng Asp*. (2010) 362:8–19. doi: 10.1016/j.colsurfa.2010.03.023
 36. Longo C, Patanarut A, George T, Bishop B, Zhou W, Fredolini C, et al. Core-shell hydrogel particles harvest, concentrate and preserve labile low abundance biomarkers. *PLoS ONE*. (2009) 4:e4763. doi: 10.1371/journal.pone.0004763
 37. Longo C, Gambara G, Espina V, Luchini A, Bishop B, Patanarut AS, et al. A novel biomarker harvesting nanotechnology identifies Bak as a candidate melanoma biomarker in serum. *Exp Dermatol*. (2011) 20:29–34. doi: 10.1111/j.1600-0625.2010.01187.x
 38. Barclay RA, Schwab A, DeMarino C, Akpamagbo Y, Lepene B, Kassaye S, et al. Exosomes from uninfected cells activate transcription of latent HIV-1. *J Biol Chem*. (2017) 292:14764. doi: 10.1074/jbc.A117.793521

Conflict of Interest: AP and BL are employed by the company Ceres Nanosciences, Inc. KK-H is a member of the Scientific Advisory board at Ceres Nanosciences, Inc. BL is a shareholder at Ceres Nanosciences, Inc. The nanoparticles used in this study were research grade and provided by Ceres Nanosciences, Inc. which are not commercially available products. The authors declare that Tasso Inc had no role in the design of the study and collection, analysis, interpretation of the data or writing of the manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Akhrymuk, Lin, Sun, Patnaik, Lehman, Altamura, Minogue, Lepene, van Hoek and Kehn-Hall. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Serological Evidence for Japanese Encephalitis and West Nile Virus Infections in Domestic Birds in Cambodia

Heidi Auerswald^{1*}, Anne-Sophie Ruget^{2,3}, Helena Ladreyt^{3,4,5}, Saraden In¹, Sokthearom Mao¹, San Sorn⁶, Sothyra Tum⁷, Veasna Duong¹, Philippe Dussart¹, Julien Cappelle^{2,3,4,8†} and Véronique Chevalier^{2,3,4†}

OPEN ACCESS

Edited by:

Giovanni Savini,
Experimental Zooprophyllactic Institute
of Abruzzo and
Molise G. Caporale, Italy

Reviewed by:

Venkatramana D. Krishna,
University of Minnesota Twin Cities,
United States
Daniel Marc,
INRA Centre Val de Loire, France

*Correspondence:

Heidi Auerswald
hauerswald@pasteur-kh.org

†These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 23 September 2019

Accepted: 09 January 2020

Published: 29 January 2020

Citation:

Auerswald H, Ruget A-S, Ladreyt H,
In S, Mao S, Sorn S, Tum S, Duong V,
Dussart P, Cappelle J and Chevalier V
(2020) Serological Evidence for
Japanese Encephalitis and West Nile
Virus Infections in Domestic Birds in
Cambodia. *Front. Vet. Sci.* 7:15.
doi: 10.3389/fvets.2020.00015

¹ Virology Unit, Institut Pasteur du Cambodge, Institut Pasteur International Network, Phnom Penh, Cambodia,

² Epidemiology and Public Health Unit, Institut Pasteur du Cambodge, Institut Pasteur International Network, Phnom Penh, Cambodia, ³ Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), Unité Mixte de Recherche ASTRE, Montpellier, France, ⁴ ASTRE, Université Montpellier, CIRAD, INRAE, Montpellier, France,

⁵ Epidemiology Unit, Laboratory for Animal Health, French Agency for Food, Environmental and Occupational Health and Safety (ANSES), University Paris-Est, Maisons-Alfort, France, ⁶ General Directorate for Animal Health and Production, Ministry of Agriculture, Forestry and Fisheries, Phnom Penh, Cambodia, ⁷ National Animal Health and Production Research Institute, General Directorate for Animal Health and Production, Ministry of Agriculture, Forestry and Fisheries, Phnom Penh, Cambodia, ⁸ UMR EpiA, INRAE, VetAgro Sup, Marcy l'Étoile, France

Mosquito-borne flaviviruses with an enzootic transmission cycle like Japanese encephalitis virus (JEV) and West Nile virus (WNV) are a major public health concern. The circulation of JEV in Southeast Asia is well-documented, and the important role of pigs as amplification hosts for the virus is long known. The influence of other domestic animals especially poultry that lives in high abundance and close proximity to humans is not intensively analyzed. Another understudied field in Asia is the presence of the closely related WNV. Such analyses are difficult to perform due to the intense antigenic cross-reactivity between these viruses and the lack of suitable standardized serological assays. The main objective of this study was to assess the prevalence of JEV and WNV flaviviruses in domestic birds, detailed in chickens and ducks, in three different Cambodian provinces. We determined the flavivirus seroprevalence using an hemagglutination inhibition assay (HIA). Additionally, we investigated in positive samples the presence of JEV and WNV neutralizing antibodies (nAb) using foci reduction neutralization test (FRNT). We found 29% (180/620) of the investigated birds positive for flavivirus antibodies with an age-dependent increase of the seroprevalence (OR = 1.04) and a higher prevalence in ducks compared to chicken (OR = 3.01). Within the flavivirus-positive birds, we found 43% (28/65) with nAb against JEV. We also observed the expected cross-reactivity between JEV and WNV, by identifying 18.5% double-positive birds that had higher titers of nAb than single-positive birds. Additionally, seven domestic birds (10.7%) showed only nAb against WNV and no nAb against JEV. Our study provides evidence for an intense JEV circulation in domestic birds in Cambodia, and the first

serological evidence for WNV presence in Southeast Asia since decades. These findings mark the need for a re-definition of areas at risk for JEV and WNV transmission, and the need for further and intensified surveillance of mosquito-transmitted diseases in domestic animals.

Keywords: Japanese encephalitis virus, West Nile virus, domestic birds, poultry, Cambodia, serology

INTRODUCTION

Japanese encephalitis virus (JEV) and West Nile virus (WNV) are the most common encephalitic flaviviruses. The family of *Flaviviridae* contains more than 70 members that were originally distinguished based on the cross-reactivity of the antibodies they induce. Early investigations with polyclonal antisera revealed the antigenic relationships and allowed the separation of the mosquito-borne flaviviruses into seven subgroups, called serocomplexes (1, 2). Members of the same serocomplex are defined by the cross-neutralization of the antibodies they induces. JEV and WNV belong to the JEV serocomplex together with other viruses like Murray Valley encephalitis virus (MVEV), St Louis encephalitis virus (SLEV), and Usutu virus (USUV).

Both JEV and WNV share some ecological similarities as they maintain an enzootic transmission cycle with several bird families as natural reservoirs and mosquitoes of the *Culex* species as main vectors (3, 4). Humans and horses are generally considered dead-end hosts, as they do not develop a viremia high enough to infect mosquitoes. An exception are pigs, as they serve as amplification hosts for JEV because they develop sufficient viral titers to support further infection of mosquitoes (5–7). Although the role of ardeid birds as reservoir hosts for JEV is admitted (8, 9), the role of domestic birds as potential amplifying hosts has been little investigated so far. Several surveys implemented in different continents suggest the involvement of domestic birds, especially ducks, in WNV epidemiological cycle, either as an amplifying host or as a reservoir (10–13). With regards to JEV, two experimental studies suggest that young ducks and chickens might produce a sufficient viremia to infect mosquitoes when biting (14, 15). Because of their close association to humans, and the varying levels of seroprevalence observed in domestic birds, their role in the epidemiological cycle as secondary reservoirs may be of importance (16–18).

JEV is mainly found across Eastern, Southern, and Southeastern Asia where it is the most commonly identified pathogen for encephalitis cases in humans (19). Despite the availability of several vaccines since the 1990s, Japanese encephalitis (JE) is still a clinically important disease with around 70,000 cases per year, causing 10,000–15,000 deaths (20–22) and leaving ~30–50% of the survivors with definitive neurological or psychiatric sequelae (4). WNV is nearly globally distributed even if human outbreaks are sporadically reported because fewer than 1% of human WNV infections develop into severe disease (1, 23). However, the impact of WNV on human and animal health increased dramatically during the last two decades, particularly in the United States of America, with more than 2,000 deaths between 1999 and 2018 (24), and in Europe

(25, 26). Human WNV cases were also reported in several Asian countries (27–30) but little is known about its epidemiology and its potential impact on health in this continent.

JEV is endemic in Cambodia and the major cause of central nervous system infections leading to encephalitis and other severe clinical outcomes in children (31). In 2007, the estimated clinically-declared JE incidence in the country was 11.1 cases per 100 000 children under 15 years of age (32). A better knowledge of JE epidemiology and areas at risk would help focusing preventive measures, such as vaccination, in the future. Regarding WNV in Cambodia, there is little known besides sporadic findings in the 1960s of WNV and its subtype Kunjin virus (33).

As part of a large research program on JE epidemiology in Cambodia (ComAcross project <http://www.onehealthsea.org/comacross>), this study aimed to investigate the exposure to JEV and WNV of domestic birds sampled in three different rural provinces in Cambodia. The collected serum samples were analyzed for flavivirus antibodies by hemagglutination inhibition assay (HIA) and subsequently JEV and WNV specific antibodies by foci reduction neutralization test (FRNT).

MATERIALS AND METHODS

Study Design

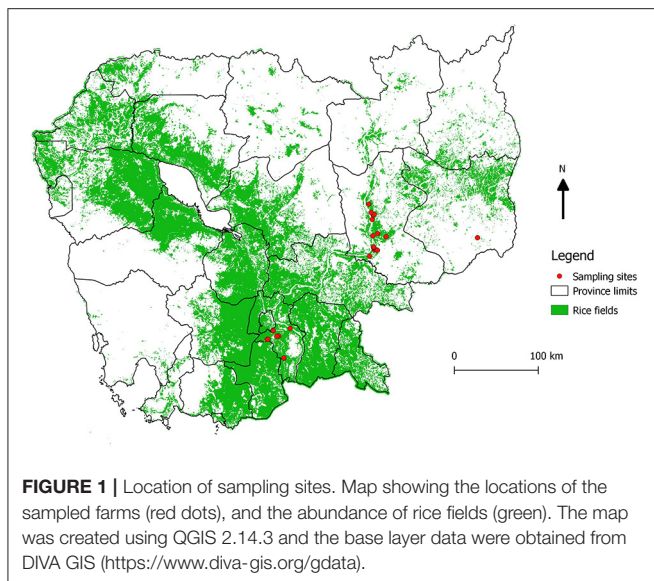
Three geographical areas of Cambodia were selected to get three ecologically contrasted areas according to their landscape characteristics (abundance of rice fields), in addition with their accessibility (distance to Institut Pasteur's laboratory in Phnom Penh) and the ability to provide sufficient number of samples. The fieldwork was conducted in three different provinces in March 2016: Kandal, being a rural area dominated by rice fields, Mondulkiri, mainly dominated by forests and Kratie as an intermediate landscape (**Figure 1**). The objective was to collect samples from at least ten farms per area to get as much representativity as possible. In each farm, animals were randomly selected and according to the owner agreement.

Ethics Statement

During this study, we followed the World Animal Health Organization (OIE) guiding principles on animal welfare included in the OIE Terrestrial Animal Health Code (34). All sampling campaigns were implemented with the supervision of the National Animal Health and Production Research Institute (NAHPRI), and local veterinary services.

Sample Collection

Only chickens and ducks born in the sampling area were included in the study. Blood samples were taken from the ulnar or



metatarsal vein. The blood was transferred into serum tube, stored on ice and at 4°C, and centrifuged later (within the sampling day or up to 5 days after sampling depending on the province) to acquire the respective serum sample. Characteristics of the farm and the GPS coordinates of each farm were collected. If known, the age of the birds was given by the farmers.

Cells and Viruses

Vero cells (ATCC CCL-81) were used for the detection of neutralizing antibodies via FRNT. They were cultivated in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, Steinheim, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) and 100 U/ml penicillin-streptomycin (Gibco) at 37°C and 5% CO₂ atmosphere. All viruses were grown in C6/36 *Aedes albopictus* cells and harvested from the supernatant. The mosquito cells were cultured in Leibovitz-15 medium (Sigma-Aldrich) supplemented with 10% FBS, 1% L-glutamine (Gibco), 10% tryptose-phosphate (Gibco) and 100 U/ml penicillin-streptomycin at 28°C.

The HIA was performed with the following flavivirus strains: JEV Nakayama (Genbank EF571853), Dengue 2 (DENV-2) strain New Guinea C (Genbank AF038403), Dengue 3 (DENV-3) H87 (Genbank M93130), and Zika (ZIKV) HD78788 (Genbank KF383039, KF383084, KF383047). The FRNT was performed with the above-mentioned JEV reference strain Nakayama and the WNV lineage 1 isolate EG101 (Genbank AF260968).

Hemagglutination Inhibition Assay (HIA)

The presence of antibodies in the serum samples was analyzed with the HIA using antigen originated from the above-mentioned JEV, DENV-2, DENV-3, and ZIKV strains. The assay followed the protocol previously described (33) adapted to 96 well microtiter plate.

Foci Reduction Neutralization Test (FRNT)

Due to the high cross-reactivity of the HIA, we aimed to characterize the flavivirus antibodies with a more virus-specific assay. Therefore, we analyzed a subset of 65 sera (39 chicken,

26 duck samples) by foci reduction neutralization tests (FRNTs) against JEV and WNV. The respective samples were chosen because (i) there was sufficient sera volume remaining to perform the FRNT, and (ii) the sample was formerly positive in the HIA for at least one of the tested viruses (JEV, DENV-2, DENV-3, and ZIKV).

The FRNT micro-neutralization assay using reference viruses for JEV and WNV determined the level of neutralizing antibodies and was performed as described previously (35). Briefly, heat inactivated serum samples were analyzed using Vero cells (ATCC CCL-81) seeded in 96 well plates. Serum samples were serially diluted and mixed with equal volume of virus. Virus-serum mixtures were incubated for 1 h at 37°C, and then used for inoculation of Vero cell monolayers. After 1 h of incubation at 37°C on Vero cells, the virus-serum mixtures were replaced by a semi-solid overlay containing 1.6% carboxymethyl cellulose (Sigma-Aldrich) in DMEM medium supplemented with 3% FBS. The plates were incubated at 37°C and 5% CO₂ atmosphere, and stained the following day. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in phosphate buffered saline (PBS) for 30 min. Afterwards, the plates were incubated sequentially with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 20 min and with 10% FBS in PBS, polyclonal anti-JEV or anti-WNV mouse hyperimmune ascites fluids (IPC, Cambodia) and anti-mouse IgG antibody conjugated to horseradish peroxidase (Bio-Rad, Marnes La Coquette, France) for 1 h each. Finally, the infected cells were visualized with TrueBlue peroxidase substrate (KPL, Gaithersburg, MD, USA). The amount of neutralizing antibodies (nAb) is expressed as the reciprocal serum dilution that induces 50% reduction of infection visualized as foci (FRNT₅₀) compared to the controls (flavivirus-negative control serum and virus dilution without added serum) and was calculated via log probit regression analysis (SPSS for Windows, Version 16.0, SPSS Inc., Chicago, IL, USA). FRNT₅₀ titers below 10 were considered negative.

Statistical Analysis

All FRNT titer calculations were performed as log probit regression by using SPSS for Windows, version 23.0. The statistical analyses were performed using R 3.6.0 (36). The arithmetic means of antibody titers were used for comparative analysis. Given the different diagnostic tests used, we considered the results from the HIA test for flavivirus prevalence, which has been carried out on all samples. Association between seroprevalence and species, age and province was first tested on the whole dataset ($n = 620$) using a Chi-square test. Age was categorized in 3-month increments (Table 1): 1–3 months old, 4–6 months old, 7–9 months old, and 10 months or older. The age was not precisely known for 128 domestic birds, however 22 of these birds which were adults, were categorized as 10 months or older.

A generalized linear model (glm) was used to assess the link between seroprevalence and age, species and province. Animals for which the exact age was unknown, including all sampled in Mondulhiri, were excluded from the multivariate analysis, and age was used as a discrete variable (age in months). Due to the sampling frame and potential overdispersion, the province (Kandal and Kratie), was incorporated in the model, either as a

TABLE 1 | Flavivirus seroprevalence based on hemagglutination inhibition assay ($n = 620$).

	Samples tested n (%)	HIA positive* n (%)			
Total	620	180 (29.0)			
Province					
Kandal	296 (47.7)	58 (19.6)			
Kratie	283 (45.6)	98 (34.6)			
Mondulkiri	41 (6.6)	24 (58.5)			
Species					
Chicken	417 (67.3)	99 (23.7)			
Duck	203 (32.7)	81 (39.9)			
Age mean (95% CI)	7.93 (7.19–8.67)		Age groups	Samples tested n (%)	HIA positive* n (%)
1 month	11 (1.8)	2 (18.2)	1–3 month	195 (31.5)	33 (16.9)
2 months	105 (16.9)	20 (19.0)			
3 months	79 (12.7)	11 (13.9)			
4 months	96 (15.5)	23 (24.0)	4–6 months	144 (23.2)	29 (20.1)
5 months	18 (2.9)	2 (11.1)			
6 months	30 (4.8)	4 (13.3)			
7 months	10 (1.6)	2 (20.0)	7–9 months	28 (4.5)	6 (21.4)
8 months	17 (2.7)	4 (23.5)			
9 months	1 (0.2)	0 -			
10 months	8 (1.3)	4 (50.0)	≥10 months [#]	147 (23.7)	75 (51.0)
11 months	1 (0.2)	0 -			
12 months	32 (5.2)	18 (56.3)			
18 months	9 (1.5)	4 (44.4)			
24 months	57 (9.2)	35 (61.4)			
27 months	2 (0.3)	0 -			
30 months	10 (1.6)	0 -			
36 months	6 (1.0)	2 (33.3)			
Unknown [#]	128 (20.6)	49 (38.3)	Unknown	106 (17.1)	37 (34.9)

*HIA titer ≥ 80 in at least one of the tested flaviviruses (JEV, DENV-2, DENV-3, ZIKV).

[#]Including 22 birds with an estimated age of <12 months.

HIA Hemagglutination inhibition assay; JEV Japanese encephalitis virus; WNV West Nile virus, DENV dengue virus, ZIKV Zika virus.

fixed or a random effect (glmm). The best model was selected according to the Akaike Information Criteria (AIC).

RESULTS

Sample Collection

In total, 620 samples were collected (Table 1) in 41 backyard farms with an average of 15 samples per farm. The collection contained 417 (67.3%) blood samples collected from chickens (*Gallus gallus domesticus*) and 203 (32.7%) from ducks (*Anas platyrhynchos domesticus*). In detail, 296 samples (47.7%) were collected in the Kandal province, 283 samples in Kratie (45.6%) and 41 samples (6.6%) in Mondulkiri. The age of the 492 (79.4%) domestic birds could be obtained, and ranged from one to 36 months (mean 7.93 months; 95% CI 7.19–8.67; Supplementary Table 1).

TABLE 2 | Results of the univariate analysis (χ^2) between seroprevalence and other factors ($n = 620$).

Variable	Df	P-value
Species	1	3.20e-5
Province	2	3.35e-8
Age group	3	4.79e-12

Flavivirus Seroprevalence Based on HIA

Overall, 180 samples (29%) were detected positive by HIA for at least one of the flaviviruses tested (JEV, DENV-2, DENV-3, and ZIKV) (Table 1). The univariate analysis (Table 2) revealed a significant higher proportion of ducks (39.9%, 81/203 samples) with anti-flavivirus antibodies compared to the amount of positive tested chickens (23.7%; 99/417). The observed flavivirus seroprevalence was also different for the investigated provinces with the lowest seroprevalence in Kandal (19.6%, 58/296 samples), and the highest for Mondulkiri (58.5%; 24/41 samples). Additionally, the seroprevalence rate increased with the age of the birds as antibodies were found in 16.9% (33/195 samples) of young birds (1–3 months old) rising to 51.0% (75/147 samples) in birds that were 10 months or older. For 22.3% (138/620) of the samples, JEV hemagglutinating antibodies were detected (Supplementary Table 1). Also, 157 samples (25.3%) were tested positive for antibodies against DENV-2 and/or DENV-3, and 63 samples (10.2%) against ZIKV. Most of the HIA positive samples showed a positive reaction against more than one of the tested viruses (Table 4), as 76.6% (138/180) of the positive samples had antibodies against JEV, and 87.2% (157/180) against DENV-2 and/or DENV-3.

Regarding the HIA titers for the individual domestic birds, the mean HIA titers differ significantly between the investigated viruses (Supplementary Figure 1). The mean JEV HIA titer was 83.47 (95% CI 58.5–108.5) and therefore significantly lower than for DENV-3 (mean 263.4; 95% CI 146.1–380.8; $p = 0.0006$; Friedman test with Dunn's multiple comparison test). The DENV-3 HIA titers were also significantly higher than for DENV-2 (mean 177.2; 95% CI 111.1–243.4; $p < 0.0001$). Overall, the mean ZIKV HIA was significantly lower than for the other three viruses (mean 39.97; 95% CI 25.28–54.65; $p < 0.0001$). The JEV HIA titers correlated moderate with the HIA titers against the other flaviviruses (DENV-2 $r = 0.62$; DENV-3 $r = 0.59$; ZIKV $r = 0.65$; Supplementary Figure 2). The DENV-2 and DENV-3 titers correlated strongly ($r = 0.90$) but less so with the closely related ZIKV (DENV-2 $r = 0.53$; DENV-3 $r = 0.49$).

According to AIC (AIC_{glm} = 553 vs. AIC_{glmm} = 558), the best generalized linear model with the flavivirus serological status based on HIA as outcome, and age, species and province as explanatory variables, incorporated the province as a fixed effect. This model confirmed the results of the univariate analysis: the seroprevalence rate is significantly higher in ducks compared to chickens (OR = 3.01, 95%CI: 1.97–4.63) and slightly increased with age (OR = 1.04, 95% CI: 1.0–21.07; Table 3, Figure 2). Domestic birds were also more exposed in Kratie than in Kandal (OR = 2.01, 95%CI: 1.31–3.09).

JEV- and WNV-Specific Seroprevalence Based on FRNT

Among all the HIA-positive samples ($n = 180$), we analyzed a subset of 65 sera by foci reduction neutralization tests (FRNTs) against JEV and WNV based on criteria previously exposed (see Materials and Methods). The comparison of FRNT and HIA results showed that from the 65 samples tested positive for flavivirus hemagglutinating antibodies, only 35 had

detectable levels of nAb for JEV and/or WNV (Table 4). From this subset, 28 samples showed nAb against JEV, including 12 sera that additionally had detectable levels of WNV nAb (Figure 3A). Interestingly, seven bird samples had nAb only against WNV and not against JEV. Most samples with detected nAb also showed HIA antibodies against more than one of the tested viruses (Table 4). Only one adult duck from Kandal with nAb against WNV was HIA positive exclusively against ZIKV.

The mean FRNT₅₀ titer of JEV nAb (21.14; SD 35.08; 95% CI 12.45–229.83) was similar to the mean WNV FRNT₅₀ titer (19.43; SD 46.89; 95% CI 7.81–31.05). The number of FRNT positive birds (Supplementary Table 2) and the levels of nAb (Supplementary Figure 3) did not significantly differ between poultry species or province of origin. Also, the nAb titers were significantly higher in the birds that were tested double positive for both JEV and WNV nAb compared to single positive sera (JEV $p = 0.002$; WNV $p = 0.014$; Mann-Whitney, Supplementary Figure 3C). We observed a weak correlation of the nAb titers

TABLE 3 | Results of generalized linear model ($n = 492^*$).

Explanatory variable	P-value	OR [IC 95%]
Intercept	<2e-16	0.11 [0.07–0.17]
Species (ref=chicken)	3.89e-7	3.01 [1.97–4.63]
Province (ref=Kandal)	1.39e-3	2.01 [1.31–3.09]
Age	7.29e-5	1.05 [#] [1.02–1.07]

*Birds with unknown or only estimated age (e.g., ">12 months") were removed from the analysis.

[#]Odds ratio of being seropositive for an additional month of age is 1.04.

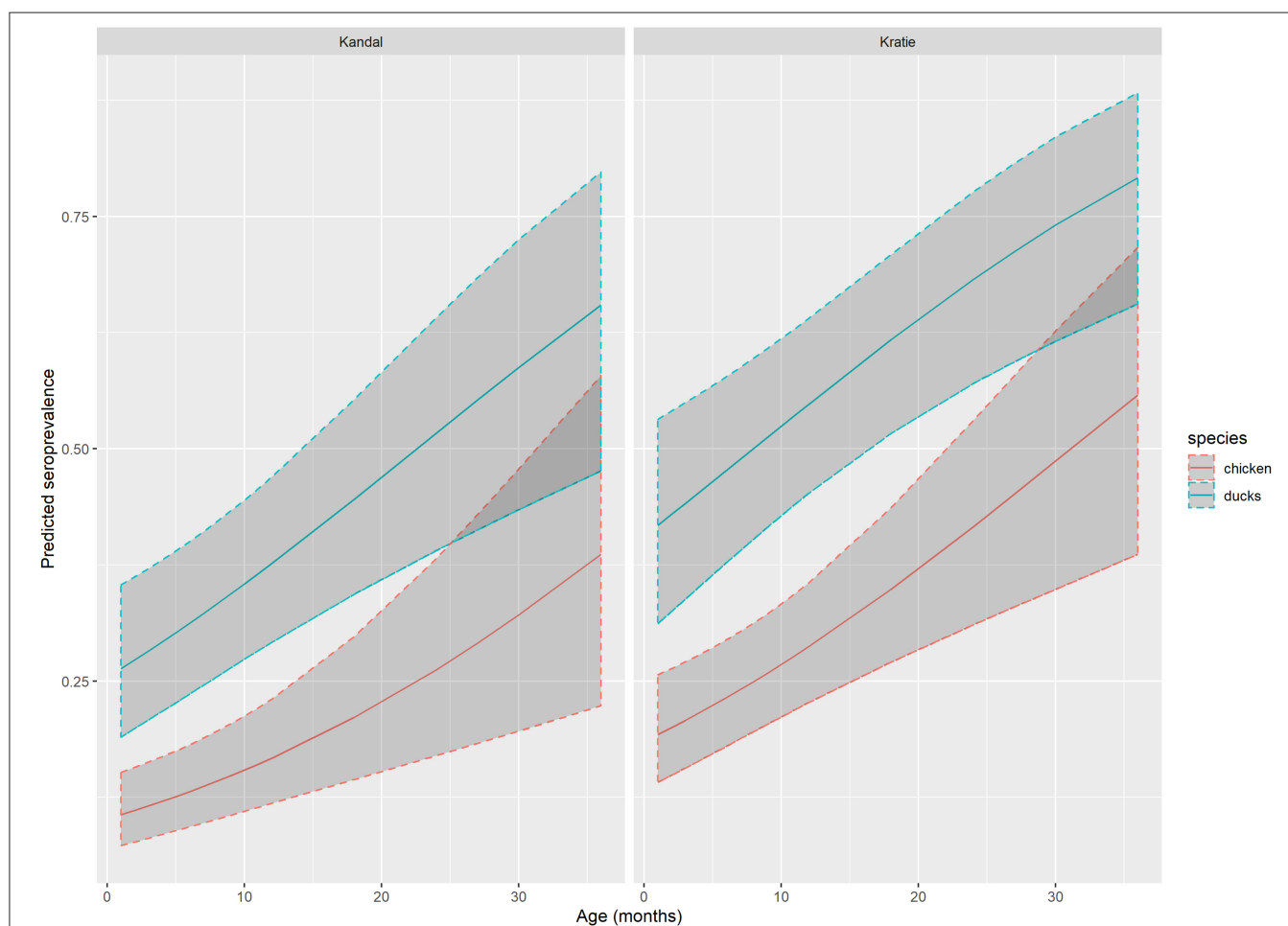
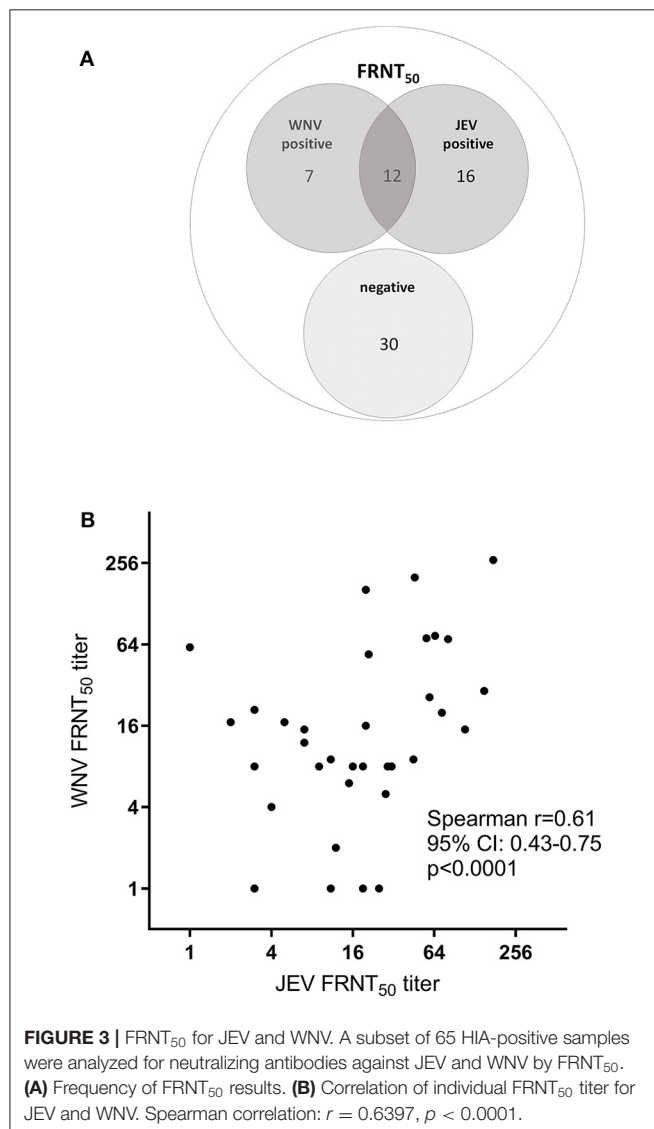


FIGURE 2 | Flavivirus seroprevalence predicted by GLM. Predicted flavivirus seroprevalence in Kandal and Kratie provinces, for chicken (red line) and ducks (blue line) by age with 95% confidence interval (dark gray area) based on the generalized linear model.

TABLE 4 | Comparison of HIA and FRNT results.

		1	2	3	4	6	7
	Number of positive results*	Flavivirus HIA	JEV HIA	DENV # HIA	ZIKV HIA	JEV FRNT ₅₀ [§]	WNV FRNT ₅₀ [§]
1	Flavivirus HIA	180	138	157	63	28	19
2	JEV HIA	138		118	60	21	18
3	DENV HIA [#]	157	118		53	26	15
4	ZIKV HIA	63	60	53		8	5
5	JEV & WNV FRNT ₅₀	35	27	31	10	28	19
6	JEV FRNT ₅₀ [§]	28	21	26	8	-	12
7	WNV FRNT ₅₀ [§]	19	18	15	5	12	-

[§] FRNT tested subset of 65 sera.* HIA titer ≥ 80 , FRNT₅₀ titer ≥ 10 .[#] HIA for DENV-2 and/or DENV-3.

between both viruses (**Figure 3B**; $r = 0.64$; $p < 0.0001$; Pearson correlation).

DISCUSSION

Our study found an overall flavivirus seroprevalence of 29% in domestic birds. This high percentage of seropositive poultry is highly likely due to the fact that JEV is endemic in Cambodia (31). This is similar to the findings of other JEV seroprevalence studies in Southeast Asia. In Bali (Indonesia) 20.6% of ducks and 36.7% of chickens were tested positive (37), and a study in Malaysia found 28.9% of the tested domestic birds positive for JEV antibodies (38). In addition, several experimental studies showed that domestic birds can be infected with JEV (5, 14, 15) and might even act as JEV reservoirs (39, 40). However, it is controversially discussed if they develop a sufficient viremia to infect mosquitoes (14, 41–43). In our study, ducks were more likely to be seropositive when they are 10 months or older than chickens (87.1% of ducks seropositive compared to 33.3% of chickens of that age). This could be due to feeding behavior of certain mosquitoes, different exposure due to distinct housing conditions or simply because ducks are usually kept longer before slaughtering than chickens.

Flavivirus detection in animals and humans especially in prevalence studies is mainly done serologically, as the viremic phase is rather short (44), e.g., JEV viremia lasts <1 week in chicks and ducklings (14). Yet, the co-circulation of several flaviviruses poses a diagnostic challenge due to the broad antibody cross-reactivity within and across the different serocomplexes (45). Indeed, extensive cross-reactivity is known for JEV and WNV even leading to reports of cross-protection (46, 47). Despite intensive attempts to develop specific diagnostic assays, the neutralization test is still considered to be the gold standard for the serological differentiation of flaviviruses (3). Due to the cross-reactivity, retrospective seroprevalence studies for flaviviruses are challenging in regions where more than one of these viruses circulate. The HIA is characterized by a high cross-reactivity which generally only allows a qualitative conclusion about the presence of flavivirus antibodies (48–51). Our HIA analysis and the moderate correlation of HIA titers among all viruses also demonstrated a high cross-reactivity especially between the two DENV serotypes 2 and 3 (Spearman $r = 0.8999$). In contrast, the correlation between the DENV HIA titers and the

other viruses was less pronounced. This could be a consequence of the degree of antigenic similarities between the viruses (52), as the closely related DENV serotypes showed a high degree of correlation whereas JEV belongs to a different serocomplex than DENV and ZIKV. Additionally, DENV infection is not reported in poultry and therefore the antibodies measured against DENV might be the result of a non-specific immune response after a JEV infection. As a consequence of the endemicity of several flaviviruses in Cambodia, we chose a high threshold of ≥ 80 for HIA positivity. For the much more specific neutralization assay (FRNT), we chose the less stringent criteria for positivity by using the FRNT₅₀ titer instead of FRNT₉₀ and a threshold of ≥ 10 for positivity. This strategy was also used in other flavivirus seroprevalence studies in birds (48, 53, 54).

A limitation of our study is the uneven sample distribution regarding species, age and province of the animals. There were much less birds sampled in Mondulkiri province and ducks were overall underrepresented in the study cohort. We also had proportionally more samples from young chickens (1–3 months old) and older ducks (≥ 10 months; see **Supplementary Table 1**). Furthermore, we did not include WNV in the HIA because WNV is not endemic in Cambodia and therefore this virus is not part of our routine serological testing. Moreover, not all HIA positive samples could be tested with FRNT because of insufficient sera volume. Additionally, this assay is time- and labor-consuming. However, the samples analyzed with FRNT were not significantly different from the samples not tested with FRNT and from all samples that were tested positive for any flavivirus HIA (**Supplementary Table 2**), even if the HIA titers are slightly lower for the subset of FRNT samples compared to all HIA positive samples.

To our knowledge, this is the first serological evidence of WNV circulation in Cambodia, where the virus was last found before the 1980s (4). However, with our study we were only able to trace nAb against WNV in 7 domestic birds in the absence of JEV nAb. The direct detection of WNV in poultry, humans or mosquitoes as thorough evidence is still missing. The global distribution of WNV in tropical and temperate regions of Europe, Africa, the Americas, Western and central Asia is well-documented (17, 18, 55). In Southeast Asia, the main encephalitic flavivirus is still JEV (56). However, concerns about the ability of WNV to spread along bird migration routes are appropriate based on the recent expansion of WNV circulation in Eurasia (57) and the explosive dissemination of WNV in the Americas since the New York city outbreak in 1999 (58, 59). Importantly, despite their high serological cross-reactivity and virological similarities, JEV and WNV show distinct ecological and epidemiological specificities. Despite that both can be transmitted by *Culex* mosquitoes, the main vector of JEV is *Culex tritaeniorhynchus*, even as it was found in over 30 other mosquito species (9), whereas WNV is mainly transmitted by females of the *Culex pipiens* complex and their hybrids. For the endemic circulation of JEV, pigs play an important role as amplification hosts (60). In contrast, WNV can exclusively replicate in birds, especially in Passerines (61, 62).

Overall, recent studies investigated intensely the role of pigs in the JEV epidemiology as these are well-known amplification hosts for the virus. However, the contribution of poultry to the

circulation of JEV remains understudied. Our study provides confirmation of a high seroprevalence for JEV in poultry as well as the first evidence of the circulation of WNV in domestic birds in the region. These findings may have consequences for the definition of areas at risk for JEV transmission, as the JEV might be able to circulate in areas with low densities of pigs or no pigs. This emphasizes the need for further and intensified surveillance of mosquito-transmitted diseases where backyard animals serve as potential amplification hosts.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because there is no national (Cambodian) animal ethics committee. We followed the World Animal Health Organization (OIE) guiding principles on animal welfare included in the OIE Terrestrial Animal Health Code. All sampling campaigns were implemented with the supervision of the National Animal Health and Production Research Institute (NAHPRI), and local veterinary services. We provided the respective letter of approval from NAHPRI to the editorial office. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

PD, JC, and VC: conceived and designed the study, the laboratory investigations and wrote the paper (comment and editing). A-SR and VC: sample collection and epidemiological analysis. HA, SI, and SM: performed the laboratory investigations. HA, A-SR, VD, and VC: analyzed the data. SS, ST, SI, VD, PD, and VC: contributed field work, reagents, materials, and analysis tools. HA, A-SR, and HL: wrote the paper (original draft).

FUNDING

This work was partial funded by ComAcross project (<http://www.onehealthsea.org/comacross>) and the Virology Unit of Institut Pasteur du Cambodge. The postdoctoral fellowship of HA was supported by the Calmette and Yersin Programme of the Institut Pasteur Department of International Affairs.

ACKNOWLEDGMENTS

The authors thank all farmers who participated in the study and allowed the sampling of their animals.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2020.00015/full#supplementary-material>

REFERENCES

- Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, et al. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol.* (1989) 70:37–43. doi: 10.1099/0022-1317-70-1-37
- De Madrid AT, Porterfield JS. The flaviviruses (group B arboviruses): a cross-neutralization study. *J Gen Virol.* (1974) 23:91–6. doi: 10.1099/0022-1317-23-1-91
- van den Hurk AF, Ritchie SA, Mackenzie JS. Ecology and geographical expansion of Japanese encephalitis virus. *Annu Rev Entomol.* (2009) 54:17–35. doi: 10.1146/annurev.ento.54.110807.090510
- Chancey C, Grinev A, Volkova E, Rios M. The global ecology and epidemiology of West Nile virus. *Biomed Res Int.* (2015) 2015:1–20. doi: 10.1155/2015/376230
- Gresser I, Hardy JL, Hu SM, Scherer WF. Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus* Giles, from infected pigs and chicks to susceptible pigs and birds. *Am J Trop Med Hyg.* (1958) 7:365–73. doi: 10.4269/ajtmh.1958.7.365
- Hurlbut HS. The Pig-Mosquito Cycle of Japanese Encephalitis Virus in Taiwan. *J Med Entomol.* (1964) 1:301–307. doi: 10.1093/jmedent/1.3.301
- Ladreyt H, Durand B, Dussart P, Chevalier V. How central is the domestic pig in the epidemiological cycle of Japanese Encephalitis Virus? A review of scientific evidence and implications for disease control. *Viruses.* (2019) 11:949. doi: 10.3390/v11100949
- Buescher EL, Scherer WF, McClure HE, Moyer JT, Rosenberg MZ, Yoshii M, et al. Ecologic studies of Japanese encephalitis virus in Japan. IV. Avian infection. *Am J Trop Med Hyg.* (1959) 8:678–88. doi: 10.4269/ajtmh.1959.8.678
- Scherer WF, Moyer JT, Izumi T, Gresser I, McCown J. Ecologic studies of Japanese encephalitis virus in Japan. VI. Swine infection. *Am J Trop Med Hyg.* (1959) 8:698–706. doi: 10.4269/ajtmh.1959.8.698
- Wojnarowicz C, Olkowski A, Schwan-Lardner K. First Canadian outbreak of West Nile virus disease in farmed domestic ducks in Saskatchewan. *Can Vet J.* (2007) 48:1270–1. Available online at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2081995/pdf/cvj48pg1270.pdf>
- Maquart M, Boyer S, Rakotoharinome VM, Ravaomanana J, Tantely ML, Heraud J-M, et al. High prevalence of West Nile Virus in Domestic Birds and Detection in 2 new mosquito species in Madagascar. *PLoS ONE.* (2016) 11:e0147589. doi: 10.1371/journal.pone.0147589
- Monastiri A, Mechri B, Vázquez-González A, Ar Gouilh M, Chakroun M, Loussaief C, et al. A four-year survey (2011–2014) of West Nile virus infection in humans, mosquitoes and birds, including the 2012 meningoencephalitis outbreak in Tunisia. *Emerg Microbes Infect.* (2018) 7:28. doi: 10.1038/s41426-018-0028-y
- Meece JK, Kronenwetter-Koepel TA, Vandermause MF, Reed KD. West Nile Virus Infection in Commercial Waterfowl Operation, Wisconsin. *Emerg Infect Dis.* (2006) 12:1451–3. doi: 10.3201/eid1209.051648
- Cleton NB, Bosco-Lauth A, Page MJ, Bowen RA. Age-related susceptibility to Japanese encephalitis virus in domestic ducklings and chicks. *Am J Trop Med Hyg.* (2014) 90:242–6. doi: 10.4269/ajtmh.13-0161
- Dhanda V, Banerjee K, Deshmukh PK, Ilkal MA. Experimental viraemia and transmission of Japanese encephalitis virus by mosquitoes in domestic ducks. *Indian J Med Res.* (1977) 66:881–8.
- Chhour YM, Ruble G, Hong R, Minn K, Kdan Y, Sok T, et al. Hospital-based Diagnosis of Hemorrhagic Fever, Encephalitis, and Hepatitis in Cambodian Children. *Emerg Infect Dis.* (2002) 8:485–9. doi: 10.3201/eid0805.010236
- Horwood PF, Duong V, Laurent D, Mey C, Sothy H, Santy K, et al. Aetiology of acute meningoencephalitis in Cambodian children, 2010–2013. *Emerg Microbes Infect.* (2017) 6:e35. doi: 10.1038/emi.2017.15
- Turner P, Suy K, Van Tan L, Sar P, Miliya T, Hong NTT, et al. The aetiologies of central nervous system infections in hospitalised Cambodian children. *BMC Infect Dis.* (2017) 17:806. doi: 10.1186/s12879-017-2915-6
- Solomon T. Control of Japanese Encephalitis — Within Our Grasp? *N Engl J Med.* (2006) 355:869–71. doi: 10.1056/NEJMp058263
- Solomon T, Dung NM, Kneen R, Gainsborough M, Vaughn DW, Khanh VT, et al. Japanese encephalitis. *J Neurol Neurosurg Psychiatry.* (2000) 68:405–15. doi: 10.1136/jnnp.68.4.405
- Hills SL, Van Cuong N, Touch S, Mai HH, Soeung SC, Lien TTH, et al. Disability from Japanese encephalitis in Cambodia and Viet Nam. *J Trop Pediatr.* (2011) 57:241–4. doi: 10.1093/tropej/fmp133
- Campbell G, Hills S, Fischer M, Jacobson J, Hoke C, Hombach J, et al. Estimated global incidence of Japanese encephalitis. *Bull World Health Organ.* (2011) 89:766–74. doi: 10.2471/BLT.10.085233
- Mansfield KL, Horton DL, Johnson N, Li L, Barrett ADT, Smith DJ, et al. Flavivirus-induced antibody cross-reactivity. *J Gen Virol.* (2011) 92:2821–9. doi: 10.1099/vir.0.031641-0
- Centers for Disease Control and Prevention. *Final Cumulative Maps & Data for 1999–2018.* (2019) Available online at: <https://www.cdc.gov/westnile/statsmaps/cumMapsData.html>
- European Centre for Disease Prevention and Control. *Epidemiological Update: West Nile Fever in Europe - Number of Infections So far Exceeds the Total Number in the Previous Five Years.* (2014) Available online at: <https://ecdc.europa.eu/en/news-events/epidemiological-update-west-nile-fever-europe-number-infections-so-far-exceeds-total>
- Holt E. West Nile virus spreads in Europe. *Lancet Infect Dis.* (2018) 18:1184. doi: 10.1016/S1473-3099(18)30616-9
- Cao L, Fu S, Lu Z, Tang C, Gao X, Li X, et al. Detection of West Nile Virus Infection in Viral Encephalitis Cases, China. *Vector-Borne Zoonotic Dis.* (2019) 19:45–50. doi: 10.1089/vbz.2018.2275
- Balakrishnan A, Thekkakare R, Sapkal G, Tandale B. Seroprevalence of Japanese encephalitis virus & West Nile virus in Alappuzha district, Kerala. *Indian J Med Res.* (2017) 146:70. doi: 10.4103/ijmr.IJMR_1638_15
- Khatun T, Chatterjee S. Emergence of West Nile virus in West Bengal, India: a new report. *Trans R Soc Trop Med Hyg.* (2017) 111:178–84. doi: 10.1093/trstmh/trx033
- Cao L, Fu S, Lv Z, Tang C, Cui S, Li X, et al. West Nile virus infection in suspected febrile typhoid cases in Xinjiang, China. *Emerg Microbes Infect.* (2017) 6:1–4. doi: 10.1038/emi.2017.27
- Touch S, Hills S, Sokhal B, Samnang C, Sovann L, Khieu V, et al. Epidemiology and burden of disease from Japanese encephalitis in Cambodia: results from two years of sentinel surveillance. *Trop Med Int Heal.* (2009) 14:1365–73. doi: 10.1111/j.1365-3156.2009.02380.x
- Mackenzie JS, Williams DT. The Zoonotic Flaviviruses of Southern, South-Eastern and Eastern Asia, and Australasia: the potential for Emergent Viruses. *Zoonoses Public Health.* (2009) 56:338–56. doi: 10.1111/j.1863-2378.2008.01208.x
- Clarke DH, Casals J. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am J Trop Med Hyg.* (1958) 7:561–73. doi: 10.4269/ajtmh.1958.7.561
- OIE. Use of Animals in Research and Education. In: *OIE Terrestrial Animal Health Code.* Available online at: http://www.oie.int/index.php?id=169&L=0&htmlfile=chapitre_aw_research_education.htm
- Auerswald H, de Jesus A, Seixas G, Nazareth T, In S, Mao S, et al. First dengue virus seroprevalence study on Madeira Island after the 2012 outbreak indicates unreported dengue circulation. *Parasit Vectors. BioMed Central.* (2019) 12:103. doi: 10.1186/s13071-019-3357-3
- R Development Core Team. *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing (2008).
- Ayu Mirah Adi AA, Astawa NM, Asri Damayanti PA, Kardena IM, Krisna Erawan IGM, et al. Seroepidemiological evidence for the presence of Japanese encephalitis virus infection in ducks, chickens, and pigs, Bali-Indonesia. *Bali Med J.* (2016) 5:189. doi: 10.15562/bmj.v5i3.343
- Kumar K, Arshad SS, Selvarajah GT, Abu J, Toung OP, Abba Y, et al. Prevalence and risk factors of Japanese encephalitis virus (JEV) in livestock and companion animal in high-risk areas in Malaysia. *Trop Anim Health Prod.* (2018) 50:741–52. doi: 10.1007/s11250-017-1490-6
- Liu W, Gibbons RV, Kari K, Clemens JD, Nisalak A, Marks F, et al. Risk factors for Japanese encephalitis: a case-control study. *Epidemiol Infect.* (2010) 138:1292–7. doi: 10.1017/S0950268810000063
- Keiser J, Maltese MF, Erlanger TE, Bos R, Tanner M, Singer BH, et al. Effect of irrigated rice agriculture on Japanese encephalitis, including challenges and opportunities for integrated vector management. *Acta Trop.* (2005) 95:40–57. doi: 10.1016/j.actatropica.2005.04.012

41. Karna A, Bowen R. Experimental evaluation of the role of ecologically-relevant hosts and vectors in Japanese encephalitis virus genotype displacement. *Viruses*. (2019) 11:32. doi: 10.3390/v11010032
42. Pant GR. A serological survey of pigs, horses, and ducks in Nepal for evidence of infection with Japanese encephalitis virus. *Ann N Y Acad Sci*. (2006) 1081:124–9. doi: 10.1196/annals.1373.013
43. Simpson DIH, Bowen ETW, Platt GS, Way H, Smith CEG, Peto S, et al. Japanese encephalitis in Sarawak: virus isolation and serology in a land Dyak village. *Trans R Soc Trop Med Hyg*. (1970) 64:503–10. doi: 10.1016/0035-9203(70)90070-2
44. Domingo C, Patel P, Linke S, Achazi K, Niedrig M. Molecular diagnosis of flaviviruses. *Future Virol*. (2011) 6:1059–74. doi: 10.2217/fvl.11.77
45. Barba-Spaeth G, Dejnirattisai W, Rouvinski A, Vaney M-C, Medits I, Sharma A, et al. Structural basis of potent Zika-dengue virus antibody cross-neutralization. *Nature*. (2016) 536:48–53. doi: 10.1038/nature18938
46. Kuno G. Serodiagnosis of flaviviral infections and vaccinations in humans. In: Chambers TJ, Monath TP, editors. *Advances in Virus Research*. San Diego, CA: Elsevier Academic Press (2003). p. 3–65. doi: 10.1016/S0065-3527(03)61001-8
47. Litzba N, Klade CS, Lederer S, Niedrig M. Evaluation of serological diagnostic test systems assessing the immune response to Japanese encephalitis vaccination. *PLoS Negl Trop Dis*. (2010) 4:e883. doi: 10.1371/journal.pntd.0000883
48. Buckley A. Serological evidence of West Nile virus, Usutu virus and Sindbis virus infection of birds in the UK. *J Gen Virol*. (2003) 84:2807–17. doi: 10.1099/vir.0.19341-0
49. Michel F, Fischer D, Eiden M, Fast C, Reuschel M, Müller K, Rinder M, et al. West Nile Virus and Usutu Virus Monitoring of Wild Birds in Germany. *Int J Environ Res Public Health*. (2018) 15:171. doi: 10.3390/ijerph15010171
50. Llopi IV, Rossi L, Di Gennaro A, Mosca A, Teodori L, Tomassone L, et al. Further circulation of West Nile and Usutu viruses in wild birds in Italy. *Infect Genet Evol*. (2015) 32:292–7. doi: 10.1016/j.meegid.2015.03.024
51. Csank T, Drzewnioková P, Korytár L, Major P, Gyuranecz M, Pistl J, et al. A Serosurvey of Flavivirus Infection in Horses and Birds in Slovakia. *Vector-Borne Zoonotic Dis*. (2018) 18:206–13. doi: 10.1089/vbz.2017.2216
52. Heinz FX, Stiasny K. Flaviviruses and their antigenic structure. *J Clin Virol*. (2012) 55:289–95. doi: 10.1016/j.jcv.2012.08.024
53. Bazanów B, Jansen van Vuren P, Szymanski P, Stygar D, Fracka A, Twardon J, et al. A survey on West Nile and Usutu viruses in horses and birds in Poland. *Viruses*. (2018) 10:87. doi: 10.3390/v10020087
54. Michel F, Sieg M, Fischer D, Keller M, Eiden M, Reuschel M, et al. Evidence for West Nile Virus and Usutu Virus Infections in Wild and Resident Birds in Germany, 2017 and 2018. *Viruses*. (2019) 11:674. doi: 10.3390/v11070674
55. Srey VH, Sadones H, Ong S, Mam M, Yim C, Sor S, et al. Etiology of encephalitis syndrome among hospitalized children and adults in Takeo, Cambodia, 1999–2000. *Am J Trop Med Hyg*. (2002) 66:200–7. doi: 10.4269/ajtmh.2002.66.200
56. Paz S, Semenza J. Environmental drivers of West Nile fever epidemiology in Europe and Western Asia—A Review. *Int J Environ Res Public Health*. (2013) 10:3543–62. doi: 10.3390/ijerph10083543
57. Petersen LR, Hayes EB. West Nile Virus in the Americas. *Med Clin North Am*. (2008) 92:1307–22. doi: 10.1016/j.mcna.2008.07.004
58. Pearce JC, Learoyd TP, Langendorf BJ, Logan JG. Japanese encephalitis: the vectors, ecology and potential for expansion. *J Travel Med*. (2018) 25:S16–26. doi: 10.1093/jtm/tay009
59. Oliveira ARS, Cohnstaedt LW, Strathe E, Etcheverry L, McVey DS, Piaggio J, et al. Meta-analyses of Japanese encephalitis virus infection, dissemination, and transmission rates in vectors. *Am J Trop Med Hyg*. (2018) 98:883–90. doi: 10.4269/ajtmh.17-0622
60. Casals J, Brown LV. Hemagglutination with arthropod-borne viruses. *J Exp Med*. (1954) 99:429–49. doi: 10.1084/jem.99.5.429
61. Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, et al. Experimental Infection of North American Birds with the New York 1999 Strain of West Nile Virus. *Emerg Infect Dis*. (2003) 9:311–22. doi: 10.3201/eid0903.020628
62. Yaremych SA, Warner RE, Mankin PC, Brawn JD, Raim A, Novak R. West Nile Virus and High Death Rate in American Crows. *Emerg Infect Dis*. (2004) 10:709–11. doi: 10.3201/eid1004.030499

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Auerswald, Ruget, Ladreyt, In, Mao, Sorn, Tum, Duong, Dussart, Cappelle and Chevalier. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Emerging Threats to Animals in the United Kingdom by Arthropod-Borne Diseases

Arran J. Folly¹, Daniel Dorey-Robinson¹, Luis M. Hernández-Triana¹, L. Paul Phipps¹ and Nicholas Johnson^{1,2*}

¹ Virology Department, Animal and Plant Health Agency (Weybridge), Addlestone, United Kingdom, ² Faculty of Health and Medicine, University of Surrey, Guildford, United Kingdom

OPEN ACCESS

Edited by:

Giovanni Savini,
Experimental Zooprophyllactic Institute
of Abruzzo and Molise G.
Caporale, Italy

Reviewed by:

Sylvie Lecollinet,
Agence Nationale de Sécurité
Sanitaire de l'Alimentation, de
l'Environnement et du Travail
(ANSES), France
Annapaola Rizzoli,
Fondazione Edmund Mach, Italy

*Correspondence:

Nicholas Johnson
nick.johnson@apha.gov.uk

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 24 September 2019

Accepted: 10 January 2020

Published: 04 February 2020

Citation:

Folly AJ, Dorey-Robinson D,
Hernández-Triana LM, Phipps LP and
Johnson N (2020) Emerging Threats
to Animals in the United Kingdom by
Arthropod-Borne Diseases.
Front. Vet. Sci. 7:20.
doi: 10.3389/fvets.2020.00020

Worldwide, arthropod-borne disease transmission represents one of the greatest threats to public and animal health. For the British Isles, an island group on the north-western coast of continental Europe consisting of the United Kingdom (UK) and the Republic of Ireland, physical separation offers a barrier to the introduction of many of the pathogens that affect animals on the rest of the continent. Added to this are strict biosecurity rules at ports of entry and the depauperate vector biodiversity found on the islands. Nevertheless, there are some indigenous arthropod-borne pathogens that cause sporadic outbreaks, such as the tick-borne louping ill virus, found almost exclusively in the British Isles, and a range of piroplasmid infections that are poorly characterized. These provide an ongoing source of infection whose emergence can be unpredictable. In addition, the risk remains for future introductions of both exotic vectors and the pathogens they harbor, and can transmit. Current factors that are driving the increases of both disease transmission and the risk of emergence include marked changes to the climate in the British Isles that have increased summer and winter temperatures, and extended the period over which arthropods are active. There have also been dramatic increases in the distribution of mosquito-borne diseases, such as West Nile and Usutu viruses in mainland Europe that are making the introduction of these pathogens through bird migration increasingly feasible. In addition, the establishment of midge-borne bluetongue virus in the near continent has increased the risk of wind-borne introduction of infected midges and the inadvertent importation of infected cattle. Arguably the greatest risk is associated with the continual increase in the movement of people, pets and trade into the UK. This, in particular, is driving the introduction of invasive arthropod species that either bring disease-causing pathogens, or are known competent vectors, that increase the risk of disease transmission if introduced. The following review documents the current pathogen threats to animals transmitted by mosquitoes, ticks and midges. This includes both indigenous and exotic pathogens to the UK. In the case of exotic pathogens, the pathway and risk of introduction are also discussed.

Keywords: emerging infectious diseases, arboviruses, mosquito, tick, midge, vector, livestock, transmission

INTRODUCTION

The threats posed to public health from vector-borne diseases are a subject of considerable investigation, particularly as changes to the climate may increase such threats (1, 2). Less attention has been paid to the threat to animals, and by animals, we include livestock, domestic pets and wildlife. To address this, and with a focus on the United Kingdom (UK), we have compiled both an inventory that includes the actual and potential vector-borne diseases that are a threat to animals and assess the risk they pose. The impact of vector-borne diseases to animals is varied. Many of the diseases considered are zoonotic so infection may not cause overt disease in animals, but their infection provides a pathogen reservoir that could eventually affect the human population. Where disease results from infection, this can lead to morbidity and mortality. In the case of livestock, certain diseases are considered notifiable (defined below) and could result in cessation of trade with other countries. This will have an economic impact that could take years to resolve and is a powerful motivating force to control disease outbreaks and limit the resulting losses. For wildlife, the emergence of disease in naïve host species could lead to a decline in population numbers that combined with anthropogenic factors that reduce available habitat or reproductive activity, could threaten species with extinction.

The definition of a notifiable disease is any disease that is required by law to be reported to a competent authority, usually governmental. The primary purpose of this, whether from a human or governmental perspective, is to prevent disease spread. In the UK the competent authority for human diseases is Public Health England within the Department of Health. For animals, this is the Department for Environment, Food and Rural Affairs (Defra). Devolution has led to the development of agencies that investigate animal disease on behalf of the devolved governments for example the Scottish Agricultural College (SAC) in Scotland and the Agri-Food Biosciences Institute (AFBI) of Northern Ireland. Veterinary investigations of livestock, poultry and equines are carried out by the Field Services Division of the Animal and Plant Health Agency (APHA). This is supplemented by veterinary services offered by university-associated Veterinary Schools of which there are six in England and two in Scotland. Other organizations offer veterinary support including The Pirbright Institute (Livestock Virology), the Institute of Zoology, and the Animal Health Trust. Domestic pets are usually dealt with by private veterinary surgeons (PVS). Wildlife monitoring, surveillance and health can involve all the above organizations and a large number of charitable bodies.

Some of the diseases discussed below are endemic. However, many are not and understanding how they can enter the UK is a key step in understanding the risk of emergence. For vector borne diseases there is the added concern of the vector and its distribution. Like diseases, not all potential vectors are present in the UK. The routes of pathogen entry are often termed pathways of introduction. For vector borne diseases this could take the form of an infected human or animal. For notifiable diseases some screening of animals for disease prior to movement is usually required to prevent importation of infected livestock or

domestic animals. Another pathway is the introduction of the vector of a particular disease. For midges, wind movements can lead to their introduction. For other vectors, such as mosquitoes and ticks, passive introduction, for example the importation of dogs infested with *Rhipicephalus sanguineus* s.l. ticks, does occur (3). Another pathway is through the movement of wildlife. For the UK, separated from the mainland of Europe by the English Channel, the main risks are associated with pathogens and vectors that are associated with migrating birds. Although not conclusively shown, it is possible that viraemic birds could expose the indigenous mosquito population to a number of viruses that would then threaten public and veterinary health. Alternatively, migrating birds are occasionally infested with ticks and this can be a route for exotic ticks, such as *Hyalomma* spp. to enter the UK. In addition, invasive mosquito species have established across Europe and are spreading further north. This spread into countries in Western Europe has been the source for importation of the Asian tiger mosquito (*Aedes albopictus*) into southern England, probably through passive transport in cars or lorries (4).

One overarching factor that could affect the risk of vector-borne disease is the impact of climate change. There is general consensus that average temperatures will rise in the UK over a timescale measured in decades. However, the impact this will have on arthropod populations is unclear as higher temperatures alone are not the only critical factor for many vector life-cycles. Both mosquitoes and ticks require moisture, mosquitoes for larval development and ticks to avoid desiccation during maturation phases between feeds. In addition, extremes of weather, such as storms or drought could have a negative effect on vector populations. One possible scenario is that indigenous vectors may become more abundant and active for longer in the year. The UK could also become colonisable to exotic species. This could lead to a larger diversity of tick, mosquito or midge species, and the potential introduction of new vectors, such as sandflies. Consequently, understanding the existing diversity and distribution of vectors, and how this evolves in response to climate change remains critical to predicting future disease threats.

Arthropod vectors are usually associated with nuisance biting. For mosquitoes there are currently no diseases that indigenous UK species transmit to humans. However, malaria was endemic in marshy areas in the east of England until the start of the twentieth century (5). Despite reintroductions after both World Wars, the parasite was eliminated, as it was from the rest of Europe until recently (6). For ticks there are a larger number of indigenous diseases of animals associated with bites, particularly from the most common tick species in the UK, *Ixodes ricinus*, which transmits louping ill virus, *Babesia divergens* and *Anaplasma phagocytophilum* in the UK. For humans, tick bites from this species can result in Lyme disease. Cases of Lyme disease have also been reported in dogs (7) and pet dogs have been proposed as a sentinel for disease risk (8). Finally, biting midges are a major vector for a number of high-impact veterinary diseases. The following sections describe and discuss the actual and potential threats to animals within the UK grouped by arthropod vector.

MOSQUITOES AND MOSQUITO-BORNE DISEASES

There are over 30 mosquito species present in the UK (listed in **Table S1**). All obtain nutrition through feeding on vertebrate hosts (**Figure 1**). Potentially the most important from a disease transmission perspective is the species *Culex pipiens*, a vector for a number of viruses including notifiable viruses, such as West Nile virus (WNV). *Cx. pipiens* is a species complex containing a number of morphologically similar forms with different bionomic properties that influence virus transmission (9). A key property of *Cx. pipiens* is its abundance across many areas of the country that put many areas at risk of virus spread. Other species, such as *Aedes detritus*, have also been associated with transmission

of a number of viruses (10–12). However, in contrast to *Cx. pipiens*, its distribution is limited to coastal sites and estuaries because of its requirement for salt water for oviposition and larval development.

A key feature of mosquitoes within the UK is their seasonality. Activity begins in early spring but most species only become abundant during the summer months (13). Activity declines during autumn and there is a complete cessation of activity with species over-wintering in a variety of forms (desiccated eggs, diapaused larvae and mature females). This restricts the period over which mosquito-borne transmission can occur and may be one of the reasons why there has been no evidence of autochthonous mosquito-borne virus transmission in the UK since the inadvertent introduction of both yellow fever virus and its vector, *Aedes aegypti*, in 1865 (14).

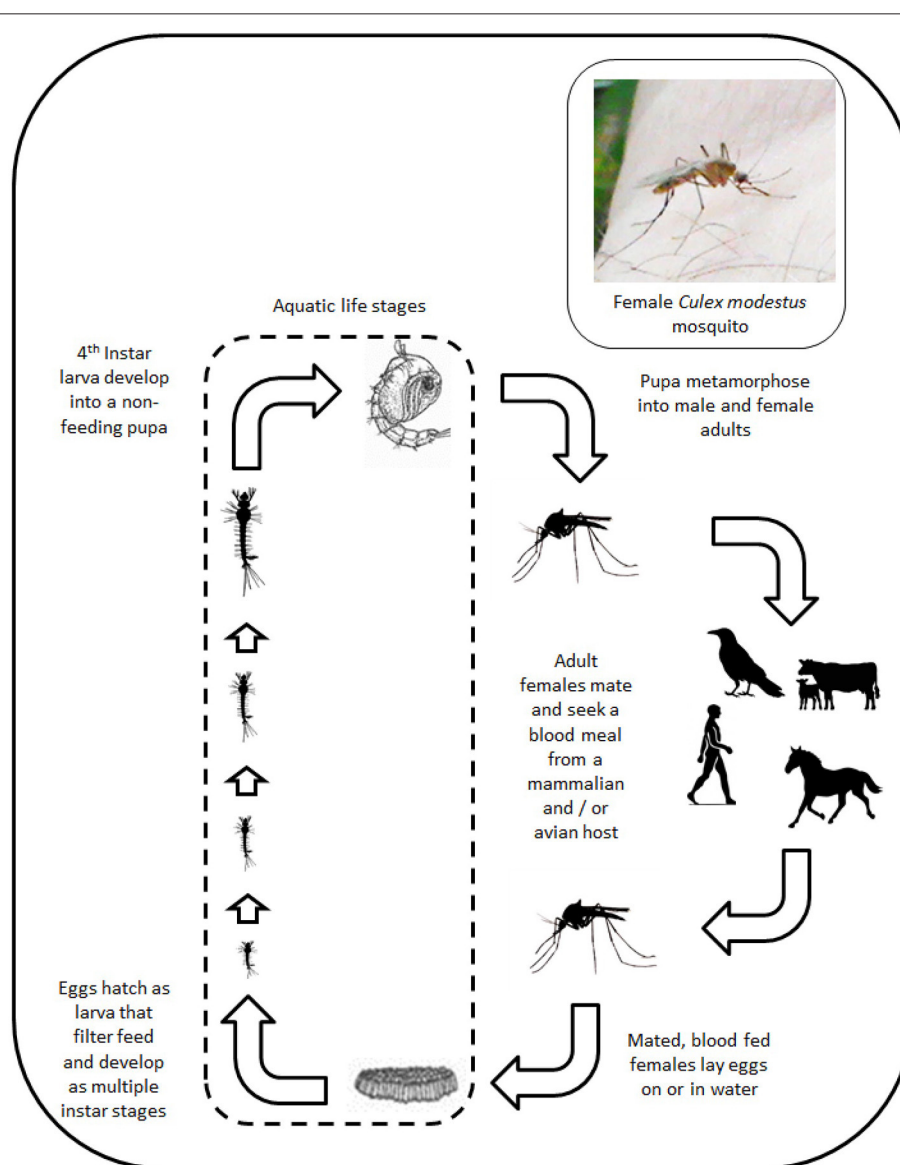


FIGURE 1 | Schematic showing the life cycle of mosquitoes.

Another key feature is the host feeding preference of mosquitoes for a vertebrate host. All species within the UK require a blood meal to provide sufficient nutrition to enable egg development and maturation. Mosquitoes generally target either a mammalian host or an avian host with this having clear implications for the ability to transmit viruses between non-conspecifics. Some mosquito species that feed on multiple hosts can act as a “bridge” vector enabling transmission of a virus that normally replicates in birds being transmitted to humans and livestock. A number of previous studies have confirmed that mosquitoes feed on a range of livestock and wildlife (15–19). In the case of some mosquito species including *Culiseta annulata* and *Anopheles messeae*, they appear to feed exclusively on large ruminants. Others, such as *Anopheles atroparvus*, are more opportunistic. The recent observation of *Culex modestus* in the Kent Estuary (20), a bridge vector for WNV in mainland Europe, has raised concerns that this could provide a vector population if the virus was introduced, although surveillance has not detected WNV in this mosquito population to date (21).

Threats From Mosquito-Borne Viruses Present in Europe

The most prominent disease threat to UK public and animal health is from those viruses that are already present in Europe as these could be more readily introduced by migratory birds. Predominantly these are flaviviruses, which are known to be transmitted by arthropod vectors and can cause disease in wildlife, livestock and in some cases humans. Below we expand on some of these economically important flaviviruses (Table 1). A number of these are currently active in Europe and capable of causing disease in wildlife, livestock and humans (22).

West Nile Fever (WNF)

Until the start of the twenty-first century, WNV caused sporadic outbreaks in Europe that affected both human and equine populations, but rapidly resolved once mosquito activity declined at the end of the summer. Notable outbreaks occurred in the Camargue region, France, in 1962 (23) and Bucharest, Romania, in 1996 (24). Figure 2 shows the

European countries that have reported cases of WNV in humans and/or horses. Domestic poultry have been affected in Europe (25), but this is not a common observation considering its prevalence and transmission by ornithophilic mosquitoes. A more common observation is disease within birds of prey (26) and these are a distinctive target for syndromic surveillance.

Over the past two decades, outbreaks due to various lineages of WNV have increased to the point where the virus is now considered endemic in some countries of southern Europe, resulting in regular outbreaks in particular regions, such as the Po Valley in Italy and the Camargue in France. This distribution changed in 2018, a year that experienced a particularly warm summer with above average temperatures for a number of months. Possibly as a result, WNV cases occurred in Germany at latitudes considerably further north than reported in previous years (27). Whether WNV establishes at these northerly latitudes and continues to spread will likely depend on the climatic conditions across northern Europe over subsequent summers. However, surveillance is critical to provide early detection of virus in arthropod and avian reservoirs prior to transmission to humans (28, 29).

WNV is notifiable in the UK in horses. The virus has not been detected in the UK although a seropositive horse (30) has been reported resulting from importation. However, the risk of introduction was recognized and a protocol put in place to investigate suspected cases without unnecessarily implementing the full range of veterinary control measures (31). Horses are considered a dead-end host, due to low viraemia and consequently are incapable of infecting other horses directly or infecting potential vectors. A risk assessment published by the Department for Environment, Food and Rural Affairs, identified eight potential pathways (Table 2) that could lead to the introduction of WNV into the UK (32). Overall, the greatest risk was associated with introduction by migratory birds and even this was considered “very low,” being defined as very rare but cannot be excluded. The caveat to that has been a dramatic change in the distribution of WNV in Europe that has led to infected birds being detected in northern Europe, reducing the potential distance that birds would need to migrate across to reach the UK.

Israel Turkey Encephalitis (ITE)

Israel Turkey encephalitis virus (ITV), the causative virus of ITE, was first reported in 1960 following descriptions of a neuro-paralytic disease of turkeys (*Melaeagris gallinavo*) in Israel (33). In addition, Bagaza virus (BAGV) was isolated from *Culex spp.* mosquitoes in the Central African Republic (34) and has since been detected across sub-Saharan Africa and India (35). Interestingly, genomic sequence analysis has shown that these viruses are very closely related flaviviruses, to the point where they are effectively the same virus species. The repeated isolation of the BAGV in mosquitoes from countries in Africa suggests that mosquitoes are the vector, a feature shared with many viruses within the genus. BAGV was detected in Europe in 2010 following the death of large numbers of red-legged partridges (*Alectoris rufa*) in southwestern Spain (36) and was coincident

TABLE 1 | Bird associated viruses within the genus *Flavivirus*.

Virus	Distribution	Susceptible vertebrates
Bagaza virus (BAGV)	Spain, sub-Saharan Africa, India	Partridge, pheasants
Israel turkey meningo-encephalitis virus (ITV)	Israel, South Africa	Turkey
Japanese encephalitis virus (JEV)	Asia	Humans, pigs, equids
Louping ill virus	United Kingdom	Sheep, cattle, grouse
St. Louis encephalitis virus (SLEV)	North America	Humans
Tembusu virus (TMUV)	Asia	Duck, goose, chicken
Usutu virus (USUV)	Africa, Europe	Passeriformes, Strigiformes
West Nile virus (WNV)	Africa, Europe, Americas, Asia	Passeriformes, Accipitriformes, humans equids

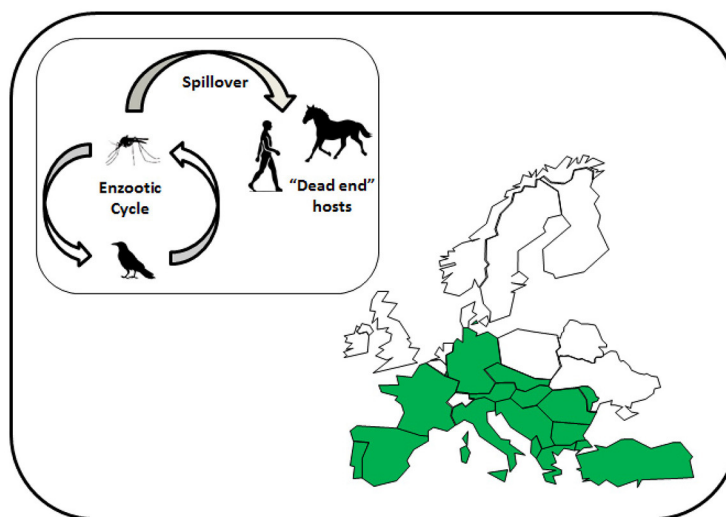


FIGURE 2 | Map of Europe showing the countries affected by West Nile virus to 2018 (marked in green). Inset shows the reservoir cycle between mosquitoes, mainly *Culex* species, and birds, and spillover into mammalian species.

TABLE 2 | Risk pathways for the introduction of West Nile virus into the UK [adapted from Defra (32)].

Pathway	Risk
Introduction by migrating birds	Very low
Importation by legal trade in horses	Very low
Importation by legal trade in biological materials (equine semen, ova, embryos)	Negligible
Importation by legal trade in poultry	Negligible
Importation by legal trade in non-equine/non-avian species	Negligible
Illegal importation of infected animal	Impossible to quantify
Importation of infected vector	Very low
Air-borne movement of vector from continental Europe	Negligible

with cases of WNV in horses in the region. Common pheasants (*Phasianus colchicus*) were also affected during the epizootic. The source of the introduction, presumably from Africa, was not identified and there have been no further reports of disease in Europe, although seroprevalence studies have suggested that the virus continues to circulate in wild bird populations in Spain (37) and thus continues to present a risk of disease to poultry if it spreads more widely.

Usutu Virus Infection

Disease caused by Usutu virus (USUV) has not been defined into a single disease entity. Infection in birds can lead to a range of disease signs and at necropsy virus is found throughout the organs of the infected animal (38), while infection in humans is rarely associated with disease. The virus is a flavivirus closely related to WNV that exists in a reservoir cycle between *Culex* spp. mosquitoes and birds. Unlike WNV, USUV is not particularly pathogenic in mammals, although occasional human infections are reported, but infection does appear to be more virulent

for avian species (39). The first reports of USUV date back to the late 1990s and retrospective analysis of bird samples has found evidence for its introduction into Italy in 1996 (40). Various strains of the virus rapidly established in parts of the Mediterranean Basin and have been repeatedly detected during surveillance for WNV (41). The viral strains have also spread north, being detected in Germany (42) and Belgium (43) often associated with increased mortality in species, such as the blackbird (*Turdus merula*). Vector competence studies have shown that *Culex pipiens* mosquitoes from the Netherlands are highly competent to transmit USUV (44) that does not appear to be reflected by those present in the UK (45). However, the introduction of USUV by short distance avian migrants from the European mainland is possible, especially during the summer months, and justifies limited surveillance in target bird species (46). The major threat of USUV would be to avian species abundance and diversity, as infection may reduce populations of susceptible species.

Infections Caused by Other Pathogens in Europe

Two further mosquito borne viruses that are implicated in disease in animals have been reported in Europe. Batai virus (BATV) is an orthobunyavirus related to Schmallenberg virus (see below). Repeated isolation of BATV suggest that mosquitoes are a competent vector for the virus (47, 48) although an association with a particular species has not been confirmed. Also the evidence that the virus causes disease in livestock is equivocal although serology studies in Germany detected evidence of infection (49) and BATV was recently implicated in the death of a harbor seal (50).

Sindbis virus (SINV) is an alphavirus that is transmitted between birds and ornithophilic mosquitoes. It is one of the most widespread viruses with evidence for its presence in Europe,

including Scandinavia, where it causes a mild febrile illness and arthralgia in humans called Ockelbo in Sweden. SINV has also been detected in Asia and South Africa. It is assumed that birds are refractory to disease, although surveillance occasionally detects the virus in bird tissues (51). There is also evidence that SINV can cause neurological disease in African horses (52), although this has not been observed in Europe.

Canine Heartworm

Canine heartworm, also known as subcutaneous dirofilariosis, is caused by the parasitic worms *Dipylaria repens* and *D. immitis* (53). Immature microfilariae circulate in the bloodstream where they can be taken up by mosquitoes and transmitted to a new host. The adult form migrates to muscular tissue where they remain, eventually leading to disease. In the early 2000s, the distribution of *D. immitis* in Europe was associated with countries around the Mediterranean Sea (54), but infections have been documented in the UK, likely following import from mainland Europe (55). Recent surveys of PVS in Western Europe provide anecdotal evidence that cases are on the increase (56), related in part to the increasing number of dogs being taken on holidays in southern Europe (57).

SIGNIFICANT MOSQUITO-BORNE VIRUSES AFFECTING ANIMALS GLOBALLY

Japanese Encephalitis

Japanese encephalitis virus (JEV) is a zoonotic virus that is found throughout Asia. Like WNV and USUV, the virus persists in a bird-mosquito cycle that can spill-over into human and livestock populations. In contrast to the other viruses, pigs can act as a vertebrate reservoir host for JEV. As its name suggests it causes severe encephalitis in humans often leaving the patient with long term neurological deficit. It is also an economic disease of pigs causing abortion, still-birth and death in piglets (58). A range of *Culex* species transmit the virus, particularly *Cx. tritaeniorhynchus*, a species found in south east Europe (59). A number of recent reports have presented evidence for JEV in Europe (60, 61). However, these results are based on detection of partial genomic sequences not a complete genome, and live virus has not been isolated and as such there is some controversy over whether these are genuine cases of infection. If they are confirmed, it would represent a dramatic translocation of the virus. However, a single case of JEV has been reported from Africa in a human co-infected with yellow fever virus (62). Overall, the threat posed by this virus to the UK is low, although continued increase in air travel from Asia could lead to viraemic humans arriving in Europe where indigenous mosquitoes are competent to transmit JEV (10, 63, 64).

Rift Valley Fever

Rift Valley fever (RVF) is a zoonotic disease of ruminants causing sporadic outbreaks among livestock caused by the Rift Valley fever virus (RVFV). RVF occurs across much of sub-Saharan Africa (65). Transmission is facilitated by bites from infected mosquitoes, although humans can become exposed through

contact with infected carcasses. In sheep and cattle, disease is initially a short-term febrile illness progressing to jaundice, hepatic failure, and hemorrhagic disease. Mortality is severe in juvenile animals reaching 90% in some outbreaks and high rates of abortion and neonatal malformation are common. Significant outbreaks have affected countries of North Africa and the translocation of infected animals has led to RVF being introduced into the Arabian Peninsula in 2000 (66–68). Other examples of its transmission beyond the African mainland include its emergence in Islands of the Indian Ocean including Madagascar (69) and Mayotte (70). In addition, serological studies have suggested that RVFV may be circulating in Turkey (71) and Iran (72). To date there has been no evidence of RVFV introduction into Europe, although some researchers have speculated that this is likely based on previous examples of translocation out of Africa and a number of studies have shown that mosquito species in Europe are competent vectors for the virus (12, 73). With the possible exception of human travel from Africa, it seems unlikely that RVFV could be introduced inadvertently in livestock or livestock products to the UK due to paucity of such trade at the current time. However, an increase in livestock trade with Africa or its introduction into mainland Europe would change this assessment. However, there is currently concern that RVFV could be introduced into the United States (US) and Europe (74).

Saint Louis Encephalitis

St. Louis encephalitis virus (SLEV) is a flavivirus that appears to occupy the same ecological niche in the New World that WNV occupies in the Old. Indeed, when WNV emerged in North America in 1999 the initial cases were suspected to be infected with SLEV. Similar to WNV the primary transmission cycle of SLEV is between mosquitoes and birds, although mammals may also contribute. In addition, the virus may cause sporadic outbreaks of human encephalitis throughout North and South America. Phylogeographic investigations have suggested that SLEV emerged in the seventeenth century in Central America and been translocated by bird migration (75). Serological surveys suggest that livestock can be infected asymptotically with SLEV (76) and there has been a report of a horse with neurological disease associated with infection with the virus (77). Critically, there is currently no evidence for SLEV infection outside of the Americas.

Equine Encephalitis

The New World also hosts a number of zoonotic alphaviruses that cause encephalitis in humans and horses. These are collectively termed the equine encephalitides and the complex is composed of three viruses: Eastern equine encephalitis virus (EEEV); Western equine encephalitis virus (WEEV); and Venezuelan equine encephalitis virus (VEEV). Each is widely distributed, transmitted by a range of mosquito species and all viruses cause severe disease in equids and humans (78). As with SLEV, there has been no evidence for these viruses outside of the Americas despite extensive intercontinental transport of horses and the risk of introduction is considered negligible.

Duck Egg-Drop Disease

Tembusu virus (TMUV) was first isolated in mosquitoes in Malaysia in 1955, and subsequently shown to cause encephalitis and growth retardation in chicks (79). Birds are the natural amplifying host and a number of wild species have been identified as playing a role in TMUV persistence. Interest in the virus has increased in recent years as it has been demonstrated as the causative agent of duck egg-drop disease in China (80). The virus has only been detected in South-east Asia and is not considered a threat to Europe currently.

The Threat Posed by Invasive Mosquito Species

Non-native or invasive mosquito species have had a dramatic impact on public health in Europe. The introduction and spread of the Asian tiger mosquito (*Aedes albopictus*) in particular has been a major factor in outbreaks of chikungunya virus in Italy, and repeated outbreaks of dengue fever in southern France (81). Surveillance for invasive mosquitoes in the UK is conducted by Public Health England (82) and there have been a number of detections in England in recent years (83). The impact on animals from the introduction of invasive mosquito species is uncertain and there is little evidence from Europe that the establishment of such mosquitoes has led to increased disease prevalence in animals. The feeding preference of *Aedes albopictus* is varied depending on the availability of potential hosts (84, 85) and there are reports of the species feeding on cattle (86, 87). However, there is no evidence that were *Ae. albopictus* to establish in the UK, there would be greater risk of disease transmission to livestock or domestic animals.

TICKS AND TICK-BORNE DISEASES

There are over 20 species of ticks indigenous to the UK (see **Table S2**) and all acquire nutrition through feeding on vertebrate hosts (**Figure 3**). Surveillance for ticks in the UK indicates that the species most often associated with tick bites to humans is the common sheep tick *Ixodes ricinus* (88, 89). Other species that feed on livestock but show limited geographical distribution include the ornate cattle tick (*Dermacentor reticulatus*) (90) and the red sheep tick (*Haemaphysalis punctata*) (91).

Louping Ill

Louping ill virus (LIV) is the only indigenous tick-transmitted virus present in the UK. The disease results from viral encephalomyelitis, mainly affecting sheep, which show signs of neurological impairment including incoordination, altered gait and ataxia. Other mammals can be infected although such cases are rare. Of further economic significance is the susceptibility of red grouse (*Lagopus lagopus scoticus*) to infection (92). LIV occurs in upland areas of the British Isles (93, 94) with sporadic reports of disease in sheep from the west of Scotland, Cumbria, Wales, and Devon.

Louping ill is classified within the family *Flaviviridae* and genus *Flavivirus*, and is closely related to tick-borne encephalitis virus, a virus found across Eurasia causing disease in humans

rather than livestock. The disease louping ill has been observed in sheep for centuries, but it was not until the late 1920s that the infectious agent was isolated from the central nervous system of sheep showing disease signs and demonstrated, through filtration, to be a virus (95). Shortly after this, the role of *Ix. ricinus* ticks in disease transmission was established (96). Since that time, most research has been directed at understanding the susceptibility of particular mammal species to LIV infection (97, 98) and the interaction of the tick vector, wild mammals and livestock in maintaining the virus within the upland ecosystem (99). Ticks can also be infected with LIV through co-feeding (defined as feeding in close proximity to another infected tick), without infection or viraemia in the host (100). This is thought to contribute to the persistence of LIV even when control measures in sheep, such as vaccination, are applied. Experimental studies in support of field observations have shown that dual infection with LIV and *Anaplasma phagocytophilum* (see below) can increase the severity of disease in sheep (101).

In addition to vaccination, alternative control measures include acaricide treatment of livestock and habitat management as means of preventing tick feeding and suppressing tick numbers, respectively. The identification of certain wildlife species that promote LIV persistence in upland areas (102) has led to the controversial management practice of culling mountain hares as a means of controlling tick abundance.

A number of viruses related to LIV are present in Europe. These are rarely reported but have very similar properties to LIV, including transmission by *Ix. ricinus* ticks and causing encephalitis in ovine species, but these viruses are restricted geographically. The most recent example of LIV-like infection was the detection of a virus causing encephalitis in goats in northern Spain (103). This was initially attributed to LIV due to genetic similarities to existing strains in the UK, but the virus has subsequently been renamed Spanish goat encephalitis virus (SGEV) based on differences across the complete genome (104) and its exclusive presence in Spain.

Babesiosis

Babesiosis is a tick-borne intraerythrocytic protozoan disease that affects mammals and is caused by species within the genus *Babesia*. The disease presents with a range of signs. Many cases may be subclinical or show mild signs of low grade fever and anorexia that may be missed. However, clinical disease results from a combination of the host immune response and hemolytic anemia caused by destruction of erythrocytes. This can lead to hemoglobinuria (classically a port wine coloration in urine). In cattle the common name for the disease in the UK is redwater fever. Overt signs include a rapid onset fever reaching 41°C and non-specific signs including anorexia, depression and weakness. Death can result from hepatic and respiratory complications, and renal congestion caused by deposition of hemoglobin in the renal tubules. Following recovery, low levels of infection may be maintained within erythrocytes of affected animals for a number of years without signs of clinical disease and which may form a reservoir of infection for feeding ticks. Calves below 9 months of age demonstrate an innate, inverse, age related

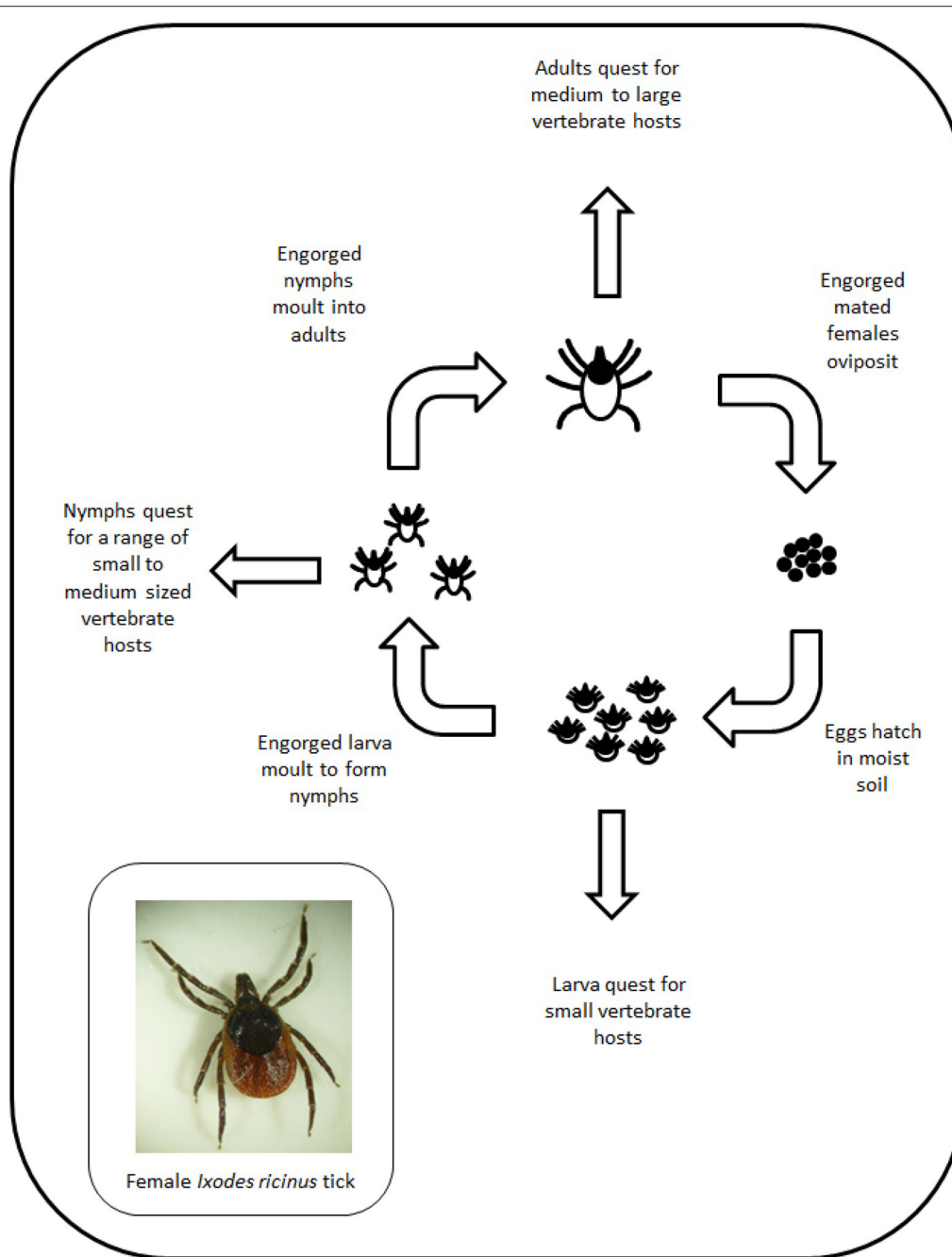


FIGURE 3 | Schematic showing the life cycle of *Ixodid* ticks.

resistance, unrelated to maternal immunity, and do not suffer clinical disease.

Babesia spp. only infect female ticks following blood feeding on infected animals and the parasites are transmitted via transovarial transmission to the next larval generation and subsequently to nymphal and adult ticks via transstadial transmission. Thus, at least one complete generation of ticks may be infected and are capable of transmitting the disease to naïve

animals. Globally, the most significant species causing babesiosis in cattle are *B. bigemina* and *B. bovis* (105) with both being found on almost all continents. The most common species causing disease in Europe is *B. divergens* (Figure 4A), which is also the most widespread *Babesia* species affecting cattle in temperate regions and was first described in England by McFadyean and Stockman (106). It was originally named *Piroplasma divergens*, referencing the pear shaped paired merozoites lying at a typically

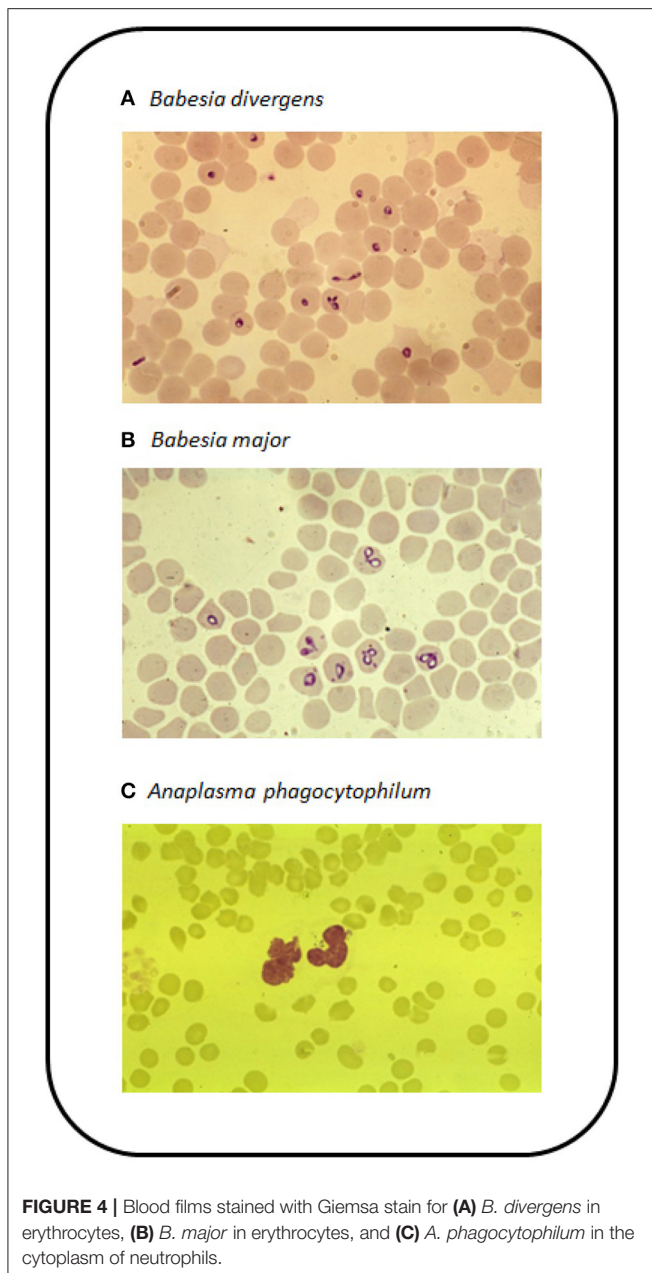


FIGURE 4 | Blood films stained with Giemsa stain for (A) *B. divergens* in erythrocytes, (B) *B. major* in erythrocytes, and (C) *A. phagocytophilum* in the cytoplasm of neutrophils.

divergent angle within the erythrocyte. Genetic evidence for the presence of *B. divergens* in British livestock has only recently been confirmed (107). Infections occur sporadically throughout Europe and may extend as far south as North Africa (108). Its distribution is defined by that of its tick vector, *Ix. ricinus*, which requires a microhabitat with at least 80% humidity to support metamorphosis and survival of life cycle stages off the host. This may include unimproved permanent pasture, rough moorland grazing, headlands and hedges of well-maintained pasture as well as forest floor. In addition *B. divergens* is zoonotic and has resulted in death in a number of humans, particularly in splenectomised or immunocompromised individuals (109). A second *Babesia* species has been detected in English cattle

(110) transmitted by *H. punctata* in the South-east and based on its morphology is now considered to be the relatively non-pathogenic species *B. major* (111, 112) (**Figure 4B**). Additional species that can infect cattle include *B. bovis*, *B. bigemina*, *B. ovata* in Eastern Asia, *B. occultans* in Africa and more recently the Mediterranean area and *B. venatorum* (formerly *Babesia* sp. EU1) (105). Treatment may include supportive therapy including intravenous administration of fluids, blood transfusion and administration of vitamins as well as anti-protozoal chemotherapy using Imidocarb dipropionate.

A threat to equines in the UK is equine piroplasmosis caused by *Babesia caballi* or *Theileria equi* infection. Historically, the UK has been considered free of equine piroplasmosis despite the presence of seropositive and pathogen positive horses resident within the country (113) and populations of one of its tick vectors, *Dermacentor reticulatus*, being present in Wales and southern England (90). Nevertheless, the risk of causative pathogens becoming established within the vector population is evident and could lead to autochthonous transmission in the future.

A range of *Babesia* species cause mild disease in sheep and goats. These include *B. ovis*, *B. motasi*, and *B. crassa* (114). Of these, *B. motasi* has been detected in Wales (115, 116) and England (117), both are associated with *H. punctata* ticks. In Europe, disease presents as hemolytic anemia and chronic wasting, although it is rare.

Canine babesiosis is caused by a small number of piroplasms (see **Table 3**). Disease can be unapparent but in severe cases, dogs can develop fatal anemia (118). All canine-associated *Babesia* species are considered exotic to the UK. However, there have been a number of reports of individual dogs infected with *B. canis* and *B. vogeli* following travel in Europe (119–121). In 2015/2016 there were reports of autochthonous transmission of *B. canis* by *D. reticulatus* ticks in Harlow, southern England (122).

Theileriosis

Theileriosis is a tick borne hemoparasitic disease of livestock including cattle, sheep, goats and equids caused by *Theileria* spp., which are apicomplexan protozoa closely related to *Babesia*. Unlike *Babesia* sp., transmission of *Theileria* spp. within the tick vector is transstadial only. Infection is acquired by larval or nymphal ticks feeding on infected animals and is maintained in the following nymphal and adult stages. No transovarial transmission of *Theileria* spp. has been demonstrated within tick vectors. Whilst both *Babesia* and *Theileria* spp. are transmitted through the bite of infected ticks, *Babesia* sp directly enter erythrocytes of infected animals whereas *Theileria* spp. initially undergo a lymphocytic phase of division (Schizogony) to produce merozoites which are released to invade host erythrocytes where further division occurs. Three species of *Theileria* cause significant economic impact on cattle farming worldwide, *T. parva* (East Coast Fever), *T. annulata* (Tropical Theileriosis), and *T. orientalis* (Far East, Australasia). Clinical bovine theileriosis is mainly reported from The Middle East, Africa, Asia (123) and most recently in Australia and New Zealand (124). East Coast Fever caused by *T. parva* is the most severe form of disease in cattle presenting with fever and

TABLE 3 | Tick-borne diseases of livestock in Europe.

Disease	Pathogen	Vector species	Susceptible species	Comment
African Swine fever	African swine fever virus	<i>Ornithodoros</i> spp.	Pig	The vector is absent from Europe, transmission is directly from pig to pig.
Louping ill	Louping ill virus	<i>Ixodes ricinus</i>	Sheep Red grouse	Restricted distribution, mainly found within the British isles.
Babesiosis	<i>Babesia bigemina</i>	<i>Rhipicephalus bursa</i>	Cattle	
	<i>Babesia bovis</i>	<i>Rh. annulatus</i>	Cattle	
	<i>Babesia major</i>	<i>Haemaphysalis punctata</i>	Cattle	
	<i>Babesia divergens</i>	<i>Ix. ricinus</i>	Cattle	
	<i>Babesia occultans</i>	<i>Hy. Marginatum</i>	Cattle	
	<i>Babesia canis</i>	<i>Rh. sanguineus</i>	Dog	
	<i>Babesia vogeli</i>	<i>Rh. sanguineus</i>	Dog	
	<i>Babesia gibsoni</i>	<i>Rh. sanguineus</i>	Dog	
	<i>Babesia ovis</i>	<i>Rh. bursa</i>	Sheep	
	<i>Babesia motasi</i>	<i>Ha. punctata</i>	Sheep	
Theileriosis	<i>Babesia caballi</i>	Various tick species	Horse	
	<i>Theileria annulata</i>	<i>Hyalomma marginatum</i>	Cattle	
	<i>Theileria lestoquardi</i>	<i>Hyalomma</i> spp.	Sheep	
	<i>Theileria equi</i>	<i>Hyalomma</i> spp.	Horse	
	<i>Theileria orientalis</i>	<i>Ha. punctata</i>	Cattle	
		<i>Ha. punctata</i>	Sheep	
Anaplasmosis	<i>Theileria luwenshuni</i>	<i>Ha. punctata</i>	Sheep	
	<i>Anaplasma phagocytophilum</i>	<i>Ix. ricinus</i>	Cattle	
	<i>Anaplasma marginale</i>	<i>Ixodes</i> and <i>Rhipicephalus</i> spp.	Cattle	
Hepatizonosis	<i>Heptatozoon canis</i>	<i>Rhipicephalus sanguineus</i> s.l.	Dog	Infection through ingestion of infected ticks

enlarged lymph nodes, particularly near tick bites (125). Other disease signs include anorexia, nasal discharge, and diarrhea with mortality reaching 100% during severe outbreaks. Blood smears show the presence of parasite in both leukocytes and in erythrocytes.

A benign form of *Theileria* has been detected in cattle in southern England transmitted by the tick *H. punctata* (126). Based on morphology of the parasite in blood smears it was identified as *T. mutans*. However, serology suggested that it was identical with *Theileria* sp. from Asia (127). Recently, there has been further evidence of *Theileria* species present in UK through the detection of the parasite in the blood meal of mosquitoes that have fed on cattle (128) grazing a known site of *H. punctata* activity. This was identified as *T. orientalis* based on genomic sequence data and although *T. orientalis* strains cause severe disease in cattle in Asia and Australasia, there have been no records of clinical bovine theileriosis in the UK. Ovine theileriosis caused by *Theileria luwenshuni* has been reported in North Kent associated with high tick burden (117).

Anaplasmosis (Tick Borne Fever, Pasture Fever)

Tick-borne fever was recognized as a discrete disease of cattle in the late 1940's (129). The causative agent is a gram negative bacterium now known as *Anaplasma phagocytophilum*

(130) a name that has replaced three synonyms, *Cytoecetes phagocytophila*, *Erhlichia phagocytophila*, *Ehrlichia equi* and is the causative agent of human granulocytic anaplasmosis (HGA). As the common disease name suggests, infection presents as a fever and anorexia. There have been repeated reports of tick-borne fever in dairy herds in the UK (131, 132) and reduction in milk yield can indicate infection. In more serious cases, abortion and stillbirth are signs of disease (133). Some animals are also affected by respiratory distress in response to infection.

In Europe *A. phagocytophilum* is transmitted by the sheep tick *Ix. ricinus*, so like *B. divergens*, its occurrence is dictated by the presence and abundance of this tick species. The disease has been reported from across the UK and Ireland. In continental Europe, cases have been reported from Spain, France, Germany and the Scandinavian countries. *Anaplasma phagocytophilum* is also present in North America and transmitted by ticks, such as *Ix. scapularis* and is more commonly identified as a cause of HGA (134). Cases of HGA in Europe are rare but do occur, often as a mild fever (135). Most outbreaks occur following the introduction of naïve cattle onto tick-infested fields (136). Following feeding by an infected tick, the bacteria are detectable in circulating granulocytes, particularly neutrophils (Figure 4C). This coincides with the onset of fever (>40°C). Due to the infection of granulocytic cells, infected animals become immunosuppressed and this can lead to increased susceptibility

to other infections, such as tick pyaemia caused by *Staphylococcus aureus* (137). This can be particularly devastating in sheep herds (138). Treatment is typically based around the administration of oxytetracycline or sulfamethazine.

EMERGING TICK-BORNE THREATS IN EUROPE AND AFRICA

A significant emerging threat to the pig production industry has been the emergence of African swine fever virus (ASFV) in Europe. The virus evolved in Africa where it is transmitted by soft ticks within the genus *Ornithodoros*. Infection in native species, such as warthogs (*Phacocherus africanus*) causes subclinical disease, whereas infection in domestic pigs can be devastating with mortality reaching 100% in some cases (139). ASFV was introduced in the Caucasus region in 2007 and spread rapidly north into the Russian Federation, presumably through movement of livestock. It then entered the wild boar population in the Baltic States and from there emerged in Western Europe in the summer of 2018 (140, 141). In Northern Europe there are no known tick vectors, so transmission is through direct contact between animals which has resulted in the culling of wild boar populations in an attempt to reduce disease spread.

Additional exotic threats include infection with Crimean-Congo hemorrhagic fever virus (CCHFV) and Nairobi sheep disease virus (NSDV). The former is transmitted by *Hyalomma* ticks to livestock that can become infected, but do not show signs of disease. The main risk is to humans that have contact with infected meat or milk, as this may lead to fatal haemorrhagic fever (142). CCHFV has a wide distribution from Spain and the Balkans in Europe, Africa and Asia (143). Nairobi sheep disease is a potentially fatal disease of ovines and found in parts of Africa where *Rhipicephalus* ticks are active (144). A variant of NSDV, Ganjam virus has been reported in India, although this represents no immediate threat to the UK.

RISKS FROM EXOTIC TICKS

The introduction and establishment of exotic ticks could lead to a change in the current risk assessment of animal diseases due to tick-borne pathogens. This could lead to either the direct introduction of a pathogen with the ticks or provide a reservoir population should a pathogen be brought in by an infected vertebrate. A range of pathways for introduction exist, perhaps the most important being those enabled by humans. The importation of ticks on animals, such as dogs, have been well-documented for *Rh. sanguineus* (3, 145) and can lead to infestation of houses. A further risk associated with this tick species is the potential for introduction of *Hepatozoon canis*, a common disease of dogs in southern Europe resulting from ingestion of infected ticks. There have been a number of recorded cases in the UK (146). However, expert opinion and surveillance suggest that this tick species cannot persist in the British climate at present. Another pathway is the introduction of ticks on migrating birds. A number of studies have reported *Hyalomma* spp. on birds migrating north through Europe (147, 148). Further

studies have confirmed that such ticks can be infected with zoonotic pathogens (149), such as Crimean-Congo haemorrhagic fever virus. But again, it is unlikely that such ticks will survive and thrive in the UK, so onward transmission of pathogens will be limited. A recent report has suggested that the presence of an adult *H. rufipes* found on an untraveled horse in the south of England could have been introduced as a nymph by migrating birds (150). This is of concern as it suggests partial completion of the ticks' lifecycle within the UK.

MIDGES AND MIDGE-BORNE DISEASES

Biting midges (**Figure 5**) within the genus *Culicoides* (Latreille, 1809) are the vectors of a number of significant diseases of livestock including bluetongue virus and African horse sickness virus (AHSV). Species within the genus are small, ranging from 1 to 3 mm in length and so morphological identification can be challenging, and with over 1,000 species within the genus a comprehensive classification is not currently available (151). However, within Europe the main species identified as responsible for virus transmission are *C. obsoletus*, *C. scoticus*, *C. dewulfi*, *C. chiopterus*, *C. pulicaris*, and *C. punctatus* (152). Whilst mosquitoes and ticks can be introduced by human interventions including cars, freight lorries, shipping, and migratory animals, midges can be moved over large distances by wind movements (153). This mechanism has been responsible for the introduction of a number of exotic livestock viruses in the UK (154). The following midge-vectoring viruses represent those that have either caused disease outbreaks in recent years, or have the potential to do so if introduced, to the UK. Bovine ephemeral fever is included in this section, although there is still uncertainty over the role of midges and mosquitoes in transmitting this virus (155).

Bluetongue

Bluetongue is a midge-borne disease caused by serotypes of the *Orbivirus*, bluetongue virus (BTV). Ruminants are susceptible to disease with cattle presenting with elevated temperature and congestion of, and discharge from, the mucous membranes. This can develop into crusts and erosion of the nasal and oral mucosa. Animals can become lame due to coronitis, inflammation of the coronary band above the hoof, and ulceration of the teats can occur. Transplacental transmission can lead to congenital deformities and developmental defects in live births (156). Diagnosis is based on serology and detection of virus using RT-PCR (157). Critically BTV serotype 8 emerged in the Netherlands in 2006 (158) and proceeded to spread across Europe. Cases of BTV infection were reported in England in 2007, likely the result of airborne spread of the midge vector across the North Sea. As a notifiable disease, control measures were introduced that eliminated the disease in the UK. In 2015, BTV serotype 8 re-emerged in France and has persisted over two winters (159). Despite its proximity, the potential of transmission to the UK is considered to be low.

Schmallenberg

Schmallenberg virus (SBV) was first reported in a herd of cattle in Germany experiencing a drop in milk yield and diarrhea (160).

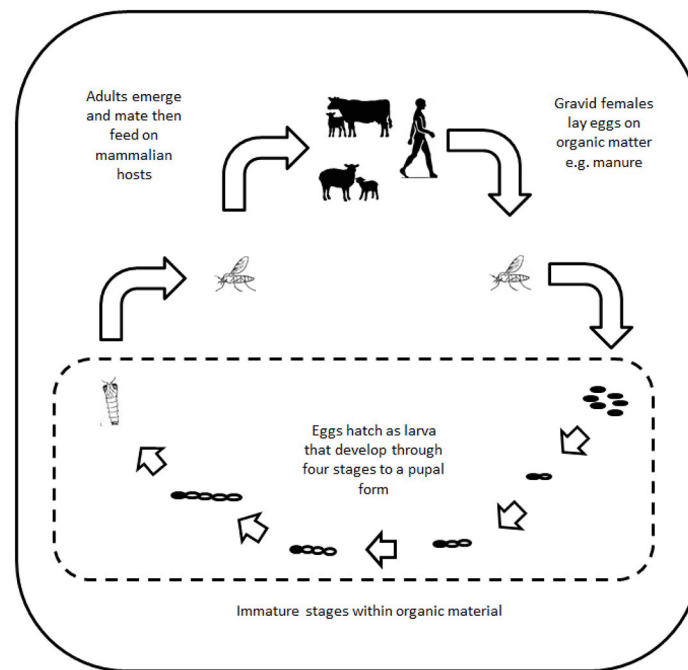


FIGURE 5 | Schematic showing the life cycle of *Culicoides* midges.

Infection with SBV in adult ruminants can be mild but infection *in utero* can lead to malformation and abortion, and this is usually how the disease presents. The virus is an *Orthobunyavirus* and is transmitted by *Culicoides* biting midges. SBV spread rapidly across Europe and the first case of disease reported in England occurred in East Anglia in April of 2012. Subsequently, there were repeated outbreaks of SBV infection in both cattle and sheep in England (161). Although currently there are no active UK outbreaks, the threat of disease remains high. Malformation in new born animals is typical of SBV infection including contraction of the limbs, arthrogryposis, and microencephaly. The diagnosis can be confirmed by detection of virus by RT-PCR (162) or detection of SBV antibodies in the mother (163).

African Horse Sickness

African horse sickness is characterized by a sudden onset fever and edema of the head and neck. A more severe form is associated with pulmonary illness that leads rapidly to death. Mortality rates can reach as high as 70%. The main vector for transmission is *C. imicola*, a species absent from northern Europe. The disease is caused by another *Orbivirus*, African horse sickness virus (AHSV) that is endemic in tropical and sub-tropical regions of Africa. Outbreaks in Europe have occurred, most notably the introduction of AHSV in a consignment of zebras brought into a safari park near Madrid in 1987 causing the disease to persist in the Iberian Peninsula until 1990 (164).

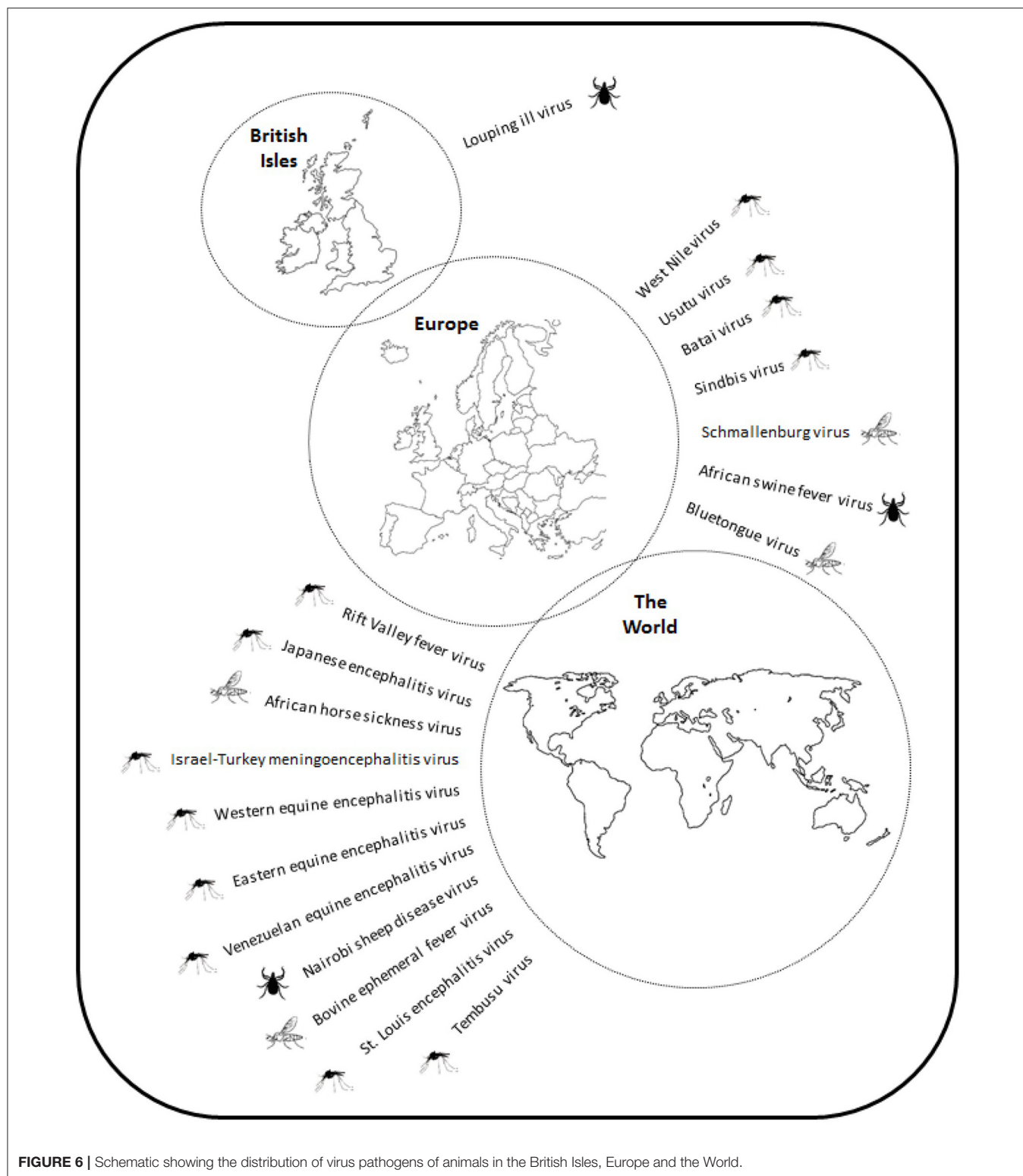
Bovine Ephemeral Fever

Bovine ephemeral fever or three-day sickness is caused by infection with bovine fever ephemerovirus (BEFV—formerly bovine ephemeral fever virus). Infection causes transient fever with ocular and nasal discharge, depression and recumbency

(165). Severe disease can lead to livestock deaths and recent outbreaks in Israel and Turkey have reported significant mortality (166, 167). The virus is transmitted by arthropod vectors although an exact association with a particular species has not been established, with BEFV being detected in both *Culicoides* midges and mosquitoes in Australia (165). Bovine ephemeral fever is either enzootic or occasionally epizootic in Africa, Asia and Australia (168–170). There have been no cases reported from Europe, with the exception of possible cases in the European region of Turkey, so currently there is a low risk of its emergence in the UK.

DISCUSSION

This review has highlighted a large number of pathogens that infect animals in the UK, and others that are at risk of introduction (Figure 6). A striking feature of this extended list is the small number of arthropod-borne viruses that are currently present in the UK, limited to one tick-borne virus, LIV. Various reasons for this have been suggested including a low level of competence of indigenous species (171) and climatic factors, such as lower mean temperatures and a shorter active season for vectors than in other parts of Europe. However, a growing number of studies have shown that indigenous species of mosquitoes are capable of transmitting viruses under experimental conditions similar to that found during the summer months (10, 12, 45). Another key factor is the absence of certain vector species, for example sandflies and tick species. *Phlebotomus* sandflies transmit *Leishmania infantum* to dogs causing canine leishmaniosis in southern Europe. The absence of sandflies in the UK means that there is no vector borne



transmission, although infected dogs are imported (172) and there is suspicion that dog to dog transmission can occur (173). The introduction of exotic ticks, such as *Hyalomma* and *Rhipicephalus spp.*, vectors of viruses, such as CCHFV and NSDV,

respectively, could lead to the introduction and establishment of these diseases. In the case of *Hyalomma* species, ticks are being introduced by migrating birds but have so far failed to establish a detectable reproductive population. Changes to the climate

may make the UK more permissive for such species. Increases in a range of parameters will influence vector populations. For example, an increase in mean temperatures throughout the year will extend the period over which vectors are active and lead to extended periods where temperatures are permissive for virus replication, which may promote vector competence. Another area of uncertainty is the ability of pathogens to survive the winter. An increase in midwinter temperatures will promote vector survival and increase the probability that infected vectors will enable overwintering of pathogens. Critically, WNV is successfully overwintering in Central Europe, which is driving repeated outbreaks of disease and increasing its distribution. For anthropophilic mosquitoes, such as *Ae. albopictus*, climate modeling suggests that only the southeast of England has suitable climatic conditions for the mosquito species to establish, coincidentally the one part of the UK where *Ae. albopictus* has been detected. The current trends in climate change, particularly to the daily temperature range will increase the areas of the UK that can accommodate the species (174).

In addition to climate change, human action in activities, such as international travel by air, livestock movements and conversion of land to agricultural use can lead to the movement of disease vectors (175, 176). The introduction of *Ae. albopictus* into southern England may have been introduced by cars or lorries entering the country. Whilst the introduction of an anthropophilic mosquito species may not change the risk of disease transmission to animals, the addition of an invasive tick may be more significant, particularly as there are already tick-borne diseases active in the UK. The emergence of the Asian long-horned tick, *Haemaphysalis longicornis*, in North America is a dramatic demonstration of how quickly an invasive tick species can establish in a new environment. As its common name suggests, the tick is a native of East Asia and had been repeatedly intercepted on quarantined livestock entering the United States. However, in 2017 it was detected in a sheep flock in New Jersey (177). Subsequent surveillance confirmed the presence of the tick in a further eight US states. Modeling has suggested that the tick could eventually spread across much of the US and Mexico (178). Of significant concern is the ability of this species to transmit a number of diseases including severe fever with thrombocytopenia syndrome in humans (179) and Theileriosis in livestock (180), in addition to transmitting existing tick-borne diseases already present in the US.

The implication of these observations are that surveillance for the introduction and spread of invasive arthropod species is necessary to offer an opportunity to prevent establishment and to predict at-risk areas well before a pathogen is introduced. Allied to this is an understanding of the assemblage, behavior, ecology and abundance of indigenous vectors. The monitoring of the mosquito species *Culex modestus* in the UK, is an example of this (181). Genetic studies suggest that this species is a recent introduction from continental Europe (182). The population has expanded across large areas of the Thames Estuary and East Anglia, and these areas are now considered at greater risk of WNV spread, were the virus to be introduced. Monitoring for other invasive arthropod vectors, such as ticks and sandflies will provide an early warning for increases in the risk of arbovirus

emergence. This reflects trends observed in southern Europe where there is push for harmonization between governments in response to an increasing risk to public health (183). In addition to field-based surveillance for vector species, there are initiatives in the UK to introduce innovative measures to detect changes to vector distribution, prevalence on animals and incidence of disease (184, 185).

CONCLUSIONS

A key driver in the application of surveillance of livestock, domestic pets, and wildlife is to detect disease before there is widespread transmission of disease. This reduces the risk of spill-over of some diseases into the human population, ameliorates the economic impact of the outbreak to industry, reduces potential harm to domestic animals and limits challenges to biodiversity within wildlife. The cost of surveillance needs to be proportionate to the risk and balanced against the cost of an outbreak. The estimated cost to the agricultural sector as a result of the 2001 outbreak of foot and mouth disease in the UK was £3.1 billion with the tourism sector being equally affected (186). It is unlikely that the introduction of an exotic arbovirus disease will be as costly in strict financial terms as this, although the ability to eliminate the disease will be highly dependent on a range of factors, including competent vector distribution and the movement of compromised animals. The experience from mainland Europe is that once established, vector-borne diseases with a wildlife reservoir are difficult to eliminate and subsequent emergence is unpredictable and challenging to control. The early identification of the source of the introduction, controlling infected vector populations and the availability of effective interventions, such as vaccination, all help to reduce the impact of disease but not continued transmission. For many disease-vector combinations, these interventions will be difficult to implement and thus elimination may not be possible and control will be replaced by prevention. The UK is in a fortunate position with respect to vector-borne diseases in animals due in part to geographical barriers. However, with changes in vector and pathogen distribution this is likely to change in the coming decades.

AUTHOR CONTRIBUTIONS

NJ conceived the idea. AF, DD-R, LH-T, LP, and NJ wrote the manuscript.

FUNDING

This review was prepared with the support of the Department for Environment, Food and Rural Affairs (Defra), the Scottish and Welsh Governments through projects SE4116 and SV3045.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2020.00020/full#supplementary-material>

REFERENCES

- Medlock JM, Leach SA. Effect of climate change on vector-borne disease risk in the UK. *Lancet Infect Dis.* (2015) 15:721–30. doi: 10.1016/S1473-3099(15)70091-5
- Baylis M. Potential impact of climate change on emerging vector-borne and other infections in the UK. *Environ Health.* (2017) 16:112. doi: 10.1186/s12940-017-0326-1
- Hansford KM, Phipps LP, Cull B, Pietzsch ME, Medlock JM. *Rhipicephalus sanguineus* importation into the UK: surveillance, risk, public health awareness and one health response. *Vet Rec.* (2017) 180:119. doi: 10.1136/vr.104061
- Vaux AGC, Dallimore T, Cull B, Schaffner F, Strode C, Pflüger C, et al. The challenge of invasive mosquito vectors in the U.K. during 2016–2018: a summary of the surveillance and control of *Aedes albopictus*. *Med Vet Entomol.* (2019) 33:443–52. doi: 10.1111/mve.12396
- Reiter P. From Shakespeare to Defoe: Malaria in England in the little ice age. *Emerg Infect Dis.* (2000) 6:1–11. doi: 10.3201/eid0601.000101
- Andriopoulos P, Economopoulou A, Spanakos G, Assimakopoulos G. A local outbreak of autochthonous *Plasmodium vivax* malaria in Laconia, Greece—a re-emerging infection in the southern border of Europe? *Int J Infect Dis.* (2013) 17:e125–8. doi: 10.1016/j.ijid.2012.09.009
- Gatellet M, Vanderheyden S, Abbe M, Adaszek L, Varloud M. A suspected case of Lyme borreliosis in a dog from Belgium. *Case Rep Vet Med.* (2019) 2019:3973901. doi: 10.1155/2019/3973901
- Smith FD, Ballantyne R, Morgan ER, Wall R. Estimating Lyme disease risk using pet dogs as sentinels. *Comp Immunol Microbiol Infect Dis.* (2012) 35:163–7. doi: 10.1016/j.cimid.2011.12.009
- Brugman VA, Hernández-Triana LM, Medlock JM, Fooks AR, Carpenter S, Johnson N. The role of *Culex pipiens* L. (Diptera: Culicidae) in virus transmission in Europe. *Int J Environ Res Public Health.* (2018) 15:389. doi: 10.3390/ijerph15020389
- Mackenzie-Impoinvil L, Impoinvil DE, Galbraith SE, Dillon RJ, Ranson H, Johnson N, et al. Evaluation of a temperate climate mosquito, *Ochlerotatus detritus* (= *Aedes detritus*), as a potential vector of Japanese encephalitis virus. *Med Vet Entomol.* (2015) 29:1–9. doi: 10.1111/mve.12083
- Blagrove MS, Sherlock K, Chapman GE, Impoinvil DE, McCall PJ, Medlock JM, et al. Evaluation of the vector competence of a native UK mosquito *Ochlerotatus detritus* (*Aedes detritus*) for dengue, chikungunya and West Nile viruses. *Parasit Vectors.* (2017) 9:452. doi: 10.1186/s13071-016-1739-3
- Lumley S, Hernández-Triana LM, Horton DL, Fernández de Marco MDM, Medlock JM, Hewson R, et al. Competence of mosquitoes native to the United Kingdom to support replication and transmission of Rift Valley fever virus. *Parasit Vectors.* (2018) 11:308. doi: 10.1186/s13071-018-2884-7
- Medlock JM, Vaux AG. Seasonal dynamics and habitat specificity of mosquitoes in an English wetland: implications for UK wetland management and restoration. *J Vector Ecol.* (2015) 40:90–106. doi: 10.1111/jvec.12137
- Meers PD. Yellow fever in Swansea, 1965. *J Hygiene.* (1986) 97:185–91. doi: 10.1017/S0022172400064469
- Service MW. Feeding behaviour and host preferences of British mosquitoes. *Bull Entomol Res.* (1971) 60:653–61. doi: 10.1017/S0007485300042401
- Danabalan R, Monaghan MT, Ponsonby DJ, Linton YM. Occurrence and host preferences of *Anopheles maculipennis* group mosquitoes in England and Wales. *Med Vet Entomol.* (2014) 28:169–78. doi: 10.1111/mve.12023
- Brugman VA, Hernández-Triana LM, Prosser S, Weland C, Westcott D, Fooks AR, et al. Molecular species identification, host preference and detection of myxoma virus in the *Anopheles maculipennis* complex (Diptera: Culicidae) in southern England, UK. *Parasit Vectors.* (2015) 8:421. doi: 10.1186/s13071-015-1034-8
- Brugman VA, Hernández-Triana LM, England ME, Medlock JM, Mertens PPC, Logan JG, et al. Blood-feeding patterns of native mosquitoes and insights into their potential role as pathogen vectors in the Thames estuary region. *Parasit Vectors.* (2017) 10:163. doi: 10.1186/s13071-017-2098-4
- Hernández-Triana LM, Brugman VA, Prosser SWJ, Weland C, Nikilova N, Thorne L, et al. Molecular approaches for blood meal analysis and species identification of mosquitoes (Insecta: Diptera: Culicidae) in rural locations in southern England. *Zootaxa.* (2017) 4250:67–76. doi: 10.11646/zootaxa.4250.1.5
- Golding N, Nunn MA, Medlock JM, Purse BV, Vaux AG, Schäfer SM. West Nile virus vector *Culex modestus* established in southern England. *Parasit Vectors.* (2012) 5:32. doi: 10.1186/1756-3305-5-32
- Vaux AG, Gibson G, Hernández-Triana LM, Cheke RA, McCracken F, Jeffries CL, et al. Enhanced West Nile virus surveillance in the North Kent marshes, UK. *Parasit Vectors.* (2015) 8:91. doi: 10.1186/s13071-015-0705-9
- Benzarti E, Linden A, Desmecht D, Garigliany M. Mosquito-borne epornitic flaviviruses: an update and review. *J Gen Virol.* (2019) 100:119–32. doi: 10.1099/jgv.0.001203
- Murgue B, Murri S, Zientara S, Durand B, Durand JP, Zeller H. West Nile outbreak in horses in southern France, 2000, return after 35 years. *Emerg Infect Dis.* (2001) 7:692–6. doi: 10.3201/eid0704.017417
- Tsai TF, Popovic F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. *Lancet.* (1998) 352:767–71. doi: 10.1016/S0140-6736(98)03538-7
- Glávits R, Ferenczi E, Ivanics E, Bakonyi T, Mató T, Zarka P, et al. Co-occurrence of West Nile fever and circovirus infections in a goose flock in Hungary. *Avian Pathol.* (2005) 34:408–14. doi: 10.1080/03079450500268039
- Erdélyi K, Ursu K, Ferenczi E, Szeredi L, Rátz F, Skáre J, et al. Clinical and pathologic features of lineage 2 West Nile virus infections in birds of prey in Hungary. *Vector Borne Zoonotic Dis.* (2007) 7:181–8. doi: 10.1089/vbz.2006.0586
- Ziegler U, Lühken R, Keller M, Cadar D, van der Ginten E, Michel F, Albrecht K, et al. West Nile virus epizootic in Germany, 2018. *Antiviral Res.* (2019) 162:39–43. doi: 10.1016/j.antiviral.2018.12.005
- Gossner CM, Marrama L, Carson M, Allerberger F, Calistri P, Dilaveris D, et al. West Nile virus surveillance in Europe: moving towards an integrated animal-human-vector approach. *Euro Surveill.* (2017) 22:30526. doi: 10.2807/1560-7917.ES.2017.22.18.30526
- Johnson N, Fernández de Marco M, Giovannini A, Ippoliti C, Danzetta ML, Svartz G, et al. Emerging mosquito-borne threats and the response of European and Eastern Mediterranean countries. *Int J Environ Res Public Health.* (2018) 15:E2775. doi: 10.3390/ijerph15122775
- Fooks AR, Horton DL, Phipps LP, Mansfield KL, McCracken F, Jeffries CL, et al. Suspect West Nile encephalitis in a horse imported into the UK from Europe. *Vet Rec Case Rep.* (2014) 2:e000104. doi: 10.1136/vetreccr-2014-000104
- Drummond R. Surveillance for West Nile virus in horses. *Vet Rec.* (2008) 162:763. doi: 10.1136/vr.162.23.763-a
- Defra. *West Nile Virus: Potential Risk Factors and the likelihood for introduction into the United Kingdom.* (2012). Available online at: <http://webarchive.nationalarchives.gov.uk/20130908115728/http://www.defra.gov.uk/animal-diseases/files/gr-a-wnv-120501.pdf> (accessed February 19, 2019).
- Komarov A, Kalmar E. A hitherto undescribed disease—turkey meningo-encephalitis. *Vet Rec.* (1960) 72:257–61.
- Digoutte J. Bagaza (BAG) virus: Dak Ar B 209. *Am J Trop Med Hyg.* (1978) 27:376–7. doi: 10.4269/ajtmh.1978.27.376
- Venter M. Assessing the zoonotic potential of arboviruses of African origin. *Curr Opin Virol.* (2018) 28:74–84. doi: 10.1016/j.coviro.2017.11.004
- Agüero M, Fernández-Pinero J, Buitrago D, Sánchez A, Elizalde M, San Miguel E, et al. Bagaza virus in partridges and pheasants, Spain, 2010. *Emerg Infect Dis.* (2011) 17:1498–501. doi: 10.3201/eid1708.110077
- Llorente F, Pérez-Ramírez E, Fernández-Pinero J, Soriguer R, Figuerola J, Jiménez-Clavero MA. Flaviviruses in game birds, southern Spain, 2011–2012. *Emerg Infect Dis.* (2013) 19:1023–5. doi: 10.3201/eid1906.130122
- Rijks JM, Kik ML, Slaterus R, Foppen R, Stroo A, Ijzer J, et al. Widespread Usutu virus outbreak in birds in the Netherlands, 2016. *Euro Surveill.* (2016) 21:30391. doi: 10.2807/1560-7917.ES.2016.21.45.30391
- Ashraf U, Ye J, Ruan X, Wan S, Zhu B, Cao S. Usutu virus: an emerging flavivirus in Europe. *Viruses.* (2015) 7:219–38. doi: 10.3390/v7010219
- Weissenböck H, Bakonyi T, Rossi G, Mani P, Nowotny N. Usutu virus, Italy, 1996. *Emerg Infect Dis.* (2013) 19:274–7. doi: 10.3201/eid1902.121191
- Engler O, Savini G, Papa A, Figuerola J, Groschup MH, Kampen H, et al. European surveillance for West Nile virus in mosquito populations. *Int J Environ Res Public Health.* (2013) 10:4869–95. doi: 10.3390/ijerph10104869
- Lühken R, Jöst H, Cadar D, Thomas SM, Bosch S, Tannich E, et al. Distribution of Usutu virus in Germany and its

- effects on breeding bird populations. *Emerg Infect Dis.* (2017) 23:1994–2001. doi: 10.3201/eid2312.171257
43. Garigliany MM, Marlier D, Tenner-Racz K, Eiden M, Cassart D, Gandar F, et al. Detection of Usutu virus in a bullfinch (*Pyrrhula pyrrhula*) and a great spotted woodpecker (*Dendrocopos major*) in north-west Europe. *Vet J.* (2014) 199:191–3. doi: 10.1016/j.tvjl.2013.10.017
 44. Fros JJ, Miessen P, Vogels CB, Gaibani P, Sambri V, Martina BE. Comparative Usutu and West Nile virus transmission potential by local *Culex pipiens* mosquitoes in north-western Europe. *One Health.* (2015) 1:31–6. doi: 10.1016/j.onehlt.2015.08.002
 45. Hernández-Triana LM, Fernández de Marco M, Mansfield LM, Thorne L, Lumley S, Marston D, et al. Assessment of vector competence of UK mosquitoes for Usutu virus of African origin. *Parasit Vectors.* (2018) 11:381. doi: 10.1186/s13071-018-2959-5
 46. Horton DL, Lawson B, Egbetade A, Jeffries C, Johnson N, Cunningham AA, et al. Targeted surveillance for Usutu virus in British birds (2005–2011). *Vet Rec.* (2013) 172:17. doi: 10.1136/vr.101275
 47. Huhtamo E, Lambert AJ, Costantino S, Servino L, Krizmancic L, Boldorini R, et al. Isolation and full genomic characterization of Batai virus from mosquitoes, Italy, 2009. *J Gen Virol.* (2013) 94:1242–8. doi: 10.1099/vir.0.051359-0
 48. Scheuch DE, Schäfer M, Eiden M, Heym EC, Ziegler U, Walther D, et al. Detection of Usutu, Sindbis, and Batai viruses in mosquitoes (Diptera: Culicidae) collected in Germany, 2011–2016. *Viruses.* (2018) 10:E389. doi: 10.3390/v10070389
 49. Hofmann M, Wiethölter A, Blaha I, Jöst H, Heinemann P, Lehmann M, et al. Surveillance of Batai virus in bovines from Germany. *Clin Vaccine Immunol.* (2015) 22:672–3. doi: 10.1128/CI.00082-15
 50. Jo WK, Pfankuche VM, Lehmbecker A, Martina B, Rubio-Garcia A, Becker S, et al. Association of Batai virus infection and encephalitis in harbour seals, Germany, 2016. *Emerg Infect Dis.* (2018) 24:1691–5. doi: 10.3201/eid2409.171829
 51. Eiden M, Ziegler U, Keller M, Müller K, Granzow H, Jöst H, et al. Isolation of Sindbis virus from a hooded crow in Germany. *Vector Borne Zoonotic Dis.* (2014) 14:220–2. doi: 10.1089/vbz.2013.1354
 52. Van Niekerk S, Human S, Williams J, van Wilpe E, Pretorius M, Swanepoel R, et al. Sindbis and Middleburg Old World alphaviruses associated with neurologic disease in horses, South Africa. *Emerg Infect Dis.* (2015) 21:2225–9. doi: 10.3201/eid2112.150132
 53. Genchi C, Kramer L. Subcutaneous dirofilariosis (*Dirofilaria repens*): an infection spreading throughout the old world. *Parasit Vectors.* (2017) 10:517. doi: 10.1186/s13071-017-2434-8
 54. Trotz-William LA, Trees AJ. Systematic review of the distribution of the major vector-borne parasitic infections in dogs and cats in Europe. *Vet Rec.* (2003) 152:97–105. doi: 10.1136/vr.152.4.97
 55. Thomas RE. A case of canine heartworm disease (*Dirofilaria immitis*) in the UK. *Vet Rec.* (1985) 117:15. doi: 10.1136/vr.117.1.14
 56. Genchi C, Bowman D, Drake J. Canine heartworm disease (*Dirofilaria immitis*) in Western Europe: a survey of veterinary awareness and perceptions. *Parasit Vectors.* (2014) 7:206. doi: 10.1186/1756-3305-7-206
 57. Fooks AR, Johnson N. Jet set pets. *Vet Med.* (2015) 6:17–25. doi: 10.2147/VMRR.S62059
 58. Mansfield KL, Hernández-Triana LM, Banyard AC, Fooks AR, Johnson N. Japanese encephalitis virus infection, diagnosis and control in domestic animals. *Vet Microbiol.* (2017) 201:85–92. doi: 10.1016/j.vetmic.2017.01.014
 59. Lytra I, Emmanouel N. Study of *Culex tritaeniorhynchus* and species composition of mosquitoes in a rice field in Greece. *Acta Tropica.* (2014) 134:66–71. doi: 10.1016/j.actatropica.2014.02.018
 60. Ravanini P, Huhtamo E, Ilaria V, Crobu MG, Nicosia AM, Servino L, et al. Japanese encephalitis virus RNA detected in *Culex pipiens* mosquitoes in Italy. *Euro Surveill.* (2012) 17:20221. doi: 10.2807/ese.17.28.20221-en
 61. Prezioso S, Mari S, Mariotti F, Rossi G. Detection of Japanese encephalitis virus in bone marrow of healthy young wild birds collected in 1997–2000 in Central Italy. *Zoonoses Public Health.* (2018) 65:798–804. doi: 10.1111/zph.12501
 62. Simon-Loriere E, Faye O, Prot M, Casademont I, Fall G, Fernandez-Garcia MD, et al. Autochthonous Japanese encephalitis with yellow fever coinfection in Africa. *N Engl J Med.* (2017) 376:1483–5. doi: 10.1056/NEJMcl1701600
 63. Huber K, Jansen S, Leggewie M, Badusche M, Schmidt-Chanasit J, Becker N, et al. *Aedes japonicus japonicus* (Diptera: Culicidae) from Germany have vector competence for Japan encephalitis virus but are refractory to infection with West Nile virus. *Parasitol Res.* (2014) 113:3195–9. doi: 10.1007/s00436-014-3983-9
 64. de Wispelaere M, Desprès P, Choumet V. European *Aedes albopictus* and *Culex pipiens* are competent vectors for Japanese encephalitis virus. *PLoS Negl Trop Dis.* (2017) 11:e0005294. doi: 10.1371/journal.pntd.0005294
 65. Mansfield KL, Banyard AC, McElhinney L, Johnson N, Horton DL, Hernández-Triana LM, et al. Rift Valley fever virus: a review of diagnosis and vaccination, and implications for emergence in Europe. *Vaccine.* (2015) 33:5520–31. doi: 10.1016/j.vaccine.2015.08.020
 66. Centers for Disease Control and Prevention (CDC). Outbreak of Rift Valley fever—Saudi Arabia, August–October, 2000. *Morb Mortal Weekly Rep.* (2000) 49:905–8. Available online at: <https://www.cdc.gov/mmwr/preview/mmwrhtml/mm4940a1.htm> (accessed January 23, 2020).
 67. Jupp PG, Kemp A, Grobbelaar A, Lema P, Burt FJ, Alahmed AM et al. The 2000 epidemic of Rift Valley fever in Saudi Arabia: mosquito vector studies. *Med Vet Entomol.* (2002) 16:245–52. doi: 10.1046/j.1365-2915.2002.00371.x
 68. Balkhy HH, Memish ZS. Rift Valley fever: an uninvited zoonosis in the Arabian Peninsula. *Int J Antimicrob Agents.* (2003) 21:153–7. doi: 10.1016/S0924-8579(02)00295-9
 69. Morvan J, Saluzzo JF, Fontenille D, Rollin PE, Coulanges P. Rift Valley fever on the east coast of Madagascar. *Res Virol.* (1991) 142:475–82. doi: 10.1016/0923-2516(91)90070-J
 70. Métras R, Cavalierie L, Dommergues L, Mérot P, Edmunds WJ, Keeling MJ, et al. The epidemiology of Rift Valley fever in Mayotte: Insight and perspectives from 11 years of data. *PLoS Negl Trop Dis.* (2016) 10:e0004783. doi: 10.1371/journal.pntd.0004783
 71. Gür S, Kale M, Erol N, Yapici O, Mamak N, Yavru S. The first serological evidence for Rift Valley fever infection in the camel, goitered gazelle and Anatolian water buffaloes in Turkey. *Trop Anim Health Pro.* (2017) 49:1531–5. doi: 10.1007/s11250-017-1359-8
 72. Fakour S, Naserabadi S, Ahmadi E. The first positive serological study on Rift Valley fever in ruminants of Iran. *J Vector Dis.* (2017) 54:348–52. doi: 10.4103/0972-9062.225840
 73. Brustolin M, Talavera S, Nuñez A, Santamaría C, Rivas R, Pujol N, et al. Rift Valley fever virus and European mosquitoes: vector competence of *Culex pipiens* and *Stegomyia albopicta* (= *Aedes albopictus*). *Med Vet Entomol.* (2017) 31:365–72. doi: 10.1111/mve.12254
 74. Rolin AI, Berrang-Ford L, Kulkarni MA. The risk of Rift Valley fever virus introduction and establishment in the United States and European Union. *Emerg Microbes Infect.* (2013) 2:e81. doi: 10.1038/emi.2013.81
 75. Kopp A, Gillespie TR, Hobelsberger D, Estrada A, Harper JM, Miller RA, et al. Provenance and geographic spread of St. Louis encephalitis virus. *mBio.* (2013) 4:e00322-13. doi: 10.1128/mBio.00322-13
 76. Thompson NN, Auguste AJ, Coombs D, Blitvich BJ, Carrington CV, da Rosa AP, et al. Serological evidence of flavivirus and alphaviruses in livestock and wildlife in Trinidad. *Vector Borne Zoonotic Dis.* (2012) 12:969–7. doi: 10.1089/vbz.2012.0959
 77. Rosa R, Costa EA, Marques RE, Oliveira TS, Furtini R, Bomfim MR, et al. Isolation of Saint Louis encephalitis virus from a horse with neurological disease in Brazil. *PLoS Negl Trop Dis.* (2013) 7:e2537. doi: 10.1371/journal.pntd.0002537
 78. Go YY, Balaasuriya UBR, Lee C-K. Zoonotic encephalitis caused by arboviruses: transmission and epidemiology of alphaviruses and flaviviruses. *Clin Exp Vaccine Res.* (2014) 3:58–77. doi: 10.7774/cevr.2014.3.1.58
 79. Kono Y, Tsukamoto K, Abd Hamid M, Darus A, Lian TC, Sam LS, et al. Encephalitis and retarded growth of chicks caused by Sitiawan virus, a new isolate belonging to the genus *Flavivirus*. *Am J Trop Med Hyg.* (2000) 63:94–101. doi: 10.4269/ajtmh.2000.63.94
 80. Zhang W, Chen S, Mahalingam S, Wang M, Cheng A. An updated review of avian-origin Tembusu virus: a newly emerging avian *Flavivirus*. *J Gen Virol.* (2017) 98:2413–20. doi: 10.1099/jgv.0.000908
 81. Succo T, Leparc-Goffart I, Ferré JB, Roiz D, Broche B, Maquart M, et al. Autochthonous dengue outbreaks in Nîmes, South of France, July to September 2015. *Euro*

- Surveill. (2016) 21:30240. doi: 10.2807/1560-7917.ES.2016.21.21.30240
82. Vaux AGC, Medlock JM. Current status of invasive mosquito surveillance in the UK. *Parasit Vector.* (2015) 8:351. doi: 10.1186/s13071-015-0936-9
 83. Medlock JM, Vaux AG, Cull B, Schaffner F, Gillingham E, Pfluger V, et al. Detection of the invasive mosquito species *Aedes albopictus* in southern England. *Lancet Infect Dis.* (2017) 17:140. doi: 10.1016/S1473-3099(17)30024-5
 84. Richards SL, Ponnusamy L, Unnasch TR, Hassan HK, Apperson CS. Host-feeding patterns of *Aedes albopictus* (Diptera: Culicidae) in relation to availability of human and domestic animals in suburban landscapes of central North Carolina. *J Med Entomol.* (2006) 43:543–51. doi: 10.1093/jmedent/43.3.543
 85. Muñoz J, Ertija R, Alcaide M, Montalvo T, Soriguer RC, Figuerola J. Host-feeding patterns of native *Culex pipiens* and invasive *Aedes albopictus* mosquitoes (Diptera: Culicidae) in urban zones from Barcelona, Spain. *J Med Entomol.* (2011) 48:956–60. doi: 10.1603/ME11016
 86. Niebylski MM, Savage HM, Nasci RS, Craig GB Jr. Blood hosts of *Aedes albopictus* in the United States. *J Am Mosquito Contr.* (1994) 10:447–50.
 87. Gomes AC, Silva NN, Marques GR, Brito M. Host-feeding patterns of potential human disease vectors in the Paraíba Valley region, State of São Paulo, Brazil. *J Vector Ecol.* (2003) 28:74–8.
 88. Abdullah S, Helps C, Tasker S, Newbury H, Wall R. Ticks infesting domestic dogs in the UK: a large-scale surveillance programme. *Parasit Vector.* (2016) 9:391. doi: 10.1186/s13071-016-1673-4
 89. Cull B, Pietzsch ME, Hansford KM, Gillingham EL, Medlock JM. Surveillance of British ticks: An overview of species records, host associations, and new records of *Ixodes ricinus* distribution. *Ticks Tick Borne Dis.* (2016) 9:605–14. doi: 10.1016/j.ttbdis.2018.01.011
 90. Medlock JM, Hansford KM, Vaux AGC, Cull B, Adullah S, Pietzsch, M, et al. Distribution of the tick *Dermacentor reticulatus* in the United Kingdom. *Med Vet Entomol.* (2017) 31:281–8. doi: 10.1111/mve.12235
 91. Medlock JM, Hanford KM, Vaux AGC, Cull B, Pietzsch ME, Gillingham EL, et al. Has the red sheep tick, *Haemaphysalis punctata*, recently expanded its range in England? *Med Vet Entomol.* (2018) 32:473–80. doi: 10.1111/mve.12335
 92. Reid HW. Experimental infection of red grouse with louping-ill virus (Flavivirus group). I. Viraemia and antibody response. *J Comp Pathol.* (1975) 85:223–9. doi: 10.1016/0021-9975(75)90063-8
 93. Jeffries CL, Mansfield KL, Phipps LP, Wakeley PR, Mearns R, Schock A, et al. Louping ill virus: an endemic tick-borne disease of Great Britain. *J Gen Virol.* (2014) 95:1005–14. doi: 10.1099/vir.0.062356-0
 94. Gilbert L. Louping ill virus in the UK: a review of the hosts, transmission and ecological consequences of control. *Exp Appl Acarol.* (2016) 68:363–74. doi: 10.1007/s10493-015-9952-x
 95. Pool WA, Brownlee A, Wilson DR. The etiology of “louping-ill”. *J Comp Pathol Therap.* (1930) 43:253–90. doi: 10.1016/S0368-1742(30)80026-2
 96. MacLeod J, Gordon WS. Studies in louping-ill, an encephalomyelitis of sheep. II. Transmission by the sheep tick, *Ixodes ricinus* L. *J Comp Pathol Therap.* (1932) 5:240–52. doi: 10.1016/S0368-1742(32)80022-6
 97. Doherty PC, Reid HW. Louping-ill encephalomyelitis in the sheep. II. Distribution of virus and lesions in nervous tissue. *J Comp Pathol.* (1971) 81:531–6. doi: 10.1016/0021-9975(71)90081-8
 98. Reid HW, Buxton D, Finlayson IP. Experimental louping-ill virus infection of cattle. *Vet Rec.* (1981) 108:497–8. doi: 10.1136/vr.108.23.497
 99. Gilbert L, Jones LD, Hudson PJ, Gould EA, Reid HW. Role of small mammals in the persistence of Louping-ill virus: field survey and tick co-feeding studies. *Med Vet Entomol.* (2000) 13:277–82. doi: 10.1046/j.1365-2915.2000.00236.x
 100. Jones LD, Gaunt M, Hails RS, Laurenson K, Hudson PJ, Reid H, et al. Transmission of louping ill virus between infected and uninfected ticks co-feeding on mountain hares. *Med Vet Entomol.* (1997) 11:172–6. doi: 10.1111/j.1365-2915.1997.tb00309.x
 101. Reid HW, Buxton D, Pow I, Brodie TA, Holmes PH, Urquhart GM. Response of sheep to experimental concurrent infection with tick-borne fever (*Cytcetes phagocytophilum*) and louping-ill virus. *Res Vet Sci.* (1986) 41:56–62. doi: 10.1016/S0034-5288(18)30572-1
 102. Laurenson MK, Norman RA, Gilbert L, Reid HW, Hudson PJ. Identifying disease reservoirs in complex systems: mountain hares as reservoirs of ticks and louping-ill virus, pathogens of red grouse. *J Anim Ecol.* (2003) 72:177–85. doi: 10.1046/j.1365-2656.2003.00688.x
 103. Balseiro A, Royo LJ, Martínez CP, Fernández de Mera IG, Höfle U, Polledo L, et al. Louping ill in goats, Spain, 2011. *Emerg Infect Dis.* (2011) 18:976–8. doi: 10.3201/eid1806.120220
 104. Mansfield KL, Morales AB, Johnson N, Ayllón N, Höfle U, Alberdi P, et al. Identification and characterization of a novel tick-borne flavivirus subtype in goats (*Capra hircus*) in Spain. *J Gen Virol.* (2015) 96:1676–81. doi: 10.1099/vir.0.000096
 105. Bock R, Jackson L, De Vos A, Jorgensen W. Babesiosis of cattle. *Parasitology.* (2004) 129:S247–69. doi: 10.1017/S0031182004005190
 106. McFadyean J, Stockman S. A new species of piroplasm found in the blood of British cattle. *J Comp Pathol.* (1911) 24:340–54. doi: 10.1016/S0368-1742(11)80062-7
 107. Lv J, de Marco MF, Phipps LP, Macrelli, M, Otter A, Inman B, et al. Molecular detection of *Babesia divergens* in Holstein cows, England. *J Vet Med Res.* (2017) 4:1078. Available online at: <https://www.jsccimedcentral.com/VeterinaryMedicine/veterinarymedicine-4-1078.pdf> (accessed January 23, 2020).
 108. Zintl A, Mulcahy G, Skerrett HE, Taylor SM, Gray JS. *Babesia divergens*, a bovine blood parasite of veterinary and zoonotic importance. *Clin Microbiol Rev.* (2003) 16:622–36. doi: 10.1128/CMR.16.4.622-636.2003
 109. Yabsley MJ, Schock BC. Natural history of zoonotic Babesia: role of wildlife reservoirs. *Int J Parasitol: Parasites Wildl.* (2013) 2:18–31. doi: 10.1016/j.ijppaw.2012.11.003
 110. Thorburn EJ. Bovine malaria—redwater. *Vet Rec.* (1902) 15:171.
 111. Brocklesby DW, Sellwood SA. Babesia major in Britain: tick transmitted infection in splenectomised calves. *Res Vet Sci.* (1973) 14:47–52. doi: 10.1016/S0034-5288(18)33937-7
 112. Purnell RE. Tick-borne diseases of British Livestock. *Vet Med Rev.* (1981) 1:58–69.
 113. Coulton RM, Phipps P, Dalley C, Lewis J, Hammond T-A, Shiels BR, et al. Equine piroplasmosis status in the UK: an assessment of laboratory diagnostic submissions and techniques. *Vet Rec.* (2019) 184:95. doi: 10.1136/vr.104855
 114. Schnittger L, Yin H, Gubbels MJ, Beyer D, Niemann S, Jongejan F, et al. Phylogeny of sheep and goat Theileria and Babesia parasites. *Parasitol Res.* (2003) 91:398–406. doi: 10.1007/s00436-003-0979-2
 115. Lewis D, Holman MR, Purnell RE, Young ER, Herbert IV, Bevan WJ. Investigations on *Babesia motasi* isolated from Wales. *Res Vet Sci.* (1981) 31:239–43. doi: 10.1016/S0034-5288(18)32501-3
 116. Alani AJ, Herbert IV. The morphometrics of *Babesia motasi* (Wales) and its transmission by *Haemaphysalis punctata* (Canestrini and Fanzago 1877) to sheep. *Vet Parasitol.* (1988) 30:87–95. doi: 10.1016/0304-4017(88)90155-0
 117. Phipps LP, Hernández-Triana LM, Gohariz H, Welchman D, Johnson N. Detection of *Theileria luwenshuni* in sheep from Great Britain. *Parasit Vectors.* (2016) 9:203. doi: 10.1186/s13071-016-1486-5
 118. Matijatko V, Torti M, Schetters TP. Canine babesiosis in Europe: how many diseases? *Trends Parasitol.* (2012) 28:99–105. doi: 10.1016/j.pt.2011.11.003
 119. Shaw SE, Lerga AI, Williams S, Beugner F, Birtles RJ, Day M, et al. Review of exotic infectious diseases in small animals entering the United Kingdom from abroad diagnosed by PCR. *Vet Rec.* (2003) 152:176–7. doi: 10.1136/vr.152.6.176
 120. Tappin S. Babesiosis in a 10-year-old British crossbreed dog returning from vacation in France. *Companion Anim.* (2009) 14:29–34. doi: 10.1111/j.2044-3862.2009.tb00551.x
 121. Johnson N, Gohariz H, Wakeley PR, Phipps LP. *Babesia vogeli* in a quarantined dog. *Vet Rec.* (2013) 172:241–2. doi: 10.1136/vr.f1329
 122. de Marco FDM, Hernández-Triana LM, Phipps LP, Hansford K, Mitchel ES, Cull B, et al. Emergence of *Babesia canis* in southern England. *Parasit Vectors.* (2017) 10:241. doi: 10.1186/s13071-017-2178-5
 123. Tait A, Hall FL. *Theileria annulata*: control measures, diagnosis and the potential use of subunit vaccines. *Rev Sci Tech.* (1990) 9:387–403. doi: 10.20506/rst.9.2.505
 124. Kamau J, de Vos AJ, Playford M, Salim B, Kinyanjui P, Sugimoto C. Emergence of new types of *Theileria orientalis* in Australian cattle

- and possible cause of theileriosis outbreaks. *Parasit Vectors*. (2011) 4:22. doi: 10.1186/1756-3305-4-22
125. Irvin AD. Clinical and diagnostic features East Coast fever (*Theileria parva*) infection of cattle. *Vet Rec*. (1983) 113:192–8. doi: 10.1136/vr.113.9.192
 126. Brocklesby DW, Barnett SF. The tick *Haemaphysalis punctata*, shown to be a vector of *Theileria mutans* in Britain. *Vet Rec*. (1972) 90:512–3. doi: 10.1136/vr.90.18.512
 127. Joyner LP, Payne RC, Takahashi K, Brocklesby DW, Irwin AD. Serological comparison of British *Theileria mutans* and Japanese *T. sergenti*. *Res Vet Sci*. (1979) 26:387–8. doi: 10.1016/S0034-5288(18)32901-1
 128. Fernández de Marco M, Brugman VA, Hernández-Triana LM, Thorne L, Phipps LP, Nikolova NI, et al. Detection of *Theileria orientalis* in mosquito blood meals in the United Kingdom. *Vet Parasitol*. (2016) 229:31–6. doi: 10.1016/j.vetpar.2016.09.012
 129. Hudson JR. The recognition of tick-borne fever as a disease of cattle. *Br Vet J*. (1950) 106:3–17. doi: 10.1016/S0007-1935(17)53027-4
 130. Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, et al. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* and *Anaplasma*, *Cowdria* with *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGA agent' as subjective synonyms of *Ehrlichia phagocytophilum*. *Int J Syst Evol Micro*. (2001) 51:2145–65. doi: 10.1099/00207713-51-6-2145
 131. Cranwell MP, Gibbons JA. Tick-borne fever in a dairy herd. *Vet Rec*. (1986) 119:531–2. doi: 10.1136/vr.119.21.531
 132. Anonymous. Tickborne fever associated with abortion outbreak in dairy cows. *Vet Rec*. (2016) 179:189–92. doi: 10.1136/vr.i4520
 133. Zintl A, Moutaillier S, Stuart P, Paredis L, Dutraive J, Gonzalez E, et al. Ticks and tick-borne disease in Ireland. *Irish Vet J*. (2017) 70:4. doi: 10.1186/s13620-017-0084-y
 134. Sanchez E, Vannier E, Wormser GP, Hu LT. Diagnosis, treatment and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: a review. *J Am Med Assoc*. (2016) 315:1767–77. doi: 10.1001/jama.2016.2884
 135. Hagerdorn P, Imhoff M, Fischer C, Domingo C, Niedrig M. Human granulocytic anaplasmosis acquired in Scotland, 2013. *Emerg Infect Dis*. (2014) 20:1079–81. doi: 10.3201/eid2006.131849
 136. Woldehiwet Z. The natural history of *Anaplasma phagocytophilum*. *Vet Parasitol*. (2010) 167:108–22. doi: 10.1016/j.vetpar.2009.09.013
 137. Dugat T, Lagrée A-C, Mallard R, Boulois H-J, Haddad N. Opening the black box of *Anaplasma phagocytophilum* diversity: current situation and future perspectives. *Front Cell Infect Microbiol*. (2015) 5:61. doi: 10.3389/fcimb.2015.00061
 138. Brodie TA, Holmes PH, Urquhart GM. Some aspects of tick-borne diseases of British sheep. *Vet Rec*. (1986) 118:415–8. doi: 10.1136/vr.118.15.415
 139. Dixon LK, Sun H, Roberts H. African swine fever. *Antiviral Res*. (2019) 165:34–41. doi: 10.1016/j.antiviral.2019.02.018
 140. Garigliany M, Desmecht D, Tignon M, Casart D, Lesenfant C, Paternostre J, et al. Phylogeographic analysis of African swine fever virus, Western Europe, 2018. *Emerg Infect Dis*. (2019) 25:184–6. doi: 10.3201/eid2501.181535
 141. Linden A, Licoppe A, Volpe R, Paternostre J, Lesenfants C, Cassart D, et al. Summer 2018: African swine fever virus hits north-western Europe. *Transbound Emerg Dis*. (2019) 66:54–5. doi: 10.1111/tbed.13047
 142. Ergonul O. Crimean-Congo hemorrhagic fever virus: new outbreaks, new discoveries. *Curr Opin Virol*. (2012) 2:215–20. doi: 10.1016/j.coviro.2012.03.001
 143. Messina JP, Pigott DM, Golding N, Duda KA, Brownstein JS, Weiss DJ, et al. The global distribution of Crimean-Congo hemorrhagic fever. *Trans R Soc Trop Med Hyg*. (2015) 109:503–13. doi: 10.1093/trstmh/trv050
 144. Davies FG. Nairobi sheep disease. *Parassitologia*. (1997) 39:95–8.
 145. Hansford KM, Pietzsch ME, Cull B, Gillingham EL, Medlock JM. Potential risk posed by importation of ticks into the UK on animals: records from the Tick Surveillance Scheme. *Vet Rec*. (2018) 182:107. doi: 10.1136/vr.104263
 146. Attipa C, Maguire D, Solano-Gallego L, Szladovits B, Barker EN, Farr A, et al. *Hepatozoon canis* in three imported dogs: a new tickborne disease reaching the United Kingdom. *Vet Rec*. (2018) 183:716. doi: 10.1136/vr.105087
 147. Capek M, Literak I, Kocianova E, Sychra O, Najer T, Trnka A, et al. Ticks of the *Hyalomma marginatum* complex transported by migratory birds into Central Europe. *Ticks Tick Borne Dis*. (2014) 5:489–493. doi: 10.1016/j.ttbdis.2014.03.002
 148. England ME, Phipps P, Medlock JM, Atkinson PM, Atkinson B, Hewson R, et al. *Hyalomma* ticks on northward migrating birds in southern Spain: implications for the risk of entry of Crimean-Congo hemorrhagic fever virus to Great Britain. *J Vector Ecol*. (2016) 41:128–34. doi: 10.1111/jvec.12204
 149. Hoffman T, Lindeborg M, Barboutis C, Erciyas-Yavuz K, Evander M, Fransson T, et al. Alkurma hemorrhagic fever virus RNA in *Hyalomma rufipes* ticks infesting migratory birds, Europe and Asia Minor. *Emerg Infect Dis*. (2018) 24:879–82. doi: 10.3201/eid2405.171369
 150. Hansford KM, Carter D, Gillingham EL, Hernández-Triana LM, Chamberlain J, Cull B, et al. *Hyalomma rufipes* on an untravelling horse: Is this the first evidence of *Hyalomma* nymphs successfully moulting in the United Kingdom. *Ticks Tick Borne Dis*. (2019) 10:704–8. doi: 10.1016/j.ttbdis.2019.03.003
 151. Harrup LE, Bellis GA, Balenghien T, Garros C. *Culicoides* Latreille (Diptera: Ceratopogonidae) taxonomy: current challenges and future directions. *Infect Genet Evol*. (2015) 30:249–66. doi: 10.1016/j.meegid.2014.12.018
 152. Carpenter S, Groschup MH, Garros C, Felipe-Bauer ML, Purse BV. *Culicoides* biting midges, arboviruses and public health in Europe. *Antiviral Res*. (2013) 100:102–13. doi: 10.1016/j.antiviral.2013.07.020
 153. Lounibos LP. Invasions by insect vectors of human disease. *Ann Rev Entomol*. (2002) 47:233–66. doi: 10.1146/annurev.ento.47.091201.145206
 154. Wilson AJ, Mellor PS. Bluetongue in Europe: past, present and future. *Philos Trans R Soc Lond B Biol Sci*. (2009) 364:2669–81. doi: 10.1098/rstb.2009.0091
 155. Walker PJ, Klement E. Epidemiology and control of bovine ephemeral fever. *Vet Res*. (2015) 46:146. doi: 10.1186/s13567-015-0262-4
 156. Williamson SM, Scholes SF, Welchman D de B, Dennison M, Batten CA, Williams DL, et al. Bluetongue virus serotype 8-associated hydranencephaly in two calves in south-eastern England. *Vet Rec*. (2010) 167:216–8. doi: 10.1136/vr.c3302
 157. Shaw AE, Monaghan P, Alpar HO, Anthony S, Darpel KE, Batten CA, et al. Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. *J Virol Meth*. (2007) 145:115–26. doi: 10.1016/j.jviromet.2007.05.014
 158. Van Wuijckhuise L, Dercksen D, Muskens J, De Bruijn J, Scheepers M, Vroenenratts R. Bluetongue in The Netherlands; description of the first clinical cases and differential diagnosis. Common symptoms just a little different and in too many herds. *Tijdschr Diergeneesk*. (2006) 131:649–54.
 159. Courtejoie N, Durand B, Bréard E, Sailleau C, Vitour D, Zientara S, et al. Serological status of BTV-8 in French cattle prior to the 2015 re-emergence. *Transbound Emerg Dis*. (2018) 65:e173–82. doi: 10.1111/tbed.12718
 160. Hoffmann B, Scheuch M, Höper D, Jungblut R, Holsteg M, Schirrmeier H, et al. Novel orthobunyavirus in cattle, Europe, 2011. *Emerg Infect Dis*. (2012) 18:469–72. doi: 10.3201/eid1803.111905
 161. Shaw AE, Mellor DJ, Purse BV, Shaw PE, McCorkell BF, Palmarini M. Transmission of Schmallenberg virus in a housed dairy herd in the UK. *Vet Rec*. (2013) 173:609. doi: 10.1136/vr.101983
 162. Bilk S, Schulze C, Fischer M, Beer M, Hilnak A, Hoffmann B. Organ distribution of Schmallenberg virus RNA in malformed newborns. *Vet Microbiol*. (2012) 159:236–8. doi: 10.1016/j.vetmic.2012.03.035
 163. Mansfield KL, La Rocca SA, Khatri M, Johnson N, Steinbach F, Fooks AR. Detection of Schmallenberg virus serum neutralising antibodies. *J Virol Meth*. (2013) 188:139–44. doi: 10.1016/j.jviromet.2012.11.031
 164. Rodriguez M, Hooghuis H, Castaño M. African horse sickness in Spain. *Vet Microbiol*. (1992) 33:129–42. doi: 10.1016/0378-1135(92)90041-Q
 165. Walker PJ. Bovine ephemeral fever in Australia and the world. *Curr Topics Microbiol Immunol*. (2005) 292:57–80. doi: 10.1007/3-540-27485-5_4
 166. Yerahim I, Yadin H, Abramovitch I, Shama Z. Bovine ephemeral fever in a dairy cattle herd in the Jordan Valley. *Vet Rec*. (2005) 156:284–6. doi: 10.1136/vr.156.9.284
 167. Tonbak S, Berber E, Yoruk MD, Azkur AK, Pestil Z, Bulut H. A large-scale outbreak of bovine ephemeral fever in Turkey, 2012. *J Vet Med Sci*. (2013) 75:1511–4. doi: 10.1292/jvms.13-0085
 168. Davies FG, Ochieng P, Walker AR. The occurrence of ephemeral fever in Kenya, 1968–1988. *Vet*

- Microbiol.* (1990) 22:129–36. doi: 10.1016/0378-1135(90)90100-A
169. Bai WB, Jiang CL, Davis SS. Preliminary observations on the epidemiology of bovine ephemeral fever in China. *Trop Anim Health Prod.* (1991) 23:22–6. doi: 10.1007/BF02361265
 170. Hirashima Y, Nojiri M, Ohtsuka Y, Kato T, Shirafuji H, Kurazono M, et al. Resurgence of bovine ephemeral fever in mainland Japan in 2015 after a 23-year absence. *J Vet Med Sci.* (2017) 19:904–11. doi: 10.1292/jvms.16-0345
 171. Gould EA, Higgs S, Buckley A, Gritsun TS. Potential arbovirus emergence and implications for the United Kingdom. *Emerg Infect Dis.* (2006) 12:549–55. doi: 10.3201/eid1204.051010
 172. Shaw SE, Langton DA, Hillman TJ. Canine leishmaniosis in the United Kingdom: a zoonotic disease waiting for a vector? *Vet Parasitol.* (2009) 163:281–5. doi: 10.1016/j.vetpar.2009.03.025
 173. Naucke TJ, Amelung S, Lorentz S. First report of transmission of canine leishmaniosis through bite wounds from a naturally infected dog in Germany. *Parasit Vectors.* (2016) 9:256. doi: 10.1186/s13071-016-1551-0
 174. Metelmann S, Caminade C, Jones AE, Medlock JM, Baylis M, Morse SP. The UK's suitability for *Aedes albopictus* in current and future climates. *J Royal Soc Interface.* (2019) 16:20180761. doi: 10.1098/rsif.2018.0761
 175. Daszak P, Cunningham AA, Hyatt AD. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. *Acta Trop.* (2001) 78:103–16. doi: 10.1016/S0001-706X(00)00179-0
 176. Lindahl JF, Grace D. The consequences of human actions on risk for infectious diseases: a review. *Infect Ecol Epidemiol.* (2015) 5:30048. doi: 10.3402/iee.v5.30048
 177. Rainey T, Occi JL, Robbins RG, Egizi A. Discovery of *Haemaphysalis longicornis* (Ixodida: Ixodidae) parasitizing a sheep in New Jersey, United States. *J Med Entomol.* (2018) 55:757–9. doi: 10.1093/jme/tjy006
 178. Ragahavan RK, Barker SC, Cobos ME, Barker D, Teo EJM, Foley DH, et al. Potential spatial distribution of the newly introduced long-horned tick, *Haemaphysalis longicornis* in North America. *Sci Rep.* (2019) 9:498. doi: 10.1038/s41598-018-37205-2
 179. Zhang YZ, Zhou DJ, Qin XC, Tian JH, Xiong Y, Wang JB, et al. The ecological, genetic diversity, and phylogeny of Huaiyangshan virus in China. *J Virol.* (2012) 86:2864–8. doi: 10.1128/JVI.06192-11
 180. Hammer JF, Emery D, Bogema DR, Jenkins C. Detection of *Theileria orientalis* genotypes in *Haemaphysalis longicornis* ticks from southern Australia. *Parasit Vectors.* (2015) 8:229. doi: 10.1186/s13071-015-0839-9
 181. Medlock JM, Vaux AG. Distribution of West Nile virus vector, *Culex modestus*, in England. *Vet Rec.* (2012) 171:278. doi: 10.1136/vr.e6123
 182. Hernández-Triana LM, Brugman VA, Pramual P, Barrero E, Nikolova NI, Ruiz-Arondo I, et al. Genetic diversity and population structure of *Culex modestus* across Europe: does recent emergence in the United Kingdom reveal a tendency for geographical spread? *J Med Entomol.* (2019). doi: 10.1111/mve.12412. [Epub ahead of print].
 183. Jourdain F, Samy AM, Hamidi A, Bouattour A, Alten B, Faraj C, et al. Towards harmonisation of entomological surveillance in the Mediterranean Area. *PLoS Negl Trop Dis.* (2019) 13:e0007314. doi: 10.1371/journal.pntd.0007314
 184. Sánchez-Vizcaino F, Wardeh M, Heayns B, Singleton DA, Tulloch JS, McGinley L, et al. Canine babesiosis and tick activity monitored using companion animal electronic health records in the UK. *Vet Rec.* (2016) 179:358. doi: 10.1136/vr.103908
 185. Tulloch JSP, McGinley L, Sánchez-Vicaino F, Medlock JM, Radford AD. The passive surveillance of ticks using companion animal electronic records. *Epidemiol Infect.* (2017) 145:2020–9. doi: 10.1017/S0950268817000826
 186. Thompson D, Muriel P, Russell D, Osborne P, Bromley A, Rowland M, et al. Economic costs of the foot and mouth disease outbreak in the United Kingdom in 2001. *Rev Sci Tech.* (2002) 21:675–87. doi: 10.20506/rst.21.3.1353

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Folly, Dorey-Robinson, Hernández-Triana, Phipps and Johnson. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Susceptibility of Chicken Embryos, Sheep, Cattle, Pigs, and Chickens to Zika Virus Infection

Aruna Ambagala^{1,2}, Thang Truong¹, Colleen Cottam-Birt¹, Yohannes Berhane^{1,2}, Volker Gerdts^{3,4}, Uladzimir Karniyshuk^{3,4}, David Safronetz⁵ and Shawn Babiuk^{1,6*}

¹ Canadian Food Inspection Agency, National Centre for Foreign Animal Disease, Winnipeg, MB, Canada, ² Department of Animal Science, University of Manitoba, Winnipeg, MB, Canada, ³ Vaccine and Infectious Disease Organization-International Vaccine Centre (VIDO-InterVac), University of Saskatchewan, Saskatoon, SK, Canada, ⁴ Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, SK, Canada, ⁵ Zoonotic Diseases and Special Pathogens, Public Health Agency of Canada, Winnipeg, MB, Canada, ⁶ Department of Immunology, University of Manitoba, Winnipeg, MB, Canada

OPEN ACCESS

Edited by:

Juergen A. Richt,
Kansas State University, United States

Reviewed by:

Richard A. Bowen,
Colorado State University,
United States
Barry Rockx,
Erasmus University
Rotterdam, Netherlands

*Correspondence:

Shawn Babiuk
shawn.babiuk@canada.ca

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 30 September 2019

Accepted: 13 January 2020

Published: 05 February 2020

Citation:

Ambagala A, Truong T, Cottam-Birt C, Berhane Y, Gerdts V, Karniyshuk U, Safronetz D and Babiuk S (2020) Susceptibility of Chicken Embryos, Sheep, Cattle, Pigs, and Chickens to Zika Virus Infection. *Front. Vet. Sci.* 7:23. doi: 10.3389/fvets.2020.00023

The susceptibility of sheep, cattle, pigs, chickens and chicken embryos to Zika virus infection was evaluated by experimental inoculation with Zika virus Thailand strain isolated from a Canadian traveler in 2013. The inoculated animals did not develop any clinical signs of disease nor evidence of Zika virus replication in peripheral blood, cerebrospinal fluid and tissues including brain and spinal cord assessed by real-time RT-PCR. Sera were also negative for Zika virus antibodies by Zika virus neutralization assays as well as Zika virus immunoperoxidase staining of Zika infected Vero cells. Chicken embryos were inoculated by different routes including yolk sac (4 day old embryos), chorioallantoic membrane (8 day old embryos), amniotic fluid (8 day old embryos) and intravenous routes (12 day old embryos). Virus replication in chicken embryos was observed in the brain and body tissues following intravenous (IV), yolk sac (YS), chorioallantoic membrane (CAM), and amniotic fluid (AF) inoculation routes. The highest mortality was observed in embryos inoculated via yolk sac. The dead embryos showed diffuse muscular hemorrhages. The yolk sac inoculated chicken embryos showed delayed hatching and displayed neurological signs immediately after hatching. These studies demonstrate that 8 week old sheep, 6 month old cattle, 4 week old pigs, and 4 week old chickens are not susceptible to Zika virus infection when inoculated experimentally and therefore unlikely to pose a risk as Zika virus reservoirs. However, chicken embryos are highly susceptible to Zika virus resulting in clinical disease of chicks after hatching. This study demonstrates that Zika virus has a tropism for embryonic tissue and that chicken embryos can be used as a model to study Zika virus replication and pathogenesis.

Keywords: Zika virus, embryo, tissue tropism, susceptibility, chicken

INTRODUCTION

Zika virus is a Flavivirus originally identified in a febrile Rhesus macaque from the Zika forest region in Africa in 1947 (1). It is spread by *Aedes aegypti*, a mosquito that can also transmit dengue fever, chikungunya and yellow fever viruses. In recent outbreaks in humans, Zika virus caused a mild self-limiting infection with clinical signs of fever, rash, conjunctivitis, arthralgia, and

arthritis during an outbreak on Yap Island of the Federated States of Micronesia in 2007 (2). Zika virus then spread to French Polynesia in 2013 (3) where it continued to spread to islands of the Pacific Ocean and then to South America (4). The spread of Zika virus in the Western Hemisphere became a public health emergency due to the link between Zika virus infection and microcephaly in infants (5–7).

Most of the arboviruses depend on nonhuman animal species for maintenance in nature. Many animal species act as reservoirs for these arboviruses, and humans are generally dead-end or accidental hosts to these viruses. Some arboviruses such as dengue virus however, have adapted completely to humans and can be maintained in a mosquito-human-mosquito transmission cycle that does not depend on nonhuman reservoirs. Antibodies to Zika virus have been detected in a number of animal populations including nonhuman primates, farm animals and wild animals (8, 9). In these studies, however, the differentiation between presumably Zika virus specific antibodies and antibodies against other closely related flaviviruses was not comprehensively performed.

Susceptibility of domestic farm animals to experimental Zika virus infection has previously been evaluated in calves, pigs, goats and chickens demonstrating these species as juveniles, young adults or adults are not susceptible (10). However, a different study demonstrated that neonatal pigs were susceptible to Zika virus infection indicating that the age of the animal may be an important factor for Zika virus infection (11). Farm animals, if susceptible to Zika virus infection, may serve as reservoirs for Zika virus increasing the risk of transmission to farm workers, veterinarians and others associated with animal husbandry. Therefore, the main goal of this study was to confirm if sheep, cattle, pigs and chickens are susceptible to Zika virus infection.

Understanding the effect of Zika virus infection on embryonic development is critical. Currently a few animal models using nonhuman primates (12, 13) and mice have been developed (14). These animal models have expanded our knowledge on Zika pathogenesis, however new animal models could provide additional information on Zika virus induced neurological lesions in susceptible hosts. Most current mouse models utilize mice lacking at least one component of the IFN-signaling pathway indicating that IFN signaling is critical for controlling Zika virus replication (15). Although pregnant mouse models using normal mice have been developed in which pups have been demonstrated to be infected with Zika virus (16, 17). In the study reported here, four different domestic animal species were experimentally infected with Zika virus, and they were closely monitored for clinical signs, viral replication and development of humoral immune response to the virus. If Zika virus replicates in these animal species to titers which are high enough to allow for transmission of the virus, they may serve as reservoir for the virus. If they develop clinical signs or pathological lesions similar to that observed in humans, they may be useful animal models to study Zika virus induced pathology.

MATERIALS AND METHODS

Animals, Zika Virus Inoculation, and Sample Collection

Six (8 week old) Rideau Arcott sheep, four (6 months old) Holstein calves, and four (4 week old) Landrace/Large White cross piglets were obtained from a high health status herd operated by a recognized commercial supplier in Manitoba, Canada. Six (2–4 week old) Leghorn chickens were obtained from a specific pathogen free status flock operated by CFIA Ottawa. The age of animals selected was based on availability to procure animals of the youngest age which were not neonates. All animals were housed in separate Biosafety Level 3 animal cubicles at the National Centre for Foreign Animal Disease (Winnipeg, Canada), and were fed a complete balanced diet and water *ad libitum* with a 1 week acclimation period. On day “0,” sheep, cattle and pigs were inoculated intradermally (0.1 ml per site for 5 sites/animal) and intravenous inoculation of 1.0 ml with Zika virus Thailand (18) at 10^6 TCID₅₀/ml propagated in Vero cells. Chickens were given five subcutaneous inoculations 0.1 ml each and 0.5 ml intravenous inoculation of Zika virus Thailand (10^6 TCID₅₀/ml). All animal experiments were conducted under the approval of the Canadian Science Centre for Human and Animal Health Animal Care Committee, which follows the guidelines of the Canadian Council on Animal Care. All animals were observed twice daily, with clinical signs recorded throughout the study. Rectal temperatures were measured prior to inoculation for baseline levels and daily from 1 to 21 days post-infection (dpi). Blood and sera, were collected from sheep on days 1, 2, 3, 6, 8, 10, 13, and 21 post-inoculation. Blood and sera were collected from cattle on days 1, 3, 6, 8, 10, 13, and 20 post-inoculation. Blood and sera were collected from pigs on days 3, 5, 6, 7, 10, 12, 14, and 21 post-inoculation. Blood and sera were collected from chickens on days 3, 4, 6, 10, 13, 17, and 21 post-inoculation. Three weeks after inoculation, sheep, cattle, pigs, and chickens were euthanized and necropsies were performed. During the necropsy, animals were assessed for gross pathology cerebrospinal fluid (CSF) as well as tissues including the brain (cortex, midbrain and cerebellum) and spinal cord were collected during the necropsies.

Egg Inoculations

Zika virus Thailand was inoculated into fertilized specific pathogen free (SPF) chicken eggs (Canadian Food Inspection Agency Fallowfield, Ottawa) at different embryonic stages using different routes of inoculations. The virus was administered using 100 μ l inoculations at various doses with the highest doses of 10^7 PFU/ml, or as 10-fold dilutions. The inoculation procedures were done as follows. For intravenous (IV) inoculations 12 day old embryonated chicken eggs (ECEs) were used. For yolk sac (YS) inoculations 4 day old ECEs were used and for chorioallantoic membrane (CAM) and amniotic fluid (AF) inoculation 8 day old ECEs were used.

Prior to inoculations, eggs were allowed to cool at room temperature for approximately 1 h. For IV inoculation, 27 gauge hypodermic needles were used. For all other routes 1½ inch 23 gauge needles were used. Once inoculated the eggs were

incubated at 33°C in a nonrocking incubator for 24 h with 55% relative humidity (RH), and transferred to a nonrocking incubator set at 37°C with 55% RH. Eggs were candled twice daily. All eggs dying within the first 24 h were discarded. The dead eggs were stored at 4°C a minimum of 1 h before the tissues (allantoic fluid, CAM, brain, heart, liver, and eye balls) were harvested. From small embryos, all organs were pooled as body parts.

Hatching Experiments

Two hatching experiments were conducted, one via CAM inoculation and the second via YS inoculation. For CAM inoculation, a group of thirty six 8 day-old embryonated eggs were inoculated with Zika virus dose of 10^4 PFU/ml and 36 control eggs were inoculated with sterile phosphate buffered saline (PBS). Eggs were incubated for 7 days in the egg incubator in the laboratory and transferred to a table top hatching incubator with turning trays located in animal pens.

For the yolk sac inoculation, a total of 50 embryonated eggs were used. Zika virus Thailand was inoculated into 30 eggs via yolk sac on day 4 using a dose of 10^4 PFU/ml, and 20 eggs with sterile PBS. The eggs were incubated for 11 days in the egg incubator and transferred to a table top hatching incubator in the animal pens.

Following inoculation, all eggs were assessed for viability by candling twice daily, and were allowed to hatch. Hatchability was determined and hatched chicks were evaluated for potential birth defects and abnormal clinical signs.

RNA Extraction and Zika Virus Real-Time RT-PCR

Total RNA was extracted from allantoic fluid, yolk, whole blood and 10% tissue homogenates using MagMax 5X Pathogen DNA/RNA kit Extraction Kit following the manufactures protocol (Applied Biosystems, USA). Two independent Zika real-time RT-PCR assays were performed to detect Zika virus RNA in the samples. Assay #1 (19) detects all known genotypes of Zika virus (FP Zika1087-5'CCGCTGCCCAACACAAG-3', Probe 1108FAM-5'-AGCCTACCTTGACAAGCAGTCAGACACTCAA-3' and RP 1163c 5'-CCACTAACGTTCTTTTGCAGACAT-3'). Assay #2 which uses FP Zika4481 FP 5'-CTGTGGCATGAACCCAATAG-3', Probe 4507cFAM 5'-CCACGCTCCAGCTGCAAAGG-3', and RP4552c 5'-ATCCCATAGAGCACTCC-3' is specific for Zika Asian genotype viruses that are currently circulating in the Western Hemisphere [CDC unpublished data, updated January 14, 2016; (20)]. All real-time RT-PCR assays were performed on an ABI 7500 Sequence Detection System (Applied Biosystems, USA). The reaction volume for the mastermix was 20 μ l per sample and contained mixture 12.5 μ l of 2 $\times$ Quantitect Probe master mix (Qiagen), 5.95 μ l of RNase-free water, 0.25 μ l Quantitect Enzyme, 0.5 μ l of 100 μ M of forward and reverse primers and 0.3 μ l of 25 μ M of TaqMan probe. To the mastermix 5 μ l of extracted RNA was added, and real-time RT-PCR assays were run under the following conditions: an initial reverse transcription step at 50°C for 30 min, followed by 95°C for 15 min and 45 cycles of amplification (15 s at 94°C and 1 min at

60°C). Zika virus Thailand spiked blood and tissue samples were used as positive controls. The data were analyzed using the 7500 software V2.3 properties (Applied Biosystems, USA).

Zika Serology

Sera were assessed for Zika virus neutralization activity using Vero cells. Animal sera from DPI 0 and DPI 21 post-inoculation were diluted 10-fold in BA-1 diluent and mixed with 200 focus-forming units of Zika virus. The virus-sera mixtures were incubated for 1 h at 37°C and transferred onto a monolayer of Vero cells in a 24-well plate at 37°C. After a 1 h incubation, the monolayers containing the virus-sera mixture were overlaid with MEM (2x) (Gibco, Life Technologies) containing 4% fetal bovine serum and 1% Noble Agar (Difco, BD). The plates were incubated at 37°C with 5% CO₂ for 4 days and a second overlay was applied containing 1% Noble agar and 0.02% neutral red vital stain (Sigma) in MEM (2x). Plates were incubated at 37°C with 5% CO₂ for an additional 3 days. Zika virus plaques were counted using a white light of a transilluminator (Fisher Scientific).

Immunoperoxidase staining: Presence of Zika virus specific antibodies in serum collected from infected animals were assessed by immune-peroxidase staining. Vero cells were infected with Zika virus using MOIs of 0.1, 0.01, 0.001 and incubated for 5 days at 37°C with 5% CO₂. The medium was removed and cells were washed three times with phosphate-buffered saline (PBS)-Tween (200 ml/well) (PBST). The cells were then fixed with 10% neutral-buffered formalin in PBS (200 ml/well) for overnight at 4°C. The fixative was removed, and the cells were washed 3 times with PBS-T (200 ml/well) and blocked with blocking buffer (Sigma) for 1 h at 37°C. The blocking buffer was removed, and wells were washed three times with PBST. Sera were diluted 10-fold in PBS and 100 μ l of diluted sera was added to the fixed cells and incubated for 1 h at 37°C. The primary antibody was removed and wells were washed three times with PBST. One hundred microliters of HRP-rec- Protein G antibody (Life Technologies) for sheep, pig, calf and donkey anti-chicken IgG (Jackson ImmunoResearch Laboratories) diluted 1:1,000 in PBS was added to each well for 1 h at 37°C. Plates were washed again three times with PBST and TrueBlue peroxidase substrate (KPL, Gaithersburg, MD) was added. After development, the substrate was removed and the plates were rinsed with water and dried. Plates were assessed for immunostaining by microscopy.

RESULTS

Susceptibility of Sheep, Cattle, Pigs, and Chickens to Zika Infection

Following inoculation with Zika virus, sheep, cattle, pigs, and chickens did not develop any clinical signs. All infected animals were clinically normal, had physiological body temperatures throughout the study, and displayed no change in appetite. Whole blood collected at various time points from sheep, cattle, pigs and chickens was assessed for the presence Zika virus genomic material using two different real-time RT-PCR assays. Zika virus RNA was not detected in any blood sample collected at any time point following Zika virus inoculation. Three weeks after inoculation the animals were euthanized and necropsies

were performed. There was no gross pathology observed during the necropsy and neural tissues (cortex, midbrain, cerebellum, and cerebrospinal fluid) from the sheep, cattle, pigs and chickens were negative for Zika virus RNA. Sheep, cattle, pigs, and chickens did not develop any detectable antibodies specific for Zika virus at any time points following inoculation by virus neutralization testing and immunoperoxidase staining.

Susceptibility of Chicken Embryos to Zika Infection via Different Routes

Zika virus Thailand strain inoculated by four different routes including IV, CAM, AF, YS were lethal to chicken embryos (**Figure 1**). YS inoculation of 4 day old embryos resulted in 100% mortality within 5 days, whereas AF inoculation resulted in 90% mortality within 4 days. Embryos inoculated via CAM route resulted in 80% and those inoculated via IV route resulted in 60% mortality within 7 days with no significant differences in mortality observed between the different inoculation routes. All the embryos that died following inoculation showed stunted growth and generalized hyperemia compared to PBS inoculated control embryos of same age (**Figure 2**). Zika genomic material was detected by real-time RT-PCR around the inoculation sites, in the brain and other tissues in embryos inoculated using the CAM and AF routes. In IV inoculated embryos, viral RNA was detected in brain and body tissues but CAM and AF samples were not tested. In yolk sac inoculated embryos, viral RNA was detected in yolk, brain and other tissues (**Table 1**).

Determining the Optimal Dose of Zika Virus Thailand for the Hatching Experiment

To determine if Zika virus infection of embryonated chicken eggs will lead to congenital defects a hatching experiment was performed. Since Zika virus is lethal to chicken embryos, a dose titration was performed to determine the optimal dose which was defined as the highest dose of Zika virus Thailand that will result in less than 25% embryo deaths. This was chosen to allow for infection of the embryos without causing high levels of killing allowing embryos to hatch. Two routes of inoculation, CAM and YS, were selected for this experiment. YS route was used as it was the earliest route that could be used to infect embryonated chicken eggs. CAM route was selected for the inoculation of 8 day old embryos. IV route was not selected because it was accessible only after 11 day of embryonation so the impact of early Zika virus infection cannot be assessed. Eight day old chicken embryos inoculated via CAM route with Zika virus at 10^5 titer resulted in 60% survival by 5 dpi and 10^6 titer resulted in 40% survival by 6 dpi. Doses 10^4 or less resulted in no mortality in inoculated embryos. Eggs inoculated with 10^4 Zika virus via YS route showed 20% mortality by 5 dpi, and 80% of the embryos survived (**Figure 3**).

Hatching Experiment

Detection of Zika viral RNA in tissues in embryonated chickens following yolk sac inoculation was performed in 20 day old embryos prior to hatching. Zika virus RNA was detected in the brain, eyes, heart, liver, CAM, and YS (**Table 2**).

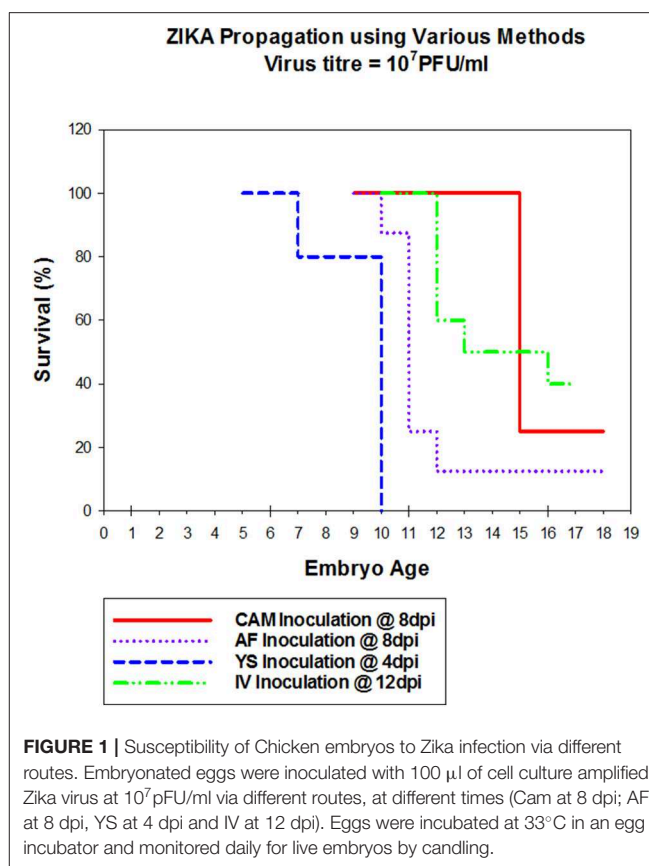


FIGURE 1 | Susceptibility of Chicken embryos to Zika infection via different routes. Embryonated eggs were inoculated with 100 μ l of cell culture amplified Zika virus at 10^7 pFU/ml via different routes, at different times (Cam at 8 dpi; AF at 8 dpi, YS at 4 dpi and IV at 12 dpi). Eggs were incubated at 33°C in an egg incubator and monitored daily for live embryos by candling.

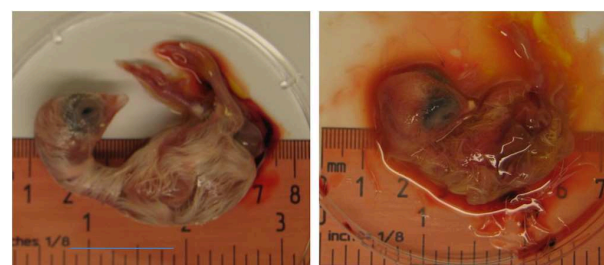


FIGURE 2 | Effect of Zika virus infection on embryo development. A dead embryo from eggs inoculated with Zika virus via CAM showing malformations and hyperemia. PBS inoculated eggs were used as controls.

The eggs started to hatch Day 21. By day 22, 15 eggs inoculated via YS route were hatched and one of the chicks (#2) showed depression, ataxia and was sitting on hocks. Another chick (#4) was mildly depressed and ataxic (**Figure 4**). On day 23 chick #2 died and chick #4 started show ataxia, depression, labored breathing, and was sitting on hocks. It was euthanized the same day and assessed for Zika virus replication. Zika virus RNA was detected in the brain, eyes and heart of both chick #2 and #4 (**Table 2**). Fifteen eggs inoculated via YS route remained unhatched by day 25, and they were necropsied. The hatched chicks were maintained until day 30. Two additional chicks died on day 26; one exhibited crusted feathers and did not gain

TABLE 1 | Detection of Zika viral RNA in tissues (CT values) of embryonated chicken following CAM, allantoic, IV, and YS inoculations.

Route	Sample	dpi	CAM	AAF	Brain	Tissues
(A)						
Chrioallantoic membrane	1	6	27.65	33.28	27.28	26.52
	2	6		31.66	27.51	31.52
	3	6		35.21	29.64	25.21
	4	6	28.37	31.56	29.11	31.97
	5	6	26.96	30.50	31.84	34.55
	6	6	28.72	34.13	32.12	34.80
Allantoic fluid	1	2	25.00	27.38	32.28	23.91
	2	2	24.14	30.83	35.23	33.69
	3	2	34.37	30.34	33.82	31.27
	4	2	27.80	28.46	30.16	28.84
	5	2	31.41	31.11	31.12	28.82
	6	3	32.69	32.15	32.20	32.22
Route	Sample	dpi	Brain		Tissues	
(B)						
Intravenous	2	2	29.87		27.11	
	3	2	27.19		25.83	
	4	2	26.08		24.73	
	5	3	25.32		27.43	
	6	6	22.49		24.61	
	7	7	25.14		25.64	
	8	7	22.33		27.87	
	9	7	24.81		27.83	
Route	Control	dpi	Yolk		Tissues	
(C)						
Yolk Sac	1	5	30.08		28.08	
	2	5	31.50		26.12	
	3	5	29.14		28.23	
	4	5	34.11		29.61	
	5	5	33.32		25.38	
	6	5	34.77		23.25	
	7	5	28.33		Path	
	8	5	34.06		Path	

weight. In control group all except 4 eggs hatched and physical deformity (crooked neck and had difficulty walking straight) was only observed in one chick.

In eggs inoculated with Zika via CAM route showed no significant differences in hatchability and health of the chicks compared to the PBS controls. The hatchability of YS inoculated embryos was lower with only 50% eggs hatching compared to control and CAM inoculated embryos which showed over 80% hatchability although not significantly significant (Figure 5).

DISCUSSION

It was recently demonstrated that pig cell line PK-15 and chicken cell line DF-1 were able to support Zika virus replication (21).

The ability of Zika virus to grow in these cell lines suggested that these hosts, pigs and chickens, have cells that are susceptible to Zika virus infection. The results from this study demonstrate that young sheep, cattle, pigs and chickens with an intact immune system were not susceptible to Zika virus infection. These findings are similar to another study where infectious virus was not detected in multiple species of animals common to North America, although Zika specific antibodies were detected in pigs and cottontail rabbits following experimental infection (10). The study used two different Zika virus strains, one human isolate from Cambodia in 2010, and the other isolated from a patient from Puerto Rico in 2015. The virus from Puerto Rico induced stronger neutralizing antibodies in pigs and rabbits compared to the Cambodian Zika virus eliciting antibodies at the limit of detection. This study demonstrated the influence of the Zika virus strain in the induction of antibodies in pigs (10). The Thailand Zika virus strain is greater than 98% similar to both the Cambodian and Puerto Rico strains previously used to infect animals (10).

The lack of viremia observed in sheep, cattle, pigs, and chickens demonstrate that these animals are not likely to serve as reservoirs for Zika virus. It was recently demonstrated that both rhesus and cynomolgus macaques are highly susceptible to Zika infection under experimental conditions (13) and that Zika virus RNA could be detected in the brains of these animals with lymph nodes and reproductive tissue having Zika virus RNA at late stages of infection (28 days following infection). Since no Zika virus RNA was detected in neural tissues of sheep, cattle, pigs and chickens 3 weeks following infection, this is further evidence that these animal species are not susceptible hosts for Zika virus. Although viral replication in these tissues cannot be excluded at earlier times since they were not evaluated. Interestingly, fetal pigs are highly susceptible to infection during gestation (22). An additional study demonstrated that 1 day old pigs could be infected with Zika virus when inoculated by intracerebral, intradermal or intraperitoneal routes (11) indicating that neonates can still have some cells which are similar to embryonic cells with respect to permissiveness to Zika virus infection. The route of inoculation likely plays an important role for successful replication of Zika virus in neonatal pigs. Unfortunately, there have been no studies in neonatal pigs using an inoculation by infected mosquitoes. This study demonstrates that nonneonate animals are not susceptible to Zika virus infection. The biological relevance of this is that embryos of nonsusceptible animals are unlikely to become infected by a natural infection since if an infected mosquito transmitted the virus into the host, the host would not replicate the virus allowing infection of the embryo. Despite this embryos of different host animals can be infected experimentally through inoculation to serve as animal models for Zika virus infection.

It has been reported that a small percentage of cows, sheep, goats and chickens had Zika specific antibodies in sero surveillance studies (8, 9). However, it needs to be understood that the antibody detection assays used in these studies were of uncertain specificity and sensitivity. In addition, since there is cross-reaction between Zika virus and other flavivirus antibodies, this further complicates the interpretation and relevance of the

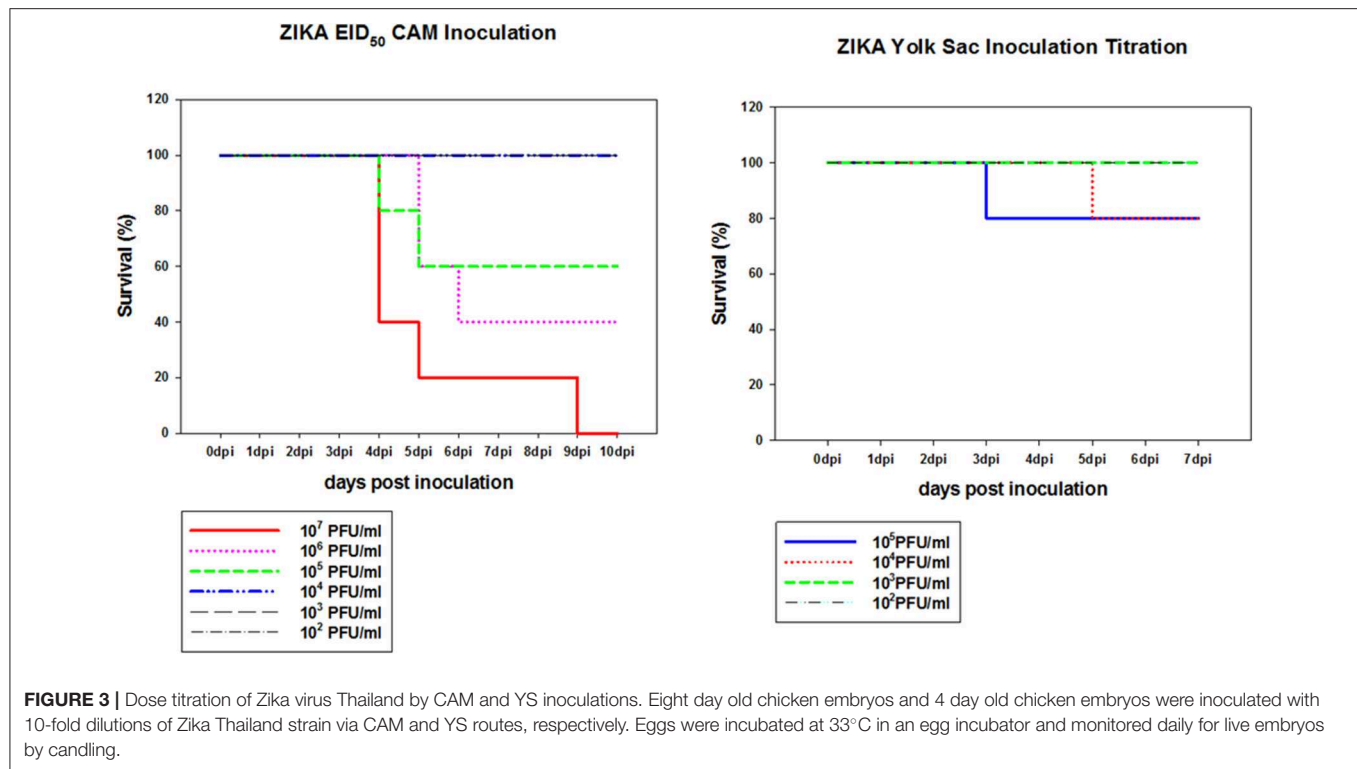


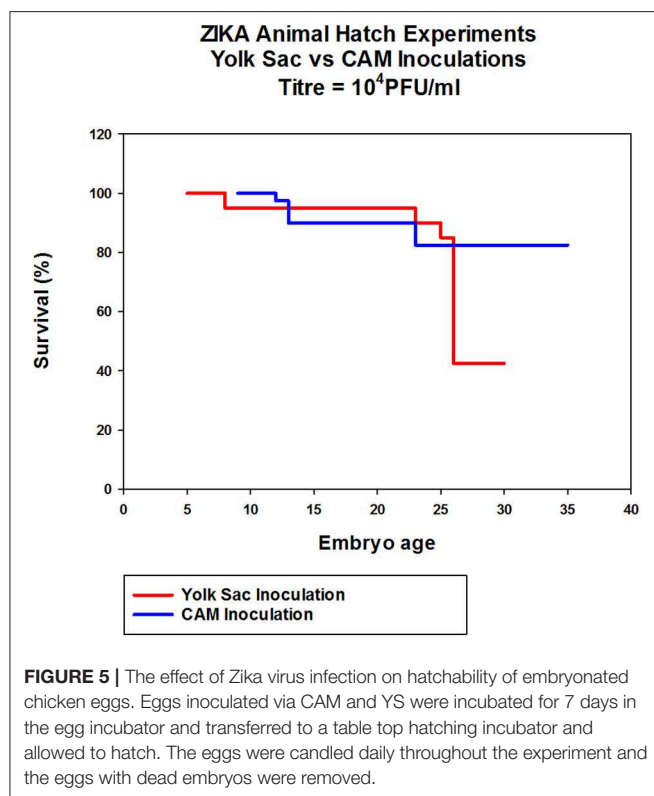
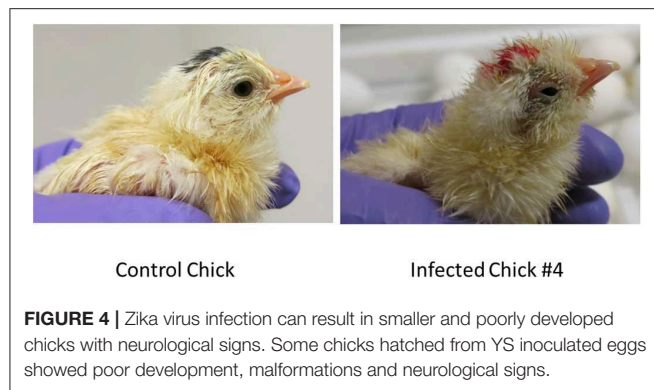
TABLE 2 | Detection of Zika viral RNA in tissues (CT values) of embryonated chickens and hatched chicks following yolk sac inoculation.

Embryo	DO	DPI	Brain	Eyes	Heart and Liver		CAM	Yolk Sac	
(A) EMBRYOS									
1	20	15	28.93	36.43	28.24/31.89		35.07	33.45	
2	20	15	26.81	35.29	28.73/32.85		34.29	37.15	
3	20	15	25.06	33.94	25.03/29.67		33.15	33.10	
4	20	15	24.73	35.31	26.52/29.47		34.90	32.68	
Chicken	Clinical signs		DPI	DO	DPH	Brain	Eyes	Heart	Liver
(B) ZIKA INOCULATED EMBRYOS WHICH HATCHED									
1	None		25	30	9	No CT	No CT	No CT	No CT
6	None		25	30	9	No CT	No CT	No CT	No CT
7	None		25	30	9	No CT	35.54	No CT	No CT
10	None		25	30	9	38.18	No CT	No CT	No CT
2	Yes/found dead		18	23	2	32.66	32.04	33.66	No CT
4	Yes		18	23	2	35.44	32.23	29.91	No CT
12	Hatched small		25	30	9	36.74	No CT	37.63	No CT

data. In the experimental infections in sheep, cattle, pigs and chickens antibodies to Zika virus were not observed at 3 weeks following infection.

Even though these animal species were not susceptible to Zika virus infection, this does not mean that embryos of these species are not susceptible (11). It has been demonstrated that Zika virus can replicate in embryonated chicken eggs (23), indicating that Zika virus has a tissue tropism for embryonic tissue. Our results demonstrate that Zika virus can replicate in embryonated

chicken eggs following several different inoculation routes. In addition, Zika virus can kill embryos when administered at high viral loads. These results agree with previous studies (23, 24). In addition, infection with African isolates caused higher embryo mortality compared to Asian-lineage isolates (25). In the current study, Zika virus RNA was detected in the brain, eye, heart and liver in embryos at 15 days post-inoculation. This is in agreement with a recent study where Zika virus was detected in brain, eye, heart and liver at 7 and 11 days post-inoculation into the brain vesicle in 5 day old embryos and that Zika virus suppressed chicken embryo development (25). In another study, it was also demonstrated that Zika virus infected embryonic chicken brains following injection of Zika virus into the neural tube of 2 day old chicken embryos (26). The results of Zika virus infection in chicken embryos demonstrate that Zika virus infects a broad range of tissues and cells; similar to what is observed in human infection during the first trimester of pregnancy although the cells types and tissues where Zika virus replication occurred were different (27). Furthermore, Zika virus RNA was detected in chickens which were hatched from inoculated embryos that displayed clinical signs. In this experiment the numbers of hatched chickens that displayed clinical signs was small because it is difficult to administer a dose that will allow the embryos to hatch without killing them, and still have enough virus replication to cause birth defects similar to embryonated chicken eggs infected with high doses of Zika virus. These studies demonstrate that Zika virus has a broad tropism for chicken embryonic tissue. In addition, Zika virus has been demonstrated to be able to infect fetuses from multiple species including pigs (22), mice (16, 28), rhesus macaques (29) and humans (30).



Therefore, it is likely that the embryos of sheep and cattle could possibly be susceptible to Zika virus directly inoculated, however further experiments are required to determine if this is true. It was demonstrated that mosquito eggs from Zika virus-infected mosquitoes had a slightly decreased hatch rate (31).

The results from this study confirms the study by Ragan et al. (10) that farm animals in North America are unlikely to be susceptible to Zika virus infection. Further study's

using animals from different regions with different genetic backgrounds are needed to confirm that these species are not susceptible. Neonatal rabbits and pigs (10) especially neonatal pigs (11) may potentially serve as sentinel species in North America where virus is transmitted by *A. albopictus*, which will feed on these species, although further evaluation of these species as sentinels is required. The use of birds as a sentinel species as a surveillance tool for West Nile virus has been previously demonstrated (32) and is of great value in monitoring rapid global spread of zoonotic pathogens (33). The results of these studies contribute to our understanding of host tropism for Zika virus and demonstration that Zika virus has a tropism for embryonic tissue. This information is required to develop appropriate risk assessments for human and animal health.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Canadian Science Centre for Human and Animal Health Animal Care Committee.

AUTHOR CONTRIBUTIONS

AA, TT, CC-B, YB, VG, UK, DS, and SB contributed conception and design of the study. AA, TT, CC-B, YB, DS, and SB performed the experimental work. AA and SB wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was funded through the Canadian Food Inspection Agency and the Public Health Agency of Canada.

ACKNOWLEDGMENTS

The animal care was supported by the National Centre for Foreign Animal Disease animal care staff, Glenn Clark, Cory Nakamura, Maggie Forbes and Jaime Bernstein and animal care veterinarian Valerie Smid. We thank Dr. Mike Drebot from the Public Health Agency of Canada for critical reading of the manuscript and Maya Traykova-Andonov and Kimberly Holloway from the Public Health Agency of Canada for technical support.

REFERENCES

1. Dick GW, Kitchen SF, Haddock AJ. Zika virus. I. Isolations and serological specificity. *Trans R Soc Trop Med Hyg.* (1952) 46:509–20. doi: 10.1016/0035-9203(52)90042-4
2. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. *N Engl J Med.* (2009) 360:2536–43. doi: 10.1056/NEJMoa0805715
3. Cao-Lormeau VM, Roche C, Teissier A, Robin E, Berry AL, Mallet HP, et al. Zika virus, French polynesia, South pacific,

2013. *Emerg Infect Dis.* (2014) J20:1085–6. doi: 10.3201/eid2006.140138
4. Campos GS, Bandeira AC, Sardi SI. Zika virus outbreak, Bahia, Brazil. *Emerg Infect Dis.* (2015) 21:1885–6. doi: 10.3201/eid2110.150847
5. Johansson MA, Mier-y-Teran-Romero L, Reefhuis J, Gilboa SM, Hills SL. Zika and the risk of microcephaly. *N Engl J Med.* (2016) 375:1–4. doi: 10.1056/NEJMp1605367
6. Kleber de Oliveira W, Cortez-Escalante J, De Oliveira WT, do Carmo GM, Henriques CM, Coelho GE, et al. Increase in reported prevalence of microcephaly in infants born to women living in areas with confirmed Zika virus transmission during the first trimester of pregnancy - Brazil, 2015. *MMWR Morb Mortal Wkly Rep.* (2016) 65:242–7. doi: 10.15585/mmwr.mm6509e2
7. Petersen LR, Jamieson DJ, Powers AM, Honein MA. Zika virus. *N Engl J Med.* (2016) 374:1552–63. doi: 10.1056/NEJMr1602113
8. Darwish MA, Hoogstraal H, Roberts TJ, Ahmed IP, Omar F. A sero-epidemiological survey for certain arboviruses (Togaviridae) in Pakistan. *Trans R Soc Trop Med Hyg.* (1983) 77:442–5. doi: 10.1016/0035-9203(83)90106-2
9. Olson JG, Ksiazek TG, Gubler DJ, Lubis SI, Simanjuntak G, Lee VH, et al. A survey for arboviral antibodies in sera of humans and animals in Lombok, Republic of Indonesia. *Ann Trop Med Parasitol.* (1983) 77:131–7. doi: 10.1080/00034983.1983.11811687
10. Ragan IK, Blizzard EL, Gordy P, Bowen RA. Investigating the potential role of North American Animals as Hosts for Zika virus. *Vector Borne Zoonotic Dis.* (2017) 17:161–4. doi: 10.1089/vbz.2016.2099
11. Darbellay J, Lai K, Babiuk S, Berhane Y, Ambagala A, Wheler C, et al. Neonatal pigs are susceptible to experimental Zika virus infection. *Emerg Microbes Infect.* (2017) 6:e6. doi: 10.1038/emi.2016.133
12. Adams Waldorf KM, Stencel-Baerenwald JE, Kapur RP, Studholme C, Boldenow E, Vornhagen J, et al. Fetal brain lesions after subcutaneous inoculation of Zika virus in a pregnant nonhuman primate. *Nat Med.* (2016) 22:1256–9. doi: 10.1038/nm.4193
13. Osuna CE, Lim SY, Deleage C, Griffin BD, Stein D, Schroeder LT, et al. Zika viral dynamics and shedding in rhesus and cynomolgus macaques. *Nat Med.* (2016) 22:1448–55. doi: 10.1038/nm.4206
14. Lazear HM, Govero J, Smith AM, Platt DJ, Fernandez E, Miner JJ, et al. A mouse model of Zika virus pathogenesis. *Cell Host Microbe.* (2016) 19:720–30. doi: 10.1016/j.chom.2016.03.010
15. Dowall SD, Graham VA, Rayner E, Atkinson B, Hall G, Watson RJ, et al. A susceptible mouse model for Zika virus infection. *PLoS Negl Trop Dis.* (2016) 10:e0004658. doi: 10.1101/042358
16. Cugola FR, Fernandes IR, Russo FB, Freitas BC, Dias JL, Guimarães KP, et al. The Brazilian Zika virus strain causes birth defects in experimental models. *Nature.* (2016) 534:267–71. doi: 10.1038/nature18296
17. Miner JJ, Cao B, Govero J, Smith AM, Fernandez E, Cabrera OH, et al. Zika virus infection during pregnancy in mice causes placental damage and fetal demise. *Cell.* (2016) 165:1081–91. doi: 10.1016/j.cell.2016.05.008
18. Fonseca K, Meatherall B, Zarra D, Drebot M, MacDonald J, Pabbaraju K, et al. First case of Zika virus infection in a returning Canadian traveler. *Am J Trop Med Hyg.* (2014) 91:1035–8. doi: 10.4269/ajtmh.14-0151
19. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg Infect Dis.* (2008) 14:1232–9. doi: 10.3201/eid1408.080287
20. Bingham AM, Cone M, Mock V, Heberlein-Larson L, Stanek D, Blackmore C, et al. Comparison of test results for Zika virus RNA in urine, serum, and saliva specimens from persons with travel-associated Zika virus disease - Florida, 2016. *MMWR Morb Mortal Wkly Rep.* (2016) 65:475–8. doi: 10.15585/mmwr.mm6518e2
21. Chan JF, Yip CC, Tsang JO, Tee KM, Cai JP, Chik KK, et al. Differential cell line susceptibility to the emerging Zika virus: implications for disease pathogenesis, non-vector-borne human transmission and animal reservoirs. *Emerg Microbes Infect.* (2016) 5:e93. doi: 10.1038/emi.2016.99
22. Darbellay J, Cox B, Lai K, Delgado-Ortega M, Wheler C, Wilson D, et al. Zika virus causes persistent infection in porcine conceptuses and may impair health in offspring. *EBioMedicine.* (2017) 25:73–86. doi: 10.1016/j.ebiom.2017.09.021
23. Goodfellow FT, Tesla B, Simchick G, Zhao Q, Hodge T, Brindley MA, et al. Zika virus induced mortality and microcephaly in chicken embryos. *Stem Cells Dev.* (2016) 25:1691–7. doi: 10.1089/scd.2016.0231
24. Willard KA, Demakovsky L, Tesla B, Goodfellow FT, Stice SL, Murdock CC, et al. Zika virus exhibits lineage-specific phenotypes in cell culture, in *Aedes aegypti* mosquitoes, and in an embryo model. *Viruses.* (2017) 9:E383. doi: 10.3390/v9120383
25. Zhang Z, Sun M, Deng J, Yu J, Yang X, Zhao W, et al. Zika virus induced more severe inflammatory response than dengue virus in chicken embryonic livers. *Front Microbiol.* (2019) 10:1127. doi: 10.3389/fmicb.2019.01127
26. Thawani A, Sirohi D, Kuhn RJ, Fekete DM. Zika virus can strongly infect and disrupt secondary organizers in the ventricular zone of the embryonic chicken brain. *Cell Rep.* (2018) 23:692–700. doi: 10.1016/j.celrep.2018.03.080
27. El Costa H, Gouilly J, Mansuy JM, Chen Q, Levy C, Cartron G, et al. ZIKA virus reveals broad tissue and cell tropism during the first trimester of pregnancy. *Sci Rep.* (2016) 6:35296. doi: 10.1038/srep35296
28. Yockey L, Varela L, Rakib T, Khoury-Hanold W, Fink SL, Stutz B, et al. Vaginal exposure to Zika virus during pregnancy leads to fetal brain infection. *Cell.* (2016) 166:1247–56.e4. doi: 10.1016/j.cell.2016.08.004
29. Nguyen SM, Antony KM, Dudley DM, Kohn S, Simmons HA, Wolfe B, et al. Highly efficient maternal-fetal Zika virus transmission in pregnant rhesus macaques. *PLoS Pathog.* (2017) 13:e1006378. doi: 10.1371/journal.ppat.1006378
30. Mlakar J, Korva M, Tul N, Popović M, Poljšak-Prijatelj M, Mraz J, et al. Zika virus associated with microcephaly. *N Engl J Med.* (2016) 374:951–8. doi: 10.1056/NEJMoa1600651
31. Chaves BA, Junior ABV, Silveira KRD, Paz ADC, Vaz EBDC, Araujo RGP, et al. Vertical transmission of Zika virus (Flaviviridae, Flavivirus) in Amazonian *Aedes aegypti* (Diptera: Culicidae) delays egg hatching and larval development of progeny. *J Med Entomol.* (2019) 6:tjz110. doi: 10.1093/jme/tjz110
32. Komar N. West Nile virus surveillance using sentinel birds. *Ann N Y Acad Sci.* (2001) 951:58–73. doi: 10.1111/j.1749-6632.2001.tb02685.x
33. Halliday JE, Meredith AL, Knobel DL, Shaw DJ, Bronsvort BM, Cleaveland S. A framework for evaluating animals as sentinels for infectious disease surveillance. *J R Soc Interface.* (2007) 4:973–84. doi: 10.1098/rsif.2007.0237

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Ambagala, Truong, Cottam-Birt, Berhane, Gerdt, Karniychuk, Safronetz and Babiuk. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Perspectives Regarding the Risk of Introduction of the Japanese Encephalitis Virus (JEV) in the United States

Ana R. S. Oliveira^{1†}, Lee W. Cohnstaedt^{2†}, Leela E. Noronha^{2†}, Dana Mitzel^{2†},
D. Scott McVey^{2†} and Natalia Cernicchiaro^{1*†}

¹ Center for Outcomes Research and Epidemiology, Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, United States, ² Arthropod-Borne Animal Diseases Research, Agricultural Research Service, United States Department of Agriculture, Manhattan, KS, United States

OPEN ACCESS

Edited by:

Michael Kogut,
United States Department of
Agriculture, United States

Reviewed by:

Mattia Calzolari,
Experimental Zooprophyllactic Institute
of Lombardy and Emilia Romagna
(IZSLER), Italy

Jason Kindrachuk,
University of Manitoba, Canada

*Correspondence:

Natalia Cernicchiaro
ncernic@vet.k-state.edu

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 27 September 2019

Accepted: 20 January 2020

Published: 07 February 2020

Citation:

Oliveira ARS, Cohnstaedt LW,
Noronha LE, Mitzel D, McVey DS and
Cernicchiaro N (2020) Perspectives
Regarding the Risk of Introduction of
the Japanese Encephalitis Virus (JEV)
in the United States.
Front. Vet. Sci. 7:48.
doi: 10.3389/fvets.2020.00048

Japanese encephalitis (JE) is a zoonotic, emerging disease transmitted by mosquito vectors infected with the Japanese encephalitis virus (JEV). Its potential for emergence into susceptible regions is high, including in the United States (US), and is a reason of economic concern among the agricultural community, and to public health due to high morbidity and mortality rates in humans. While exploring the complexities of interactions involved with viral transmission, we proposed a new outlook on the role of vectors, hosts and the environment under changing conditions. For instance, the role of feral pigs may have been underappreciated in our previous work, given research keeps pointing to the importance of susceptible populations of wild swine in naïve regions as key elements for the introduction of emergent vector-borne diseases. High risk of JEV introduction has been associated with the transportation of infected mosquitoes via aircraft. Nonetheless, no JEV outbreaks have been reported in the US to date and results from a qualitative risk assessment considered the risk of establishment to be negligible under the current conditions (environmental, vector, pathogen, and host). In this work, we discuss virus-vector-host interactions and ecological factors important for virus transmission and spread, review research on the risk of JEV introduction to the US considering the implications of risk dismissal as it relates to past experiences with similar arboviruses, and reflect on future directions, challenges, and implications of a JEV incursion.

Keywords: arbovirus, Japanese encephalitis, JEV, perspective, risk assessment

INTRODUCTION

The Japanese encephalitis virus (JEV) is a flavivirus transmitted by mosquitoes and the most important cause of viral encephalitis in Southeast Asia and the Western Pacific Rim. Affecting around 68,000 people yearly, Japanese encephalitis (JE) is a debilitating disease with no cure, although there is a vaccine available, which is used extensively in most endemic countries. The case fatality risk may approach 25% and up to 50% of the patients that survive can develop debilitating permanent neurological damage (1, 2). Chronic sequelae, including cognitive dysfunction and neurologic deficits, affect mainly children and are responsible for the high burden of disease of JE globally (3, 4).

Viral transmission is influenced by complex interactions that occur among virus, vector and host, and is driven by environmental, genetic, and ecological determinants (5). The enzootic cycle of JEV is maintained by pigs (the main JEV amplifying host) and ardeid birds, with more than 30 mosquito species identified as potential vectors (3, 6–9). Humans are dead-end hosts that do not amplify the virus nor sustain mosquito infection due to low peaks of viremia (3).

Having expanded from Japan, where it was first isolated, JEV has spread to all neighboring countries, now covering most regions in Southeast Asia. Besides the wide distribution of JEV, recent evidence of geographical genotype displacement has pointed to the changing dynamics of JEV transmission, raising public health concern regarding virus spread to susceptible regions of the globe (4, 10–12). Japanese encephalitis virus genetic material has already been identified in mosquitoes and birds collected in northern Italy, where human cases are unreported to date (13, 14); concurrently, other arboviruses have been emerging in previously unaffected areas, with one of the most recent examples being the occurrence of outbreaks of Zika (although humans are reservoir of this virus) virus in South America (15). In the United States (US), specifically, the introduction of the West Nile virus (WNV) has demonstrated the vulnerability for the emergence of exotic pathogens (16). Moreover, the presence of competent vectors and hosts, the apt weather and climatic conditions in most US states, the non-existence of active JEV surveillance programs and cross-reactivity of JEV with other flaviviruses in diagnostic testing, as well as the increased international travel and trade, make the US a suitable region for JEV introduction and spread (7, 16–18).

Geographical expansion of the virus depends on biotic and abiotic factors which are not static; changes in those factors, such as vector and host population abundance, distribution, and composition, can influence forecasted local transmission cycles. Thus, the aim of this article is to: (1) discuss current advances in virus-vector-host interactions and ecological factors important for virus transmission and spread with a review of research addressing the risk of introduction of JEV in the US, and (2) consider future directions, challenges and implications for JEV introduction, including potential surveillance, and vector mitigation strategies.

CURRENT ADVANCES

Virus-Vector-Host-Environment Interactions

Lessons Learned Regarding Virus-Vector-Host Interactions

Our previous studies focused on the relative role that various vectors and hosts have on the epidemiology of JEV (7–9). Mosquito vectors other than *Culex tritaeniorhynchus* were found to have higher pooled proportions of JEV infection (7, 8), as well as infection and transmission risks (9). To date, *Culex tritaeniorhynchus* has been considered the most important JEV vector in Southeastern Asia (6); however, this may be the result of an overrepresentation of this species in the literature due to

issues related to study and sampling design (19). In fact, the highest pooled infection rate estimates were observed in *Culex annulirostris*, *Culex sitiens*, and *Culex fuscocephala* (9). *Aedes japonicus* has also been identified as a vector with high JEV infection¹ (90%) and transmission² rates (75%), pointing to its importance as a potential vector species for the spread of JEV to susceptible regions where it is also present, such as the US (21) and Europe (22). Furthermore, reported pooled estimates of JEV transmission risk in *C. tritaeniorhynchus* are as low as 36% (9), which is much lower than estimates for other mosquito species that are not commonly associated with JEV infection or transmission.

Despite being the primary mammalian amplifying host for JEV (6), meta-regression modeling did not identify domestic pigs as the host species with the highest proportion of JEV infection (7). Nonetheless, North American domestic pigs were shown to be susceptible to JEV experimental infection (23–25) and although the majority of pigs in the US are housed indoors, commercial housing does not preclude mosquito exposure (26–28).

Other hosts, including wild pigs [i.e., pigs that have escaped or been released in the wild (GISD)], have greater pooled proportion of infection estimates when compared to domestic pigs (53 vs. 41%) (7)³. This could be related to the intensification of industrial pig farming and biosecurity measures, as well as the decrease in backyard pig rearing in Asia (4). Conversely, increasingly higher populations of wild swine have been identified in certain regions of Asia, potentiating the role of these animals in the ecology of JEV (29–32). Wild pigs are known to play a role in the transmission of several disease agents, including JEV (32), and represent a rapidly growing, free-range population of vertebrate hosts that is expanding worldwide (32–36). In the US, this species has expanded to 35 states due to their adaptability to geographic and climatic conditions and the lack of natural predators (37). The potential of wild pigs as reservoirs and drivers of disease is further increased due to their destructive behavior, which has created new mosquito larval habitats (38), and the possible vector-free JEV transmission between pigs (39, 40).

The estimated proportion of JEV infection in ardeid birds such as herons, although lower than in swine, was reported to be 28% (7). In the US, national surveys from 1966 to 2015 showed that some ardeid bird populations are increasing annually (41). This includes ring-bill gulls (*Larus delawarensis*) and great egrets (*Ardea alba*), which are susceptible to JEV under experimental conditions, with virus shedding via oral and cloacal secretions (17). The epidemiological significance of the latter is not yet known, but like the recent evidence of vector-independent

¹Infection rate being defined as the sum of individual mosquitoes that test positive for JEV (or pools of mosquitoes, if applicable) divided by the total number of mosquitoes (or pools) tested (9).

²Transmission rate is the proportion of mosquitoes that were orally exposed to JEV and transmitted the virus on refeeding or contained the virus in their saliva or salivary glands (9, 20).

³Global Invasive Species Database (GISD). Invasive Species Specialist Group. Available online at: <http://www.iucngisd.org/gisd/species.php?sc=73> (accessed September 26, 2019).

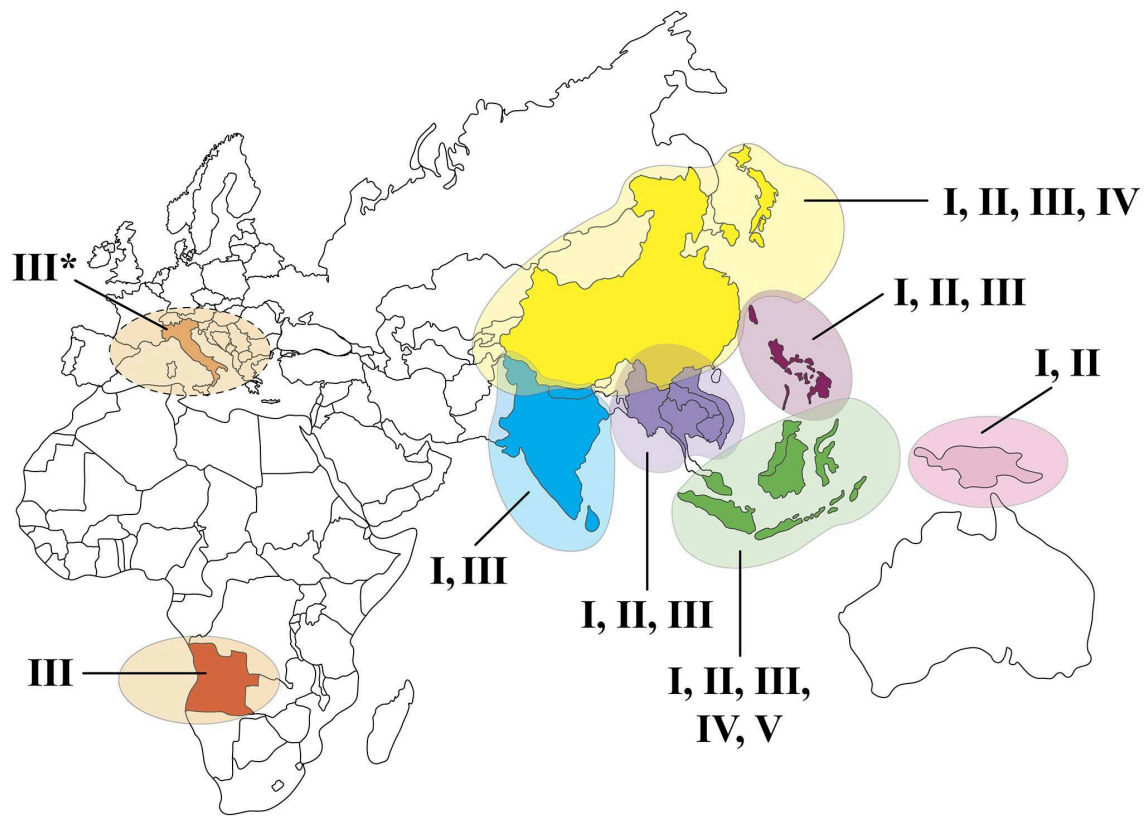


FIGURE 1 | Map of the geographic distribution of JEV genotypes. *No human cases of JE have been reported in Italy to date.

transmission in pigs, it highlights fundamental knowledge gaps surrounding JEV transmission.

JEV Genomics and Phylogeny

The JEV strains that have been isolated since its discovery can be classified into one of five JEV genotypes [genotype I (GI) to V (GV)] (42). Historically, JEV circulated throughout most of Asia, but various genotypes have spread geographically or have re-emerged in recent years (**Figure 1**). In 1995, JEV (genotype GII) demonstrated to spread outside of Asia with widespread activity in the Torres Strait of the Australasia region for the first time (43). Approximately 5 years later, a new JEV genotype (GI) was isolated from sentinel pigs and mosquitoes found in the same area as the previous outbreak (44).

Genotype V virus was first isolated in Malaysia in the 1940s and then went undetected until 2009, when it was isolated from a pool of mosquitoes in Tibet and then again in 2010 in the Republic of Korea (45, 46). Genotype V is not a common genotype, with only three isolates having been detected. However, the question arises if the re-emergence after so many years is indicative of genotypic shift in the area.

In addition to the geographic spread, changes in the molecular epidemiology of JEV have occurred throughout Asia. Until the 1990s, GIII was the dominant genotype in Asia, however, surveillance data revealed that GI gradually replaced GIII as the most frequent genotype in many Asian countries. Sequence

analysis identified a few variations in the genome that may have played a role in the phenotypic change (42). However, further research is needed to determine if these genetic changes provided an advantage for the virus to survive and thrive in the temperate area. Other studies compared replication efficiency of GI isolates to GIII isolates. Depending on the study, GI was shown to replicate more efficiently in pig, avian, and mosquito cells than GIII (10, 47, 48). Genotype I had a higher infection rate and shorter extrinsic incubation period than GIII during *in vivo* studies using *C. quinquefasciatus* (12). Whereas, these studies help to explain how GI might have displaced the previous genotype, other host and environmental factors, such as effects of immunity of a population to the different genotypes, changes in farming, and animal husbandry practices, and changes in migratory patterns of birds, may have also contributed to the emergence of GI. The recent spread and displacement of JEV demonstrates the importance of understanding how small changes in viral genetics or the introduction of a different strain can lead to an expansion in host range, enhanced vector competence, and hence, arboviral emergence, and increase transmission potential (49).

Ecological Factors Important for Viral Transmission and Spread

Emergence of arboviruses frequently follows change in one or various ecological or environmental factors. For JEV, these

include precipitation, humidity, temperature, altitude, as well as aspects related to vegetation, land usage, and agricultural practices (5, 50, 51).

Gould et al. (50) discussed the impact of urbanization due to the increase in population densities, which have led to a higher exposure of humans to mosquito vectors and to changes in the interaction patterns occurring among virus, vectors, and hosts. The intensification of deforestation, agriculture, and animal production is the natural response to the pressures of a growing urbanized population. Likewise, the domestication of arthropods in order to adapt to the modern human environment is rampant, as is the invasion of humans into areas that were previously only inhabited by wild flora and fauna, hence changing completely pre-existing dynamics (50). Increased urbanization can also lead to concentration of susceptible human hosts, which depending on their socioeconomic status, can also be conducive to enhanced transmission (49).

Geographic expansion of the virus can result from viral adaptation and displacement. Vector and host population growth and expansion, and improved viral amplification in vertebrate hosts may be related to elongation of seasons, shortening of gonotrophic cycles, and creation of new niches that are associated with environmental changes (e.g., global warming). Invasion and expansion of hosts and vectors through dispersal or migration, are also facilitated by tropical storms or other natural disasters (e.g., flooding) (51).

FUTURE DIRECTIONS AND CHALLENGES

Future Directions and Implications for the Risk of Introduction

Assessing the Risk of Introduction of JEV

Several epidemiological studies have been conducted to quantify vector and host parameters and to evaluate the risk of emergence of JEV in the US (5, 7–9, 52, 53). Risk assessment, as a decision tool, is a method to make decisions under uncertainty (54). This implies that approximations and assumptions often need to be made using data that are available, rather than ideal data (55).

The risk of introduction of JEV in the US, evaluated using a risk assessment framework, through infected adult mosquito vectors was predicted to be very high: there is a 0.95 median probability (95% CI: 0.80–0.99) of at least one infected mosquito, and a median of three infected mosquitoes (95% CI: 1–7), being introduced during March to October via aircraft, the most likely pathway of entry, to the US from JEV-affected countries (52). Mediterranean California and Eastern Temperate Forests ecoregions (covering all US states on the East Coast, except Southern Florida, the Midwest, and the Southeast), which are similar to the ecosystems found among the regions at risk, were the areas in the US with the highest risk of JEV introduction via infected mosquitoes transported in aircraft (52).

When considering other pathways of entry (e.g., birds, hosts, vaccines, other biologicals), the risk of JEV introduction was considered negligible. The risk of transmission was considered variable and the risk of establishment negligible given current conditions (53). Changing aspects and preconditions related to

the introduction and transmission of JEV will also imply a change in probability estimation. Thus, revisiting the pathways of introduction and considering paths that were previously deemed as non-important (e.g., domestic and wild pigs) can lead to different assumptions and therefore, different probability estimates.

As discussed elsewhere (53), bird migration (e.g., flyways coming from Asia into the US through Alaska) was considered a negligible pathway for JEV introduction into the US. Short viremia in avian hosts [2–4 days (56)] and their long migration flights, life-long immunity after infection, the low probability of co-occurrence of an infectious migrant bird with competent vectors and susceptible birds, low number of competent vectors (e.g., *Aedes vexans*) in Alaska, where flyways coming from Asia and heading south to the US overlap, and Alaska's short mosquito season, are factors contributing to the dismissal of this pathway for JEV to enter and establish in the US (17, 53). When disregarding the entry of viremic migratory birds as a potential pathway of introduction for JEV, we may have not considered the role of climate change and land perturbation, which could push birds toward new habitats, with new mosquito vectors, and modulate pathogen dynamics.

Legal and illegal importation of potentially infected birds was deemed not important. Legal import of birds is regulated through the U.S. Department of Agriculture, Animal and Plant Health Inspection Service and the U.S. Fish and Wildlife Service. Although quarantine procedures are unlikely to support virus transmission to mosquitoes and to other birds, illegally imported birds, if infected, not subjected to quarantine or examination would be more likely to transmit the virus to mosquitoes and other birds (57).

It is important to note that all pathways of JEV introduction assessed in both the qualitative and quantitative risk assessment models (52, 53) pertained to inadvertent and intentional sources. Despite being considered of low, negligible or unknown risk, most intentional (e.g., illegal importation of animals) causes should not be disregarded. However, the scarcity and uncertainty of empirical data on movement of increasing populations of potentially infected competent vertebrate host animals (e.g., feral swine or ardeid birds) or illegal importation of animals, make these routes extremely challenging to be examined (51, 58).

Parallels With Other Arboviruses

Bluetongue virus, and Venezuelan equine encephalitis virus are examples of arboviruses whose emergence has been associated with the dispersal of vector species, introduction of animal hosts, climate effects, urbanization and globalization, among other factors (49). Similarly, WNV, a closely related flavivirus, was introduced to and became endemic in North America over a period of a few years (59). During the summer of 1999, several *Culex pipiens* complex mosquitoes were identified as the principal vector and house sparrows as important maintenance hosts (60–64). American crows (and some other corvids) suffered fulminating systemic disease and were deemed critical amplifying hosts (65–69). Previous experience in temperate regions of Europe suggested that introduced strains of WNV from Africa or the Mediterranean did not persist, and re-introduction was

necessary for repeated outbreaks of disease (70); however, WNV is now endemic in Europe (as well as in the Middle East, Africa, Asia and Australia) (71). In North America, where WNV is also endemic, virus persistence was achieved, and sustained by long-term infections of both mosquitoes and birds (72, 73). Factors such as normal migration and legal or illegal importation of zoo, pet, domestic, or wild birds have been hypothesized to have played a role in the introduction of the WNV to the western hemisphere, whereas complex ecological factors determined its geographic spread (57). It is important to note that the North American introduction and establishment of the WNV overcame similar unfavorable circumstances to the ones faced with JEV, given sufficient time and introduction opportunities.

Challenges

The recent decline in overall arboviral surveillance capacity (and lack of JEV surveillance in particular) in the US can compromise our ability to rapidly detect and respond to existing and emerging threats (74). There have been 14 travel-associated JE human cases reported among US citizens from 1973 to 2008, with cases most likely being acquired in Thailand, the Philippines, Vietnam, Singapore, Japan and China (75). Cases occurred among military personnel, tourists visiting friends and relatives, and expatriates. Since then, two additional cases were recorded, one fatal case in a US child that visited the Philippines, and a refugee traveling from Thailand to the US (76). All cases, thus far, have been imported.

Despite an estimated high risk of entry into the US via infected adult mosquitoes by aircraft (52, 53), no evidence of JEV emergence, transmission, or establishment has been reported up until now in the US *under current conditions* related to virus, vector, host and environment. Potential hypotheses for explaining the non-emergence of JEV in the US include: (1) the fragility of JEV in the environment, which is easily destroyed by heat, UV light and common detergents (52, 77); (2) potentially low mosquito distribution and host density in airport and seaport areas (which are considered the most likely pathways of US introduction) (52, 53, 78); (3) short apparent periods of viremia in pigs and ardeid birds, ranging from 3–4 days (56, 79); (4) insufficient contact rates between hosts and vectors; (5) cross-protection of JEV with other endemic flaviviruses, such as WNV and St. Louis encephalitis virus; and (6) a potentially limited infection capacity of mosquitoes during establishment.

Co-circulation and strain displacement are not new to flaviviruses as they have occurred in multiple areas for dengue virus and in the US for WNV (80–83). Gould et al. (50) speculated that given the vectors' widespread geographic range and high adaptability toward changing environmental conditions, another genotype could emerge in new regions (50). Similarly, the possible movement of vectors and hosts associated with urbanization, carried by tropical storms, or other natural disasters, could increase rates of contact and hence, transmission potential.

Future genotype displacement or genetic modifications can compromise current cross-protection, and in turn threaten vaccine effectiveness, current immunization and other public health programs (12). Other challenges associated with emergence or reemergence of JEV genotypes could

include changes in transmission paths, disease burden, or host demographics (11, 84).

Although viremia in the amplifying host is short, recent studies pointing at transmission via oronasal secretions between pigs without the involvement of vectors (39), suggest a previously unrecognized mechanism of transmission may exist. Incomplete knowledge regarding JEV transmission in wild and domestic pigs may cause the role of these species in the epidemiology of JEV to be underestimated.

Japanese encephalitis is a vaccine-preventable disease, but recent research suggests that currently available vaccines (both inactivated and attenuated) may not provide complete protection against GV infection (85). Additionally, and because humans are dead-end hosts, JEV vaccination does not provide herd immunity (3). Whether or not new vaccines are needed to deal with this challenge is still under debate. Moreover, the introduction of JEV could have devastating public health consequences, especially in locations with naïve and aging populations such as in the US, usually affected by chronic diseases (immunocompromised population), where there is potentially no herd immunity against JEV. In addition to vaccines, reducing contact between mosquito vectors with humans and animal reservoirs would limit the duration and extent of viral outbreaks in the environment (5). In JE endemic countries, larval habitat treatment of rice fields by chemical or mechanical manipulation (86, 87) and adult aerial spraying (88) are the main methods used for management of mosquito vectors; these methods are also used by mosquito and vector control districts throughout the US. The public perception of the health and environmental effects associated with the use of pesticides, however, has greatly impacted the area coverage and the type of products used for mosquito mitigation. Larval habitat treatments with *Bacillus thuringiensis israelensis*, spinosad, and other dipteran-specific larvicides are largely unimpacted, but adulticidal treatments are heavily regulated by US state and federal agencies. States like California limit the application of some adulticide active ingredients in riparian zones (e.g., coastal marshes) where endangered species are found. Mosquito vector control districts in this state must consult the Pesticide Regulation's Endangered Species Custom Realtime Internet Bulletin Engine or PRESCRIBE dataset, prior to pesticide application in public areas, however, pesticide application in residential areas does not have such restrictions (89). These limitations could make proper and timely mitigation of vectors very difficult.

Although JEV has not established in the US, the conditions are rapidly changing. Reduced mosquito control in areas at highest risk (i.e., west coast), no active surveillance for JEV in place, increasing populations of vector species and host reservoirs, and emerging viral genotypes that may change the probability of establishment, may dictate the future emergence, and subsequent spread of JEV in the US. Similarly, the increase in population density and in human and animal movement, coupled with climate effects, habitat modification and other anthropogenic factors, emphasize the need for early detection of arboviral diseases through surveillance in areas at higher risk. Hence, we propose monitoring changes in host or vector

population composition or dynamics, and/or environmental configuration that can be beneficial for virus introduction, in US areas at higher risk. Similarly, ongoing identification of emerging disease risks through surveillance (e.g., detection of virus in vectors and hosts) efforts will increase the speed by which US officials can detect pathogen emergence. Rapid response to outbreaks can be achieved by increasing preparedness efforts including education of citizens (e.g., through citizen science campaigns), clinicians and laboratory diagnosticians on disease recognition and prevention, and improvement of laboratory detection capabilities. Lastly, conducting an economic assessment linking disease risk at the wildlife-livestock interface and comparing the benefits and costs of risk management (e.g., surveillance, biosecurity) in both livestock and wildlife, as well as determining where public health efforts are required, can reduce the vulnerability and potential consequences of a JEV incursion in the US. The potential impact of the emergence of arboviral diseases, in particular JEV, a disease with high morbidity and mortality rates in humans, in a susceptible region such as the Americas and the US specifically, which has an increasingly globalized commerce and tourism as well as concentrated and interconnected livestock production, is large

and can lead to long lasting effects on public health, economies, and production systems.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) [Project number 5430-32000-008-05S] and the College of Veterinary Medicine (Cernicchiaro start-up funds) at Kansas State University.

ACKNOWLEDGMENTS

We would like to acknowledge Dr. William Walton from University of California, Riverside for providing information regarding mitigation strategies applied in the state of California.

REFERENCES

- Weaver S, Barrett ADT. Transmission cycles, host range, evolution and emergence of arboviral disease. *Nat Rev Microbiol.* (2004) 2:789–801. doi: 10.1038/nrmicro1006
- Campbell GL, Hills SL, Fischer M, Jacobson JA, Hoke CH, Hombach JM, et al. Estimated global incidence of Japanese encephalitis: a systematic review. *Bull World Health Organ.* (2011) 89:766–74. doi: 10.2471/BLT.10.085233
- Solomon T. Control of Japanese encephalitis – within our grasp? *N Engl J Med.* (2006) 355:869–71. doi: 10.1056/NEJMp058263
- Erlanger TE, Weiss S, Keiser J, Utzinger J, Wiedenmayer K. Past, present and future of Japanese encephalitis. *Emerg Infect Dis.* (2009) 15:1–7. doi: 10.3201/eid1501.080311
- Oliveira ARS, Cohnstaedt LW, Cernicchiaro N. Japanese encephalitis virus: Placing disease vectors in the epidemiologic triad. *Ann Entomol Soc Am.* (2018) 111:295–303. doi: 10.1093/aesa/say025
- Le Flohic G, Porphyre V, Barbazan P, Gonzalez JP. Review of climate, landscape, and viral genetics as drivers of the Japanese encephalitis virus ecology. *PLoS Negl Trop Dis.* (2013) 7:e2208. doi: 10.1371/journal.pntd.0002208
- Oliveira ARS, Cohnstaedt LW, Strathe E, Hernandez LE, McVey DS, Piaggio J, et al. Meta-analyses of the proportion of Japanese encephalitis virus infection in vectors and vertebrate hosts. *Parasit Vect.* (2017) 10:418. doi: 10.1186/s13071-017-2354-7
- Oliveira ARS, Strathe E, Etcheverry L, Cohnstaedt LW, McVey DS, Piaggio J, et al. Assessment of data on vector and host competence for Japanese encephalitis virus: a systematic review of the literature. *Prev Vet Med.* (2018) 154:71–89. doi: 10.1016/j.prevetmed.2018.03.018
- Oliveira ARS, Cohnstaedt LW, Strathe E, Etcheverry L, McVey DS, Piaggio J, et al. Meta-analyses of Japanese encephalitis virus infection, dissemination, and transmission rates in vectors. *Am J Trop Med Hyg.* (2018) 98:883–90. doi: 10.4269/ajtmh.17-0622
- Xiao C, Li C, Di D, Cappelle J, Liu L, Wang X, et al. Differential replication efficiencies between Japanese encephalitis virus genotype I and III in avian cultured cells and young domestic ducklings. *PLoS Negl Trop Dis.* (2018) 12:e0007046. doi: 10.1371/journal.pntd.007046
- Gao X, Liu H, Li X, Fu S, Cao L, Shao N, et al. Changing geographic distribution of Japanese encephalitis virus genotypes, 1935–2017. *Vector Borne Zoonotic Dis.* (2019) 19:35–44. doi: 10.1089/vbz.2018.2291
- Karna AK, Bowen RA. Experimental evaluation of the role of ecologically-relevant hosts and vectors in Japanese encephalitis virus genotype displacement. *Viruses.* (2019) 11:1–16. doi: 10.3390/v11010032
- Platonov AE, Rossi G, Karan LS, Mironov KO, Busani L, Rezza G. Does the Japanese encephalitis virus (JEV) represent a threat for human health in Europe? Detection of JEV RNA sequences in birds collected in Italy. *Euro Surveill.* (2012) 17:20241. doi: 10.2807/ese.17.32.20241-en
- Ravanini P, Huhtamo E, Ilaria V, Crobu MG, Nicosia AM, Servino L, et al. Japanese encephalitis virus RNA detected in *Culex pipiens* mosquitoes in Italy. *Euro Surveill.* (2012) 17:20221. doi: 10.2807/ese.17.28.20221-en
- Baud D, Gubler DJ, Schaub B, Lanteri MC, Musso D. An update on Zika virus infection. *Lancet.* (2017) 390:2099–109. doi: 10.1016/S0140-6736(17)31450-2
- Nett RJ, Campbell GL, Reisen WK. Potential for the emergence of Japanese encephalitis virus in California. *Vect Borne Zoonotic Dis.* (2009) 9:511–7. doi: 10.1089/vbz.2008.0052
- Nemeth N, Bosco-Lauth A, Oesterle P, Kohler D, Bowen R. North American birds as potential amplifying hosts of Japanese encephalitis virus. *Am J Trop Med Hyg.* (2012) 87:760–7. doi: 10.4269/ajtmh.2012.12-0141
- Huang YJS, Harbin JN, Hettenbach SM, Maki E, Cohnstaedt LW, Barrett ADT, et al. Susceptibility of a North American *Culex quinquefasciatus* to Japanese encephalitis virus. *Vector Borne Zoonotic Dis.* (2015) 15:709–11. doi: 10.1089/vbz.2015.1821
- Lord JS, Al-Amin HM, Chakma S, Alam MS, Gurley ES, Pulliam JRC. Sampling design influences the observed dominance of *Culex tritaeniorhynchus*: considerations for future studies of Japanese encephalitis virus transmission. *PLoS Negl Trop Dis.* (2016) 10:e0004249. doi: 10.1371/journal.pntd.0004249
- Golnar AJ, Turell MJ, LaBeaud AD, Kading RC, Hamer GL. Predicting the mosquito species and vertebrate species involved in the theoretical transmission of Rift valley fever virus in the United States. *PLoS Negl Trop Dis.* (2015) 8:e3163. doi: 10.1371/journal.pntd.0003163
- Darsie RF Jr, Ward RA. *Identification and Geographical Distribution of the Mosquitoes of North America, North of Mexico.* Gainesville, FL: University Press of Florida (2005).

22. ECDC/EFSA. *European Centre for Disease Prevention and Control and European Food Safety Authority*. Stockholm: ECDC (2018). Available online at: <https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps> (accessed June 12, 2019).
23. Konishi E, Pincus S, Paoletti E, Laegreid WW, Shope RE, Mason PW. A highly attenuated host range-restricted vaccinia virus strain, NYVAC, encoding the prM, E, and NS1 genes of Japanese encephalitis virus prevents JEV viremia in swine. *Virology*. (1992) 190:454–8. doi: 10.1016/0042-6822(92)91233-K
24. Lyons AC, Huang YS, Park SL, Ayers VB, Hettenbach SM, Higgs S, et al. Shedding of Japanese Encephalitis virus in oral fluid of infected swine. *Vector Borne Zoonotic Dis.* (2018) 18:469–74. doi: 10.1089/vbz.2018.2283
25. Park SL, Huang YS, Lyons AC, Ayers VB, Hettenbach SM, McVey DS, et al. North American domestic pigs are susceptible to experimental infection with Japanese encephalitis virus. *Sci Rep.* (2018) 8:7951. doi: 10.1038/s41598-018-26208-8
26. Otake S, Dee SA, Rossow KD, Moon R, Pijoan C. *Identification of Porcine Reproductive and Respiratory Syndrome Virus in Mosquitoes (Culicidae)*. International Symposium on Swine Disease Eradication (2001).
27. Otake S, Dee SA, Rossow KD, Moon RD, Pijoan C. Mechanical transmission of porcine reproductive and respiratory syndrome virus by mosquitoes, *Aedes vexans* (Meigen). *Can J Vet Res.* (2002) 66:191–5.
28. Schurrer JA, Dee SA, Moon RD, Deen J, Pijoan C. Evaluation of three strategies for insect control on a commercial swine farm. *J Swine Health Prod.* (2006) 14:76–81.
29. Saito M, Koike F, Momose H, Mihira T, Uematsu S, Ohtani T, et al. Forecasting the range expansion of a recolonising wild boar *Sus scrofa* population. *Wildl Biol.* (2012) 18:383–92. doi: 10.2981/11-110
30. Yang DK, Kim HH, Hyun BH, Lim SI, Nam YK, Nah JJ, et al. Detection of neutralizing antibody against Japanese encephalitis virus in wild boars of Korea. *J Bacteriol Virol.* (2012) 42:353–6. doi: 10.4167/jbv.2012.42.4.353
31. Nidaira M, Kyan H, Taira K, Okano S, Oshiro T, Kato T, et al. Survey of Japanese Encephalitis virus in pigs and wild boards on Ishigaki and Iriomote islands in Okinawa, Japan. *Epidemiol Infect.* (2014) 142:856–860. doi: 10.1017/S.0950268813001611
32. Ruiz-Fons F. A review of the current status of relevant zoonotic pathogens in wild swine (*Sus scrofa*) populations: changes modulating the risk of transmission to humans. *Transbound Emerg Dis.* (2017) 64:68–88. doi: 10.1111/tbed.12369
33. Root TL, Price JT, Hall KR, Schneiders SH, Rosenzweig C, Pounds JA. Fingerprints of global warming on wild animals and plants. *Nature.* (2003) 421:57–60. doi: 10.1038/nature01333
34. Hutton T, DeLiberto T, Owen S, Morrison, B. *Disease Risks Associated With Increasing Feral Swine Numbers and Distribution in the United States*. Report for the Midwest Association of Fish and Wildlife Agencies, Wildlife and Fish Health Committee (2006). Available online at: https://www.michigan.gov/documents/emergingdiseases/Hutton_Pig_Paper_177657_7.doc (accessed June 12, 2019).
35. Rosvold J, Andersen R. *Wild boar in Norway – is Climate a Limiting Factor? Norges Teknisk-Naturvitenskapelige Universitet, Vitenskapsmuseet, Rapport Zoologisk Serie 2008–1*. (2008). Available online at: https://www.ntnu.no/c/document_library/get_file?uuid=e2c9a9d6-cd49-4b50-95c7-cbe0ad520156&groupId=10476 (accessed June 12, 2019).
36. Campbell TA, Long DB. Feral swine damage and damage management in forested ecosystems. *For Ecol Manage.* (2009) 257:2319–26. doi: 10.1016/j.foreco.2009.03.036
37. USDA-APHIS United States Department of Agriculture, Animal and Plant Health Inspection Service. *History of Feral Swine in the Americas*. (2018). Available online at: <https://www.aphis.usda.gov/aphis/ourfocus/wildlifedamage/operational-activities/feral-swine/sa-fs-history> (accessed September 9, 2019).
38. Ahumada JA, Lapointe D, Samuel MD. Modeling the population dynamics of *Culex quinquefasciatus* (Diptera: Culicidae), along an elevational gradient in Hawaii. *J Med Entomol.* (2004) 41:1157–70. doi: 10.1603/0022-2585-41.6.1157
39. Ricklin ME, García-Nicolás O, Brechbühl D, Python S, Zumkehr B, Nougairé A, et al. Vector-free transmission and persistence of Japanese encephalitis virus in pigs. *Nat Commun.* (2016) 7:10832. doi: 10.1038/ncomms10832
40. García-Nicolás O, Braun RO, Milona P, Lewandowska M, Dijkman R, Alves MP, et al. Targeting of the nasal mucosa by Japanese encephalitis virus for non-vector-borne transmission. *J Virol.* (2018) 92:1–16. doi: 10.1128/JVI.01091-18
41. Sauer JR, Ziolkowski D Jr, Pardieck KL, Smith AC, Hudson MA, Rodriguez V, et al. The first 50 years of the North American breeding bird survey. *Condor.* (2017) 119:576–93. doi: 10.1650/CONDOR-17-83.1
42. Schuh AJ, Ward MJ, Leigh Brown AJ, Barrett ADT. Phylogeography of Japanese encephalitis virus: genotype is associated with climate. *PLoS Negl Trop Dis.* (2013) 7:e2411. doi: 10.1371/journal.pntd.0002411
43. Hanna JN, Ritchie SA, Phillips DA, Shield J, Bailey MC, Mackenzie JS, et al. An outbreak of Japanese encephalitis in the Torres Strait, Australia, 1995. *Med J Aus.* (1996) 165:256–60. doi: 10.5694/j.1326-5377.1996.tb124960.x
44. Pyke AT, Williams DT, Nisbet DJ, Van den Hurk AF, Taylor CT, Johansen CA, et al. The appearance of a second genotype of Japanese encephalitis virus in the Australasian region. *Am J Trop Med Hyg.* (2001) 65:747–53. doi: 10.4269/ajtmh.2001.65.747
45. Li MH, Fu SH, Chen WX, Wang HY, Guo YH, Liu QY, et al. Genotype V Japanese encephalitis virus is emerging. *PLoS Negl Trop Dis.* (2011) 5:e1231. doi: 10.1371/journal.pntd.0001231
46. Takhampunya R, Kim HC, Tippayachai B, Kengluetcha A, Klein TA, Lee WJ, et al. Emergence of Japanese encephalitis virus genotype V in the Republic of Korea. *Virol J.* (2011) 8:449. doi: 10.1186/1743-422X-8-449
47. Schuh AJ, Ward MJ, Leigh Brown AJ, Barrett ADT. Dynamics of the emergence and establishment of a newly dominant genotype of Japanese encephalitis virus throughout Asia. *J Virol.* (2014) 88:4522–32. doi: 10.1128/JVI.02686-13
48. Do LP, Bui TM, Phan NT. Mechanism of Japanese encephalitis virus genotypes replacement based on human, porcine and mosquito-originated cell lines model. *Asian Pac J Trop Med.* (2016) 9:333–6. doi: 10.1016/j.apjtm.2016.03.007
49. Weaver SC, Reisen WK. Present and future of arboviral threats. *Antiviral Res.* (2010) 85:328–45. doi: 10.1016/j.antiviral.2009.10.008
50. Gould E, Pettersson H, Higgs S, Charrel R, de Lamballerie X. Emerging arboviruses: Why today? *One Health.* (2017) 4:1–13. doi: 10.1016/j.onehlt.2017.06.001
51. Esser HJ, Mogling R, Cleton NB, van der Jeugd H, Sprong H, Stroo A, et al. Risk factors associated with sustained circulation of six zoonotic arboviruses: a systematic review for selection of surveillance sites in non-endemic areas. *Parasit Vectors.* (2019) 12:265. doi: 10.1186/s13071-019-3515-7
52. Oliveira ARS, Piaggio J, Cohnstaedt LW, McVey DS, Cernicchiaro N. A quantitative risk assessment (QRA) of the risk of introduction of the Japanese encephalitis virus (JEV) in the United States via infected mosquitoes transported in aircraft and cargo ships. *Prev Med Vet.* (2018) 160:1–9. doi: 10.1016/j.prevetmed.2018.09.020
53. Oliveira ARS, Piaggio J, Cohnstaedt LW, McVey DS, Cernicchiaro N. Introduction of the Japanese encephalitis virus (JEV) in the United States – A qualitative risk assessment. *Transbound Emerg Dis.* (2019) 66:1558–74. doi: 10.1111/tbed.13181
54. de Vos C, Hoek M, Fischer E, de Koeijer A, Bremmer J. *Risk Assessment Framework for Emerging Vector-Borne Livestock Diseases*. AMB Express (2011).
55. Zepeda Sein C. Risk analysis: a decision support tool for the control and prevention of animal diseases. In: *Proc. 70th General Session of the International Committee*. Paris: World Organization for Animal Health (OIE) (2002). p. 26–31.
56. Gresser I, Hardy JL, Hu SMK, Scherer WF. Factors influencing transmission of Japanese B encephalitis virus by a colonized strain of *Culex tritaeniorhynchus* Giles, from infected pigs and chicks to susceptible pigs and birds. *Am J Trop Med Hyg.* (1958) 7:365–73. doi: 10.4269/ajtmh.1958.7.365
57. Rappole JH, Derrickson SR, Hubalek Z. Migratory birds and spread of West Nile Virus in the Western Hemisphere. *Emerg Infect Dis.* (2000) 6:319–28. doi: 10.3201/eid0604.000401
58. Miller RS, Sweeney SJ, Sloodmaker C, Grear DA, Di Salvo PA, Kiser D, et al. Cross-species transmission potential between wild pigs, livestock, poultry, wildlife, and humans: implications for disease risk management in North America. *Sci Rep.* (2017) 7:7821. doi: 10.1038/s41598-017-07336-z
59. McVey DS, Wilson WC, Gay CG. West Nile virus. *Rev Sci Tech.* (2015) 34:431–9. doi: 10.20506/rst.34.2.2369

60. Komar N. West Nile virus surveillance using sentinel birds. *Ann N Y Acad Sci.* (2001) 951:58–73. doi: 10.1111/j.1749-6632.2001.tb02685.x
61. Komar N, Panella NA, Burns JE, Duszka SW, Mascarenhas TM, Talbot TO. Serologic evidence for West Nile virus infection in birds in the New York City vicinity during an outbreak in 1999. *Emerg Infect Dis.* (2001) 7:621–5. doi: 10.3201/eid0704.017403
62. Nasci RS, Savage HM, White DJ, Miller JR, Cropp BC, Godsey MS, et al. West Nile virus in overwintering *Culex* mosquitoes, New York City, 2000. *Emerg Infect Dis.* (2001) 7:742–4. doi: 10.3201/eid0704.017426
63. Komar N. West Nile virus: epidemiology and ecology in North America. *Adv Virus Res.* (2003) 61:185–234. doi: 10.1016/S0065-3527(03)61005-5
64. Wheeler SS, Vineyard MP, Woods LW, Reisen WK. Dynamics of West Nile virus persistence in House Sparrows (*Passer domesticus*). *PLoS Negl Trop Dis.* (2012) 6:e1860. doi: 10.1371/journal.pntd.0001860
65. Eidson M, Komar N, Sorhage F, Nelson R, Talbot T, Mostashari F, et al. Crow deaths as a sentinel surveillance system for West Nile virus in the northeastern United States, 1999. *Emerg Infect Dis.* (2001) 7:615–20. doi: 10.3201/eid0704.017402
66. Brault AC, Langevin SA, Bowen RA, Panella NA, Biggerstaff BJ, Miller BR, et al. Differential virulence of West Nile strains for American crows. *Emerg Infect Dis.* (2004) 10:2161–8. doi: 10.3201/eid1012.040486
67. Brault AC, Huang CY, Langevin SA, Kinney RM, Bowen RA, Ramey WN, et al. A single positively selected West Nile viral mutation confers increased virogenesis in American crows. *Nat Genet.* (2007) 39:1162–6. doi: 10.1038/ng2097
68. LaDeau SL, Kilpatrick AM, Marra PP. West Nile virus emergence and large-scale declines of North American bird populations. *Nature.* (2007) 447:710–3. doi: 10.1038/nature05829
69. Nemeth NM, Thomsen BV, Spraker TR, Benson JM, Bosco-Lauth AM, Oesterle PT, et al. Clinical and pathologic responses of American crows (*Corvus brachyrhynchos*) and fish crows (*C. ossifragus*) to experimental West Nile virus infection. *Vet Pathol.* (2011) 48:1061–74. doi: 10.1177/0300985811398249
70. Hubalek Z. European experience with the West Nile virus ecology and epidemiology: could it be relevant for the New World? *Viral Immunol.* (2000) 13:415–26. doi: 10.1089/vim.2000.13.415
71. Murray KO, Mertens E, Desprès P. West Nile virus and its emergence in the United States of America. *Vet Res.* (2010) 41:1–14. doi: 10.1051/vetres/2010039
72. Reisen WK, Fang Y, Lothrop HD, Martinez VM, Wilson J, Oconnor P, et al. Overwintering of West Nile virus in Southern California. *J Med Entomol.* (2006) 43:344–55. doi: 10.1093/jmedent/43.2.344
73. Reisen WK. Ecology of West Nile virus in North America. *Viruses.* (2013) 5:2079–105. doi: 10.3390/v5092079
74. Hadler JL, Patel D, Nasci RS, Petersen LR, Hughes JM, Bradley K, et al. Assessment of arbovirus surveillance 13 years after introduction of West Nile Virus, United States. *Emerg Infect Dis.* (2015) 21:1159–66. doi: 10.3201/eid2107.140858
75. Hills SL, Griggs AC, Fischer M. Japanese Encephalitis in travellers from non-endemic countries, 1973–2008. *Am J Trop Med Hyg.* (2010) 82:930–6. doi: 10.4269/ajtmh.2010.09-0676
76. Centers for Disease Control and Prevention (CDC). *Morbidity and Mortality Weekly Report.* (2011). *Japanese Encephalitis in Two Children — United States, 2010.* Available online at: <https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6009a3.htm> (accessed on September 17, 2019).
77. OIE Technical Disease Cards. *Japanese Encephalitis.* (2018). Available online at: www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/JAPANESE_ENCEPHALITIS.pdf (accessed June 14, 2019).
78. Mier-y-Teran-Romero L, Tatem AJ, Johansson MA. Mosquitoes on a plane: disinsection will not stop the spread of vector-borne pathogens, a simulation study. *PLoS Negl Trop Dis.* (2017) 11:1–13. doi: 10.1371/journal.pntd.0005683
79. Misra UK, Kalita J. Overview: Japanese encephalitis. *Prog Neurobiol.* (2010) 91:108–20. doi: 10.1016/j.pneurobio.2010.01.008
80. Ebel GD, Carricaburu J, Young D, Bernard KA, Kramer LD. Genetic and phenotypic variation of West Nile virus in New York, 2000–2003. *Am J Trop Med Hyg.* (2004) 71:493–500. doi: 10.4269/ajtmh.2004.71.493
81. Moudy RM, Meola MA, Morin LLL, Ebel GD, Kramer LD. A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by *Culex* mosquitoes. *Am J Trop Med Hyg.* (2007) 77:365–70. doi: 10.4269/ajtmh.2007.77.365
82. Hanley KA, Nelson JT, Schirtzinger EE, Whitehead SS, Hanson CT. Superior infectivity for mosquito vectors contributes to competitive displacement among strains of dengue virus. *BMC Ecol.* (2008) 8:1. doi: 10.1186/1472-6785-8-1
83. Quiner CA, Parameswaran P, Ciota AT, Ehrbar DJ, Dodson BL, Schlesinger S, et al. Increased replicative fitness of a dengue virus 2 clade in native mosquitoes: potential contribution to a clade replacement event in Nicaragua. *J Virol.* (2014) 88:13125–34. doi: 10.1128/JVI.01822-14
84. Sunwoo JS, Jung KW, Lee ST, Lee SK, Chu K. Reemergence of Japanese Encephalitis in South Korea, 2010–2015. *Emerg Infect Dis.* (2016) 22:1841–3. doi: 10.3201/eid2210.160288
85. Cao L, Fu S, Gao X, Li M, Cui S, Li X, et al. Low protective efficacy of the current Japanese Encephalitis vaccine against the emerging genotype 5 Japanese Encephalitis virus. *PLoS Negl Trop Dis.* (2016) 10:e0004686. doi: 10.1371/journal.pntd.0004686
86. Keiser J, Maltese MF, Erlanger TE, Bos R, Tanner M, Singer BH, et al. Effect of irrigated rice agriculture on Japanese encephalitis, including challenges and opportunities for integrated vector management. *Acta Trop.* (2005) 95:40–57. doi: 10.1016/j.actatropica.2005.04.012
87. Ben-Dov E. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins.* (2014) 6:1222–43. doi: 10.3390/toxins6041222
88. Karunaratne SHPP, Hemingway J. Insecticide resistance spectra and resistance mechanisms in populations of Japanese encephalitis vector mosquitoes, *Culex tritaeniorhynchus* and *Cx. gelidus*, in Sri Lanka. *Med Vet Entomol.* (2000) 14:430–6. doi: 10.1046/j.1365-2915.2000.00252.x
89. California Department of Pesticide Regulation (2019). Available online at: <https://calpip.cdpr.ca.gov/main.cfm> (accessed September 9, 2019).

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation, though no other collaboration, with several of the authors LC, LN, DM, and DSM.

Copyright © 2020 Oliveira, Cohnstaedt, Noronha, Mitzel, McVey and Cernicchiaro. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Identification and Characterization of Bluetongue Virus Serotype 14 in Russia

Andrei Koltsov*, Sodnom Tsybanov, Andrey Gogin, Denis Kolbasov and Galina Koltsova*

Federal Research Center for Virology and Microbiology, Pokrov, Russia

OPEN ACCESS

Edited by:

Stéphan Zientara,
Agence Nationale de Sécurité
Sanitaire de l'Alimentation, de
l'Environnement et du Travail
(ANSES), France

Reviewed by:

Alessio Lorusso,
Experimental Zooprophyllactic Institute
of Abruzzo and Molise G.
Caporale, Italy
Muhammad Zubair Shabbir,
University of Veterinary and Animal
Sciences, Pakistan

*Correspondence:

Andrei Koltsov
kolcov.andrew@gmail.com
Galina Koltsova
lila5757@yandex.ru

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 30 September 2019

Accepted: 13 January 2020

Published: 28 February 2020

Citation:

Koltsov A, Tsybanov S, Gogin A,
Kolbasov D and Koltsova G (2020)
Identification and Characterization of
Bluetongue Virus Serotype 14 in
Russia. *Front. Vet. Sci.* 7:26.
doi: 10.3389/fvets.2020.00026

This paper reports a case of bluetongue virus (BTV) infection in the Smolensk and Kaluga regions of Russia in 2011–2012. The virus was initially detected in heifers transferred in Russia from Germany through Poland and Belarus in 2011. On day 27 of quarantine, RNA and infectious viruses of BTV were detected in four heifers, but five were serologically positive. However, on day 3 before shipment, all heifers were seronegative and PCR-negative for BTV. Thus, a few animals from this consignment were viremic without any evident subclinical infection. Based on Seg-2 (VP2 gene) and Seg-5 (NS1 gene) sequencing, the recovered virus had 99.86–100% nucleotide identity with BTV-14-like viruses such as the vaccine BTV-14 strain RSArrrr/BTV 14 and the BTV-14 isolates detected in Lithuania and Poland in 2012. Subsequently, BTV-14 was also reported in local animals in two regions of Russia. During the monitoring survey, 1623 local animals within a 300-km radius were tested, of which 471 tested positive by ELISA and 183 by PCR for BTV-14 RNA. No other serotypes were identified in either imported or aboriginal animals within that radius. The *Culicoides* midges trapped at the site of the outbreak in May 2012 tested positive for the BTV-14 genome, indicating that the possible mechanism of spread most likely occurs via vector bites. However, further investigation is required to confirm this hypothesis, which would provide an improved understanding of the circulation and overwintering of BTV in northern latitudes.

Keywords: bluetongue virus, *Orbivirus*, phylogenetic analysis, serotype 14, blood-sucking midge vectors, spread, Russia

INTRODUCTION

Historically, bluetongue (BT) was considered endemic within Africa and was first discovered after fine-wool sheep imported from Europe (1). The affected sheep exhibited the typical symptoms of swollen lips and tongue accompanied by a characteristic tongue cyanosis, giving a name to the disease (2).

The etiologic agent of BT was later discovered to be a double-stranded RNA virus with a segmented genome of the genus *Orbivirus* and family *Reoviridae* (3, 4). The virus is transmitted by blood-sucking midges of the genus *Culicoides* (5–8). Ticks have also been implicated as a vector (9). A total of 27 serotypes were recently recognized, with another few pending (10–16). The serotypes exhibit various patterns of transmission, including direct contact (17, 18), which contradicts the accepted concept that bluetongue virus (BTV) is only transferred via a *Culicoides* vector (19).

BTV is endemic throughout Africa within the range of its primary vector *Culicoides imicola*. The virus was first confirmed outside Africa in India (20) and then in Australia, where the virus was isolated from *Culicoides* collected near Darwin in 1975 (21). The distribution of BTV has been substantially expanding, although historically, before 1998, its range was confined to regions between the latitudes of 35°S and 40°N (7).

BTV was never expected to reach areas far beyond its traditional northernmost latitude. That could be explained by the relatively cold climate and short period of seasonal midge activity preventing this virus from establishment [reviewed by (22)]. However, in 2008, BT arrived in northern Europe and caused unprecedented outbreaks (7). The arrival of BTV could be attributed to the movement of infected ruminants or the wind dispersal of infected midges (23). Notably, the key role in the transmission of BTV in northern Europe was played by novel Palaearctic vectors, the *Obsoletus* and *Pulicaris* complexes (24). Considering the abundance of vectors and the recent changes in climate, it is not surprising that new BTV strains have appeared. These strains include reassortants and serotypes from the Mediterranean region, which are now considered endemic, and new strains are expected to continue to regularly appear (18, 25, 26), putting Eastern Europe and the European part of Russia at risk.

In Russia, the first historical outbreak of BTV among sheep caused morbidity of 58.3% and mortality of 66.3% in 1993 in Eastern Siberia (Republic of Buryatia) (27). Vaccination successfully contained the outbreak. The serotype was identified as BTV16, which is related to the eastern topotype (unpublished data). Considering that Europe has recently experienced multiple incursions of different serotypes (25), the risks of BTV introduction into European Russia has increased dramatically owing to cattle trading between Russia and Europe. The first cases of BTV-8 infection were identified in cattle imported from Germany and the Netherlands in 2008 (28). Notably, BTV-8 was only isolated from imported animals. BTV-8 infection among local animals was prevented as all PCR and seropositive animals were immediately slaughtered (unpublished data). In addition, as reported here, in 2011 in the Smolensk region, another set of imported animals imported from Germany was identified as carrying the BTV genome and anti-BTV antibodies during

the quarantine period, although the laboratory results obtained before shipment into Russia were negative.

In this study, the BTV-14 strain isolated in the 2011 outbreak in the Smolensk region in Russia was characterized. Serotype/topotype analysis of the Russian BTV isolate relied on the comparison of Seg-2 and Seg-5 sequences and conventional serotyping. To determine the source of the infection and investigate its spread within the country, phylogenetic and evolutionary divergence data, as well as data from monitoring studies in the Smolensk region and neighboring areas, were analyzed.

MATERIALS AND METHODS

Sample Collection

Serum and blood samples of 71 heifers from the farm “Smolensky Gallovei” in the Smolensk region of Russia (55°00′05.0″N, 34°28′49.1″E) were submitted in September 2011 to the Federal Research Center for Virology and Microbiology (Pokrov, Russia) for routine testing during quarantine in accordance with Russian legislation. The 71 young heifers of the Galloway breed were imported from Germany (Jurgen Greiner Ginsterweg) into the Smolensk region, following transport through Poland and Belarus. Before shipment, the animals were declared seronegative and PCR-negative for BTV (veterinary certificate 132 no. 0007170-0007171, dated 29.08.11).

The farm “Smolensky Gallovei” is typical in that it has a barn with open windows and animals are grazed daily in the field and rounded up twice a day for milking.

According to the BTV testing protocol, the animals were sampled for PCR and ELISA twice, at days 3 (05.09.2011) and 27 (29.09.2011) of the quarantine. Following the identification of infection, the quarantine was extended, and additional samples from the seropositive animals were collected on days 73, 104, 138, 189, and 207 (Table 1).

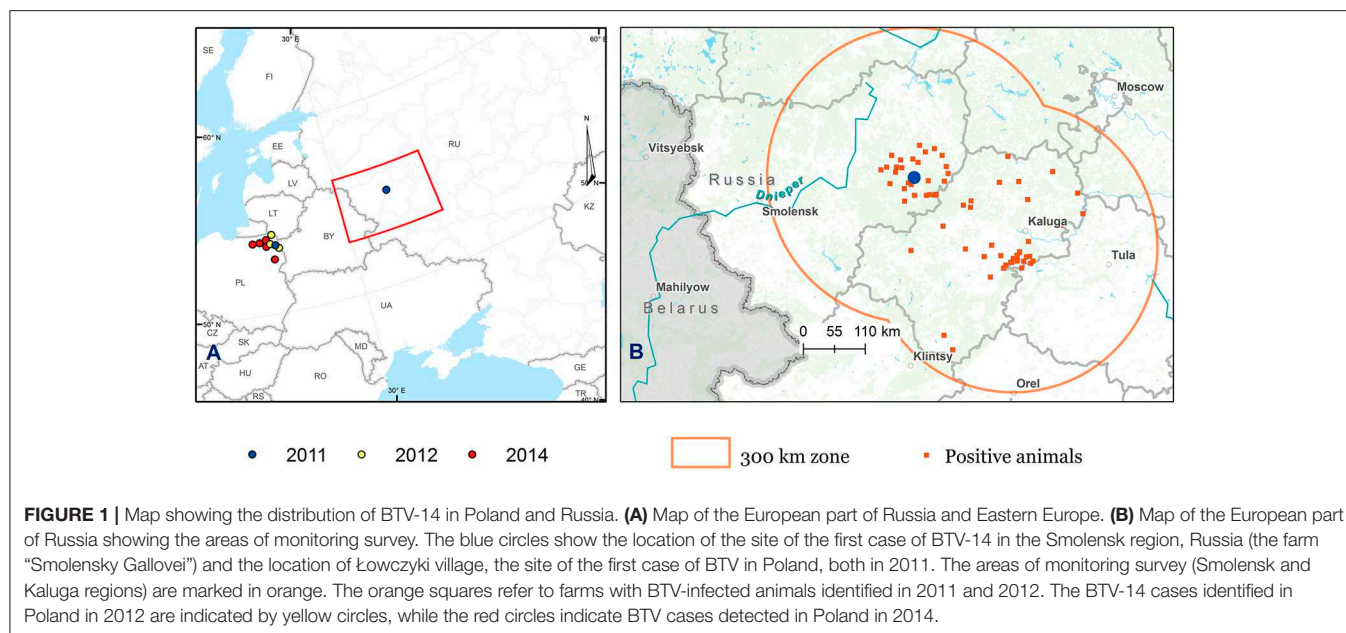
As part of the study, in 2012, an additional 1623 serum and whole-blood samples were collected from clinically healthy cattle, sheep, and goats from different farms in the Kaluga and Smolensk regions within a radius of 300 km from the outbreak site (Figure 1).

TABLE 1 | Examination of the samples from the imported cattle for the presence of BTV genome and anti-BTV antibodies made during the quarantine (3–207 days) on the territory of Russia by real-time RT-PCR and ELISA.

No animal	3 days		27 days		73 days		104 days		138 days		189 days		207 days	
	RT-PCR, Ct	ELISA	RT-PCR, Ct	ELISA	RT-PCR, Ct	ELISA	RT-PCR, Ct	ELISA	RT-PCR, Ct	ELISA	RT-PCR, Ct	ELISA	RT-PCR, Ct	ELISA
75981	Neg	Neg	27.8	Pos	30.3	Pos	27.3	Pos	25.7	Pos	Neg	Pos	Neg	Pos
01051	Neg	Neg	24.9	Pos	27.9	Pos	23.8	Pos	24.2	Pos	Neg	Pos	Neg	Pos
94835	Neg	Neg	Neg	Pos	neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos
01244	Neg	Neg	26.3	Pos	28.6	Pos	25.9	Pos	24.9	Pos	Neg	Pos	Neg	Pos
10055	Neg	Neg	25.6	Pos	30.0	Pos	26.7	Pos	25.7	Pos	Neg	Pos	Neg	Pos

Results are representative of three independent experiments.

RT-PCR, real-time reverse transcription-PCR; Ct, cycle threshold; Pos, positive study result; Neg, negative study result.



Virus Isolation

Blood was used for the first round of virus isolation in 10- to 12-day-old chicken embryos, according to the protocol described by Clavijo et al. (29). The inoculation of embryonating chicken eggs is more sensitive than cell culture, allowing detection of BTV in samples containing a low viral concentration (30). The blood (1/10 dilution) was intravenously inoculated into an embryo vein, incubated at $33.5 \pm 0.5^\circ\text{C}$, and monitored daily for 7 days. Chicken embryos with pathological signs were further processed for downstream inoculation onto Vero cells, which were inoculated with filtered material in a T25 flask. Cytopathic effects (CPE) in the cell monolayers were used to detect the virus. The development of CPE was observed daily for up to 7 days post-inoculation. The virus was titrated by visualization of CPE in Vero and BHK-21/13 cell cultures. Titers were expressed as median tissue culture infectious dose (TCID₅₀), according to the Reed–Muench method (31).

Laboratory Diagnosis and Virus Identification

Laboratory diagnosis of infection was performed using enzyme-linked immunosorbent assay (ELISA) and reverse-transcription polymerase chain reaction (RT-PCR). Blood serum was tested for anti-VP7 antibodies by using an ID Screen Bluetongue Competition ELISA kit (IDVet, France) following the manufacturer's recommendations. The signal-to-noise ratio ($S/N\% = \text{OD Sample} / \text{OD NC} \times 100$) was calculated, and a sample with a ratio greater than or equal to 40% was considered negative, whereas a sample with a ratio less than 40% was considered positive. Then, serum samples with inconclusive results were retested.

Total RNA was extracted from the samples with TRIzol (Thermo Fisher Scientific, USA), precipitated with isopropanol, and washed with 75% ethanol, according to the manufacturer's

protocol. Universal real-time RT-PCR directed against the non-structural protein 3 (NS3) fragment of the BTV genome was initially used to detect virus dsRNA in blood samples (32). The serotype 14 was identified using serotype-specific RT-PCR for genome segment 2 (Seg-2) (33). For both assays, the cutoff was set at a Ct-value of 38.

To exclude the other epidemiologically significant BTV serotypes in Russia, serotype-specific RT-PCR was used to test for BTV 1, 2, 4, 6, 8, 9, 11, and 16 (34).

To confirm the identified serotype, virus neutralization tests (VNT) against reference antisera to each of the 25 BTV serotypes (1–24, 26) were conducted. The VNT was performed using Vero and BHK-21/13 cell cultures as described by Koltsov (35).

Sequencing and Phylogenetic Analysis

The serotype was confirmed by sequencing of the cDNA amplicons and by phylogenetic comparisons with previously characterized reference strains of each serotype.

Then, the samples that were identified as positive by universal real-time RT-PCR were subjected to VP2 (Seg-2, 2,922 nt) and NS1 (Seg-5, 1,772 nt) sequencing. Seg-2 and Seg-5 were chosen as serotype- and topotype-determining genes. Seg-2, together with Seg-5, encodes the proteins that define the BTV serotype, with the major contribution coming from VP2 (36). The complete gene sequences of VP2 and NS1 were determined by amplification of overlapping PCR products (three for segment 2 and two for segment 5) in the PCR assay, as previously described (35).

The cDNA was synthesized with primers specific to the ends of the BTV genome segments, using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, USA). The PCR was performed using specific primers and Quick-Load® Taq 2X Master Mix (NEB, USA). The PCR products were purified using a MinElute Gel Extraction Kit (Qiagen, USA). Sanger sequencing was used to assemble the complete sequences of Seg-2 and

Seg-5 from overlapping PCR amplicons. The sequencing was conducted using the same primers in both directions.

SeqScape® Software for Mutation Profiling v. 2.5 (Applied Biosystems, USA) was used to obtain a consensus sequence. The VP2 and NS1 gene sequences were aligned using ClustalX (37). The full-length VP2 and NS1 sequence data of a BTV isolate were subjected to blast analysis using the NCBI BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and were compared with sequences of commonly used reference strains and other strains represented in the GenBank (<https://www.ncbi.nlm.nih.gov/genbank>). Phylogenetic trees were constructed using the maximum likelihood method in which phylogenetic distances were estimated using Kimura's two-parameter model (K2P) (38) in the MEGA 7 program (39).

Entomological Surveillance

Entomological investigations were conducted in the territory of the Smolensk region in 2012. *Culicoides* biting midges were collected in May from farms where BTV-14-infected animals were detected, and trapped using a CDC light trap with UV light (OVI traps) using a common technique. The trap was elevated 1.5 m above the ground near the cows and was operated from dusk (1800) to dawn (0800).

The collected midges were cooled, pooled, and morphologically identified into the *Obsoletus* and *Pulicaris* complexes using the keys described by Glukhova (40).

To identify the complexes at the species level, midge pools were tested using PCR assays based on mitochondrial cytochrome c oxidase subunit I gene amplification (41). RNA from insect pools (100 midges) was isolated using Trizol (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations.

Statistical Analyses

Statistical analyses were performed in the program VassarStats (<http://vassarstats.net/>).

RESULTS

Blood and serum samples of the 71 heifers from the same consignment in the Smolensk region were tested for the BTV genome using real-time RT-PCR and for anti-BTV antibodies using ELISA at days 3, 27, 73, 104, 138, 189, and 207 of quarantine. At day 3 of quarantine, all the animals were tested negative by real-time RT-PCR and ELISA (Table 1). At day 27, as well as at days 73, 104, and 138, antibodies were detected in five animals (nos. 01051, 01224, 75981, 94835, and 10055), four of which (the exception was no. 94835) were RT-PCR positive for the BTV genome at 138 days (Table 1).

At days 189 and 207, BTV RNA was no longer detected in the blood of the five animals, although anti-BTV antibodies were still detected.

Virus Isolation

In the positive blood samples collected on day 27 of quarantine, the virus was isolated by inoculation into chicken embryos, followed by cultivation on Vero cells. After a 7-day incubation,

the virus was harvested at 5.0 lg TCID₅₀/cm³. A total of four BTV isolates were isolated.

The isolated BTV was capable of replication in different cell cultures (Vero, BHK-21/13, SK). The viral titer, depending on the cell line and the multiplicity of infection, ranged from 5.5 to 7.5 lg TCID₅₀/cm³. Viral reproduction led to the destruction of the cell monolayer at 3–5 days post-infection.

Virus Identification

The highest neutralization index (3.0 lg) was observed when the viruses isolated from the four animals (01051, 01224, 75981, and 10055) tested positive for reference BTV serotype 14 antiserum in the VNT. Simultaneously, the neutralization indexes when testing the virus samples with other reference sera did not exceed 0.75 lg, indicating that only the one serotype was present in the field.

The BTV-14 genome was detected in all blood samples of infected animals, as well as in the samples of infected chicken embryos and cell cultures that previously tested positive by universal real-time RT-PCR based on the NS3 protein. In the serotype-specific RT-PCR testing, the results were negative for BTV 1, 2, 4, 6, 8, 9, 11, and 16.

Thus, the virus isolated from the infected animals in the Smolensk region was identified as serotype 14 of BTV.

Molecular Characterization

The VP2 and NS1 gene sequences of the G244/11 strain were deposited in GenBank under the accession numbers KR233814.1 and MN746787, respectively. The Seg-2 and Seg-5 nucleotide sequences of the four isolated viruses were all identical. In the phylogenetic analysis based on Seg-2 sequences of the G244/11 strain and previously characterized reference strains, the result of virus typing by VNT and serotype-specific real-time RT-PCR was confirmed. Thus, in the maximum likelihood tree, the G244/11 strain was grouped with BTV strains of serotype 14 with a bootstrap confidence value of 54–100% (Figure 2). Phylogenetic inference was used to place the G244/11 strain in a group comprising serotypes 6, 14, and 21 (Nucleotide C).

The sequence of the VP2 gene (Seg-2) had 99.93% nucleotide identity with that of the VP2 of the RSArrrr/BTV 14 (AJ585135) strain that was part of a polyvalent vaccine preparation, which included serotypes 1, 4, 6, 12, and 14 (Onderstepoort Biological Products, South Africa). The Seg-2 sequence was also similar to the sequences of European BTV-14 strains SPA2012/01 (isolated in Spain from animals imported from Lithuania) and POL2012/01 (isolated in Poland), with nucleotide identity of 99.86 and 99.90%, respectively.

The NS1 gene analysis showed that G244/11 belonged to the western topotype, sharing 100% nucleotide identity with RSArrrr/14 and BT87/59, both being BTV-14.

Monitoring Survey

In 2011, BTV-14 was detected for the first time in the Russian Federation. As described previously, the virus was isolated only from cattle imported from Germany, during the quarantine period. To evaluate the incidence of BTV-14 among local livestock, local cattle, sheep, and goats grazing within a 300-km

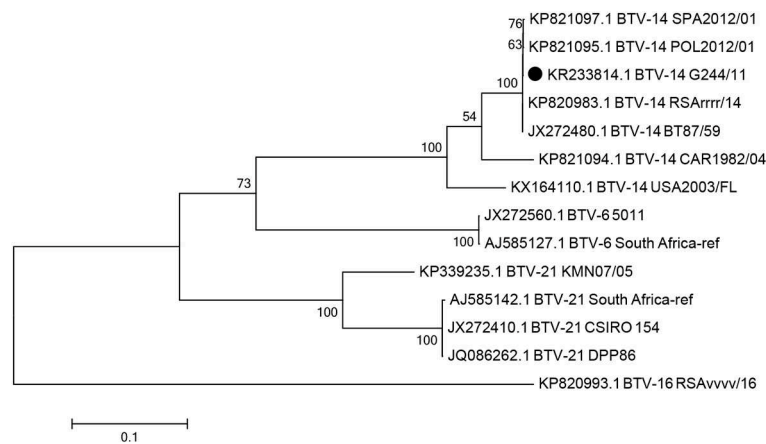


FIGURE 2 | The maximum likelihood phylogenetic tree showing the relationships of the complete Seg-2 sequences (2,922 nt) of the G244/11 strain and other BTV strains of the Nucleotype C (BTV-21, BTV-6, BTV-14). The phylogenetic tree was constructed using MEGA 7. The percentage of replicated trees in which the associated taxa clustered together in the bootstrap test (500 replicates) with a percentage higher than 54 are shown next to the branches. The Russian BTV G244/11 strain isolated in the Smolensk region in 2011 is indicated by the black circle.

TABLE 2 | Serological and virological investigation of local animals in the Smolensk and Kaluga regions during 2011–2012.

Region	Year	Animals	Number of animals	Seroprevalence	Number of PCR-positive animals (Ct)
Smolensk	2012	Cattle	1065	17.46%	85 (Ct 26.1–37.6)
		Sheep		(186/1065,	
		Goats		CI = 15.25–19.9)	
Kaluga	2012	Cattle	558	51.08%	98 (Ct 26.8–37.7)
		Sheep		(285/558,	
		Goats		CI = 46.85–55.29)	
Total			1623	29.02% (471/1623, CI = 26.83–31.31)	183 (Ct 26.1–37.7)

radius were sampled. To assess the prevalence of BTV infection, a serological and virological survey was conducted in local cattle, sheep, and goats from farms in the Smolensk and Kaluga regions between 2011 and 2012 (Figure 1).

A serological survey was conducted between 2011 and 2012 for specific antibodies against BTV using ELISA. The results for the seroprevalence of BT among animals in these regions are presented in Table 2. The seropositivity of cattle, sheep, and goats for BTV was 29.02% (471 of 1,623 animals, 95% CI = 26.83–31.31%). The highest seroprevalence was among ruminants in the Kaluga region (51.08%; 285 of 558 animals, 95% CI = 46.85–55.29%). Additionally, the BTV-14 genome was detected in 183 of the serologically positive animals (Ct values ranged from 26.1 to 37.6) by serotype-specific real-time RT-PCR and by universal real-time RT-PCR, whose results were 100% correlated (Table 2).

The negative results of the serotype-specific RT-PCR tests for BTV 1, 2, 4, 6, 8, 9, 11, and 16 eliminated the other serotypes and indicated that only the BTV-14 serotype was actively circulating in the populations of local animals in the studied regions.

Entomological Investigation

A total of 4,963 midges were collected, of which 366 (7.37%) belonged to the *Obsoletus* complex and 4,597 (92.63%) to the *Pulicaris* complex. Molecular analysis confirmed that the four species of the *C. pulicaris* complex (*C. pulicaris* s.s., *C. punctatus*, *C. impunctatus*, and *C. griseus*) were present in the pools of biting midges.

Four pools of *Obsoletus* complex midges and 46 pools of *Pulicaris* complex midges were tested. Among them, only three pools of *Pulicaris* complex midges tested positive for BTV-14 RNA, with Ct values ranging from 31.3 to 33.9. The *Obsoletus* complex midges had no detectable BTV genome. When Seg-2 was sequenced, the sequences of the positive midge pools proved identical to those of the animals, indicating the same virus.

DISCUSSION

The key factors driving the incursions, transmission, and overwintering of BTV in Europe remain poorly understood (42). In particular, the unexpected incursions into new territories, of which the arrival of BTV-8 in Northern Europe is a good example (43), require elucidation.

The Russian territory bordering Europe experiences prolonged, cold winters followed by relatively short summers, which do not seem to favor the spread of BTV and make overwintering unlikely (44). Nevertheless, midges are abundant across the country (45). The first peaks of blood-sucking midge activity occur as early as late May, and secondary peaks, although less abundant, occur in July in Western Russia, dwindling dramatically in August (44, 46). However, the genetics and vectorial capacity of Russian midges remain to be poorly understood to conduct a risk analysis.

The serendipitous detection of BTV-14 in the cattle in the Smolensk region in 2011–2012 represents the first historical incidence of BTV in the European part of Russia in which

antibodies and live viruses were identified in local livestock. The circulation of the live virus was confirmed by detection of BTV-14 in the neighboring Smolensk and Kaluga regions in 2012 (Figure 1, Table 2), as well as in Poland in 2012 and 2014 (47).

Sequencing of the VP2 (Seg-2) and NS1 (Seg-5) genes resulted in the Russian BTV-14 strain being grouped with a vaccine-like BTV-14 strain used in African countries (Figure 2). In addition to these findings, a related BTV-14 strain was also documented in Poland in animals near the Polish–Belarusian and Polish–Lithuanian borders in 2012 (47). Notably, Russian strain G244/11, strain SPA2012/01 detected in cattle in Spain imported from Lithuania, and Polish strain POL2012/01 shared approximately 99.9% identity based on the VP2 gene. However, the Russian strain carried two non-synonymous substitutions at position 1622 (C/T) and position 2427 (A/G) that were missing from the Polish and Lithuanian strains. This observation indicates the circulation of a live BTV-14 vaccine strain that spread asymptotically and evolved in the field, likely in midges, before its detection in the Russian territory in 2011. The different BTV-14 strains were likely derived from a common source. However, when compared with the North American strains BTV-14, CAR1982/04, and USA2003/FL, Seq-2 of G244/11 shared 87.4 and 87.2% identity, respectively.

According to data obtained from Rosselkhozinform (Russia), there was no evidence of infection in local animals before 2011 or after 2012 (<http://fsvps.ru/fsvps/iac/rf/reports.html>). However, 58 seropositive animals were identified in the Kaluga region in 2010 during monitoring studies in Russia ($n = 39,113$ during 2009–2010) (<http://fsvps.ru/fsvps/iac/rf/reports.html>). In blood serum samples ($n = 1963$) collected in 2011 from different regions of Poland, antibodies against BTV were detected in 494 animals. However, no virus or viral genome was detected (47). Currently, no information is available on the prevalence of the disease in past decades in Belarus. Thus, the initial source of infection remains unclear.

Notably, the animals did not present with any clinical signs and remained asymptomatic throughout the outbreak, which significantly limited understanding of BTV epidemiology in the moderate continental climate. The third day of quarantine occurred in early September 2011, whereas the 27th day (when the virus could still be recovered) occurred in late September. The average temperature at dusk between the dates was 7–13°C. This range comprised temperatures that include the minimum temperature or even outside the optimal range for flight activity of midges at this time of the year in Smolensk (44–46). In addition, little actual flight activity was recorded during this period. Thus, the entomological evidence does not support the hypothesis that the outbreak originated in Russia with the animals becoming infected during quarantine. Moreover, although the infectious virus was recovered in late September (day 27), for infection to occur, the midges had to be active and abundant, which was not the case in Smolensk in September 2011. Therefore, the most likely scenario is that the heifers were infected en route in Poland or Belorussia, which have a milder climate and where, from May to September, the highest midge activity is observed (47). The failure to detect the virus at day 3 of the quarantine may be attributable to the incubation period of the

disease. In this study, BTV RNA could be detected for only 111 days (Table 1), although the maximum period for which RNA can be detected in infected animals is up to 160 days (48).

Due to this outbreak and the following report by Russia in 2011 (Ref. OIE: 11439, Report Date: 30/12/2011, Country: Russia), the attention of the World Organization for Animal Health (OIE)¹ was drawn and BTV-14 became the focus of attention in Eastern Europe beginning in 2012 (47, 49). Although Russia was the first to report BTV-14, the report followed the import and testing of the consignment of heifers and therefore does not mean that the infection first appeared in Russia. The infection may have appeared elsewhere, and spread over a long distance via midges that are not bound by national borders.

Importantly, a live vaccine containing BTV-14 is not available on either the EU or the Russian market, which indeed suggests previous illegal use of a BTV-14-based vaccine. Although no evidence is available to support this contention, according to Nomikou et al. (49, 50), whole-genome sequencing data suggest that BTV-14 strains that have spread in Europe and Russia are reassortants containing genome segments derived from different reference vaccine strains originating from South Africa (49, 50). Nevertheless, although a possible vaccine incident is suggested as the source of the infection, consistent with the sequencing data presented herein, evidence of the illegal use of a live vaccine is lacking, as previously noted. Moreover, the use of a polyvalent vaccine preparation that included serotypes 1, 4, 6, 12, and 14 (Onderstepoort Biological Products, South Africa) does not seem logical, because the greatest threat to this area is associated with the BTV-8 serotype.

A possible alternative explanation for the present incidence is infection via BTV-contaminated fetal bovine serum, which is used as a semen extender for artificial insemination of cattle (51). Unfortunately, data on the extent of its use in the farming industry are not available. In addition, contaminated serum or other components may have been used to produce other live vaccines for cattle or small ruminants, which have already shown potential for contamination with BTV, as reported for a sheep-pox vaccine (16). Following vaccination, the contaminating BTV can escape and begin to circulate in the field. This route seems most plausible for the reported case; however, this hypothesis lacks evidence on what type of vaccine may have been contaminated.

Collectively, the literature indicates that blood-sucking insects drive BTV transmission across countries (8, 19). The reassortant nature of BTV-14 in Eastern Europe suggests that midges are most likely responsible for the BTV diversity (49, 52). Nevertheless, the vector species for the virus in Western Europe remains to be determined.

Entomological surveillance conducted in the Smolensk and Kaluga regions in 2012 demonstrated detection of the BTV-14 genome in midges of the *C. pulicaris* complex but not in those of the *C. obsoletus* complex. This study is the first to show that midges in Russia can potentially serve as a vector for BTV. However, this claim is made with the following caveats. In this

¹https://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Disease_cards/BLUETONGUE.pdf.

study, midges were not sorted into nulliparous and engorged midges, and the focus was only on PCR positivity. Therefore, the results could indicate a positive blood meal rather than infected midge heads. This finding warrants further study to determine whether Russian midges can potentially be infected and subsequently transmit BTV. Another interesting observation was that *Pulicaris complex* midges dominated the catch, although Sprygin et al. (44) reported that *Obsoletus complex* midges are very abundant in the Smolensk region. In addition, although BTV RNA was not detected in *Obsoletus complex* midges, only four pools were tested.

Another limitation of the current study is that archived samples approximately 1 year before importation of the animals could not be accessed. This information would have provided insight into the actual prevalence of BTV-14 in the area during that period.

To obtain an update on the epidemiology of BTV in Russia, including serotype 14, an annual monitoring survey has been conducted. According to published reports by Rosselkhozinform, no infected animals were detected from 2013 to 2019 in Russia ($n = 217,286$) (<http://fsvps.ru/fsvps/iac/rf/reports.html>).

In summary, this study describes the detection of asymptomatic BTV-14 infection in cattle in the Smolensk region of Russia in 2011. The identified BTV strain was genetically related to a vaccine BTV-14 strain, based on Seg-2 sequencing. However, the origin of the outbreak remains speculative. Although the possible mechanism of spread seems to be via vector bites because the collected midges tested positive for BTV dsRNA, this assumption merits further investigation. Elucidation of the mechanism involved would provide crucial insights into the circulation and overwintering of BTV in northern latitudes.

REFERENCES

1. Spreull J. Malarial catarrhal fever (bluetongue) of sheep in South Africa. *J Comp Pathol Ther.* (1905) 18:321–37. doi: 10.1016/s0368-1742(05)80073-6
2. Mellor PS, Baylis M, Peter PC, Mertens PP. *Bluetongue, 1st Ed.* London: Academic Press Elsevier (2009). p. 167–96.
3. Gouet P, Diprose JM, Grimes JM, Malby R, Burroughs JN, Zientara S, et al. The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography. *Cell.* (1999) 97:481–90. doi: 10.1016/S0092-8674(00)80758-8
4. Mertens P, Diprose J, Singh K, Attoui H, Maan S, Samuel A. Bluetongue virus replication, molecular and structural biology. *Vet Ital.* (2004) 40:426–37.
5. Mellor PS. The replication of bluetongue virus in Culicoides vectors. *Curr Top Microbiol Immunol.* (1990) 162:143–61.
6. Mellor PS, Boorman J, Baylis M. Culicoides biting midges: their role as arbovirus vectors. *Annu Rev Entomol.* (2000) 45:307–40. doi: 10.1146/annurev.ento.45.1.307
7. Wilson AJ, Mellor PS. Bluetongue in Europe: past, present and future. *Philos Trans R Soc Lond B Biol Sci.* (2009) 364:2669–81. doi: 10.1098/rstb.2009.0091
8. Koenraadt CJM, Balenghien T, Carpenter S, Ducheyne E, Elbers ARW, Fife M, et al. Bluetongue, Schmallenberg - what is next? Culicoides-borne viral diseases in the 21st century. *BMC Vet Res.* (2014) 10:77. doi: 10.1186/1746-6148-10-77
9. Bouwknegt C, van Rijn PA, Schipper JJM, Hölzel D, Boonstra J, Nijhof AM, et al. Potential role of ticks as vectors of bluetongue virus. *Exp Appl Acarol.* (2010) 52:183–92. doi: 10.1007/s10493-010-9359-7
10. Maan S, Maan NS, Nomikou K, Batten C, Antony F, Belaganahalli MN, et al. Novel bluetongue virus serotype from Kuwait. *Emerg Infect Dis.* (2011) 17:886–9. doi: 10.3201/eid1705.101742
11. Zientara S, Sailleau C, Viarouge C, Höper D, Beer M, Jenckel M, et al. Novel bluetongue virus in goats, Corsica, France, 2014. *Emerg Infect Dis.* (2014) 20:2123–5. doi: 10.3201/eid2012.140924
12. Jenckel M, Breard E, Schulz C, Sailleau C, Viarouge C, Hoffmann B, et al. Complete coding genome sequence of putative novel bluetongue virus serotype 27. *Genome Announc.* (2015) 3:e00016-15. doi: 10.1128/genomeA.00016-15
13. Schulz C, Bréard E, Sailleau C, Jenckel M, Viarouge C, Vitour D, et al. Bluetongue virus serotype 27: detection and characterization of two novel variants in Corsica, France. *J Gen Virol.* (2016) 97:2073–83. doi: 10.1099/jgv.0.000557
14. Sun EC, Huang LP, Xu QY, Wang HX, Xue XM, Lu P, et al. (2016). Emergence of a Novel Bluetongue Virus Serotype, China 2014. *Transbound Emerg Dis.* 63:585–9. doi: 10.1111/tbed.12560
15. Savini G, Puggioni G, Meloni G, Marcacci M, Di Domenico M, Rocchigiani AM, et al. Novel putative Bluetongue virus in healthy goats from Sardinia, Italy. *Infect Genet Evol.* 51:108–17. (2017). doi: 10.1016/j.meegid.2017.03.021
16. Bumarov V, Golender N, Jenckel M, Wernike K, Beer M, Khinich E, et al. Characterization of bluetongue virus serotype 28. *Transbound Emerg Dis.* (2019) 67:171–82. doi: 10.1111/tbed.13338
17. Menzies FD, McCullough SJ, McKeown IM, Forster JL, Jess S, Batten C, et al. Evidence for transplacental and contact transmission of bluetongue virus in cattle. *Vet Rec.* (2008) 163:203–9. doi: 10.1136/vr.163.7.203

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the <https://www.ncbi.nlm.nih.gov/nuccore/MN746787>, <https://www.ncbi.nlm.nih.gov/nuccore/KR233814.1>.

ETHICS STATEMENT

An ethical review was not required for this study, according to local and national legislation, because this work does not contain any experiments on animals. Blood and serum samples from cattle, sheep and goats were collected using standard procedures to avoid the suffering of animals and followed the guidelines of the State Veterinary Service of the Russian Federation. Farmers consented for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

ST and DK contributed to the conception and the design of the study. AK conducted field and laboratory work. AG participated in epidemiological data analysis. ST, AK, and GK performed laboratory data analysis. GK and AK wrote and reviewed the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We thank Agnessa Panferova for assistance with the molecular genetic research and Marina Novikova for help with virus isolation and conducting the VNT. The authors thank all persons involved in BT laboratory diagnosis at the Federal Research Center for Virology and Microbiology.

18. Batten CA, Maan S, Shaw AE, Maan NS, Mertens PPC. A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment. *Virus Res.* (2008) 137:56–63. doi: 10.1016/j.virusres.2008.05.016
19. Maclachlan NJ, Mayo CE. Potential strategies for control of bluetongue, a globally emerging, Culicoides-transmitted viral disease of ruminant livestock and wildlife. *Antiviral Res.* (2013) 99:79–90. doi: 10.1016/j.antiviral.2013.04.021
20. Sapre SN. An outbreak of bluetongue in goats and sheep. *Vet Rev.* (1964) 15:78–80.
21. George TDS, Standfast HA, Cybinski DH, Dyce AL, Muller MJ, Doherty RL, et al. The isolation of a bluetongue virus from Culicoides collected in the Northern Territory of Australia. *Aust Vet J.* (1978) 54:153–4. doi: 10.1111/j.1751-0813.1978.tb05539.x
22. Sperlova A, Zendulkova D. Bluetongue: a review. *Vet Med.* (2011) 56:430–52.
23. Saegerman C, Berkvens D, Mellor PS. Bluetongue epidemiology in the European Union. *Emerg Infect Dis.* (2008) 14:539–44. doi: 10.3201/eid1404.071441
24. Meiswinkel R, Baldet T, de Deken R, Takken W, Delécolle JC, Mellor PS. The 2006 outbreak of bluetongue in northern Europe—The entomological perspective. *Prev Vet Med.* (2008) 87:55–63. doi: 10.1016/j.prevetmed.2008.06.005
25. Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PPC, Baylis M. Climate change and the recent emergence of bluetongue in Europe. *Nat Rev Microbiol.* (2005) 3:171–81. doi: 10.1038/nrmicro1090
26. Maclachlan NJ. Global implications of the recent emergence of bluetongue virus in Europe. *Vet Clin North Am Food Anim Pract.* (2010) 26:163–71. doi: 10.1016/j.cvfa.2009.10.012
27. Vishnyakov IF, Strizhakov AA, Novikova MB, Lunitsin AV, Kurinnov BV, Karpov GM, et al. Identification and typing of sheep catarrh fever virus (in Russian). *Vet.* (1995) 4:20–5.
28. Vyalykh IV, Fedorov GP, Nogina IV, Kurinnov VV, Novikova MB. Bluetongue virus isolation from imported cattle (in Russian). *Vet.* (2010) 8:23–6.
29. Clavijo A, Heckert RA, Dulac GC, Afshar A. Isolation and identification of bluetongue virus. *J Virol Methods.* (2000) 87:13–23. doi: 10.1016/S0166-0934(00)00150-6
30. Wechsler SJ, Luedke AJ. Detection of bluetongue virus by using bovine endothelial cells and embryonated chicken eggs. *J Clin Microbiol.* (1991) 29:212–4.
31. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. *Am J Epidemiol.* (1938) 27:493–7. doi: 10.1093/oxfordjournals.aje.a118408
32. Hofmann MA, Renzullo S, Mader M, Chaignat V, Worwa G, Thuer B. Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. *Emerg Infect Dis.* (2008) 14:1855–61. doi: 10.3201/eid1412.080818
33. Panferova AV, Koltsov AY, Tsybanov SZh, Krasochko PA, Kolbasov DV. Development of real time RT-PCR to identify bluetongue serotype 14 virus (in Russian). *Biotechnology.* (2013) 3:81–5.
34. Panferova AV. *Genotyping of bluetongue virus serotypes* (dissertation). Russian Federation, Pokrov, Russia (2012).
35. Koltsov AY. *Development of molecular genetic analysis tools for Bluetongue virus 14 serotype isolated in the Russian federation* (dissertation). Russian Federation, Volginsky, Russia (2017).
36. Maan S, Maan NS, Samuel AR, Rao S, Attoui H, Mertens PPC. Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. *J Gen Virol.* (2007) 88:621–30. doi: 10.1099/vir.0.82456-0
37. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* (1997) 25:4876–82. doi: 10.1093/nar/25.24.4876
38. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol.* (1980) 16:111–20. doi: 10.1007/BF01731581
39. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. *Mol Biol Evol.* (2016) 33:1870–4. doi: 10.1093/molbev/msw054
40. Glukhova VM. *Blood-sucking Midges Genera Culicoides and Forcipomyia (Ceratopogonidae)*. Fauna of the USSR. Insects, Diptera. Volume 3, Issue 5. Leningrad, Nauka. (in Russian). (1989).
41. Nolan DV, Carpenter S, Barber J, Mellor PS, Dallas JF, Mordue AJ, et al. Rapid diagnostic PCR assays for members of the Culicoides obsoletus and Culicoides pulicaris species complexes, implicated vectors of bluetongue virus in Europe. *Vet Microbiol.* (2007) 124:82–94. doi: 10.1016/j.vetmic.2007.03.019
42. Jacquot M, Nomikou K, Palmarini M, Mertens P, Biek R. Bluetongue virus spread in Europe is a consequence of climatic, landscape and vertebrate host factors as revealed by phylogeographic inference. *Proc R Soc Lond B Biol Sci.* (2017) 284:20170919. doi: 10.1098/rspb.2017.0919
43. Dal Pozzo F, Saegerman C, Thiry E. Bovine infection with bluetongue virus with special emphasis on European serotype 8. *Vet J.* (2009) 182:142–51. doi: 10.1016/j.tvjl.2009.05.004
44. Sprygin AV, Fiodorova OA, Babin YY, Kononov AV, Karaulov AK. Blood-sucking midges from the genus Culicoides (diptera:ceratopogonidae) act as field vectors of human and animal diseases (review). *Sel'skokhozyaistvennaya Biologiya.* (2015) 50:183–97. doi: 10.15389/agrobiol.2015.2.183eng
45. Sprygin AV, Fiodorova OA, Babin YY, Elatkin NP, Mathieu B, England ME, et al. Culicoides biting midges (Diptera, Ceratopogonidae) in various climatic zones of Russia and adjacent lands. *J Vector Ecol.* (2014) 39:306–15. doi: 10.1111/jvec.12105
46. Sprygin A, Fiodorova O, Babin Y, Pavelko V, Bellis G, Kononov A. Seasonal population fluctuations of Culicoides midges in the Smolenskaya, Pskovskaya and Vladimirskaia provinces of Western Russia in 2013. *Vet Ital.* (2016) 52:231–4. doi: 10.12834/VetIt.254.2305.3
47. Orłowska A, Trebas P, Smreczak M, Marzec A, Zmudzinski JF. First detection of bluetongue virus serotype 14 in Poland. *Arch Virol.* (2016) 161:1969–72. doi: 10.1007/s00705-016-2857-0
48. Katz J, Alstad D, Gustafson G, Evermann J. Diagnostic analysis of the prolonged bluetongue virus RNA presence found in the blood of naturally infected cattle and experimentally infected sheep. *J Vet Diagn Invest.* (1994) 6:139–42. doi: 10.1177/104063879400600201
49. Nomikou K, Batten C, Morecroft E, Vigo Martin M, Kolbasov D, Frost L, et al. Molecular characterization of BTV-14 in North Europe. In: *Bluetongue and Related Orbiviruses. IV International Conference*. Rome (2014).
50. Nomikou K, Hughes J, Wash R, Kellam P, Breard E, Zientara S, et al. Widespread reassortment shapes the evolution and epidemiology of bluetongue virus following European invasion. *PLoS Pathog.* (2015) 11:e1005056. doi: 10.1371/journal.ppat.1005056
51. Evermann JF. Letter to the Editor, regarding *Bluetongue virus* and canine abortions. *J Vet Diagn Invest.* (2013) 25:670. doi: 10.1177/1040638713504535
52. Caporale M, Di Gialleonardo L, Janowicz A, Wilkie G, Shaw A, Savini G, et al. Virus and host factors affecting the clinical outcome of bluetongue virus infection. *J Virol.* (2014) 88:10399–411. doi: 10.1128/jvi.01641-14

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Koltsov, Tsybanov, Gogin, Kolbasov and Koltsova. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Bluetongue Serotype 3 in Israel 2013–2018: Clinical Manifestations of the Disease and Molecular Characterization of Israeli Strains

Natalia Golender^{1*}, Velizar Bumbarov¹, Avi Eldar¹, Alessio Lorusso², Gabriel Kenigswald³, Joseph Seffi Varsano⁴, Dan David¹, Shani Schainin³, Ilan Dagoni³, Iosef Gur³, Alon Kaplan³, Anna Gorohov¹, Ori Koren³, Eldad Oron³, Yevgeny Khinich¹, Ily Sclamovich³, Abraham Meir³ and Giovanni Savini²

¹ Department of Virology, Kimron Veterinary Institute, Bet Dagan, Israel, ² OIE Reference Laboratory for Bluetongue, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM), Campo Boario, Teramo, Italy, ³ Hachaklait Veterinary Services, Caesarea, Israel, ⁴ Indi-Vet Ltd., Ashdod, Israel

OPEN ACCESS

Edited by:

Jesus Hernandez,
Centro de Investigación en
Alimentación y Desarrollo
(CIAD), Mexico

Reviewed by:

Semmannan Kalaiyarasu,
ICAR-National Institute of High
Security Animal Diseases
(ICAR-NIHSAD), India
Muhammad Zubair Shabbir,
University of Veterinary and Animal
Sciences, Pakistan

*Correspondence:

Natalia Golender
golendern@moag.gov.il

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 29 September 2019

Accepted: 13 February 2020

Published: 06 March 2020

Citation:

Golender N, Bumbarov V, Eldar A, Lorusso A, Kenigswald G, Varsano JS, David D, Schainin S, Dagoni I, Gur I, Kaplan A, Gorohov A, Koren O, Oron E, Khinich Y, Sclamovich I, Meir A and Savini G (2020) Bluetongue Serotype 3 in Israel 2013–2018: Clinical Manifestations of the Disease and Molecular Characterization of Israeli Strains. *Front. Vet. Sci.* 7:112. doi: 10.3389/fvets.2020.00112

In this paper, the results of the diagnostic activities on Bluetongue virus serotype 3 (BTV-3) conducted at Kimron Veterinary Institute (Beit Dagan, Israel) between 2013 and 2018 are reported. Bluetongue virus is the causative agent of bluetongue (BT), a disease of ruminants, mostly transmitted by competent *Culicoides* species. In Israel, BTV-3 circulation was first detected in 2013 from a sheep showing classical BT clinical signs. It was also evidenced in 2016, and, since then, it has been regularly detected in Israeli livestock. Between 2013 and 2017, BTV-3 outbreaks were limited in sheep flocks located in the southern area only. In 2018, BTV-3 was instead found in the Israeli coastal area being one of the dominant BTV serotypes isolated from symptomatic sheep, cattle and goats. In Israeli sheep, BTV-3 was able to cause BT classical clinical manifestations and fatalities, while in cattle and goats infection ranged from asymptomatic forms to death cases, depending on either general welfare of the herds or on the occurrence of viral and bacterial co-infections. Three different BTV-3 strains were identified in Israel between 2013 and 2018: ISR-2019/13 isolated in 2013, ISR-2153/16 and ISR-2262/2/16 isolated in 2016. Sequencing and phylogenetic analysis of these strains showed more than 99% identity by segment (Seg) 2, 5, 6, 7, and 8 sequences. In contrast, a wide range of diversity among these strains was exhibited in other viral gene segments, implying the occurrence of genome reassortment between these local circulating strains and those originating from Africa. The genome sequences of the BTV-3 isolated in 2017 and 2018 were most closely related to those of the ISR-2153/16 strain suggesting their common ancestor. Comparison of BTV-3 Israeli strains with those recently detected in the Mediterranean region uncovered high percentage identity (98.19–98.28%) only between Seg-2 of all Israeli strains and the BTV-3 Zarzis/TUN2016 strain. A 98.93% identity was also observed between Seg-4 sequences of ISR-2019/13 and the BTV-3 Zarzis/TUN2016 strain. This study demonstrated that BTV-3 has been circulating in the Mediterranean region at least since 2013, but, unlike the other Mediterranean strains, Israeli BTV-3 were able to cause clinical signs also in cattle.

Keywords: orbivirus, sheep, cattle, clinical signs, phylogeny, descriptive epidemiology

INTRODUCTION

Bluetongue (BT) is a non-contagious, arthropod-borne viral disease of domestic and wild ruminants, listed as a notifiable disease by the World Organization for Animal Health (OIE). The Bluetongue virus (BTV) is the prototype member of the *Orbivirus* genus within the *Reoviridae* family (1, 2).

BTV is a double-stranded (ds) RNA virus (3), its genome consists of ten segments (Seg-1 to Seg-10) of linear dsRNA coding 7 structural (VP1–VP7) and 5 non-structural (NS1, NS2, NS3/NS3a, NS4, and NS5) proteins (4). At present twenty-eighth distinct BTV serotypes have been officially recognized based on Seg-2 gene sequence (5, 6). Other putative novel BTV serotypes have also been described (7–11).

Transmission between mammalian hosts and spread of the infection rely mostly on competent *Culicoides* species (12–14), so the presence of the disease is then strictly related to the distribution of competent vectors (2). Even though they don't seem to be epidemiologically important, vertical and horizontal transmissions have also been described (15–18). For BTV-26, BTV-27 v02 and, probably, BTV-X ITL2015 and BTV-28 transmission by direct contact has been demonstrated or hypothesized (6, 10, 19, 20).

Clinical signs of BT are more severe and most commonly observed in sheep or in white-tailed deer, often leading to animal fatality especially in naïve animals (1, 2). In cattle, BTV infection is usually asymptomatic, although symptoms were reported after infection with some strains (21–24).

As a RNA virus with a segmented genome, BTV can undergo reassortment which can occur when a cell is simultaneously infected with more than one BTV strain and involves the packaging, into a single virion, of full length of genomic segments of different ancestry. Reassortment in BTV is very flexible, and can involve any genome segment (25–27). However, the genome sequence of BTV isolates generally reflects their geographic origins (28–30).

Seg-2 and Seg-6 encoded the BTV outer capsid proteins VP2 and VP5. They represent the primary target for neutralizing antibodies generated during infection of the mammalian host (31–33). Their highly variable sequences are associated with virus serotype (particularly in VP2/segment) (34–36) and, within each serotype, with the geographic origin of the virus strain (28, 34–37).

Among BTV serotypes, Seg-2/VP2 sequences of BTV-3, BTV-16, and BTV-13 are closely related. All of them are included within the nucleotide B reflecting a serological relationship (3). BTV-3 strains can be subdivided into at least two main clusters. 1st cluster includes strains originated from Africa, Mediterranean Basin and North America (western topotypes, w); 2nd-cluster includes strains originated from Japan, India, and Australia (eastern topotypes, e). The nucleotide (nt) identity between Western and Eastern topotype Seg-2 can be as small as 71.5% (7, 38–43).

Between 2016 and 2018, two novel BTV-3 western strains have been identified in two different geographical areas of Tunisia: one in the north-eastern part of the country (Peninsula of Cap Bon, prototype BTV-3 TUN2016) and the other in the South-East near by the border with Libya (prototype strain BTV-3

TUN2016/Zarzis). The BTV-3 TUN2016 spread in 2017 to Italy infecting a single 3-year-old female crossbred sheep belonging to a flock located in the municipality of Trapani, Sicily, which are 150 km distant from Peninsula of Cap Bon (7, 39, 40) and in 2018 in the Southern area of Sardinia causing numerous outbreaks (38). Clinical signs in infected sheep included depression, fever, nasal discharge, submandibular edema, and crusted discharge around the nostrils. Four animals died because of the severity of infection (38). In 2016, another BTV-3 strain closely related to TUN2016/Zarzis strain was detected in Egypt (38).

The present paper reports the results of the diagnostic activities on BTV conducted at Kimron Veterinary Institute between 2013 and 2018 and the evidence of BTV-3 circulation in Israel. Clinical signs of infected sheep, goats and cattle along with the genetic characterization and phylogenetic analysis of the BTV-3 strains are also described.

MATERIALS AND METHODS

Field Samples

During years 2013, 2016, 2017, and 2018, 3,149 samples (714 in 2013, 669 in 2016, 744 in 2017, and 1,022 in 2018) from domestic and wild ruminants were collected and examined at the Kimron Institute, Beit Dagan, Israel (KVI). Samples included whole blood from symptomatic animals, spleen or/and lung samples from dead animals and spleen, lung, placenta and brain samples from aborted fetuses. Details on number, samples, species from which samples were collected are shown in **Table 1**.

Clinical and Epidemiological Follow Up

Farms where domestic clinically ill ruminants were confirmed by laboratory tests as BTV-3 infected were recorded and numbered in **Table 3**. Information in **Table 3** included location of the farm or animal grazing place, type of farming, breed, and number of animals in the farm/group. All clinical events observed in the farms were followed and reported (**Table 4**).

Laboratory Tests

Internal organs were examined for aerobic and anaerobic bacteria and *Salmonella* spp. growth according to standard procedures (45).

From all field samples and virus isolates (chicken embryo homogenate and tissue culture supernatant) viral RNA was extracted using Invisorb Spin Virus RNA Mini Kit (STRATEC Molecular, Berlin, Germany), and by MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific), according to recommendations of manufacturers. To detect bovine ephemeral fever virus (BEF), the RNA extraction and RT-qPCR were performed on white blood cells from whole EDTA blood samples of cattle origin according to Erster et al. (46).

Presence of Malignant Catarrhal Fever virus (MCFV) DNA was determined according to the method described by Cunha (47). Most samples from cattle were also tested for the presence of Epizootic Hemorrhagic disease virus (EHDV) RNA by EHDV Real-Time PCR Kit (Applied Biosystems™, Thermo Fisher Scientific Inc., Lissieu, France) according to manufacturer's instructions. The method described by Boxus (48) was used to

TABLE 1 | Field samples tested by Pan-BTV RT-qPCR from different kinds of domestic and wild/zoo ill or dead animals and subsequent virus isolations during 2013, and 2016–2018.

Species		Cattle			Sheep			Goat			Wild ruminants			Total samples	Total VI
Year		w.b.	s/l	a.f.	w.b.	s/l	a.f.	w.b.	s.	a.f.	w.b.	s.	a.f.		
2013	No. of tested samples	564	10	3	68	28	3	6	12	2	0	16	2	714	
	No. of pos. samples	113	3	1	18	13	2	0	5	1	0	2	0	158	
	No. of isolated BTV-2	1													1
	No. of isolated BTV-3				1										1
	No. of isolated BTV-4	1			6										7
	No. of isolated BTV-16	3			3										6
2016	No. of tested samples	484	41	10	73	20	18	1	4	5	0	11	2	669	
	No. of pos. samples	159	13	1	49	5	0	0	1	0	0	0	0	228	
	No. of isolated BTV-2				3										3
	No. of isolated BTV-3				3										3
	No. of isolated BTV-4	1													1
	No. of isolated BTV-8	24	1		26										51
2017	No. of tested samples	402	35	7	173	41	10	9	24	0	17	23	3	744	
	No. of pos. samples	93	6	0	51	9	0	0	6	0	1	2	0	168	
	No. of isolated BTV-2	1													1
	No. of isolated BTV-3				4										4
	No. of isolated BTV-4	1			12										13
	No. of isolated BTV-6	6			10										16
2018	No. of tested samples	629	18	53	72	32	156	8	21	14	4	13	3	1,022	
	No. of pos. samples	217	6	1	36	0	6	3	2	0	0	0	0	271	
	No. of isolated BTV-2	1													1
	No. of isolated BTV-3	1			6			1							8
	No. of isolated BTV-4	1			9										10
	No. of isolated BTV-6				2										2
	No. of isolated BTV-15	9													9

No., number; w.b., whole blood samples; s/l, spleen or lung samples; s., spleen samples; a.f., aborted fetus; VI, virus isolation. Data from 2017 was also used in publication (44).

detect Bovine Respiratory Syncytial virus (BRSV) RNA from lung cattle samples, while an in-house RT-qPCR (unpublished) and Kishimoto et al. (49) methods were used for detecting bovine Parainfluenza 3 (BPI3) and bovine Corona virus (BCoV) RNAs, respectively. A specific RT-qPCR was used for detection and identification Simbu serogroup RNA viruses in plasma samples (50) and, finally, the VetMAXTM BTV NS3 All Genotypes Kit (Applied BiosystemsTM, Thermo Fisher Scientific Inc., Lissieu, France) RT-qPCR kit targeting Seg-10 of the BTV genome was used for detecting BTV RNA.

Those samples which were positive to the BTV RT-qPCR with Cycle Threshold (Ct) values ≤ 33 , were further tested for determining the serotype. Virotype[®] BTV pan/4 RT-PCR and Virotype[®] BTV pan/8 RT-PCR kits (QIAGEN, Leipzig, Germany) were applied directly to RNA extracted from diagnostic samples to detect and identify BTV-4 and BTV-8 serotypes, respectively. The same samples were also retroactively tested for the presence of BTV-3 by an in-house specific RT-qPCR according to the method described by Lorusso et al. (7).

In an attempt to isolate BTV, all BTV RT-qPCR positive samples were inoculated in 9 to 11 day old embryonated chicken

eggs (ECE) according to the method described by Komarov and Goldsmith (51) and adopted by Golender et al. (52). In the homogenate of the ECE organs, the presence of BTV was confirmed by BTV RT-qPCR. BTV-positive ECE homogenates were subsequently tested for the presence of the RNA of all BTV serotypes that have circulated in Israel and neighboring countries during the recent decades (BTV-2, -3, -4, -5, -8, -12, -15, -16, and -24) by using a conventional specific in-house RT-PCR (primers are listed in **Table S1**) using a One-Step RT-PCR kit (Qiagen, Hilden, Germany).

Primers 3VP2-1F (5'-GTT AAA AAC GCT GTC CCG AGA-3') and 3VP2-637R (5'-GAG CGC CCA CTC TAA ATT CCT C-3') targeting a portion of 658 bp of the Seg-2 of BTV-3 by RT-PCR were used for BTV-3 identification. All positive in in-house conventional serotype specific RT-PCR tested BT virus isolates from all serotypes were consequently sequenced.

All viral-segment amplicons of Israeli BTV-3 were sequenced by standard Sanger methods with an ABI 3730xl DNA Analyzer (Hylabs, Rehovot, Israel). The cDNA fragments were purified with a MEGAquick-spin Total Fragment DNA Purification Kit (iNtRON Biotechnology, Gyeonggi-do, South

TABLE 2 | Field samples tested by BTV serotype specific RT-qPCRs from different kinds of domestic ill or dead animals during 2018.

Species		Cattle		Sheep	Goat		Total
Specific RT-qPCR		w. b.	s/l	w. b.	w. b.	s.	
BTV-3	No. of tested samples	133	5	32	3	2	175
	No. of positive samples	13	2	8	1	1	25
BTV-4	No. of tested samples	133	5	32	3	2	175
	No. of positive samples	11	2	18	1	0	32
BTV-8	No. of tested samples	133	5	32	3	2	175
	No. of positive samples	0	0	0	0	0	0

No., number; w.b., whole blood samples, s/l, spleen or lung samples; s., spleen samples.

Korea). The resulting nucleotide sequences were assembled and nucleotide (nt) and amino acid (aa) sequences were aligned and pairwise compared by using Geneious version 9.0.5 (Biomatters, Auckland, New Zealand). Phylogenetic trees were constructed with the Mega 7.1 software (53).

RESULTS

BTV Detection by RT-qPCR From Field Samples

Data regarding the presence of BTV in the samples examined by BTV RT-qPCR between 2013 and 2018 are shown in **Table 1**. A total of 3,149 samples were tested for the presence of BTV. Of these 792 were found positive by BTV RT-qPCR. Of the 714 samples collected in 2013, 125 were found positive for BTV, 228 of the 669 collected in 2016, 168 of the 744 collected in 2017, and 271 of the 1,022 collected in 2018. Details on the BTV results obtained from the tested samples are also shown in **Table 1**.

Field Samples Serotyping by Specific RT-qPCR, 2018

Of the 271 field samples collected during 2018 and found positive for BTV by RT-qPCR, 175 (138 from cattle, 32 from sheep and 5 from goats) had Ct values of ≤ 33 . These positive samples were further tested by BTV-4, BTV-8 and BTV-3 specific RT-qPCR. Of the 175 BTV positive samples, 25 were found positive for BTV-3 (15 whole blood samples from 14 cattle; from one cattle blood samples were collected twice), 8 from sheep and 2 from goats) and 32 for BTV-4 (13 from cattle, 18 from sheep and 1 from a goat). Additional 2 samples, one newborn calf and one placenta from case of abortion in sheep, were found positive to in-house conventional RT-PCR unveiling Israeli BTV-4 strain in the both cases. One whole blood sample from sheep and 2 samples from cattle (one whole blood and one spleen samples), were contemporaneously positive for BTV-3 and BTV-4 in specific RT-qPCRs. All samples positive for BTV-4 were received between March and December 2018, while all samples positive for BTV-3 were received between August and December 2018. No BTV-8 positive samples were found in 2018 (**Table 2**).

Virus Isolation

Of the 792 BTV RT-PCR samples inoculated into ECE, 136 BTV strains were isolated, 15 were isolated from the batch of samples

collected in 2013, 59 from the batch of samples collected in 2016, 40 from the samples collected in 2017 and 25 from the samples collected in 2018. The 15 BTV strains isolated in 2013 were identified as BTV-2 ($n = 1$), BTV-3 ($n = 1$), BTV-4 ($n = 7$), and BTV-16 ($n = 6$). The 59 strains isolated in 2016 included BTV-2 ($n = 3$); BTV-3 ($n = 3$), BTV-4 ($n = 1$), BTV-8 ($n = 51$), and BTV-15 ($n = 1$). In one sample BTV-3 and BTV-8 mixed infection was found. The 40 BTV strains isolated in 2017 comprised BTV-2 ($n = 1$), BTV-3 ($n = 4$), BTV-4 ($n = 13$), BTV-6 ($n = 16$), and BTV-15 ($n = 6$) (**Table 1**). The 30 BTV isolated in 2018 included BTV-2 ($n = 1$), BTV-3 ($n = 8$), BTV-4 ($n = 10$), BTV-6 ($n = 2$), and BTV-15 ($n = 9$). In 4 occasions BTV-3 and BTV-4 mixed infection was observed (**Tables 1, 3, 4**). Notably, where only one out of four samples was positive in BTV-4 specific RT-qPCR, from which mixed BTV-3 and BTV-4 were isolated, probably pointing out undetected cases by BTV-4 RT-qPCR test.

Clinical and Epidemiological Follow Up Associated With BTV-3 Infection

In this study, BTV-3 was detected in 20 farms numbered from 1 to 21 (**Tables 3, 4**). From 2013 till 2017, BT-like symptoms were recorded from sheep flocks located in Southern Israel, only. In 2018, BTV-3 was detected in sheep, goat and cattle farms situated in southern and central areas of Israel (**Figure 1, Tables 3, 4**).

The main clinical signs in sheep included pyrexia, nasal discharge, hyperemia and ulceration of the oral and/or nasal mucosa, facial and/or thorax and hindlimbs edema, fatigue, apnea, recumbency, and few abortions. Morbidity in flocks was seen both in young and adult animals and ranged from 5 to 33.3%, with case mortality ranging from 0 to 30% (**Table 3**). In farms number 1, 2, 6, and 9, no additional pathogen was identified, while in farms number 3, 4, 5, 10, and 11 additional BTV serotypes were contemporaneously identified or/and isolated in the farm or/and in the same animals (BTV-4, 6, 8, **Tables 2, 3**). In one farm (number 17), where the only clinical sign was abortion, additionally to BTV-3, Shuni virus (SHUV) was detected in plasma sample from an ewe after abortion. Interestingly, in sheep farm from the southern part of Israel, BTV-3 recurrent infection was observed in fall period of 2017 and 2018 (farms number 4 and 9, **Tables 3, 4**). Moreover, cases of acutely affected animal showing classical BT clinical signs

TABLE 3 | Geographic localities and epidemiological aspects on sheep and cattle farms, where BTV-3 was identified.

Year	Locality/geographic zone/distinct	Farm	Species/No. of animals in the farm	Breed	Affected group	No. of dead animals/No. of ill animals/total animals in affected group	Morbidity/case mortality (%)
2013	Pri Gan/Negev desert/Southern Distinct	1	Sheep/no data	Merino, Romanov, Charolais	No data	No data	No data
2016	Moshav Tkuma/Negev desert/Southern Distinct	2	Sheep/400	Fin, Merino and Afek (Borulla)	All ages	10/50/400	12.5/20
2016	Mikne Dekel/Negev desert/Southern Distinct	3	Sheep/1250	Charolais	Adult ewes and 5-month old lambs	60/150/no data	No data/40
2017	Moshav Lachish/ Negev Desert/Southern Distinct	4	Sheep/750	Merino, Romanov, Asaf, Puld-Dorset	Lambs 4–15 month and primipara ewes	20–30/100/no data	No data/20–30
2017	Sde David/ Negev desert/Southern distinct	5	Sheep/450	Asaf x Merino	Lambs of different ages	10/50/150	33.3/20
2017	Rahat/Negev desert/Southern distinct	6	Sheep/no data	No data	No data	No data	No data/high
2018	Kfar Silver/Sothorn distinct	7	Goat/700	Alpine	Adult doe	1/1/350	0,14/100
2018	Moshav Avigdor/ Southern distinct	8	Cattle/ 550	Holstein-Friesian	Pregnant calf (24 m) and adult cows	4/4/550	0,72/100
2018	Moshav Lachish/ Negev Desert/Southern Distinct	9	Sheep/ 900	Merino, Romanov, Asaf, Puld-Dorset	Lambs 4–12 month	30–40/200/400	10/15–20
2018	Mikne Dekel/Negev desert/Southern Distinct	10	Sheep/1250	Charolais	Lambs and adult ewes	5/no data/1250	<1/no data
2018	Moshav Nordia/Sharon plain/Central distinct	11	Sheep/2200	Asaf	Lambs and adult ewes	No data/no data/2200	No data
2018	Kfar Maimun/ Southern Distinct	12	Goat/400	Saanen	Pregnant does	0/40/150	0/0
2018	Kibbutz Kvutsat Yavne/ Southern coastal plate/Sothorn distinct	13	Cattle/760	Holstein-Friesian	2 adult pregnant cows	2/2/400	0.5/100
2018	Moshav Beer Tuvya/ Shfela/Southern Distinct	14	Cattle/ no data	Holstein-Friesian	Adult bulls	No data	No data
2018	Moshav Nir Galim/ Southern coastal plain/ Southern distinct	15	Cattle/ 800	Holstein-Friesian	4 month old calves	15/20/400	5/75
2018	Kfar Vitkin/ Hefer Valley/ Central distinct	16a	Cattle/185	Holstein-Friesian	18-month old female calf	0/1/30	3.3/0
2018	Kfar Vitkin/ Hefer Valley/ Central distinct	16b	Cattle/400	Holstein-Friesian	18-month old female calf	0/1/180	0.56/0
2018	Havat Shikmim/ Negev desert/Southern distinct	17	Sheep/4000	Charolais	Adult ewes	0/100/2000	5/0
2018	Beit Yitzhak/ Central distinct	18	Cattle/200	Holstein-Friesian	One-year-old female calf and one adult cow	2/2/200	1/100
2018	Kfar Shmuel/ Shfela/ Central distinct	19	Cattle/120	Holstein-Friesian	An adult cow	0/1/70	1.3/0
2018	Kibbutz Berot Ithak/ Central distinct	20	Cattle/574	Holstein-Friesian	A calf and an adult milking cow	0/2/574	0.35/50
2018	Kibbutz Yad Mordechai/Southern Distinct	21	Cattle/1200	Holstein-Friesian	A single one-year-old female calf	0/1/100	1/0

were seen in this farm during 2 month period and confirmed by successful virus isolation (Table 4).

In 2018, BTV-3 was also detected in two goat farms (Tables 3, 4). One was detected in the spleen of a sudden dead goat and the other in a blood sample collected from a doe right after abortion. In the first case, the low BTV-3 load (Ct 32) found in the spleen was the only laboratory

finding as neither bacteriological nor toxicological investigation was conducted. Regarding the farm with abortion cases, BTV-4 was also detected in the blood of another doe after abortion. In both farms the pathogen was detected in adult does.

BTV-3 was also identified in field samples collected from 10 cattle farms situated in southern and central areas of the

TABLE 4 | Clinical signs, virus isolation, and additional laboratory diagnoses found in BTV-3 affected domestic ruminants.

Farm	Beginning of disease	Clinical signs	Sample date	Pos/total	BTV-3 strain	Other laboratory investigations/method	Pos laboratory tests/method
1	Beg-Nov-2013	Classical clinical manifestations of BT disease	Beg-Nov-2013	2/3	ISR-2019/13	VI	No
2	End-Sep-2016	Fever, nasal discharge, hyperemia and ulcers in the oral and/or nasal mucosa, face and/or thorax edema, fatigue, and apnea	28-Sep-2016	1/1	ISR-2153/16	VI	No
3	Oct-2016	Fever, nasal discharge, severe hyperaemia, ulceration of oral and/or nasal mucosa, severe face and/or thorax edema, recumbency, and apnea	16-Oct-2016	3/3	ISR-2262/2/16	VI	BTV-8/VI from another two samples
4	Mid-Sep-2017	Fever, followed by lameness and stiffness in legs and back muscles, conjunctivitis, nasal discharge, ulceration of oral and nasal mucosa, recumbency, fatigue, mild respiratory distress, and a few abortions	14-Sep-2017; 28-Nov-2017	3/3; 2/2	ISR-2107/2/17; ISR-2396/2/17	VI	BTV-6/VI from another sample
5	Beg-Sep-2017	Fever followed by skin erythema	6-Sep-2017	3/3	ISR-2079/2/17	VI	BTV-6/VI
6	Oct-2017	Fever, fatigue, reluctance to move, inappetence, pneumonia, and subsequent high mortality	16-Oct-2017	1/1	ISR-2219/17	VI	No
7	23-Aug-2018	Abrupt death	23-Aug-2018	1/1	No	BTV-4,-8/PCR; VI	No
8	Beg-Sep-2018	Fever, recumbency with following death	2-Sep-2018	1/1	No	BTV-4,-8/PCR; BEF/PCR; BEF/SN; VI; bacterial culture	<i>Clostridium perfringens</i> (calf) and few cases in the farm
9	Beg-Sep-2018	Classical clinical manifestations of BT disease	13-Sep-2018; 29-Oct-2018	2/2	ISR-1965/18; ISR-2210/18	BTV-4,-8/PCR; VI	No
10	Mid-Sep-2018	Fever, facial, neck, and hind limbs edema, stiff gate	17-Sep-2018	3/3	ISR-1975/1/18; ISR-1975/2/18	BTV-4,-8/PCR; VI	BTV-4/PCR; BTV-4/VI
11	Beg-Oct-2018	Fever, lips and facial edema, stiff gate	10-Oct-2018	3/4	ISR-2078/2/18; ISR-2078/4/18	BTV-4,-8/PCR; VI	BTV-4/PCR; BTV-4/VI
12	Oct-2018	Abortions	21-Oct-2018	2/2	ISR-2144/2/18	BTV-4,-8/PCR; VI; pan-Simbu/PCR	BTV-4/PCR (in another goat sample)
13	End-Oct-2018	Recumbency, death	24-Oct-2018	1/1	No	BEF/PCR; BEF/SN; BTV-4,-8/PCR; VI	No
14		No data	24-Oct-2018; 4-Nov-2018; 21-Nov-2018; 19-Dec-2018	1/8; 1/4; 2/10; 1/10	No	BTV-4,-8/PCR; VI	No
15	End-Oct-2018	Recumbency and death after few hours from beginning of illness. Post mortem examination- pneumonia	25-Oct-2018	1/1	No	BTV-4,-8/PCR; VI; BSRV/PCR; BPI-3/PCR; BCoV/PCR; bacterial culture	<i>Salmonella</i> spp.
16	End-Oct-2018	Fever, recumbency, tremor/stiffness of neck muscles	25-Oct-2018; 31-Oct-2018	2/2	No	BTV-4,-8/PCR; pan-Simbu/PCR; EHDV/PCR; VI	No
17	Oct-2018	Abortions	28-Oct-2018	2/3	No	BTV-4,-8/PCR; pan-Simbu/PCR; VI	SHUV/PCR
18	Beg-Nov-2018	Hypersalivation, neural ketosis, neck tense muscles (adult cow); fever, fatigue, conjunctival hyperemia (calf)	28-Oct-2018; 5-Nov-2018	1/1	ISR-2255/18	BTV-4,-8/PCR; EHDV/PCR; VI-adult cow; BTV-4,-8/PCR;BEF/PCR; EHDV/PCR; BLV/ID; VI- calf	BEF/PCR (calf)
19	Beg-Nov-2018	Fever, indifference, inappetence, sharp decrease in milk production	4-Nov-2018	1/1	No	BTV-4,-8/PCR; MCFV/PCR VI	No

(Continued)

TABLE 4 | Continued

Farm	Beginning of disease	Clinical signs	Sample date	Pos/total	BTV-3 strain	Other laboratory investigations/method	Pos laboratory tests/method
20	End-Nov-2018	Foamy salivation, nystagmus, hypothermia, mucosal cyanosis and death (calf). Post parturient ketosis, sharp blindness, endometritis, tachycardia (adult cow); after symptomatic treatment- recovery. Generally in farm- cases of diarrhea and abortions	29-Nov-2018; 16-Dec-2018	1/1	No	BTV-4,-8/PCR; VI; MCFV/PCR	BTV-4/PCR (calf)
21	Mid-Dec-2018	Bloody-purulent nasal discharge	13-Dec-2018	1/1	No	BTV-4,-8/PCR; VI; IBRV/PCR; BEF/PCR; EHDV/PCR; IBRV/ELISA-Ab	BTV-4/PCR

Pos/total, number of positive and number of total samples received from the farm; VI, virus isolation; BEF, bovine ephemeral fever; EHDV, epizootic hemorrhagic disease virus; MCFV, malignant catarrhal fever virus; BLV, bovine leukemia virus, pan-Simbu- panel of Simbu serogroup viruses (Peribunyaviridae family); ID, immune diffusion; BRSV, bovine respiratory syncytial virus; BPI-3, bovine parainfluenza 3 virus; BCoV, bovine corona virus; SHUV, Shuni virus; IBRV, infectious bovine rhinotracheitis virus.

country (Figure 1; Tables 3, 4). Except for the cattle farm number 14, where clinically healthy animals were tested for commercial purposes, BTV-3 was detected in samples of sick and dead cattle received from the beginning of September till the middle of December 2018. In two cattle farms (farms 8 and 15) with unusual mortality rates, *Clostridium perfringens* and *Salmonella* spp. were also found in calves, respectively (Tables 3, 4). In farm number 18, a post parturient BTV-3 positive adult cow was euthanized 2 days after showing hypersalivation, neural ketosis, and neck tense muscle. In addition, a one-year-old calf died after suffering from fever, fatigue, and conjunctival hyperemia. From blood sample of this calf, BEFV and BTV-3 were detected and BTV-3 was successfully isolated. In farm number 20, the clinical cases associated with BTV-3 detection included a calf which died after showing foamy salivation, nystagmus, hypothermia, and mucosal cyanosis and an adult cow showing post parturient ketosis, sharp blindness, endometritis, and tachycardia, which recovered after treatment with antibiotics and anti-inflammatory drugs. BTV-4 was also identified by RT-qPCR in tested samples. Similarly, BTV-3 and BTV-4 mixed infection was detected in a cow with bloody-purulent nasal discharge in farm number 21. Oppositely to previously described cases, no additional pathogens other than BTV-3 were found in farms number 13 and 19. In farm number 13, two adult pregnant cows showed recumbency before dying several days after appearance of clinical signs. No additional laboratory investigations were done for identifying the reason of the death. In farm 19, BTV-3 was detected in an adult cow which recovered after showing fever, indifference, inappetence, and sharp decrease in milk production.

Field samples from ill cattle were not investigated for BTV-15 by RT-qPCR, due to absence of in-house or commercial RT-qPCR validated with currently circulating Israeli strains.

Sequence, BLAST, Pairwise and Phylogenetic Analysis of the Israeli BTV-3

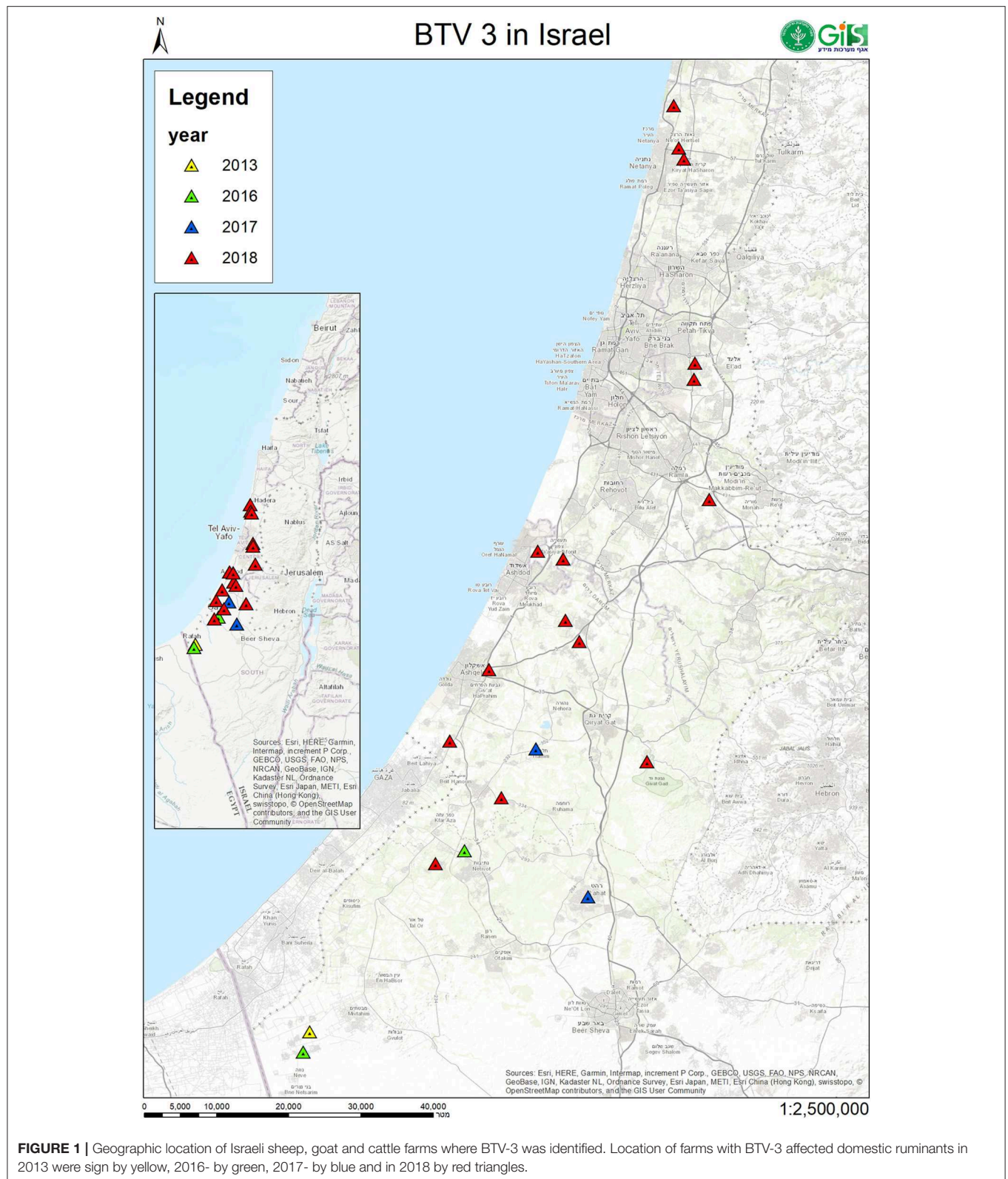
The three BTV-3 strains isolated in 2013 and 2016 were fully sequenced. Partial sequencing of Seg-2 of the four BTV-3 isolated in 2017 and partial sequencing of Seg-2 of the eight BTV-3

isolated in 2018 revealed very close relationship between strains isolated during the same year. For this reason, two out of four BTV-3 strains isolated in 2017 from two different sheep farms and two out of eight strains isolated in 2018 (one from sheep from the southern distinct and one from cattle from central distinct of Israel) were selected for further study. The coding regions of all 10 segments of ISR-2019/13, ISR-2153/16, ISR-2262/2/16, ISR-2219/17, ISR-2210/18, and ISR-2255/18 (accession numbers of NCBI GenBank and length of sequenced region of every sequence are shown in Table S2) were almost full sequenced, when most segments of ISR-2396/2/17 strain were sequenced partially. Partial sequences of segment 2 (Seg-2) of all other Israeli BTV-3 isolates were also submitted to NCBI GenBank (accession numbers MH107823, MH107824, MN398282- MN398287) (<https://www.ncbi.nlm.nih.gov/nucleotide/>).

Israeli BTV-3 strain sequences were also compared and data on nt and aa substitutions were summarized in the Table 5. Due to very close relationship (99.45–99.92% of nt identity in all genome segments) between BTV-3 strains isolated during 2017–2018 and ISR-2153/16 strain isolated in 2016, only sequences of the ISR-2153/16 strain were considered in the genetic analyses when nt sequences were compared to those of the global BTV strains (Table 6). First Israeli BTV-3 isolate (ISR-2019/13) was used as a prototype Israeli BTV-3 strain. Based on the data on number nt and aa substitutions, as well as on the identity with local or global BTV sequences, genome sequences were considered as homologous, or reassorted (Table 5).

Seg-1

According to BLAST and pairwise analyses, the ISR-2019/13 and the ISR-2262/2/16 strains were closely related (99.59% nt and 99.61 aa identity, Table 5). They also showed high degree of nt sequence identity with Tunisian (TUN2000/01) and French (FRA2001/01) BTV-2 strains, with which they shared 97.63–97.71% identity, respectively. The ISR-2153/16 strain was instead most closely related to the BTV-22 84/184 strain, with which it shared 96.66% nt identity (Table 6, Figure S1A).



The strains clustered with ISR-2019/13 and the ISR-2262/2/16 between 2001 and 2004 while the ISR-2153/16 strains isolated in France, Spain and Morocco African strains. BTV-2 and BTV-4 strain clustered with BTV-16, -22, and -24

TABLE 5 | Comparison between nucleotide (nt) and amino acid (aa) sequences of Israeli BTV-3 strains and ISR-2019/13 strains, which was considered as the prototype Israeli BTV-3 strain.

Strain	ISR-2262/2/16	ISR-2153/16	ISR-2219/17	ISR-2396/2/17	ISR-2210/18	ISR-2255/18
Segment	type/nt/aa	type/nt/aa	type/nt/aa	type/nt/aa	type/nt/aa	type/nt/aa
Seg-1	H/16/5	R/220/18	R/215/16	R/NA	R/225/16	R/227/16
Seg-2	H/16/3	H/20/5	H/22/4	H/NA	H/23/4	H/23/4
Seg-3	H/7/0	R/74/1	R/75/0	R/NA	R/75/1	R/76/0
Seg-4*	R/36/8	R/105/9	R/107/9	R/108/9	R/107/9	R/106/9
Seg-5	H/5/2	H/6/2	H/6/2	H/NA/NA	H/6/2	H/8/2
Seg-6	H/1/0	H/2/0	H/6/1	H/NA/1	H/10/1	H/11/2
Seg-7	H/3/1	H/4/1	H/4/1	H/NA/NA	H/6/1	H/4/1
Seg-8*	H/6/2	H/5/2	H/6/3	H/7/3	H/7/3	H/6/3
Seg-9	R/38/12/0	H/7/2/0	H/8/3/0	**H/8/3/0	H/9/4/0	H/9/3/0
Seg-10	R/101/8/16	R/129/12/19	R/131/12/19	R/129/12/19	R/131/14/19	R/131/13/19

*Segments 4 and 8 of ISR-2019/13 strain are partial. The comparison was done between the same genome regions. **The sequenced region of BTV-3 ISR-2396/2/17 strain is partial and considered from nucleotide position 85. NA, not analyzed. The results were shown in the next sequence: type/-type of origin (homologous (H) or reassorted (R)/nt- number of nucleotide substitutions/ aa- number of amino acid substitutions). In case of segments 9 and 10, where two different open reading frame coding two proteins, aa substitutions in both proteins are shown.

Seg-2

The pairwise analysis showed that all isolated BTV-3 Israeli strains were closely related, sharing 99.39–99.52% nt and 99.47–100% aa identity (Table 5). Israeli BTV-3 strains isolated in 2017 (ISR-2219/17 and ISR-2396/2/17) and 2018 (ISR-2210/18 and ISR-2255/18) were most closely related to the ISR-2153/16 strain. According to the pairwise and BLAST analyses, the Israeli BTV-3 nt sequences showed a very high identity (98.12–98.28%) with the BTV-3 TUN2016/Zarzis strain (Table 6).

The phylogenetic analysis showed that Israeli and Tunisian BTV-3 belonged to the same western topotype/lineage; based on Seg-2 analyses, two different subclusters of closely related BTV-3 isolates circulating in Africa and the Mediterranean region were revealed. One included the closely related BTV-3 Israeli and the BTV-3 TUN-Zarzis/2016 isolates, the second subcluster included BTV-3TUN2016 and the BTV-3 ZIM2002/01 strain from Zimbabwe (Figure 2A).

Seg-3

The BLAST and the pairwise analyses showed that the BTV-3 ISR-2019/13 and the ISR-2262/2/16 strains had almost identical nt sequences (99.75% nt and 100% aa identity, Table 5). They showed also high identity (97.73–97.83%) with the South African BTV-3 strain 3/O. aries-tc/ZAF/2017/Smithfield_VR33 (Table 6). The ISR-2153/16 and South African BTV-5 O. aries-tc/ZAF/2011/Benoni_01012015 strains were most closely related to each other, sharing 98.41% of nt identity (Table 6).

The phylogenetic analysis of Seg-3 nt sequences also revealed two sub-clusters. The ISR-2019/13 and the ISR-2262/2/16 strains grouped with South African BTV-3 and BTV-16 strains isolated in 2017 (3/O. aries-tc/ZAF/2017/Smithfield_VR33 and 16/O. aries-tc/ZAF/2017/Bethal_VR08 strains, respectively, Figure S1B), while the ISR-2153/16, ISR-2219/17, ISR-2296/2/17, ISR-2210/18, and ISR-2255/18 strains clustered with the South African BTV-4 O. aries-tc/ZAF/2017/Smithfield VR30 strains

and BTV-5 aries-tc/ZAF/2011/Benoni_01012015 strains (Figure S1B).

Seg-4

The BLAST and pairwise analyses showed a very high nt sequence identity (98.93%) between the BTV-3 ISR-2019/13 and TUN2016/Zarzis strains. The ISR-2153/16 strain was most closely related to Chinese, South African, and Nigerian BTV-1 and BTV-16 (strains SZ97/1, NIG1982/10, and 5012, respectively), with which it shared 95.39–95.74% nt identity. The ISR-2262/2/16 strain was most closely related to Israeli BTV-24 (the representative strain ISR2008/02), with which it shared 99.39% nt identity (Table 6, Figure S1C).

Except for the ISR-2153/16 -like isolates, which formed a separate branch, the phylogenetic analysis showed that Tunisian and the other Israeli BTV-3 strains were closely related and formed a single cluster with some BTV-2, -4, and -24 strains that circulated in the Mediterranean Basin between 2000 and 2010. The phylogenetic analysis also confirmed the very high nucleotide sequence identity between the ISR-2019/13 and the TUN-Zarzis/2016 strains, which clustered together, and between the ISR-2262/2/16 and Israeli BTV-24 strains (Figure S1D).

Seg-5

The BLAST and the pairwise analyses confirmed that all Israeli BTV-3 strains had high nt and aa sequences (99.64–99.71% and 99.47–100% respectively, Table 5). They also showed high nt (98.19–98.31%) identity with BTV-1 and BTV-4 strains from the Mediterranean Basin (Table 6). The phylogenetic analysis of Seg-5 resulted in a single cluster grouping Israeli BTV-3 strains and BTV-1 and BTV-4 strains circulating in the Mediterranean Basin between 2006 and 2010 (Figure S1E).

Seg-6

The pairwise analysis showed that the three Israeli BTV-3 strains were closely related, sharing 99.82–99.94% nt and 99.61–100% aa identity (Table 5). According to the BLAST and the pairwise

TABLE 6 | Comparison between nt composition of Israeli BTV-3 strains and the closest global BTV strains.

Strain	Segment	Closest sequence-nt identity (%)
ISR-2019/13	Seg-1	KP820917/BTV-2/TUN2000/01/2000-97.71
	Seg-2	MF124293/BTV-3/TUN2016/Zarzis/2016-98.28
	Seg-3	MG255621/BTV-3/ZAF/ <i>O.aries-tc</i> /ZAF/2017/Smithfield_VR33/2017-97.83
	Seg-4	MF124295/BTV-3/TUN2016/Zarzis/2016-98.93
	Seg-5	JX861492/BTV-1/FRA2007/18*-98.19
	Seg-6	AJ586694/BTV-16/NIG1982/10-97.00
	Seg-7	MG255625/BTV-3/ <i>O.aries-tc</i> /ZAF/2017/Smithfield_VR33/2017-98.17
	Seg-8	KP821732/BTV-1/LIB2007/06-97.61
	Seg-9	KP821904/BTV-24(4)/ISR2008/02-98.95
	Seg-10	KP821951/BTV-16/CYP2006/01-99.87
ISR-2153/16	Seg-1	JX272389/BTV-22/ZAF/84/184-96.66
	Seg-2	MF124293/BTV-3/TUN2016/Zarzis/2016-98.19
	Seg-4	JN848762/BTV-1/CHN/SZ97/1-95.74
	Seg-5	JX861492/BTV-1/FRA2007/18*-98.27
	Seg-6	AJ586694/BTV-16/NIG1982/10-96.75
	Seg-7	MG255625/BTV-3/ <i>O.aries-tc</i> /ZAF/2017/Smithfield_VR33/2017-97.99
	Seg-8	KP821732/BTV-1/LIB2007/06-97.59
	Seg-9	KP821904/BTV-24(4)/ISR2008/02-98.76
	Seg-10	MG255688/BTV-12/ZAF/ <i>O.aries-tc</i> /ZAF/2017/Queenstown_VR55/2017-97.13
ISR-2262/2/16	Seg-1	KP820917/BTV-2/TUN2000/01/2000-97.66
	Seg-2	MF124293/BTV-3/TUN2016/Zarzis/2016-98.19
	Seg-3	MG255621/BTV-3/ZAF/ <i>O.aries-tc</i> /ZAF/2017/Smithfield_VR33/2017-97.73
	Seg-4	KP821302/BTV-24(4)/ISR2008/02/2008-99.39
	Seg-5	JX861492/BTV-1/FRA2007/18*-98.31
	Seg-6	AJ586694/BTV-16/NIG1982/10-96.94
	Seg-7	MG255625/BTV-3/ <i>O.aries-tc</i> /ZAF/2017/Smithfield_VR33/2017-97.93
	Seg-8	KP821732/BTV-1/LIB2007/06-97.44
	Seg-9	KP821870/BTV-2/FRA2001/03-97.13
	Seg-10	FJ713330/BTV-19/600579-92.47

*Strain sharing the same nt identity with several other BTV strains belonging to BTV-1 and BTV-4.

analyses, sequences of the Israeli strains showed up to 97% identity with the Nigerian BTV-16 NIG1982/10 strain (**Table 6**, **Figure 2B**).

Phylogenetic analysis of Seg-6 showed that the Israeli BTV-3 strains formed a separate branch which clustered with Nigerian BTV-16 (NIG1982/10), ZIM2002/01 BTV-3 strain from Zimbabwe, German and Israeli BTV-6 strains (GER2008/05 and ISR-2095/3/17, respectively) and Tunisian BTV-3 TUN2016 and TUN2016/Zarzis strains (**Figure 2B**).

Seg-7

The BLAST and the pairwise analyses showed that Israeli BTV-3 strains had almost identical nt and aa sequences (99.65–99.73% and 99.41–100%, respectively, **Table 5**). They also showed highest identity with the South African BTV-3

3/*O. aries-tc*/ZAF/2017/Smithfield_VR33 strain (97.93–98.17%) (**Table 6**). Phylogenetic analysis confirmed the close relationship between Israeli BTV-3 and South African BTV-3 3/*O. aries-tc*/ZAF/2017/Smithfield_VR33 strains (**Figure S1E**).

Seg-8

The BLAST and the pairwise analyses also showed a very high nt and aa sequence identity between Israeli BTV-3 strains (99.14–99.82% and 98.85–100%, respectively, **Table 5**). A high nt identity (97.30–97.61%) was also observed with the Libyan BTV-1 LIB2007/06 strain (**Table 6**). Phylogenetic analysis showed that Israeli BTV-3 strains formed a separate branch which clustered with Libyan BTV-1 strain LIB2007/06, BTV-2 French and Tunisian strains (TUN2000/01 and FRA2001/03, respectively), BTV-4 SUD1983/01 strain from Sudan and BTV-5 South African strain 5/ZAF/*O.aries-tc*/ZAF/2011/Benoni_01012015 (**Figure S1F**).

Seg-9

The pairwise and the BLAST analyses showed a very high nt and aa sequence identity (99.33–99.39%, respectively) between BTV-3 ISR-2019/13 and ISR-2153/16 strains (**Table 5**). They also showed high nt sequence identity with the Israeli BTV-24 ISR2008/02 strain (98.76–98.95%, **Table 6**). Conversely, the BTV-3 ISR-2262/2/16 strain was most closely related to the BTV-2 FRA2001/03 French strain with which it shared 97.13% nt identity (**Tables 5, 6**).

Phylogenetic analysis showed that Israeli BTV-3 ISR-2019/13 and ISR-2153/16 strains formed a separate sub-cluster with the Israeli BTV-24 2008/02 strain. The ISR-2262/2/16 strain instead clustered with the British BTV-8 8UKG2007/06 strain (**Figure S1G**).

Seg-10

The BLAST and pairwise analysis of Seg-10 nt sequences showed that the BTV-3 ISR-2019/13 strain was closely related to Israeli BTV-5, 16 and 24 (strains ISR-1405/11; ISR-1794/4/12; ISR-3027/6/10, respectively), and to Cypriot BTV-16 CYP2006/01 strain, sharing 99.62–99.87% nt identity (**Table 6**, the only CYP2006/01 strain is shown). The BTV-3 ISR-2153/16 strain was closely related to some European BTV-8 (UKR2007/06 and NET2006/04 strains), BTV-12 *O.aries-tc*/ZAF/2017/Queenstown_VR55 and South African BTV-18 (BT32/76 and 600578 strains), sharing 97.13% nt identity (only BTV-12 12/ZAF/*O.aries-tc*/ZAF/2017/Queenstown_VR55 strain is shown in **Table 6**). The BTV-3 ISR-2262/2/16 strain had the closest relationship with BTV-19 (600579 and RA 19 OP strains), sharing 91.88–92.47% nt identity (**Table 6**).

The phylogenetic analysis showed that the ISR-2019/13 strain clustered with local (Israeli and Cypriot) BTV-5, -16, and -24 strains. ISR-2262/2/16 strain clustered with BTV-19 strains, while the ISR-2153/16 strain clustered with South African BTV-12, -14 and 18 (strains 12/ZAF/*O.aries-tc*/ZAF/2017/Queenstown_VR55, BT87/59, and BT32/76, respectively; **Figure S1H**).

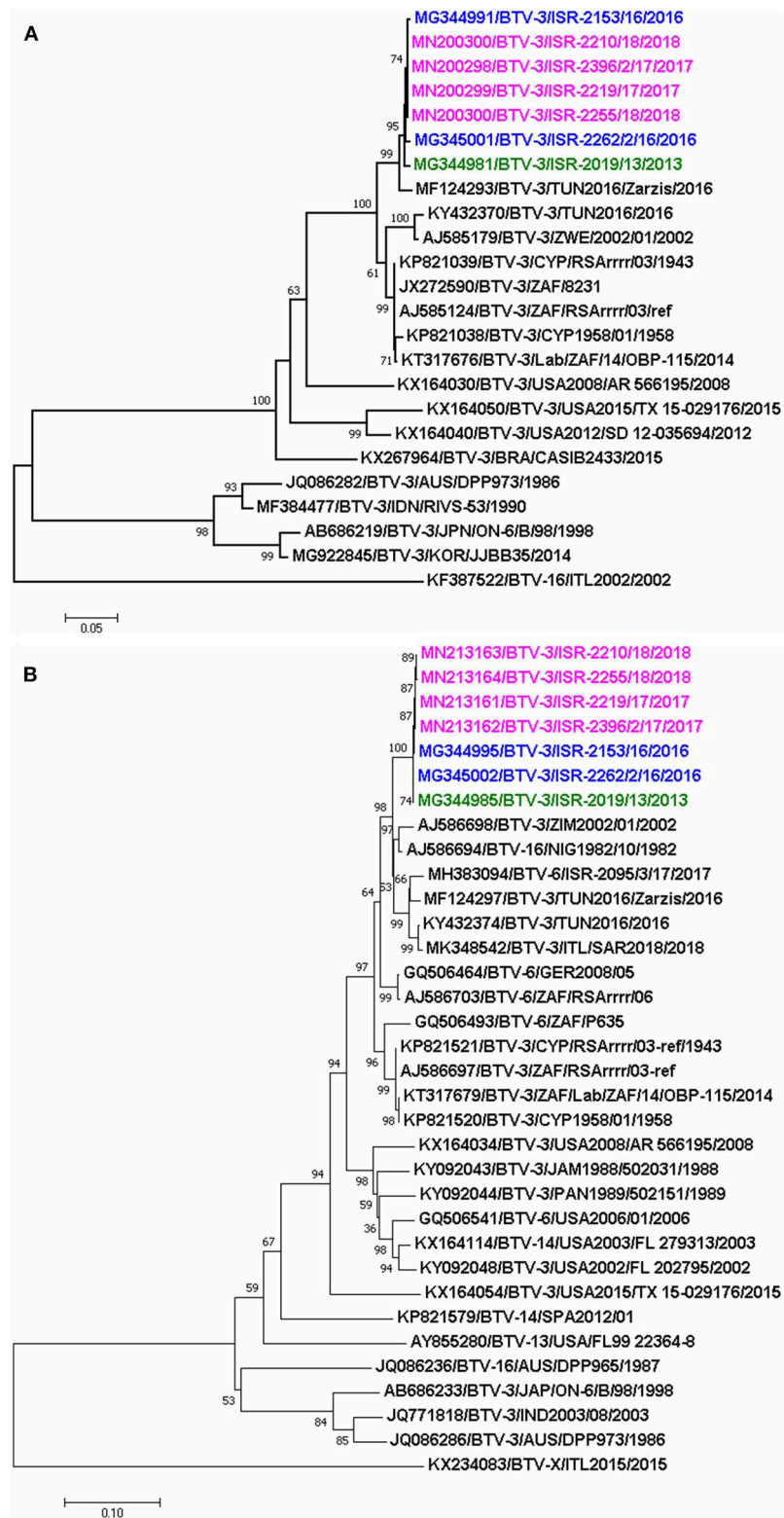


FIGURE 2 | Phylogenetic trees of segment 2 and 6 of Israeli BTV-3 isolates compared with globally published, closely related BTV sequences **(A)** phylogenetic analyses of segment-2; **(B)** phylogenetic analyses of segment 6. Sequences were analyzed and phylogenetic relationships were inferred by using the Neighbor-Joining method. Numbers below branches indicate bootstrap values, based on 1,000 replicates. As outgroup BTV-16 (ITL2002) was used for phylogenetic tree of segment 2, when BTV-X ITL2015 was used as outgroup for phylogenetic tree of segment 6. Israeli BTV-3 strain from 2013 is signed in green, 2016- in blue, and 2017–2018- in pink colors. Viruses are identified by accession number/serotype/isolate/location/year.

DISCUSSION

The first recorded cases of BTV-3 in the Mediterranean Basin date back in the middle of 20th century, when BTV-3 was isolated from sheep samples in 1943 and 1958 (www.ncbi.nlm.nih.gov/pubmed). For the next several decades, no evidence of BTV-3 circulation was reported. BTV-3 circulation has been recently reported in Tunisia, Egypt and Italy. Even though detected either in goats or cattle (7, 38, 54) in these countries clinical signs associated to BTV-3 infection were observed and described in sheep only (7, 38, 39). According to the retrospective findings of this study, during 2013–2017 BTV-3 outbreaks were restricted to Negev Desert area, in the southern district, while in 2018 it spread among most coastal and some central areas of Israel (Figure 1, Table 3). An additional difference between 2018 and 2013–2017 BTV-3 outbreaks was the exposure of Israeli goats and cattle in 2018 outbreaks. In this year, in fact, BTV-3 infection was occasionally associated with BT typical and atypical clinical signs, occurrence which was never observed before either in Israel or in the Mediterranean region (7, 38, 54). These events may imply an increased infectivity and pathogenicity of the BTV-3 Israeli strains circulating in 2018 or/and their adaptation to the local vectors. However, because of the exiguous number of samples from diseased and clinically healthy animals received from the BTV-3 affected farms, it was hard to estimate the real exposure of ruminants and real effect BTV-3 infection on these animals. In Israeli goats and cattle, making any conclusion regarding the role of BTV-3 in causing illness and death was even harder due to involvement of different bacterial and viral agents in the clinical cases or to the absence of bacterial and toxicological investigations in some fatal cases. According to what observed in this survey, all Israeli BTV-3 strains were definitely capable of inducing classical manifestations of BT disease in sheep. These symptoms were more severe in case of BTV mixed infections (BTV-3 and BTV-4, BTV-6, or BTV-8). Notably, cases of acutely affected animal showing classical BT clinical signs were seen in one sheep farm during two-month period and confirmed by successful virus isolation. This fact illustrated presence of newly infected animals and probably infected vector during prolonged period during two seasons 2017 and 2018 (farms number 4 and 9, Table 4).

Interestingly, in some cattle and sheep farms, BTV-3 as well as BTV-4 were detected in fetal tissues, placenta and in newborn animals. Although these findings were not sufficient to establish the definitive involvement of BTV-3 in determining reproductive failures, its presence in fetal tissues and/or newborn animals, provided evidence of its capability to infect placenta and probably also cross the placental barrier. As far as we know, apart from BTV-8, causing numerous abortions and malformations, and lastly BTV-1, this capability is proper of vaccine or lab derived strains (18, 55).

Higher susceptibility observed in sheep reflected the number of successful virus isolations achieved in this species (33%) compared to cattle (7.7%), which, in turn, may imply a higher number of sheep acutely infected by BTV-3 than cattle.

Two different strains named TUN2016 and TUN2016/Zarzis have been identified as responsible of the Northern African and

Italian BTV-3 outbreaks (7, 38, 39, 54). This study revealed that three additional BTV-3 strains have been circulating in the Mediterranean Basin and in Israel, in particular, at least since 2013. Genome comparison allowed a tentative reconstruction of the ancestral viral genome of these strains. When the Israeli BTV-3 strain sequences were compared, it was evident that Seg-2, 5, 6, 7 and 8 of the Israeli strains derived from a common ancestor. According to Seg-2 phylogenetic analyses, the BTV-3 Israeli strains also shared common ancestors with the Tunisian TUN2016/Zarzis strain. For the ISR-2019/13 strain, this was evident for Seg-4 too.

In all these strains, reassortment phenomena were also found. When compared with the prototype ISR-2019/13 strain, the ISR-2262/2/16 and the ISR-2153/16-like strains have 3 and 4 reassorted segments, respectively. Moreover, their Seg-4, Seg-9, and Seg-10 sequences clearly evidenced a different origin. “Traces” of untyped South African strains and BTV serotypes (BTV-18, BTV-19, BTV-22) exotic for the region in the Israeli BTV-3 genomes were clear, at least in their last segments. These results may indicate the circulation of exotic strain/serotypes in the region.

Between 2013 and 2017, only a low proportion of the BTV strains isolated from sick domestic ruminants was identified as BTV-3. Thus, in 2013 only one out of 15, in 2016, 3 out of 58, in 2017 4 out of 40 virus isolates were BTV-3. In these years, the BTV-3 circulation was limited to southern Israel only. In 2018, the number of BTV-3 isolates among the total number of BTV isolated, sharply increased (8 out of 25). The BTV-3 circulation also spread along all coastal area of Israel, suggesting an increased infectivity of the BTV-3 ISR-2153/16 strain among susceptible Israeli domestic ruminants which can be explained by point mutations both in coding and non-coding regions (not shown in this work) or possible introduction closely related viruses to ISR-2153/16 strain into Israel.

In spite of a long history of BTV-3 infections in ruminants in South Africa, India, Caribbean and Northern America (41, 42, 56, 57), epidemiological data as well as information on pathogenicity and infectivity of BTV-3 infection from these regions are absent or scarce. Even if it was not possible to evaluate their pathogenicity in cattle and goats, this paper still gives important information on the possible origin of the BTV-3 strains circulating in the Mediterranean basin, elucidating their possible routes of incursion. However, further investigation is needed to improve our understanding on their biological properties and their impact on livestock.

DATA AVAILABILITY STATEMENT

The data analyzed in this study can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

This study did not involve any human participants and animals. Ethical approval was not required for this study according to national legislation.

AUTHOR CONTRIBUTIONS

GK, JV, IS, SS, AM, ID, IG, AK, OK, and EO collected field samples and epidemiological data. NG, DD, and VB performed diagnostic tests of field samples. NG developed in-house PCR systems, performed PCR tests for sequencing, analyzed and summarized data of sequencing, and phylogenetic analyses. VB, AG, and NG performed isolations of the viruses in eggs and tissue cultures. AE and AL reviewed the manuscript. NG and GS drafted the manuscript, while VB, AE, and YK discussed the results and commented on the manuscript.

FUNDING

This study was supported by H2020 PALE BLU project, Proposal ID 727393-2.

ACKNOWLEDGMENTS

We thank Dr. Anita Kovtunen for technical assistance and Mrs. Marisol Guini-Rubinstein, Mrs. Limor

Weisbelith, and Dr. Marcelo Gabriel Fleker for performing laboratory tests.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2020.00112/full#supplementary-material>

Figure S1 | Phylogenetic trees of segment 1, 3–5, and 7–10 Israeli BTV-3 isolates compared with globally published, closely related BTV sequences. **(A)** phylogenetic analyses of segment 1; **(B)** phylogenetic analyses of segment 3; **(C)** phylogenetic analyses of segment 4; **(D)** phylogenetic analyses of segment 5; **(E)** phylogenetic analyses of segment 7; **(F)** phylogenetic analyses of segment 8; **(G)** phylogenetic analyses of segment 9; **(H)** phylogenetic analyses of segment 10. Sequences were analyzed and phylogenetic relationships were inferred by using the Neighbor-Joining method. Numbers below branches indicate bootstrap values, based on 1,000 replicates. As outgroup BTV-26 (KUW2010) was used for phylogenetic trees of most segments. Israeli BTV-3 strain from 2013 is signed in green, 2016- in blue, and 2017–2018- in pink colors. Viruses are identified by accession number/serotype/isolate/location/year.

Table S1 | List of segment 2 primers used for identification and sequencing of circulating Israeli bluetongue serotypes.

Table S2 | Accession numbers of Israeli BTV-3 and sequenced regions.

REFERENCES

1. MacLachlan NJ, Drew CP, Darpel KE, Worwa G. The pathology and pathogenesis of bluetongue. *J Comp Pathol.* (2009) 141:1–16. doi: 10.1016/j.jcpa.2009.04.003
2. MacLachlan NJ, Mayo CE, Daniels PW, Savini G, Zientara S, Gibbs EP. Bluetongue. *Rev Sci Tech.* (2015) 34:329–40. doi: 10.20506/rst.34.2.2360
3. Attoui H, Maan S, Anthony SJ, Mertens PPC. Bluetongue virus, other orbiviruses and other reoviruses: their relationships and taxonomy. In: Mellor PS, Baylis M, Mertens PPC, editors. *Bluetongue Monograph*. 1st ed. London: Elsevier/Academic Press (2009). p. 23–52. doi: 10.1016/B978-012369368-6.50007-1
4. Stewart M, Hardy A, Barry G, Pinto RM, Caporale M, Melzi E, et al. Characterization of a second open reading frame in genome segment 10 of bluetongue virus. *J Gen Virol.* (2015) 96:3280–93. doi: 10.1099/jgv.0.000267
5. Belbis G, Zientara S, Bréard E, Sailleau C, Caignard G, Vitour D, et al. Bluetongue virus: from BTV-1 to BTV-27. *Adv Virus Res.* (2017) 99:161–97. doi: 10.1016/bs.aivir.2017.08.003
6. Bumbarov V, Golender N, Jenckel M, Wernike K, Beer M, Khinich E, et al. Characterization of bluetongue virus serotype 28. *Transbound Emerg Dis.* (2019) 67:171–82. doi: 10.1111/tbed.13338
7. Lorusso A, Sghaier S, Di Domenico M, Barbria ME, Zaccaria G, Megdich A, et al. Analysis of bluetongue serotype 3 spread in Tunisia and discovery of a novel strain related to the bluetongue virus isolated from a commercial sheep pox vaccine. *Infect Genet Evol.* (2018) 59:63–71. doi: 10.1016/j.meegid.2018.01.025
8. Wright IM. *Serological and Genetic Characterisation of Putative New Serotypes of Bluetongue Virus and Epizootic Haemorrhagic Disease Virus Isolated from an Alpaca*. MSc Thesis. Potchefstroom: North-West University. (2014).
9. Sun EC, Huang LP, Xu QY, Wang HX, Xue XM, Lu P, et al. Emergence of a novel Bluetongue virus serotype, China 2014. *Transbound Emerg Dis.* (2016) 63:585–9. doi: 10.1111/tbed.12560
10. Savini G, Puggioni G, Meloni G, Marcacci M, Di Domenico M, Rocchigiani AM, et al. Novel putative Bluetongue virus in healthy goats from Sardinia, Italy. *Infect Genet Evol.* (2017) 51:108–17. doi: 10.1016/j.meegid.2017.03.021
11. Marcacci M, Sant S, Mangone I, Gorla M, Dondo A, Zoppi S, et al. One after the other: a novel Bluetongue virus strain related to Toggenburg virus detected in the Piedmont region (North-western Italy), extends the panel of novel atypical BTV strains. *Transbound Emerg Dis.* (2018) 65:370–4. doi: 10.1111/tbed.12822
12. Carpenter S, Wilson A, Mellor PS. Culicoides and the emergence of bluetongue virus in northern Europe, Trends. *Microbiol.* (2009) 17:172–8. doi: 10.1016/j.tim.2009.01.001
13. Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PP, Baylis M. Climate change and the recent emergence of bluetongue in Europe. *Nat Rev Microbiol.* (2005) 3:171–81. doi: 10.1038/nrmicro1090
14. MacLachlan NJ. Bluetongue: history, global epidemiology, and pathogenesis. *Prev Vet Med.* (2011) 102:107–11. doi: 10.1016/j.prevetmed.2011.04.005
15. López-Olvera JR, Falconi C, Fernández-Pacheco P, Fernández-Pinero J, Sánchez MA, Palma A, et al. Experimental infection of European red deer (*Cervus elaphus*) with bluetongue virus serotypes 1 and 8. *Vet Microbiol.* (2010) 145:148–52. doi: 10.1016/j.vetmic.2010.03.012
16. Backx A, Heutink R, van Rooij E, van Rijn P. Transplacental and oral transmission of wild-type bluetongue virus serotype 8 in cattle after experimental infection. *Vet Microbiol.* (2009) 138:235–43. doi: 10.1016/j.vetmic.2009.04.003
17. Darpel KE, Barber J, Hope A, Wilson AJ, Gubbins S, Henstock M, et al. Using shared needles for subcutaneous inoculation can transmit bluetongue virus mechanically between ruminant hosts. *Sci Rep.* (2016) 6:20627. doi: 10.1038/srep20627
18. Savini G, Lorusso A, Paladini C, Migliaccio P, Di Gennaro A, Di Provvido A, et al. Bluetongue serotype 2 and 9 modified live vaccine viruses as causative agents of abortion in livestock: a retrospective analysis in Italy. *Transbound Emerg Dis.* (2014) 61:69–74. doi: 10.1111/tbed.12004
19. Batten C, Darpel K, Henstock M, Fay P, Veronesi E, Gubbins S. Evidence for transmission of bluetongue virus serotype 26 through direct contact. *PLoS ONE.* (2014) 9:e96049. doi: 10.1371/journal.pone.0096049
20. Bréard E, Schulz C, Sailleau C, Bernelin-Cottet C, Viarouge C, Vitour D, et al. Bluetongue virus serotype 27: experimental infection of goats, sheep and cattle with three BTV-27 variants reveal atypical characteristics and likely direct contact transmission BTV-27 between goats. *Transbound Emerg Dis.* (2018) 65:e251–63. doi: 10.1111/tbed.12780
21. Elbers ARW, van der Spek AN, van Rijn PA. Epidemiologic characteristics of bluetongue virus serotype 8 laboratory-confirmed outbreaks in the Netherlands in 2007 and a comparison with the situation in 2006. *Prev Vet Med.* (2009) 92:1–8. doi: 10.1016/j.prevetmed.2009.08.007

22. Szmargd C, Wilson A, Carpenter S, Mertens PP, Mellor PS, Gubbins S. Mortality and case fatality during the recurrence of BTV-8 in northern Europe in 2007. *Vet Rec.* (2007) 161:571–2. doi: 10.1136/vr.161.16.571-e
23. Golender N, Panshin A, Brenner J, Rotenberg D, Oura C, Khinich E, et al. Bluetongue virus serotype 24 (BTV-24) in Israel: phylogenetic characterization and clinical manifestation of the disease. *Vet Ital.* (2016) 52:333–41. doi: 10.12834/VetIt.643.3163.3
24. Bumbarov V, Golender N, Rotenberg D, Brenner J. Unusual clinical manifestations in Israeli ruminant populations infected with Orbiviruses. *Vet Ital.* (2016) 52:343–51. doi: 10.12834/VetIt.641.3154.2
25. Coetzee P, Van Vuuren M, Stokstad M, Myrmel M, van Gennip RG, van Rijn PA, et al. Viral replication kinetics and *in vitro* cytopathogenicity of parental and reassortant strains of bluetongue virus serotype 1, 6 and 8. *Vet Microbiol.* (2014) 171:53–65. doi: 10.1016/j.vetmic.2014.03.006
26. Shaw AE, Ratnir M, Nunes SF, Nomikou K, Caporale M, Golder M, et al. Reassortment between two serologically unrelated bluetongue virus strains is flexible and can involve any genome segment. *J Virol.* (2013) 87:543–57. doi: 10.1128/JVI.02266-12
27. Nomikou K, Hughes J, Wash R, Kellam P, Breard E, Zientara S, et al. Widespread reassortment shapes the evolution and epidemiology of bluetongue virus following European invasion. *PLoS Pathogens.* (2015) 11:e1005056. doi: 10.1371/journal.ppat.1005056
28. Gould AR, Pritchard LI. Genomic sequences amongst bluetongue viruses revealed by comparisons of capsid and outer coat protein nucleotide sequences. *Virus Res.* (1990) 17:31–52. doi: 10.1016/0168-1702(90)90078-P
29. Pritchard LI, Gould AR. Relationships amongst bluetongue viruses revealed by comparisons of capsid and outer coat protein nucleotide sequences. *Virus Res.* (1990) 17:31–52. doi: 10.1016/0168-1702(90)90078-p
30. Pritchard LI, Sendow I, Lunt R, Hassan SH, Kattenbelt J, Gould AR, et al. Genetic diversity of bluetongue viruses in south east Asia. *Virus Res.* (2004) 101:193–201. doi: 10.1016/j.virusres.2004.01.004
31. Cowley JA, Gorman BM. Cross-neutralization of genetic reassortants of bluetongue virus serotypes 20 and 21. *Vet Microbiol.* (1989) 19:37–51. doi: 10.1016/0378-1135(89)90089-8
32. Huisman H. The use of recombinant DNA technology for the development of a bluetongue virus subunit vaccine. *Onderstepoort J Vet Res.* (1985) 52:149–51.
33. Mertens PPC, Pedley S, Cowley J, Burroughs JN, Corteyn AH, Jeggo MH, et al. Analysis of the roles of bluetongue virus outer capsid proteins VP2 and VP5 in determination of virus serotype. *Virology.* (1989) 170:561–5. doi: 10.1016/0042-6822(89)90447-9
34. Maan S, Maan NS, Samuel AR, Rao S, Attoui H, Mertens PP. Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. *J Gen Virol.* (2007) 88:621–30. doi: 10.1099/vir.0.82456-0
35. Mertens PP, Maan NS, Prasad G, Samuel AR, Shaw AE, Potgieter AC, et al. Design of primers and use of RT-PCR assays for typing European bluetongue virus isolates: differentiation of field and vaccine strains. *J Gen Virol.* (2007) 88:2811–23. doi: 10.1099/vir.0.83023-0
36. Singh KP, Maan S, Samuel AR, Rao S, Meyer AJ, Mertens PP. Phylogenetic analysis of bluetongue virus genome segment 6 (encoding VP5) from different serotypes. *Vet Ital.* (2004) 40:479–483.
37. Bonneau KR, Zhang N, Zhu J, Zhang F, Li Z, Zhang K, et al. Sequence comparison of the L2 and S10 genes of bluetongue viruses from the United States and the People's Republic of China. *Virus Res.* (1999) 61:153–60. doi: 10.1016/S0168-1702(99)00034-9
38. Cappai S, Rolesu S, Loi F, Liciardi M, Leone A, Marcacci, et al. Western Bluetongue virus serotype 3 in Sardinia, diagnosis and characterization. *Transbound Emerg Dis.* (2019) 66:1426–31. doi: 10.1111/tbed.13156
39. Sghaier S, Lorusso A, Portanti O, Marcacci M, Orsini M, Barbria ME, et al. A novel Bluetongue virus serotype 3 strain in Tunisia, November 2016. *Transbound Emerg Dis.* (2017) 64:709–15. doi: 10.1111/tbed.12640
40. Lorusso A, Guercio A, Purpari G, Cammà C, Calistri P, D'Alterio N, et al. Bluetongue virus serotype 3 in Western Sicily, November 2017. *Vet Ital.* (2017) 53:273–5. doi: 10.12834/VetIt.251.520.178
41. Gerdes GH. A South African overview of the virus, vectors, surveillance and unique features of bluetongue. *Vet Ital.* (2004) 40:39–42.
42. Schirtzinger EE, Jaspersen DC, Ostlund EN, Johnson DJ, Wilson WC. Recent US bluetongue virus serotype 3 isolates found outside of Florida indicate evidence of reassortment with co-circulating endemic serotypes. *J Gen Virol.* (2018) 99:157–68. doi: 10.1099/jgv.0.000965
43. Silva TG, Lima MS, Spedicato M, Carmine I, Teodori L, Leone A, et al. (editors). In: *Abstracts of the 11th EPIZONE Annual Meeting*. Paris (2017). Abstract P1 12
44. Golender N, Eldar A, Ehrlich M, Khinich Y, Kenigswald G, Varsano JS, et al. Emergence of a Novel Reassortant Strain of Bluetongue Serotype 6 in Israel, 2017: Clinical Manifestations of the Disease and Molecular Characterization. *Viruses.* (2019) 11:E633. doi: 10.3390/v11070633
45. Quinn PJ, Carter ME, Markey B, Carter GR. *Clinical Veterinary Microbiology*. 2nd edn. Edinburgh: Harcourt Publishers Ltd., Mosby (1994).
46. Erster O, Stram R, Menasherow S, Rubinstein-Giuni M, Sharir B, Khinich E, et al. High-resolution melting (hrm) for genotyping bovine ephemeral fever virus (bevf). *Virus Res.* (2017) 229:1–8. doi: 10.1016/j.virusres.2016.11.030
47. Cunha CW, Otto L, Taus NS, Knowles DP, Li H. Development of a multiplex real-time PCR for detection and differentiation of malignant catarrhal fever viruses in clinical samples. *J Clin Microbiol.* (2009) 47:2586–9. doi: 10.1128/JCM.00997-09
48. Boxus M, Letellier C, Kerkhofs P. Real Time RT-PCR for the detection and quantitation of bovine respiratory syncytial virus. *J Virol Methods.* (2005) 125:125–30. doi: 10.1016/j.jviromet.2005.01.008
49. Kishimoto M, Tsuchiaka S, Rahpaya SS, Hasebe A, Otsu K, Sugimura S, et al. Development of one-run real-time PCR detection system for pathogens associated with bovine respiratory disease complex. *J Vet Med Sci.* (2017) 79:517–23. doi: 10.1292/jvms.16-0489
50. Golender N, Bumbarov VY, Erster O, Beer M, Khinich Y, Wernike K. Development and validation of a universal S-segment-based real-time RT-PCR assay for the detection of Simbu serogroup viruses. *J Virol Methods.* (2018) 261:80–5. doi: 10.1016/j.jviromet.2018.08.008
51. Komarov A, Goldsmit L. A disease similar to Blue Tongue in cattle and sheep in Israel. *Ref Vet.* (1951) 8:96–100.
52. Golender N, Khinich Y, Gorohov A, Abramovitz I, Bumbarov V. Epizootic hemorrhagic disease virus serotype 6 outbreak in Israeli cattle in 2015. *J Vet Diagn Invest.* (2017) 29:885–8. doi: 10.1177/1040638717726826
53. Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol.* (2016) 33:1870–4. doi: 10.1093/molbev/msw054
54. Ahmed S, Mahmoud MAE, Viarouge C, Sailleau C, Zientara S, Breard E. Presence of bluetongue and epizootic hemorrhagic disease viruses in Egypt in 2016 and 2017. *Infect Genet Evol.* (2019) 73:221–6. doi: 10.1016/j.meegid.2019.04.033
55. Chauhan HC, Biswas SK, Chand K, Rehman W, Das B, Dadawala AI, et al. Isolation of bluetongue virus serotype 1 from aborted goat fetuses. *Rev Sci Tech.* (2014) 33:803–12. doi: 10.20506/rst.33.3.2319
56. Maan S, Maan NS, Batra K, Kumar A, Gupta A, Rao PP, et al. Reverse transcription loop-mediated isothermal amplification assays for rapid identification of eastern and western strains of bluetongue virus in India. *J Virol Methods.* (2016) 234:65–74. doi: 10.1016/j.jviromet.2016.04.002
57. Maan S, Maan NS, Nomikou K, Anthony SJ, Ross-smith N, Singh KP, et al. Molecular epidemiology studies of bluetongue virus. In: Mellor PS, Baylis M, Mertens PPC, editors. *Bluetongue*. 1st ed. London: Elsevier/Academic Press (2009). p. 135–166. doi: 10.1016/B978-012369368-6.50011-3

Conflict of Interest: NG was employed by Kimron Veterinary Institute, Israel.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Golender, Bumbarov, Eldar, Lorusso, Kenigswald, Varsano, David, Schainin, Dagoni, Gur, Kaplan, Gorohov, Koren, Oron, Khinich, Sclamovich, Meir and Savini. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Endemic and Emerging Arboviruses in Domestic Ruminants in East Asia

Tohru Yanase^{1*†}, Katsunori Murota¹ and Yoko Hayama²

¹ Kyushu Research Station, National Institute of Animal Health, NARO, Kagoshima, Japan, ² Viral Disease and Epidemiology Research Division, National Institute of Animal Health, NARO, Tsukuba, Japan

OPEN ACCESS

Edited by:

William C. Wilson,
United States Department of
Agriculture, United States

Reviewed by:

Diego G. Diel,
Cornell University, United States
Sushila Maan,
Lala Lajpat Rai University of Veterinary
and Animal Sciences, India

*Correspondence:

Tohru Yanase
tyanase@affrc.go.jp

†ORCID:

Tohru Yanase
orcid.org/0000-0003-0838-9156

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 05 October 2019

Accepted: 10 March 2020

Published: 07 April 2020

Citation:

Yanase T, Murota K and Hayama Y
(2020) Endemic and Emerging
Arboviruses in Domestic Ruminants in
East Asia. *Front. Vet. Sci.* 7:168.
doi: 10.3389/fvets.2020.00168

Epizootic congenital abnormalities caused by Akabane, Aino, and Chuzan viruses have damaged the reproduction of domestic ruminants in East Asia for many years. In the past, large outbreaks of febrile illness related to bovine ephemeral fever and Ibaraki viruses severely affected the cattle industry in that region. In recent years, vaccines against these viruses have reduced the occurrence of diseases, although the viruses are still circulating and have occasionally caused sporadic and small-scaled epidemics. Over a long-term monitoring period, many arboviruses other than the above-mentioned viruses have been isolated from cattle and *Culicoides* biting midges in Japan. Several novel arboviruses that may infect ruminants (e.g., mosquito- and tick-borne arboviruses) were recently reported in mainland China based on extensive surveillance. It is noteworthy that some are suspected of being associated with cattle diseases. Malformed calves exposed to an intrauterine infection with orthobunyaviruses (e.g., Peaton and Shamonda viruses) have been observed. Epizootic hemorrhagic disease virus serotype 6 caused a sudden outbreak of hemorrhagic disease in cattle in Japan. Unfortunately, the pathogenicity of many other viruses in ruminants has been uncertain, although these viruses potentially affect livestock production. As global transportation grows, the risk of an accidental incursion of arboviruses is likely to increase in previously non-endemic areas. Global warming will also certainly affect the distribution and active period of vectors, and thus the range of virus spreads will expand to higher-latitude regions. To prevent anticipated damages to the livestock industry, the monitoring system for arboviral circulation and incursion should be strengthened; moreover, the sharing of information and preventive strategies will be essential in East Asia.

Keywords: arthropod-borne virus, cattle, *Culicoides*, mosquito, orbivirus, orthobunyavirus, rhabdovirus

INTRODUCTION

Arthropod-borne (arbo-) viruses are transmitted by hematophagous arthropods. The arboviruses include various taxonomic groups, such as *Rhabdoviridae*, *Nairoviridae*, *Peribunyaviridae*, *Phenuiviridae*, *Orthomyxoviridae*, *Flaviviridae*, *Togaviridae*, *Reoviridae*, and *Asfarviridae* (1, 2). They cause a broad spectrum of illness ranging from asymptomatic to severe and fatal disease in vertebrate hosts. Because some of the arboviruses have affected human and domestic animal health, the efforts to prevent and control the diseases caused by arboviruses continue, but novel arboviruses

have continuously emerged and have sometimes been involved in human and animal diseases. The battle thus seems to have no end.

Bluetongue virus (BTV; the genus *Orbivirus*, the family *Reoviridae*) is probably the most well-known arbovirus affecting livestock ruminant productions in the world (3). The virus causes severe illness in ruminants and its presence can act as a barrier for international trades of animals and animal products. Since end of the last century, the global range of BTV has remarkably changed (4). The incursion of BTV serotype 8 into northern Europe caused large economic damage to the livestock industry. Another important arbovirus, epizootic hemorrhagic disease virus (EHDV; the genus *Orbivirus*), also has been regarded as a threat to European counties in recent years, because its outbreaks has been reported in the countries bordering the region (3, 5). The ruminant diseases caused by both viruses have been listed as notifiable terrestrial animal diseases by World Organization for Animal Health (OIE) and well-investigated through the world. In addition, recent appearance of an emerging arbovirus, Schmallenberg virus (SBV), in northern Europe (6) has caught the world's attention (7). However, many other arboviruses, which are not listed by OIE, also have been involved in ruminant illness and their epizootic might have severely impacted on the livestock industry in each region. Unfortunately, the situation of arbovirus infections of ruminant livestock remains uncertain in many parts of the world.

East Asia, i.e., the eastern subregion of Asia, has the largest human population in the world, and the livestock industry is an important component of the lives of the region's populations. The estimated global distribution data for livestock animals indicated that East Asia is one of the regions with the highest population density of domestic ruminants in the world (8). Significant concern about the health and safety of livestock for consumption has thus been expressed. Unfortunately, along with other important diseases, a variety of arbovirus infections has probably damaged the production of domestic ruminants (Tables 1, 2), although there is insufficient statistical data at this time to draw any conclusion.

Akabane disease and bovine ephemeral fever (BEF) are still problematic in East Asia, despite the widespread use of vaccines against them. Outbreaks of other endemic arbovirus infections such as Aino virus infection and Ibaraki disease have occasionally been reported. In addition, recent progress in the research tools for virus hunting have enhanced the detection of the circulation of arboviruses that were previously unrecognized in East Asia (Table 1). Notably, some of the arboviruses are suspected of being associated with ruminant diseases such as epizootic congenital abnormalities, febrile illness, and neurological disorders (Table 3). Many reports about arboviruses infecting domestic ruminants have been provided from mainland China, Japan, the Republic of Korea, and Taiwan, which are part of East Asia. This review provides a compilation of the available knowledge about the arboviruses that are associated with domestic ruminants in the East Asia region at present. Gaps in the research that remain to be addressed and future research directions are also discussed.

HISTORY OF ENDEMIC AND EMERGING ARBOVIRUSES AFFECTING DOMESTIC RUMINANTS

Bovine Ephemeral Fever

BEF is characterized by sudden fever, anorexia, depression, and anastasia, and it can be regarded as the first recognized arbovirus infection in domestic ruminants in East Asia. Due to the cessation of lactation in dairy cattle and the loss of condition in beef cattle, BEF has had considerable economic impact (9). The etiological virus is bovine ephemeral fever virus (BEFV; species *Bovine fever ephemerovirus*, genus *Ephemerovirus*, family *Rhabdoviridae*). The virus was widely distributed through Africa, the Middle East, Asia, and Australia. Phylogenetic analysis based on the surface glycoprotein (G) gene demonstrated that BEFV isolates are grouped into four lineages with different geographical origins: Africa, the Middle East, Asia, and Australia (9, 10). Recently, several BEFV isolates clustered into Asian lineage have been appeared in Turkey and Iran, indicating its expansion to the westward (11, 12). Outside of East Asia, large BEF epizootics have been reported in Israel, Turkey, and Australia (11, 13, 14).

Old records indicated that epidemics of BEF occurred in the central and western parts of Japan in the late nineteenth century (15). Between 1949 and 1951, ~700,000 cases and 10,000 deaths were reported in the same region (16). The disease was initially called "bovine epizootic fever," but the etiological virus was later identified as BEFV based on the serological relationship between the Japanese isolate and BEFVs isolated in Australia and South Africa (17). Large- and medium-scale outbreaks of BEF repeatedly occurred in Japan until 1989 (18, 19). Since then, the epidemics had been limited to the southwestern islands of Japan, which are close to Taiwan (20, 21). However, in 2015 a reoccurrence of BEF was reported in the southern end of Kyushu, which is one of the main islands of Japan (22).

From 1955 to 2011, BEF epidemics were often recorded in mainland China (23). China's nationwide sero-surveillance in cattle between 2012 and 2014 demonstrated that the range of BEFV covered most of the area of mainland China (24). Epidemics of BEF have also occurred in Korea, and some of them were probably linked to those in Japan (19). Meteorological analysis demonstrated that a low-jet stream blew from mainland China, along the Korean Peninsula, to western Japan at the peak of BEF epidemic in China and Korea in 1988 and 1991 (19, 25, 26). The stream probably brought the infected vectors and contributed to the BEF expansion to Japan. The seroprevalence of BEFV without clinical cases was demonstrated in Korea between 2009 and 2012 (27). Several intervals of BEF epidemic have been documented in Taiwan between 1967 and 2014 (28).

Although the mortality rate of BEF is known to be <1% (9), a high case-fatality rate due to a novel genogroup of BEFV was observed in several recent outbreaks in mainland China and Taiwan (23, 29). This novel genogroup of BEFV was first isolated in mainland China in 2011 and was then detected in Taiwan and Japan 2 and 4 years later, respectively (22, 23, 30). These reports indicate that the virus rapidly spread in East Asia. The impact of

TABLE 1 | Arboviruses associated with domestic ruminants in East Asia.

Family	Genus	Species	Virus/serotype (abbreviation)	Arthropod vectors	Geographical location of isolation/detection*
<i>Peribunyaviridae</i>	<i>Orthobunyavirus</i>	<i>Akabane orthobunyavirus</i>	Akabane virus (AKAV)	<i>Culicoides</i> spp.	CHN, JPN, ROK, TWN
		<i>Aino orthobunyavirus</i>	Aino virus (AINOV)	<i>Culicoides</i> spp.	<i>CHN, JPN, ROK, TWN</i>
		<i>Peaton orthobunyavirus</i>	Peaton virus (PEAV)	<i>Culicoides</i> spp.	<i>CHN, JPN, ROK, TWN</i>
		<i>Schmallenberg orthobunyavirus</i>	Sathuperi virus (SATV)	<i>Culicoides</i> spp.	JPN, ROK
			Shamonda virus (SHAV)	<i>Culicoides</i> spp.	JPN, ROK
<i>Phenuiviridae</i>	<i>Banyangvirus</i>	<i>Batai orthobunyavirus</i>	Batai virus (BATV)	Mosquitoes	CHN, JPN
		<i>Huaiyangshan banyangvirus</i>	Severe fever with thrombocytopenia syndrome virus (SFTSV)	Ticks	CHN, JPN, ROK
<i>Nairoviridae</i>	<i>Orthonairovirus</i>	<i>Crimean-Congo hemorrhagic fever orthonairovirus</i>	Crimean-Congo hemorrhagic fever virus (CCHFV)	Ticks	CHN
<i>Reoviridae</i>	<i>Orbivirus</i>	<i>Epizootic hemorrhagic virus</i>	Epizootic hemorrhagic disease virus 1 (EHDV-1)	<i>Culicoides</i> spp.	JPN
			Epizootic hemorrhagic disease virus 2 (EHDV-2)	<i>Culicoides</i> spp.	JPN, ROK, TWN
			Epizootic hemorrhagic disease virus 6 (EHDV-6)	<i>Culicoides</i> spp.	<i>JPN</i>
			Epizootic hemorrhagic disease virus 7 (EHDV-7)	<i>Culicoides</i> spp.	CHN, JPN
			Epizootic hemorrhagic disease virus 10 (EHDV-10)	<i>Culicoides</i> spp.	JPN
		<i>Bluetongue virus</i>	Bluetongue virus 1 (BTV-1)	<i>Culicoides</i> spp.	CHN, JPN, ROK
			Bluetongue virus 2 (BTV-2)	<i>Culicoides</i> spp.	CHN, JPN, ROK, TWN
			Bluetongue virus 3 (BTV-3)	<i>Culicoides</i> spp.	CHN, JPN, ROK
			Bluetongue virus 4 (BTV-4)	<i>Culicoides</i> spp.	CHN, ROK
			Bluetongue virus 5 (BTV-5)	<i>Culicoides</i> spp.	CHN
			Bluetongue virus 7 (BTV-7)	<i>Culicoides</i> spp.	CHN, ROK
			Bluetongue virus 9 (BTV-9)	<i>Culicoides</i> spp.	CHN, JPN
			Bluetongue virus 12 (BTV-12)	<i>Culicoides</i> spp.	CHN, JPN, TWN
			Bluetongue virus 15 (BTV-15)	<i>Culicoides</i> spp.	CHN, ROK
			Bluetongue virus 16 (BTV-16)	<i>Culicoides</i> spp.	CHN, JPN, ROK
			Bluetongue virus 20 (BTV-20)	<i>Culicoides</i> spp.	<i>JPN</i>
			Bluetongue virus 21 (BTV-21)	<i>Culicoides</i> spp.	CHN, JPN
			Bluetongue virus 24 (BTV-24)	<i>Culicoides</i> spp.	CHN
			Unclassified strain XJ1407	<i>Culicoides</i> spp.	CHN
		<i>Palyam virus</i>	Chuzan virus (CHUV)	<i>Culicoides</i> spp.	CHN, JPN, ROK, TWN
			D'Aguilar virus (DAGV)	<i>Culicoides</i> spp.	JPN
			Bunyip Creek virus (BCV)	<i>Culicoides</i> spp.	JPN
		<i>Yunnan orbivirus</i>	Yunnan orbivirus (YUOV)	Mosquitoes	CHN
		Unclassified	Guangxi orbivirus (GXOV)	NR	CHN
		Unclassified	Tibet orbivirus (TIBOV)	Mosquitoes, <i>Culicoides</i> spp.	CHN
		Unclassified	Sathuvachari virus (SVIV)	Mosquitoes	JPN
	<i>Seadornavirus</i>	<i>Banna virus</i>	Banna virus (BAV)	Mosquitoes	CHN
<i>Rhabdoviridae</i>	<i>Ephemerovirus</i>	<i>Bovine fever ephemerovirus</i>	Bovine ephemeral fever virus (BEFV)	Mosquitoes/ <i>Culicoides</i> spp.	CHN, JPN, ROK, TWN
		Unclassified	KSB-1/P/03	NR	JPN
	<i>Ledantavirus</i>	<i>Fukuoka ledantavirus</i>	Fukuoka virus (FUKV)	Mosquitoes/ <i>Culicoides</i> spp.	JPN
<i>Flaviviridae</i>	<i>Flavivirus</i>	<i>Japanese encephalitis virus</i>	Japanese encephalitis virus (JEV)	Mosquitoes	CHN, JPN, ROK, TWN
		<i>West Nile virus</i>	West Nile virus (WNV)	Mosquitoes	CHN, ROK

(Continued)

TABLE 1 | Continued

Family	Genus	Species	Virus/serotype (abbreviation)	Arthropod vectors	Geographical location of isolation/detection*
<i>Togaviridae</i>	Unclassified	<i>Kyasanur Forest disease virus</i>	Kyasanur Forest disease virus (KFDV)	Ticks	CHN
		<i>Tick-borne encephalitis virus</i>	Tick-borne encephalitis virus (TBEV)	Ticks	CHN, JPN, ROK
		Unclassified	Jingmen tick virus (JMTV)	Ticks	CHN
	<i>Alphavirus</i>	<i>Getah virus</i>	Getah virus (GETV)	Mosquitoes	CHN, JPN, ROK, TWN

NR, none reported; CHN, mainland China; JPN, Japan; ROK, Republic of Korea; TWN, Taiwan.

*Locations with virus isolation (bold letter), serological evidence (italics), genetic detection (underline) or the latter two (italics and underline).

TABLE 2 | Important endemic arbovirus infections in ruminant livestock in East Asia.

Disease	Etiological virus	Clinical signs (affected ruminant species)
Akabane disease	AKAV	Abortion, premature birth, stillbirth, congenital malformations (cattle, buffalo, sheep, goat) Neurological disorders caused by encephalomyelitis (postnatally infected cattle)
Aino virus infection	AINOV	Abortion, premature birth, stillbirth, congenital malformations (cattle, buffalo, sheep, goat)
Chuzan disease	CHUV	Congenital malformations (cattle)
Bovine ephemeral fever	BEFV	Sudden fever, anorexia, depression, tachypnea, lacrimation, foamy salivation, arthritis, limping, anastasia (cattle, buffalo)
Ibaraki disease	EHDV-2 (Ibaraki strain)	Fever, anorexia, lachrymation, salivation, nasal discharge, conjunctival injection, swallowing difficulty (cattle)
Bluetongue	BTV	Fever, nasal discharge, tachypnea, lethargy, hemorrhages (cattle, sheep, goat) Congenital malformations (cattle)

BEF caused by the novel genogroup on dairy and beef production should thus be evaluated.

At laboratory diagnosis, the virus neutralization test (VNT) is generally used to detect rising titers of neutralization antibody in paired acute and convalescent sera from affected cattle which show the typical clinical signs of BEF (14). For serological detection of BEFV, an indirect-ELISA with the recombinant protein of BEFV G gene was also developed (31). In recent years, conventional RT-PCRs targeting the G gene was available to detect BEFV in the diseased cattle (32–34). Real-time RT-PCR and reverse-transcription, loop-mediated isothermal amplification (LAMP) assays were also established for rapid and sensitive detection of BEFV gene (32, 35, 36).

Vaccination is now the current gold standard to prevent BEF. Commercial attenuated and killed vaccines have been developed and widely used in Japan, Korea, China and Taiwan (23, 27, 33,

TABLE 3 | Emerging arboviruses suspected to cause diseases in domestic ruminants in East Asia.

Virus	Clinical signs (affected ruminant species)
PEAV	Congenital malformations (cattle, experimentally in sheep)
SHAV	Congenital malformations (cattle)
SATV	Congenital malformations (cattle)
EHDV-6	Anorexia, fever, cessation of rumination, salivation, swallowing difficulty (cattle)
EHDV-7	Abortion, stillbirth, salivation, swallowing difficulty (cattle)
DAGV	Congenital malformations (cattle)
YUOV	Neurological disorders (cattle, sheep)

TABLE 4 | Zoonotic arboviruses infecting domestic ruminants in East Asia.

Virus	Symptoms in humans	Symptoms in domestic ruminants (species)
BATV	Mild flu-like illness	Mild illness (sheep, goat)
SFTSV	Severe fever with thrombocytopenia syndrome	NR
CCHFV	Hemorrhagic fever	NR
BAV	Encephalitis	NR
JEV	Encephalomyelitis	Encephalomyelitis (cattle)
WNV	Febrile illness, encephalitis, meningitis	Lymphoplasmacytic meningoencephalitis (sheep)
KFDV	Hemorrhagic fever	NR
TBEV	Febrile illness, encephalitis	NR

NR, none reported.

37–39). Repetitive outbreaks of BEF in East Asia indicate that continuous vaccination program is essential to provide sufficient immunity against BEFV among naïve population and minimize economical loss caused by BEF. Antigenic association between the vaccine strains and recent field isolates of BEF should be assessed to guarantee the vaccine efficacy.

Ibaraki Disease

During a large epidemic of an acute febrile disease in 1959 and 1960 in Japan, deglutitive difficulty based in the esophageal and laryngopharyngeal musculature was observed among affected

cattle (40). A total of 43,793 cases and 4,298 deaths were reported during the epidemic. That febrile disease was at one time called “bluetongue-like disease” because its clinical signs resembled those caused by BTV. The etiological agent was isolated from naturally and experimentally infected cattle and was designated as Ibaraki virus (IBAV), which denotes the location from which it was isolated (40, 41). The clinical signs of IBAV were exhibited by the experimentally infected cattle. IBAV was later identified as a strain of EHDV serotype 2 (EHDV-2) based on a virus neutralization test (42). Smaller outbreaks were reported in southern Japan in 1982 and 1987 (43–45), but no case report was known between 1988 and 2012. A resurgence of IBAV was observed in southern Japan in 2013, although only a few cattle showed the typical symptoms (46).

An occurrence of Ibaraki disease was recognized in Korea in 1982, when the epidemic was observed in southern Japan (47). In Taiwan, IBAV was initially obtained in 1990 from the cerebellum of a calf with non-suppurative encephalitis, and an occurrence of Ibaraki disease was observed at several farms in the same year (48). IBAV was also isolated from the affected cattle and from asymptomatic cattle from the same cowsheds where the affected cattle were raised. The seroprevalence (25–85%) of IBAV in dairy cattle was revealed in Taiwan between 1987 and 1990. There is no available information about Ibaraki disease outside of the above-mentioned regions.

At laboratory diagnosis, detection of neutralization antibody to IBAV and virus isolation from diseased cattle are usually tested (41, 43–46). IBAV-specific RT-PCR based on segment 2 is available to detect the virus from infected cattle (46). A real-time RT-PCR assay for typing of EHDV would be able to detect the presence of IBAV in diagnostic samples (49). Currently, commercial attenuated and killed vaccines to IBAV are prepared in Japan (50, 51).

Akabane Disease

A large outbreak of abortions, premature births, still births, and congenital malformations in calves was reported in Japan between 1972 and 1975 (52). The number of affected calves reached ~42,000 during the outbreak. The malformed calves had congenital lesions characterized by arthrogryposis-hydranencephaly (A-H) syndrome (53, 54). A serological survey of affected calves demonstrated that Akabane virus (AKAV; species *Akabane orthobunyavirus*, genus *Orthobunyavirus*, family *Peribunyaviridae*) was a causative agent of the disease (54). The virus is widely distributed in Africa, the Middle East, Asia and Australia (55) and severe outbreaks of Akabane disease have sometimes occurred in Japan, Australia, and Israel (52, 56–59).

Although AKAV was first isolated in 1959 from mosquitoes (60), its relationship with ruminant disease had been unknown until the outbreak. The etiology of AKAV in bovine and caprine fetuses was demonstrated by experimental infections of pregnant cows and goats (61, 62). Stillbirths and congenital malformations of neonate lambs caused by AKAV were observed in northern Japan between late 1985 and early 1986 (63). The isolation of AKAV from *Culicoides* biting midges, cattle, and a piglet has also been successful (64–67). In 1984, AKAV was isolated from a

postnatally infected calf with encephalitis during a small outbreak in Japan; it was a suspected causative agent (68).

Outbreaks of encephalomyelitis caused by postnatal AKAV infection occurred in 2006 and 2011 with 180 and 165 deaths, respectively (67, 69). The AKAVs isolated in Japan since 1959 are clustered into two genogroups (I and II) (67, 70). Genogroup I AKAVs have frequently been detected and/or isolated from affected cattle with encephalomyelitis (67, 69). These isolates probably have high neurotropic and/or neurovirulence and cause the neurological disorders in cattle. Recent monitoring in Japan revealed that the frequent incursion of genogroup I AKAVs increased the number of postnatal encephalitis cases (65). There have been repeated epidemics of Akabane disease including both *in utero* and postnatal infections in Japan (52, 67).

The first recognition of Akabane disease in Korea was in 1979 (71). Several AKAV isolates were then obtained from aborted fetuses, affected cattle with neurological disorders, and sentinel cattle between 1993 and 2010 (72–75). Serological surveillance of domestic ruminants revealed that frequent incursions of AKAV had occurred, and the virus occasionally spread widely (27, 76–78). Ruminants affected by AKAV have been reported since the first recognition (52, 79). In 2010, a large outbreak of epizootic encephalomyelitis in postnatally infected cattle also occurred in the southern part of Korea (74). The causative AKAV isolates were classified into genogroup I (75). Before that outbreak, several sporadic cases of bovine encephalomyelitis were reported in 2000 and 2001 (80).

In 1992, AKAV was isolated in Taiwan from affected calves with non-suppurative encephalitis and healthy calves (81). The 307 sera collected from 341 affected calves with encephalitis in 1989 and 1990 contained the specific antibodies to AKAV (82). A high seroprevalence (94.3%) of AKAV was also observed in 1994 (82). Since 1998, AKAV has been isolated from mosquitoes, bamboo rats (*Rhizomys pruinosus*), and a goat in the southern part of mainland China (83–87). Large-scale serological surveillance demonstrated that AKAV infection was relatively common in cattle and sheep in most of mainland China (83). However, no detailed description of AKAV outbreaks in mainland China can be obtained from published references, and the impacts of AKAV on the Chinese livestock industries remain unknown.

Diagnosis of Akabane disease is usually conducted by VNT for detection of specific antibodies to AKAV in fetal fluids or precolostrum sera of malformed newborns (55). ELISAs were also established for serological tests to detect antibodies to AKAV (88, 89) and the commercial ELISA kits are often used for the diagnosis and serological surveillance (90–92). The virus may be isolated from aborted fetuses (93). Because the neutralization antibodies to AKAV probably clear the virus and virus infected cells before the delivery of affected neonates, virus isolation is not successful from them. The virus gene is also unable to be detected from most of the affected calves. However, conventional and real-time RT-PCR assays can provide a sensitive detection of the AKAV-specific gene in central nervous system of the postnatally infected cattle with encephalomyelitis (69, 74, 94). Attenuated and inactivated vaccines against AKAV for breeding cows have

been developed (95–97) and commercially provided in Japan and Korea.

Aino Virus Infection

Aino virus (AINOV; species *Aino orthobunyavirus*, genus *Orthobunyavirus*) was first isolated from mosquitoes in Japan in 1964 (98). *Culicoides* biting midges are currently regarded as incriminate vectors for AINOV (65). During the outbreak of bovine congenital abnormalities caused by AKAV in Japan in 1972–1975, the precolostral sera of several affected calves with A-H syndrome contained antibodies to AINOV, but not to AKAV (53). Since then, cases suspected of being associated with AINOV have been sporadically reported in the western part of Japan (99–102). AINOV was also obtained from an aborted bovine fetus with brain lesions (103, 104). These findings indicated that AINOV was involved in abnormal deliveries in the region. Later, teratogenicity characterized by arthrogryposis, hydranencephaly, and cerebellar hypoplasia was demonstrated in neonate calves intrauterinely injected with AINOV (105). Several outbreaks (from 17 to 700 cases) of Aino virus infection in Japan were reported between 1995 and 2006 (52). All known Japanese AINOVs likely belong to a single genogroup, but genetically distant from Australian AINOVs (106–108).

A few cases of Aino virus infection were also reported in Korea, and the virus was isolated from a healthy cow in 1999 (52). The seroprevalence of AINOV was demonstrated in cattle ($\leq 33.2\%$) and goats (13.3%) in Korea (27, 77, 78). Although there has been no report of Aino virus infection in China, 10 of 50 AKAV-positive cattle sera collected during the nationwide surveillance of AKAV in mainland China neutralized AINOV (83). A partial genome of AINOV was detected from *Culicoides* biting midges and mosquitoes collected at dairy farms in Taiwan between 2012 and 2015 (109).

In Australia, AINOV was also isolated (110, 111) and its association with congenital abnormalities was reported (112). Shuni virus, which is closely related with AINOV and distributed in Africa and the Middle East, caused abortions and congenital malformations in sheep, goat, and cattle (113), and neurological disorders in cattle (114).

VNT for precolostral sera of the affected neonates is the most common diagnostic test at present. Commercial inactivated vaccines for breeding cattle are available in Japan and Korea (51, 115).

Chuzan Disease

From the winter of 1985 to the spring of 1986, an epizootic of congenital abnormalities of calves with hydranencephaly-cerebellar hypoplasia (HCH) syndrome occurred in southern Japan (116). Unlike Akabane disease, the affected calves did not show arthrogryposis. Approximately 2,400 affected calves were reported during the epizootic. In the autumn of 1985, a virus classified as a member of the *Orbivirus* genus was isolated from *Culicoides* biting midges and bovine blood samples collected in the epizootic region; it was tentatively designated as Chuzan virus (CHUV) (117). The etiological role of CHUV in the epizootic was suggested by serological tests for the affected calves (116). In addition, a newborn calf with HCH syndrome was delivered from a dam experimentally infected with CHUV,

and the disease caused by CHUV was named Chuzan disease (118). It was later reported that CHUV is serologically cross-reacted with Kasba virus (KASV), which was isolated in India in 1957 and is a serotype of the species *Palyam virus* (119). Recent genetical characterization of Palyam virus group also demonstrated the closed relationship between CHUV, KASV, and Vellore virus, which was isolated in India in 1956 (120, 121). After the large 1985–1986 outbreak in southern Japan, the disease has sporadically occurred there.

The isolation of the virus and its seroprevalence (91%) among dairy cattle in Taiwan in 1993 were reported (122). In Korea, the prevalence of CHUV has been recognized since 1993, but only a few sporadic cases of affected calves have been reported in recent years (27, 52, 77). Two isolates of CHUV were obtained from sentinel cattle in the southern part of mainland China in 2012 (123, 124). CHUV was also detected from yaks in the Qinghai-Tibetan Plateau in 2016–2017, although no affected case was reported (125). The impact of CHUV in mainland China remains unclear.

Presence of antibody to CHUV in precolostral serum from abnormal calves indicates its association with lesions in central nervous system (126). For serological diagnosis, VNT is appropriate. Inactivated vaccines against CHUV for breeding cows was developed and are commercially available in Japan and Korea (51, 115, 127).

Bluetongue

Bluetongue is an infectious hemorrhagic disease in domestic ruminants and is caused by BTV, which belongs to the genus *Orbivirus* and consists of at least 27 serotypes (128). *Culicoides* biting midges play a principal role in its transmission. The virus is widely distributed through the world. The circulation of a variety of BTV serotypes has been detected in East Asia. In 1974, bovine sera shown to be BTV-positive by a complement-fixation (CF) test were obtained from cattle in southern Japan (129). Thereafter, at least eight BTV serotypes have been found in Japan based on virus isolation and serological surveillance (130–132). In 1994 and 2001, a total of 23 and 45 sheep, respectively, on the same farm in central Japan developed typical symptoms of bluetongue (131). Twenty-three cattle also developed bluetongue in the same region in 1994.

Suspected bluetongue cases in sheep were first reported in the southern part of mainland China in 1974, and the virus was isolated in 1979 during an outbreak of bluetongue-like disease (133, 134). The prevalence of 12 BTV serotypes in mainland China was determined based on virus isolation (135). A probable novel serotype of BTV spread in the northwestern part of China in 2014 (136). Extensive research including risk analyses of BTV spread were conducted in areas with large sheep populations (137–139).

The first BTV isolation in Taiwan was in 2003 (140). The circulation of two BTV serotypes in Taiwan has been identified to date (141). A retrospective study revealed that antibodies to BTV have been present in goat sera in Taiwan since 1989. However, no clinical cases of bluetongue have been observed. In Korea, BTV-1 was isolated from a bovine blood sample collected at an abattoir in 2012 (142), and thereafter, the circulation of BTV was confirmed in goats, deer, and dairy cattle by serological

surveillance (143–145). Seven BTV serotypes have been detected in Korea to date. Recently, BTV-3 was also obtained from a blood sample of cattle reared in Korea (146).

The genomic analysis of BTV isolates in East Asia demonstrated that most of them cluster within the eastern BTV topotype from Asia and Australia (132, 135, 146–150). However, a part of segments of Chinese BTV-7 isolate probably originated from those of African BTV isolates which cluster within the western BTV topotype (151). The finding suggests that genetic reassortment events between different topotypes have occurred in regions neighboring to East Asia.

Various serological and molecular assays have been developed and used for laboratory diagnosis (152). Competitive ELISA, VNT, and conventional/real-time RT-PCR assays have been applied for BTV surveillances in mainland China and Korea (137–139, 141, 143–145). The agar gel immunodiffusion (AGID) test for detection of antibodies to BTV also had been used (131). However, cross-reactions between IBV and BTV may result in false-positive to BTV-AGID (153). There is no available information for applicable BTV vaccines in East Asia.

Other Orthobunyaviruses

Around the year 2000, the circulation of three orthobunyaviruses (genus *Orthobunyavirus*) which were new in East Asia was identified. In 1999, Peaton virus (PEAV; species *Peaton orthobunyavirus*), which was initially isolated in Australia in 1976 (154), was obtained from sentinel cattle and *Culicoides* biting midges in Japan (155). A stocked virus isolated in 1987 was also identified as PEAV (107). Australian researchers reported that the experimental infection of a pregnant ewe with PEAV induced congenital defects in its lamb (156). Precolostral sera obtained from 31 malformed calves between 1996 and 2016 were positive for PEAV, but negative for known teratogenic viruses (157). These findings suggested that, like AKAV and AINOV, PEAV causes congenital malformations in calves and lambs. Antibodies to PEAV were detected in cattle in mainland China during large-scale sero-surveillance for AKAV (83). The seroprevalence (1.1%) of PEAV among cattle was also demonstrated in Korea during 2013–2015 surveillance (78). In Taiwan, a partial sequence of PEAV was obtained from *Culicoides* biting midges (109). The PEAV circulation was also observed in Israel (158). During the epizootic, PEAV specific gene was detected from a calf with hydranencephaly (159).

Sathuperi virus (SATV; species *Schmallenberg orthobunyavirus*) was isolated from mosquitoes in India in 1957, and its prevalence in Nigeria in the 1960s was identified (160, 161). The virus has been isolated from sentinel cattle and *Culicoides* biting midges since 1999, and an association between SATV and congenital abnormalities in calves was suggested because precolostral sera from several calves with congenital malformations neutralized SATV (162, 163). The seroprevalence (4.9%) of SATV in Korea was also reported (78).

Shamonda virus (SHAV; species *Schmallenberg orthobunyavirus*) was initially isolated in Nigeria in 1960s (161), and since then there had been no report of it for a long while. In 2002, SHAV suddenly emerged in southern Japan, which is geographically far from Africa (164). Precolostral sera from several malformed calves delivered after the SHAV spread

in the epizootic region contained neutralization antibodies to SHAV (164–166). Between the winter of 2015 and the spring of 2016, over 20 malformed calves exposed to intrauterine infection with SHAV were reported in southern Japan (166, 167). Antibodies to SHAV (5.6%) were also detected in Korea, but there is no information regarding its association with abnormal deliveries (78).

The pathogenicity of these orthobunyaviruses is poorly understood, because no experimental infections to cattle successfully developed clinical signs that are observed in the clinical cases in the field. The reported cases that were probably affected by PEAV, SATV, or SHAV were highly sporadic after their epizootic in Japan. The congenital lesions in the brains of calves infected with PEAV or SHAV were milder than those of calves infected with AKAV or AINOV (157, 166). It is probable that the viruses currently circulating in East Asia have relatively low pathogenicity. However, like other segmented viruses, a genetic shift of orthobunyaviruses with three segmented genomes potentially strengthens their virulence to ruminant hosts (168).

It is of interest that the “S” and “L” RNA segments of SBV have a high degree of sequence identity with that of SHAV, but the sequence of the “M” RNA segment of SBV was genetically close to that of SATV (163, 169). *In vitro* coinfection assays in mammalian and insect cells easily generate reassortants between SATV and SHAV (170). The genetic reassortment might have also occurred among other orthobunyaviruses isolated from ruminants in East Asia and Australia in their evolutionary history (70, 107). Although the impacts of PEAV, SATV, and SHAV remain unclear, these viruses should be listed among the teratogenic viruses in ruminants, and their circulation and the suspected cases associated with them should be monitored in East Asia.

Orbiviruses Other Than IBV, CHUV, and BTV

In 1997, over 1,000 cases of abortion and stillbirths in cattle occurred in southern Japan. A potential etiological agent was isolated from aborted fetuses, sentinel cattle, and *Culicoides* biting midges and was identified as a strain of EHDV (65, 171, 172). The EHDV strain was initially regarded as a variant of IBV, because the virus was cross-reacted with IBV by a virus neutralization test, and the sequence of its segment 2 was reported to show a degree of similarity to that of IBV (172, 173). Later research demonstrated that this virus belongs to the serotype 7 group of EHDV, based on phylogenetic studies of segment 2 sequences encoding the outermost capsid protein (46, 174). Ibaraki disease-like symptoms (e.g., swallowing difficulty) in cattle were also reported during the epizootic. In 2013, EHDV-7 was also isolated in the southern part of mainland China from sentinel cattle, but its pathogenicity remains unknown (175).

In 2015, an outbreak of febrile illness resembling Ibaraki disease in cattle occurred in a limited part of mainland Japan (176). The EHDV serotype 6 (EHDV-6) gene was detected in all affected cattle by serotype-specific RT-PCR. Although EHDV serotype 1 has been detected and isolated in Japan (46, 174, 177), its association with clinical cases in ruminants has not been demonstrated. A strain of EHDV that was isolated in the southwestern islands of Japan was serologically and genetically

distinct from the known EHDV serotypes and was tentatively assigned to the new serotype 10 (174).

D'Aguilar virus (DAGV), which is a member of the species *Palyam virus*, was first isolated in Australia in 1968 (110). The analysis of stocked viruses revealed that the incursion of DAGV occurred in Japan's southwestern islands in 1987 at the least (178). DAGV spread in southern Japan in the autumn of 2001, and affected calves with Chuzan disease-like symptoms were observed during the following winter and spring. During the epizootic period, no CHUV was isolated from cattle in the affected region. Precolostral sera of these calves clearly neutralized DAGV, suggesting its association with congenital malformations. The virus has been repeatedly isolated in the southern part of mainland Japan and Japan's southwestern Islands (179, 180).

Bunyip Creek virus (BCV), which is another serotype of species *Palyam virus*, was isolated from cattle and *Culicoides* biting midges in Japan in 2008 and 2009 (179, 180). There is no evidence that BCV was associated with ruminant disease in Japan or in Australia, where BCV was initially isolated (181).

Yunnan orbivirus (YUOV) was first isolated from mosquitoes collected in the southern part of mainland China in 1997, and it was identified as a member of a new orbivirus species, *Yunnan orbivirus*, in 2005 (182). A second serotype of *Yunnan orbivirus* (YOUV-2), which is also called Middle Point virus, was obtained from healthy cattle in Australia in 1998 (183, 184). In addition, another orbivirus related with YOUV was isolated from a blood sample of cattle in the southern part of mainland China in 2015 and was designated as Guangxi orbivirus (185). Interestingly, YOUV was also isolated from cattle, sheep, a donkey, and a dog in Peru in 1997 (186). The infected animals showed neurological signs. Although no evidence of an association between YOUV and animal disease in East Asia has been described, this finding suggests its potential ability to cause neurological disease in domestic ruminants.

ZOONOTIC ARBOVIRUSES

Mosquito-Borne Arboviruses

Several zoonotic arboviruses of importance to human health have been detected from domestic ruminants in East Asia (Table 4). Some of these arboviruses cause clinical signs in both infected humans and ruminants, but the others produce asymptomatic and negligible mild disease in ruminants.

Japanese encephalitis virus (JEV; species *Japanese encephalitis virus*, genus *Flavivirus*, family *Flaviviridae*) caused severe encephalitis not only in humans but also in horses and cattle (187). The JEV infection resulted in abortion and stillbirth in sows and aspermia and disruptions of spermatogenesis in boars (187). Although JEV has spread across a wide area in Japan (188), only a few affected cases of cattle have been sporadically recorded in Japan (189–193).

Outbreaks of West Nile virus (WNV; species *West Nile virus*, genus *Flavivirus*) infection have occurred across the Mediterranean region, Eastern Europe, Africa, Asia, and North America (194). Human cases of WNV infection were also reported in western China in 2011, and WNV was simultaneously

isolated from mosquitoes in the affected area (195). The WNV gene was detected from domestic pigeons in Korea in 2014 (196). It was recently demonstrated that sporadic cases of severe lymphoplasmacytic meningoencephalitis were associated with WNV in California (197). However, ruminants are not considered hosts severely affected by WNV, and no ruminant illness associated with WNV has been recorded in East Asia.

Banna virus (BAV), which belongs to the species *Banna virus* of the genus *Seadornavirus* within the family *Reoviridae*, was first isolated in mainland China in 1987 from a patient with meningoencephalitis (198, 199). Since then, BAV has been obtained from pigs, cattle, ticks, mosquitoes, and *Culicoides* biting midges over a wide range of mainland China (200, 201). The virus is thought to cause encephalitis in humans, and many cases associated with it might have been misdiagnosed as Japanese encephalitis (200). At present, there is no evidence of a relationship between BAV and ruminant illness.

Batai virus (BATV), which belongs to the species *Batai orthobunyavirus* of the genus *Orthobunyavirus*, has been isolated in Japan and mainland China since 1994 (202–205). BATV is widely distributed in Asia and Europe, and its infection has been observed in cattle, sheep, and goats (202, 206, 207). BATV was reported to cause a mild flu-like illness in humans and mild illness among sheep and goats. At present, no affected case associated with BATV has been reported in East Asia. Ngari virus, which is a possible reassortant between Bunyamwera virus and BATV, has been implicated in human hemorrhagic fever in Africa (202, 208, 209). In the future, BATV may obtain a higher virulence by genetic reassortment with other related orthobunyaviruses. The potential risk of BATV to public health in East Asia should be considered.

Tick-Borne Arboviruses

Four medically important tick-borne arboviruses that infect domestic ruminants are prevalent in East Asia (Table 4). Crimean-Congo hemorrhagic fever virus (CCHFV; species *Crimean-Congo hemorrhagic fever orthonavirus*, genus *Orthonairovirus*, family *Nairoviridae*) was involved in human clinical cases in the northwestern part of mainland China (210). Although infected domestic ruminants play a crucial role as reservoirs of CCHFV in an endemic cycle of transmission, they do not develop any symptomatic illness after CCHFV infection (211).

Tick-borne encephalitis virus (TBEV; species *Tick-borne encephalitis virus*, genus *Flavivirus*, family *Flaviviridae*) is widely distributed in mainland China, Japan, and Korea (212). This virus sometimes causes severe encephalitis in humans. In Europe, TBEV infection in humans following the consumption of unpasteurized milk and cheese from domestic ruminants has been reported (213). However, the role of domestic ruminants in TBEV endemics in East Asia remains unknown. Another zoonotic tick-borne flavivirus, Kyasanur Forest disease virus (KFDV), was isolated from a febrile patient in the southern part of mainland China in 1989 (214). Although domestic ruminants may be infected with KFDV (215), their role in the viral transmission remains unclear.

Severe fever with thrombocytopenia syndrome virus (SFTSV; species *Huaiyangshan banyangvirus*, genus *Banyangvirus*, family *Phenuiviridae*) was recognized in mainland China, Japan, and Korea (216–218). Although this tick-borne virus has probably been circulating in East Asia for many years, its association with the severe illness in humans was not recognized until 2011. The seroprevalence of SFTSV in domestic ruminants in mainland China, Japan, and Korea was reported (219–222), and epidemiological studies suggested that farmers in rural areas are at risk of SFTSV infection (223). The continuous monitoring for SFTSV in domestic ruminants is therefore necessary to assess the ruminants' contribution to SFTSV endemics in the affected regions.

Compared to mosquito/*Culicoides*-borne viruses, tick-borne viruses are not likely to expand their range within a short period of time. However, it was suggested that migratory birds play an important role as long-distance transporters for ticks infected with arboviruses (224, 225). The role of birds in any sudden incursion and occurrence of tick-borne virus infections in a region that was previously free of the infections should thus be considered.

OTHER ARBOVIRUSES

Several arboviruses with unknown impacts have been confirmed to infect domestic ruminants. The infected ruminants usually do not develop apparent clinical signs, and thus these viruses have been neglected for many years. Nevertheless, the recent progress in genetic analyses can characterize their properties and assign their taxonomy.

Getah virus (GETV; species *Getah virus*, genus *Alphavirus*, family *Togaviridae*) is an etiological agent of fever, rash, and leg edema in horses (226) and of fetal death and reproduction disorders in pigs (227, 228). The prevalence of GETV has been confirmed in mainland China, Japan, Korea, and Taiwan (229, 230). Outbreaks of GETV infection have occurred among Japanese racehorses (231, 232). It is known that GETV also infects cattle, although there is no evidence of illness in the infected cattle (226, 233, 234). However, cattle may contribute to a GETV endemic as a reservoir.

Fukuoka virus (FUKV) is assigned to the species *Fukuoka ledantavirus* of the genus *Ledantavirus* within the family *Rhabdoviridae* and was initially isolated from *Culicoides punctatus* and the mosquito *Culex tritaeniorhynchus* in southern Japan in 1982 (235). The virus was first regarded as a member of the ephemeral fever serogroup but was subsequently classified as a member of the Kern Canyon serogroup viruses (236, 237). FUKV was later isolated from sentinel cattle in a different region in Japan and, simultaneously, the seroprevalence of FUKV in cattle was demonstrated (238). Although cattle were identified as a susceptible host of FUKV, the pathogenicity of this virus in cattle remains uncertain. Full-genome analysis of ledantaviruses demonstrated that FUKV has a closed relationship (79.5% nucleotide identity) with Barur virus which has been isolated from ticks, fleas, mosquitoes and a rodent in India and Africa (239). Nishimuro virus from a wild boar in Japan (240) is also genetically similar to FUKV. Further studies are needed to determine their vertebrate hosts and arthropod vectors.

Sathuvachari virus (SVIV) is a member of the genus *Orbivirus* and was initially isolated in 1963 from starlings (*Brahminy myna*) collected in southern India (241). The virus was also isolated from an unidentified source collected in Vietnam over 50 years ago (242). The genetic characterization of SVIV suggested its close relationship with other mosquito-borne orbiviruses (241). An unassigned virus isolated from bovine blood collected in Japan's southwestern islands in 2005 was recently identified as SVIV (243). No illness in the infected cattle was reported when their blood samples were collected, but the finding demonstrated that cattle are a susceptible host of SVIV and indicated that the virus has a wider host range than previously thought.

An ephemerovirus tentatively called KSB-1/P/03 was isolated from sentinel cattle in southern Japan in 2003 (179). The phylogenetic analysis based on the L protein of rhabdoviruses revealed that this virus is clustered with other ephemeroviruses. The infected cattle did not demonstrate any clinical signs. Other than BEFV, no ephemerovirus had been isolated in East Asia. Further genetic analyses should be conducted to determine the taxonomic assignment of KSB-1/P/03.

A virus isolated from mosquitoes in Tibet, China in 2009 was identified as a novel species of the genus *Orbivirus* (244). Designated as *Tibet Orbivirus* (TIBOV), the virus has been obtained from mosquitoes and *Culicoides* biting midges in other regions of mainland China (86, 245, 246). Phylogenetic analysis of inner core (T2) protein of known orbiviruses demonstrated that TIBOV is clearly categorized into the *Culicoides*-borne group with BTV, EHDV, and PALV (244, 245). The amino acid sequence diversity among outermost capsid proteins of TIBOV isolates indicated that the species consists of at least two serotypes (246). The neutralization antibodies to TIBOV were detected in cattle reared in the southern border region of mainland China (246).

A novel segmented RNA virus which is genetically related to flaviviruses was detected in ticks collected in mainland China in 2010 and was designated as Jingmen tick virus (JMTV) (247). Together with insect viruses that have a similar genome composition, JMTV is tentatively referred as the Jingmenvirus group (248). Cattle sera collected in mainland China were reported to contain antibodies against JMTV (247). The genome of JMTV was also detected in a part of the seropositive samples by RT-PCR screening. JMTV was recently identified as a potential human pathogen, because patients infected with the virus showed clinical manifestations such as fever, headache, and myalgia (249).

ARTHROPOD VECTORS OF IMPORTANT RUMINANT ARBOVIRUSES

The identification of the vectors of arboviruses is essential to understand the epidemiology of the arboviruses and to develop control measures against them. In this section, we focus on the potential vectors of arboviruses that affect domestic ruminants in East Asia. Many orbiviruses (e.g., BTV and EHDV) and orthobunyaviruses (e.g., AKAV and SBV) that are pathogenic to domestic ruminants were reported to be transmitted by *Culicoides* biting midges (250, 251). Long-term monitoring for virus detection in *Culicoides* biting midges in southern Japan

TABLE 5 | Isolation sources of hematophagous invertebrates for arboviruses affecting domestic ruminants in East Asia.

Virus	Mosquitoes	<i>Culicoides</i> biting midges
AKAV	<i>Aedes vexans</i> , <i>Culex tritaeniorhynchus</i> , <i>Cx. quinquefasciatus</i> , <i>Anopheles</i> <i>sinensis</i> , <i>An. vagus</i>	<i>C. oxystoma</i>
AINOV	<i>Cx. tritaeniorhynchus</i>	<i>C. oxystoma</i> , <i>C. punctatus</i>
PEAV	NR	<i>C. jacobsoni</i>
SHAV	NR	<i>C. tainanus</i>
SATV	NR	<i>C. oxystoma</i>
CHUV	NR	<i>C. oxystoma</i>
DAGV	NR	<i>C. oxystoma</i> , <i>C. sumatrae</i>
BCV	NR	<i>C. oxystoma</i>
EHDV-1	NR	<i>C. punctatus</i>
EHDV-2	NR	<i>C. oxystoma</i> , <i>C.</i> <i>lungchiensis</i> , <i>C. punctatus</i>
EHDV-7	NR	<i>C. oxystoma</i>
BTV-2	NR	<i>Culicoides</i> spp.
BTV-9	NR	<i>C. asiana</i>
BTV-12	NR	<i>Culicoides</i> spp.
BTV-16	NR	<i>C. tainanus</i>
YUOV	<i>Cx. tritaeniorhynchus</i>	NR

NR, none reported.

demonstrated that various arboviruses such as AKAV, AINOV, SATV, CHUV, DAGV, BCV, and EHDV were preferentially isolated from *C. oxystoma* (Table 5) (65, 179).

Although *Culicoides* biting midges are regarded as incriminate vectors of AKAV, the virus has been obtained from three major mosquito genera (*Aedes*, *Culex*, and *Anopheles*) in mainland China and Japan (Table 5) (60, 84, 86). A small portion of mosquitoes that feed on infected cattle during viremia may be infected with AKAV, as observed in the experimental infection of *Culex* mosquitoes with SBV (252).

Culicoides species such as *C. imicola* and *C. sonorensis*, which play the principal role for ruminant arbovirus transmission in other regions, are not distributed in East Asia (253). The distribution of *C. brevitarsis*, which is a incriminate vector of ruminant arboviruses in Australia (254) was described in southern Japan and Taiwan (255–257). However, molecular analyses using mitochondrial and nuclear DNA indicated that the specimens collected in southern Japan were distinct from the *C. brevitarsis* specimens collected in Australia, and thus a new species was recorded: *Culicoides asiana* (258, 259). The isolation of BTV from field-collected *C. asiana* was successful (260), and an experimental infection indicated that this species is susceptible to AKAV (261).

These findings suggested the vector capacity of *C. asiana* as with *C. brevitarsis*. The above-cited studies also suggested that *C. brevitarsis* and *C. asiana* were co-distributed throughout Southeast Asia and its neighboring regions, including Hainan Island, which is in a subtropical zone in China (258, 259). The role of these two species in arbovirus transmission should be further evaluated in their distribution ranges.

A laboratory rearing system of *Culicoides* biting midges was successfully established for limited species distributed in North America and Europe (251, 253). This limitation hampers the progress in the assessments of the vector capacity of various other *Culicoides* species. Instead, field-collected midges have often been used for checking the oral susceptibility and vector competence of ruminant arboviruses (262–266). Unfortunately, the vector competence of *Culicoides* biting midges distributed in East Asia is poorly understood. The viral isolations from pools of field-collected midges which were identified at the species level have helped estimate vector species (65, 179).

The oral susceptibility of several Japanese *Culicoides* species for AKAV was demonstrated (261). As the virus isolation from field-collected midges suggested, *C. oxystoma* was susceptible to AKAV, and the viral replication and dissemination were demonstrated in the infected midges. Interestingly, *C. tainanus* and *C. punctatus*, which have not been considered AKAV-competent vectors, were also orally infected with AKAV. During the AKAV endemic in northern Japan in 2010, *C. tainanus* and *C. punctatus* were dominant/subdominant species at cowsheds in the endemic region, but *C. oxystoma* has been never collected there (267). These species, which are common in East Asia (268–271) probably contributed to the AKAV transmission during that period.

BEFV has been isolated from several mosquito and *Culicoides* species in Africa, Australia, and Central Asia (9), but there has been no record of the isolation of BEFV from invertebrate vectors in East Asia so far (28). A laboratory experimental infection of *Culicoides* biting midges indicated that, at the least, they are not efficient vectors of BEFV (272). The vector search will be essential to understand the epidemiology of BEFV in East Asia.

In light of the BTV and SBV endemic experiences in northern Europe, it appears that the *Culicoides* species that are indigenous in northern temperate regions have the potential to transmit ruminant arboviruses (273, 274). It is necessary to assess the vector capacity of *Culicoides* species which are distributed in the higher-latitude regions in East Asia. It is also important to determine their distribution range and seasonality to gain a greater understanding of the potential risks of arbovirus spreads, even in previous arbovirus-free regions.

RESEARCH GAPS AND FUTURE DIRECTIONS

Vaccinations are probably the most effective preventive measures for arbovirus infections in domestic ruminants. The numbers of animals affected by AKAV, AINOV, CHUV, IBAV, and BEFV were dramatically reduced in Japan after the spread of vaccinations (52). However, limited effectiveness of the current vaccination strategy for BEFV was suggested in Taiwan and Israel (33, 275). It might be difficult to confer long-lasting effective immunity in vaccinated and infected cattle (276, 277), and this probably caused the repetition of BEF occurrence in the above-mentioned regions. It remains difficult to prevent the encephalomyelitis caused by postnatal AKAV infection (67). Vaccines for AKAV are generally administered to breeding cows but not other cattle

such as fattening cattle and bulls. There is not yet enough preparation for the pathogens that have recently emerged, such as PEAV, SATV, SHAV, and YUOV, which are suspected to cause ruminant illnesses. Probable loss and future threat caused by these viruses also remain uncertain. Depending on the situation, the development of preventive measures for these arboviruses that have newly emerged in East Asia would be necessary.

The genogroup shifts of AKAV in Japan clearly reflected the change in the types of disease (67). The newly emerged clade of BEFV seems to be more virulent than those of previously endemic strains (28). In addition, the reassortment of segmented RNA viruses (such as orthobunyaviruses and orbiviruses) may increase the viral fitness in vertebrate hosts and/or invertebrate vectors and enhance the virulence in vertebrate hosts and/or the transmission efficacy by invertebrate vectors. Therefore, continuous monitoring of the virulence and pathogenicity of endemic arboviruses should be conducted in East Asia.

The recent emergence of “new” arboviruses in East Asia has attracted interest in their origins and incursion routes. In addition, genogroup shifts of endemic arboviruses in the region have probably been induced by the introductions of viruses from a different genetic pool in other regions. Extensive surveillance in Australia obtained many arboviruses, and some of them are also endemic or were recently emerged in East Asia (278). However, phylogenetic studies of orthobunyaviruses and BEFV indicated that they evolved independently in separate gene pools with different geographical origins (22, 108, 279). In contrast, BEFV, BTV, BANV, and JEV strains, which were isolated in Southeast Asia, often have closed genetic relationships with those isolated in East Asia (280–283).

The circulation of other ruminant arboviruses such as AKAV, AINOV, PEAV, and BATV has also been demonstrated in Southeast Asian countries by serological surveillance and virus isolations (284–287). Unfortunately, there are not enough reports to determine the current status of these arboviruses in Southeast Asia. Further virological surveillance and genetic characterizations of isolated viruses are essential to elucidate the epidemiological linkage of ruminant arboviruses between East Asia and Southeast Asia.

The long-range dispersal of vector insects by winds is considered a principal cause of the introduction of arboviruses to geographically distant locations, including previously arbovirus-free areas (288). Historically, windborne introductions of *Culicoides*-borne animal diseases from distant endemic areas have been estimated based on meteorological data (289, 290). Plausible dispersal events from eastern Indonesia, Timor-Leste, and southern Papua New Guinea explored by using an atmospheric dispersion model helped elucidate the incursion of exotic *Culicoides* species and BTVs into northern Australia (291). Atmospheric dispersion models would be useful to assess the potential sources of arbovirus incursions from outside of East Asia and their spread within the region.

As a pioneering study, a backward-trajectory analysis of the BEF epidemic in 2012 in Japan's southwestern islands showed that the estimated potential sources of infected vectors were Southeast Asian countries such as Vietnam and Philippine (21).

Reassessments of historical outbreaks of ruminant arbovirus infections with an atmospheric dispersal model could also help determine their incursion sources and spread patterns.

With the exception of BEFV, which has a higher incident rate in cattle (9), almost all of the arboviruses generally result in a low morbidity, asymptomatic/mild illnesses or no apparent acute phase in domestic ruminants, and thus they silently circulate in the endemic regions. Active surveillance such as sentinel and vector surveillance systems are therefore needed for the efficient detection of the arboviruses' circulation in the field prior to disease outbreaks (292). The recent increase in the number of emerging arboviruses in East Asia indicates that other undetected arboviruses may have circulated. The expansion of global transportation may also enhance the risk of arbovirus introductions from geographically distant areas. Global warming will certainly affect the distribution and active period of vectors, and thus the ranges of virus spread will expand to higher-latitude regions.

Although sensitive detection systems are essential to identify the incursion and circulation of arboviruses, the current serological and genetic techniques cannot cover all arboviruses, especially if they are novel. Next-generation sequencing (NGS) technologies will support the detection and characterization of such viruses (293). The findings revealed by NGS will contribute to the development of diagnostic/detection systems suitable for the arboviruses and to the progress of etiological studies.

Clearly, knowledge gaps remain regarding the transmission cycles of arboviruses and vector ecology in East Asia. To the best of our knowledge, there is no detailed distribution map of vectors in the East Asia region. The vector capacity for arboviruses of veterinary importance has been evaluated for only a limited number of species. Further studies of the systematics, biology, and vector competence of mosquitoes, *Culicoides* biting midges, and ticks should be conducted. The knowledge obtained from vector studies will contribute to increased accuracy of epidemiological analyses based on landscape and meteorological data. Risk assessments based on such analyses will enable the development of better surveillance and preventive measures for arbovirus infections which threaten the production of ruminant livestock, as suggested in earlier investigations (294, 295).

Finally, the construction of a network in East Asia and its neighboring countries would be essential to promote research on arbovirus infections of importance to livestock. The sharing of epidemiological information, diagnostic tools, and control/preventive strategies are highly important because neighboring countries are likely to experience the same problems due to arboviruses. Language barriers may present a challenge to information exchanging and sharing. Many scientific reports and public records from East Asian countries have been written in national languages such as Chinese, Japanese, and Korean, but a limited portion of them might have been translated in English. Unfortunately, the status of arbovirus infections in domestic ruminants in Mongolia and North Korea remains unclear. Regional cooperation would therefore be a crucial key to clarify the current status of arbovirus infections

and minimize their impacts on the livestock industries in East Asia.

AUTHOR CONTRIBUTIONS

TY executed a first draft. YH and KM helped draft the manuscript. All authors contributed equally to the final draft and editing of the manuscript.

REFERENCES

1. Arrigo NC, Weaver SC, Calisher, CH. The taxonomy of arboviruses. In: Vasilakis N, Gubler DJ, editors. *Arboviruses*. Norfolk: Caister Academic Press (2016). p. 9–29. doi: 10.21775/9781910190210.02
2. Abudurexiti A, Adkins S, Alioto D, Alkhovsky SV, Avšič-Županc T, Ballinger MJ, et al. Taxonomy of the order *Bunyavirales*: update 2019. *Arch Virol*. (2019) 164:1949–65. doi: 10.1007/s00705-019-04253-6
3. Maclachlan NJ, Zientara S, Wilson, WC, Richt, JA, Savini, G. Bluetongue and epizootic hemorrhagic disease viruses: recent developments with these globally re-emerging arboviral infections of ruminants. *Curr Opin Virol*. (2019) 34:6–62. doi: 10.1016/j.coviro.2018.12.005
4. Wilson AJ, Mellor PS. Bluetongue in Europe: past, present and future. *Philos Trans R Soc Lond B Biol Sci*. (2009) 364:2669–81. doi: 10.1098/rstb.2009.0091
5. Savini G, Afonso A, Mellor P, Aradaib I, Yadin H, Sanaa M, et al. Epizootic hemorrhagic disease. *Res Vet Sci*. (2011) 91:1–17. doi: 10.1016/j.rvsc.2011.05.004
6. Hoffmann B, Scheuch M, Höper D, Jungblut R, Holsteg M, Schirrmeier H, et al. Novel orthobunyavirus in cattle, Europe, 2011. *Emerg Infect Dis*. (2012) 18:469–72. doi: 10.3201/eid1803.111905
7. Endalew AD, Faburay B, Wilson WC, Richt, JA. Schmallenberg disease—a newly emerged *Culicoides*-borne viral disease of ruminants. *Viruses*. (2019) 11:1065. doi: 10.3390/v11111065
8. Gilbert M, Nicolas G, Cinarli G, Van Boeckel TP, Vanwambeke, SO., et al. Global distribution data for cattle, buffaloes, horses, sheep, goats, pigs, chickens and ducks in 2010. *Sci Data*. (2018) 5:180227. doi: 10.1038/sdata.2018.227
9. Walker PJ, Klement E. Epidemiology and control of bovine ephemeral fever. *Vet Res*. (2015) 46:124. doi: 10.1186/s13567-015-0262-4
10. Dacheux L, Dommergues L, Chouanibou Y, Doméon L, Schuler C, Bonas S, et al. Co-circulation and characterization of novel African arboviruses (genus *Ephemerovirus*) in cattle, Mayotte island, Indian Ocean, 2017. *Transbound Emerg Dis*. (2019) 66:2601–4. doi: 10.1111/tbed.13323
11. Oguzoglu TC, Ertürk A, Çizmeçi SG, Koç BT, Akça Y. A report on bovine ephemeral fever virus in Turkey: antigenic variations of different strains of BEFV in the 1985 and 2012 outbreaks using partial glycoprotein gene sequences. *Transbound Emerg Dis*. (2015) 62:e66–70. doi: 10.1111/tbed.12187
12. Almasi S, Bakhshesh M. Antigenic variation of bovine ephemeral fever viruses isolated in Iran, 2012–2013. *Virus Genes*. (2019) 55:654–9. doi: 10.1007/s11262-019-01688-6
13. Aziz-Boaron O, Klausner Z, Hasoksuz M, Shenkar J, Gafni O, Gelman B, et al. Circulation of bovine ephemeral fever in the Middle East—strong evidence for transmission by winds and animal transport. *Vet Microbiol*. (2012) 158:300–7. doi: 10.1016/j.vetmic.2012.03.003
14. Walker PJ. Bovine ephemeral fever in Australia and the world. *Curr. Top. Microbiol Immunol*. (2005) 292:57–80. doi: 10.1007/3-540-27485-5_4
15. Sato K. A history of bovine influenza in Japan. *Jpn J Vet Hist*. (2016) 53:14–23. doi: 10.3201/eid2208.160362
16. Inaba Y. Bovine epizootic fever. *JARQ*. (1968) 3:36–42.
17. Inaba Y, Sato K, Tanaka Y, Ito H, Omori T. Serological identification of bovine epizootic fever virus as ephemeral fever virus. *Jpn J Microbiol*. (1969) 13:388–9. doi: 10.1111/j.1348-0421.1969.tb00484.x
18. Omori T. Ibaraki disease: a bovine epizootic disease resembling bluetongue. *Nat Anim Health Q*. (1970) 10:45–55.

FUNDING

This study was conducted under the research project on Development of the Management Technologies for the Risk of Introduction of Livestock Infectious Diseases and Their Wildlife-borne Spreads in Japan funded by the Ministry of Agriculture, Forestry and Fisheries of Japan (FY2018–2022).

19. Shirakawa H, Ishibashi K, Ogawa T. A comparison of the epidemiology of bovine ephemeral fever in South Korea and south-western Japan. *Aust Vet J*. (1994) 71:50–2. doi: 10.1111/j.1751-0813.1994.tb06153.x
20. Aizawa M, Takayoshi K, Kokuba T, Kato T, Yanase T, Yamakawa M, et al. Molecular epidemiological analysis of bovine ephemeral fever virus isolated in Okinawa Prefecture. *J Jpn Vet Med Assoc*. (2008) 61:363–6. doi: 10.12935/jvma1951.61.363
21. Hayama Y, Moriguchi S, Yanase T, Suzuki M, Niwa T, Ikemiyagi K, et al. Epidemiological analysis of bovine ephemeral fever in 2012–2013 in the subtropical islands of Japan. *BMC Vet Res*. (2016) 12:47. doi: 10.1186/s12917-016-0673-0
22. Hirashima Y, Nojiri M, Ohtsuka Y, Kato T, Shirafuji H, Kurazono M, et al. Resurgence of bovine ephemeral fever in mainland Japan in 2015 after a 23-year absence. *J Vet Med Sci*. (2017) 79:904–11. doi: 10.1292/jvms.16-0345
23. Zheng F, Qiu C. Phylogenetic relationships of the glycoprotein gene of bovine ephemeral fever virus isolated from mainland China, Taiwan, Japan, Turkey, Israel and Australia. *Virol J*. (2012) 9:268. doi: 10.1186/1743-422X-9-268
24. Li Z, Zheng F, Gao S, Wang S, Wang J, Liu Z, et al. Large-scale serological survey of bovine ephemeral fever in China. *Vet Microbiol*. (2015) 176:155–60. doi: 10.1016/j.vetmic.2014.12.008
25. Ogawa T. Epidemiological investigation of bovine ephemeral fever outbreaks in Kyushu Island in Japan during the fall of 1988. *Prev Vet Med*. (1992) 14:69–76. doi: 10.1016/0167-5877(92)90085-T
26. Ogawa T, Satoh C, Yamane I, Inoue T. Epidemiology of bovine ephemeral fever in China. *Bull Natl Inst Anim Health*. (1992) 100:29–35.
27. Kim YH, Oem JK, Lee EY, Lee KK, Kim SH, Lee MH, et al. Seroprevalence of five arboviruses in sentinel cattle as part of nationwide surveillance in South Korea, 2009–2012. *J Vet Med Sci*. (2015) 77:247–50. doi: 10.1292/jvms.14-0163
28. Lee F. Bovine ephemeral fever in Asia: recent status and research gaps. *Viruses*. (2019) 11:412. doi: 10.3390/v11050412
29. Yang SJ, Wu L. Tainan cattle farms hit by bovine ephemeral fever. *Focus Taiwan* (2014). Available Online at: <http://m.focustaiwan.tw/news/aall/201404150029.aspx>
30. Ting LJ, Lee MS, Lin YL, Cheng MC, Lee F. Invasion of exotic bovine ephemeral fever virus into Taiwan in 2013–2014. *Vet Microbiol*. (2016) 182:15–17. doi: 10.1016/j.vetmic.2015.10.025
31. Zheng FY, Lin GZ, Qiu CQ, Zhou JZ, Cao XA, Gong, et al. Serological detection of bovine ephemeral fever virus using an indirect ELISA based on antigenic site G1 expressed in *Pichia pastoris*. *Vet J*. (2010) 185:211–5. doi: 10.1016/j.tvjl.2009.06.002
32. Zheng F, Lin G, Zhou J, Wang G, Cao X, Gong X, et al. A reverse-transcription, loop-mediated isothermal amplification assay for detection of bovine ephemeral fever virus in the blood of infected cattle. *J Virol Methods*. (2011) 171:306–9. doi: 10.1016/j.jviromet.2010.10.028
33. Ting LJ, Lee MS, Lee SH, Tsai HJ, Lee F. Relationships of bovine ephemeral fever epizootics to population immunity and virus variation. *Vet Microbiol*. (2014) 173:241–8. doi: 10.1016/j.vetmic.2014.07.021
34. Niwa T, Shirafuji H, Ikemiyagi K, Nitta Y, Suzuki M, Kato T, et al. Occurrence of bovine ephemeral fever in Okinawa Prefecture, Japan, in 2012 and development of a reverse-transcription polymerase chain reaction assay to detect bovine ephemeral fever virus gene. *J Vet Med Sci*. (2015) 77:455–60. doi: 10.1292/jvms.14-0492
35. Finlaison DS, Read AJ, Zhang J, Paskin R, Kirkland PD. Application of a real-time polymerase chain reaction assay to the diagnosis of bovine ephemeral

- fever during an outbreak in New South Wales and northern Victoria in 2009–10. *Aust Vet J.* (2014) 92:24–7. doi: 10.1111/avj.12139
36. Gao S, Du J, Tian Z, Niu Q, Huang D, Wang J, et al. A SYBR green I-based quantitative RT-PCR assay for bovine ephemeral fever virus and its utility for evaluating viral kinetics in cattle. *J Vet Diagn Invest.* (2019) 32:44–50. doi: 10.1177/1040638719895460
 37. Inaba Y, Kurogi H, Sato K, Goto Y, Omori T, Matumoto M. Formalin-inactivated, aluminum phosphate gel-adsorbed vaccine of bovine ephemeral fever virus. *Arch Gesamte Virusforsch.* (1973) 42:42–53. doi: 10.1007/bf01250506
 38. Inaba Y, Kurogi H, Takahashi A, Sato K, Omori T. Vaccination of cattle against bovine ephemeral fever with live attenuated virus followed by killed virus. *Arch Gesamte Virusforsch.* (1974) 44:121–32. doi: 10.1007/bf01250220
 39. Wang FI, Hsu AM, Huang KJ. Bovine ephemeral fever in Taiwan. *J Vet Diagn Invest.* (2001) 13:462–7. doi: 10.1177/104063870101300602
 40. Omori T, Inaba Y, Morimoto T, Tanaka Y, Ishitani R. Ibaraki virus, an agent of epizootic disease of cattle resembling bluetongue. I. Epidemiologic, clinical and pathologic observations and experimental transmission to calves. *Jpn J Microbiol.* (1969) 13:139–57. doi: 10.1111/j.1348-0421.1969.tb00447.x
 41. Omori T, Inaba Y, Morimoto T, Tanaka Y, Kono M. Ibaraki virus, an agent of epizootic disease of cattle resembling bluetongue. II. Isolation of the virus in bovine cell culture. *Jpn J Microbiol.* (1969) 13:159–68. doi: 10.1111/j.1348-0421.1969.tb00448.x
 42. Campbell CH, Barber TL, Jochim MM. Antigenic relationship of Ibaraki, bluetongue, and epizootic hemorrhagic disease viruses. *Vet Microbiol.* (1978) 3:15–22. doi: 10.1016/0378-1135(78)90011-1
 43. Uchikoshi N, Enaga N, Shimohira H, Aoki Y, Minamikawa F, Tokunaga M, et al. An outbreak of Ibaraki disease in Kyushu, Japan in 1982. *J Jpn Vet Med Assoc.* (1983) 36:648–52. doi: 10.12935/jvma1951.36.648
 44. Kitano Y, Yamashita S, Fukuyama T. Pathological observations on cattle that died of Ibaraki disease. *J Jpn Vet Med Assoc.* (1988) 41:884–8. doi: 10.12935/jvma1951.41.884
 45. Iwasaki S, Goto Y, Miura Y, Nagatomo M, Kono Y. An outbreak of Ibaraki disease in Miyazaki Prefecture. *J Jpn Vet Med Assoc.* (1990) 43:244–8. doi: 10.12935/jvma1951.43.244
 46. Hirashima Y, Kato T, Yamakawa M, Shirafuji H, Okano R, Yanase T. Reemergence of Ibaraki disease in southern Japan in 2013. *J Vet Med Sci.* (2015) 77:1253–9. doi: 10.1292/jvms.15-0039
 47. Bak UB, Choi HI, Lee CW, Oh HS, Rhee YO, Cheong CK, et al. An outbreak of Ibaraki disease in Korea. *Korean J Vet Res.* (1983) 23:81–9.
 48. Liao YK, Lu YS, Huang ST, Lee SH. The etiologic and epidemiological studies on the Ibaraki disease in Taiwan. *Exp Rep Taiwan Anim Health Res Inst.* (1996) 32:49–57.
 49. Maan NS, Maan S, Potgieter AC, Wright IM, Belaganahalli M, Mertens PPC. Development of real-time RT-PCR assays for detection and typing of epizootic haemorrhagic disease virus. *Transbound Emerg Dis.* (2017) 64:1120–32. doi: 10.1111/tbed.12477
 50. Hanaki T. Attenuated vaccine to Ibaraki disease. *J Jpn Vet Med Assoc.* (1980) 33:451–3. doi: 10.12935/jvma1951.33.451
 51. Sekiguchi H, Kozasa T, Makie H. Current status of animal vaccines in Japan. *J Jpn Vet Med Assoc.* (2010) 63:234–41. <http://nichiju.lin.gr.jp/mag/06304/a4.pdf>
 52. Forman S, Hungerford N, Yamakawa M, Yanase T, Tsai HJ, Joo YS, et al. Climate change impacts and risks for animal health in Asia. *Rev Sci Tech.* (2008) 27:581–97. doi: 10.20506/rst.27.2.1814
 53. Miura Y, Hayashi S, Ishihara T, Inaba Y, Omori T. Neutralizing antibody against Akabane virus in precolostral sera from calves with congenital arthrogryposis-hydranencephaly syndrome. *Arch Gesamte Virusforsch.* (1974) 46:377–80. doi: 10.1007/bf01240082
 54. Kurogi H, Inaba Y, Goto Y, Miura Y, Takahashi H. Serologic evidence for etiologic role of Akabane virus in epizootic abortion-arthrogryposis-hydranencephaly in cattle in Japan, 1972–1974. *Arch Virol.* (1975) 47:71–83. doi: 10.1007/bf01315594
 55. Kirkland PD. Akabane virus infection. *Rev Sci Tech.* (2015) 34:403–10. doi: 10.20506/rst.34.2.2366
 56. Haughey KG, Hartley WJ, Della-Porta AJ, Murray MD. Akabane disease in sheep. *Aust Vet J.* (1988) 65:136–40. doi: 10.1111/j.1751-0813.1988.tb14440.x
 57. Kirkland PD, Barry RD, Harper PA, Zelski RZ. The development of Akabane virus-induced congenital abnormalities in cattle. *Vet Rec.* (1988) 122:582–6. doi: 10.1136/vr.122.24.582
 58. Jagoe S, Kirkland PD, Harper PA. An outbreak of Akabane virus-induced abnormalities in calves after agistment in an endemic region. *Aust Vet J.* (1993) 70:56–8. doi: 10.1111/j.1751-0813.1993.tb15139.x
 59. Brenner J, Rotenberg D, Jaakobi S, Stram Y, Guini-Rubinstein M, Menasherov S, et al. What can Akabane disease teach us about other arboviral diseases. *Vet Ital.* (2016) 52:353–62. doi: 10.12834/VetIt.547.2587.2
 60. Oya A, Okuno T, Ogata T, Kobayashi I, Matsuyama T. Akabane, a new arbor virus isolated in Japan. *Jpn J Med Sci Biol.* (1961) 14:101–8. doi: 10.7883/yoken1952.14.101
 61. Kurogi H, Inaba Y, Takahashi E, Sato K, Satoda K. Congenital abnormalities in newborn calves after inoculation of pregnant cows with Akabane virus. *Infect Immun.* (1977) 17:338–43.
 62. Kurogi H, Inaba Y, Takahashi E, Sato K, Goto Y. Experimental infection of pregnant goats with Akabane virus. *Natl Inst Anim Health Q.* (1977) 17:1–9.
 63. Nakamura K, Morita J, Hanamatsu K, Tomabechi M, Sasaki M, Saito S, et al. A collective outbreak of Akabane disease on sheep farms. *J Jpn Vet Med Assoc.* (1987) 40:513–8. doi: 10.12935/jvma1951.40.513
 64. Kurogi H, Akiba K, Inaba Y, Matumoto M. Isolation of Akabane virus from the biting midge *Culicoides oxystoma* in Japan. *Vet Microbiol.* (1987) 15:243–8. doi: 10.1016/0378-1135(87)90078-2
 65. Yanase T, Kato T, Kubo T, Yoshida K, Ohashi S, Yamakawa M, et al. Isolation of bovine arboviruses from *Culicoides* biting midges (Diptera: Ceratopogonidae) in southern Japan: 1985–2002. *J Med Entomol.* (2005) 42:63–7. doi: 10.1093/jmedent/42.1.63
 66. Yamakawa M, Yanase T, Kato T, Tsuda T. Chronological and geographical variations in the small RNA segment of the teratogenic Akabane virus. *Virus Res.* (2006) 121:84–92. doi: 10.1016/j.virusres.2006.04.007
 67. Yanase T, Kato T, Hayama Y, Akiyama M, Itoh N, Horiuchi S, et al. Transition of Akabane virus genogroups and its association with changes in the nature of disease in Japan. *Transbound Emerg Dis.* (2018) 65:e434–43. doi: 10.1111/tbed.12778
 68. Miyazato S, Miura Y, Hase M, Kubo M, Goto Y, Kono Y. Encephalitis of cattle caused by Iriki isolate, a new strain belonging to Akabane virus. *Nihon Juigaku Zasshi.* (1989) 51:128–36. doi: 10.1292/jvms1939.51.128
 69. Kono R, Hirata M, Kaji M, Goto Y, Ikeda S, Yanase T, et al. Bovine epizootic encephalomyelitis caused by Akabane virus in southern Japan. *BMC Vet Res.* (2008) 4:20. doi: 10.1186/1746-6148-4-20
 70. Kobayashi T, Yanase T, Yamakawa M, Kato T, Yoshida K, Tsuda T. Genetic diversity and reassortments among Akabane virus field isolates. *Virus Res.* (2007) 130:162–71. doi: 10.1016/j.virusres.2007.06.007
 71. Bak UB, Lim CH, Cheong CK, Hwang WS, Cho MR. Outbreaks of Akabane disease of cattle in Korea. *Korean J Vet Res.* (1980) 20:65–78.
 72. Cho JJ, Lee CG, Park BK, Chang CH, Chung CW, Cho IS, et al. Isolation of Akabane virus and its molecular diagnosis by reverse transcription polymerase chain reaction. *Korean J Vet Res.* (2000) 40:42–8. Available online at: <http://kjvr.org/upload/06902772.pdf>
 73. Yang DK, Kim YH, Kim BH, Kweon CH, Yoon SS, Song JY, et al. Characterization of Akabane virus (KV0505) from cattle in Korea. *Korean J Vet Res.* (2008) 48:61–6. Available online at: <http://kjvr.org/upload/06903826.pdf>
 74. Oem JK, Lee KH, Kim HR, Bae YC, Chung JY, Lee OS, et al. Bovine epizootic encephalomyelitis caused by Akabane virus infection in Korea. *J Comp Pathol.* (2012) 147:101–5. doi: 10.1016/j.jcpa.2012.01.013
 75. Oem JK, Yoon HJ, Kim HR, Roh IS, Lee KH, Lee OS, et al. Genetic and pathogenic characterization of Akabane viruses isolated from cattle with encephalomyelitis in Korea. *Vet Microbiol.* (2012) 158:259. doi: 10.1016/j.vetmic.2012.02.017
 76. Lim SI, Kweon CH, Tark DS, Kim SH, Yang DK. Sero-survey on Aino, Akabane, Chuzan, bovine ephemeral fever and Japanese encephalitis virus of cattle and swine in Korea. *J Vet Sci.* (2007) 8:45–9. doi: 10.4142/jvs.2008.9.4.381
 77. Yang DK, Hwang IJ, Kim BH, Kweon CH, Lee KW, Kang MI, et al. Serosurveillance of viral diseases in Korean native goats (*Capra hircus*). *J Vet Med Sci.* (2008) 70:977–9. doi: 10.1292/jvms.70.977

78. Jun K, Yanaka T, Lee KK, Lee JB. Seroprevalence of bovine arboviruses belonging to genus *Orthobunyavirus* in South Korea. *J Vet Med Sci.* (2018) 80:1619–23. doi: 10.1292/jvms.17-0542
79. Yang DK, Kim SY, Kim HH, Kang MS, Nah JJ, Choi SS, et al. The follow up study after massive outbreak of Akabane and bovine ephemeral fever viruses in Korea. *Korean J Vet Serv.* (2013) 36:151–5. doi: 10.7853/kjvs.2013.36.3.151
80. Lee JK, Kim JH, Park BK, Yoo HS, Lee BC, Kim DY. Akabane viral encephalitis in calves in South Korea. *Vet Rec.* (2007) 161:236–8. doi: 10.1136/vr.161.7.236
81. Liao YK, Lu YS, Goto, Y Inaba Y. The isolation of Akabane virus (Iriki strain) from calves in Taiwan. *J Basic Microbiol.* (1996) 36:33–9. doi: 10.1002/jobm.3620360108
82. Liao YK, Chang CY, Lu YS. The serological investigation of Akabane disease in Taiwan. *J Chin Soc Vet Sci.* (1996) 22:121–8.
83. Wang J, Blasdel KR, Yin H, Walker PJ. A large-scale serological survey of Akabane virus infection in cattle, yak, sheep and goats in China. *Vet Microbiol.* (2017) 207:7–12. doi: 10.1016/j.vetmic.2017.05.014
84. Feng Y, He B, Fu S, Yang W, Zhang Y, Tu C, et al. Isolation and identification of the Akabane virus from mosquitoes in Yunnan Province, China. *Bing Du Xue Bao.* (2015) 31:51–7.
85. Tang HB, Chen F, Rao G, Bai A, Jiang J, Du Y, et al. Characterization of Akabane virus from domestic bamboo rat, Southern China. *Vet Microbiol.* (2017) 207:280. doi: 10.1016/j.vetmic.2017.06.018
86. Cao Y, Fu S, Song S, Cai L, Zhang H, Gao L, et al. Isolation and genome phylogenetic analysis of arthropod-borne viruses, including Akabane virus, from mosquitoes collected in Hunan Province, China. *Vector Borne Zoonotic Dis.* (2019) 19:62–72. doi: 10.1089/vbz.2018.2267
87. Tang HB, Ren P, Qin S, Lin J, Bai A, Qin S, et al. Isolation, genetic analysis of the first Akabane virus from goat in China. *J Vet Med Sci.* (2019) 81:1445–9. doi: 10.1292/jvms.18-0602
88. Tsuda T, Yoshida K, Yanase T, Ohashi S, Yamakawa M. Competitive enzyme-linked immunosorbent assay for the detection of the antibodies specific to Akabane virus. *J Vet Diagn Invest.* (2004) 16:571–6. doi: 10.1177/104063870401600613
89. Lunt RA, White JR, Newberry KM, Pourquier P, Comtet L. Development of a commercial ELISA for the detection of serum antibody to Akabane virus. In: *Poster Presented at the 2007 WAVLD Meeting.* Melbourne (2007).
90. Kittelberger R, McFadden AM, Kirkland PD, Hannah MJ, Orr D, Bueno R, et al. Evaluation of two commercial enzyme-linked immunosorbent assay kits for the detection of serum antibodies against Akabane virus in cattle. *J Vet Diagn Invest.* (2013) 25:645–8. doi: 10.1177/1040638713500496
91. Oem JK, Kim YH, Kim SH, Lee MH, Lee KK. Serological characteristics of affected cattle during an outbreak of bovine enzootic encephalomyelitis caused by Akabane virus. *Trop Anim Health Prod.* (2014) 46:261–3. doi: 10.1007/s11250-013-0468-2
92. Li X, Jing H, Liu X, Wang Q, Qiu S, Liu D, et al. Comparative evaluation of two commercial ELISA kits for detection of antibodies against Akabane virus in cattle serum. *BMC Vet Res.* (2019) 15:408. doi: 10.1186/s12917-019-2156-6
93. Kurogi H, Inaba Y, Takahashi E, Sato K, Omori T, Miura Y, et al. Epizootic congenital arthrogryposis-hydranencephaly syndrome in cattle: isolation of Akabane virus from affected fetuses. *Arch Virol.* (1976) 51:67–74. doi: 10.1007/bf01317835
94. Shirafuji H, Yazaki R, Shuto Y, Yanase T, Kato T, Ishikura Y, et al. Broad-range detection of arboviruses belonging to Simbu serogroup lineage 1 and specific detection of Akabane, Aino and Peaton viruses by newly developed multiple TaqMan assays. *J Virol Methods.* (2015) 225:9–15. doi: 10.1016/j.jviromet.2015.08.021
95. Kurogi H, Inaba Y, Takahashi E, Sato K, Goto Y, Satoda K, et al. Development of inactivated vaccine for Akabane disease. *Natl Inst Anim Health Q.* (1978) 18:97–108.
96. Kurogi H, Inaba Y, Takahashi E, Sato K, Akashi H, Satoda K, et al. An attenuated strain of Akabane virus: a candidate for live virus vaccine. *Natl Inst Anim Health Q.* (1979) 19:12–22.
97. Kim YH, Kweon CH, Tark DS, Lim SI, Yang DK, Hyun BH, et al. Development of inactivated trivalent vaccine for the teratogenic Aino, Akabane and Chuzan viruses. *Biologicals.* (2011) 39:152–7. doi: 10.1016/j.biologics.2011.02.004
98. Takahashi K, Oya A, Okazda T, Matsuo R, Kuma M. Aino virus, a new member of Simbu group of arbovirus from mosquitoes in Japan. *Jpn J Med Sci Biol.* (1968) 21:95–101. doi: 10.7883/yoken1952.21.95
99. Egashira T, Itamaru H, Yoshinaga N, Enaga N, Utikoshi N, Minamikawa R. Abnormal birth of cattle suggesting an infection of Aino virus. *J Jpn Vet Med Assoc.* (1989) 42:94–6. doi: 10.12935/jvma1951.42.94
100. Fukutomi T, Ouchi M, Okuda K, Maruno F, Tabayashi K. An abnormal calf birth suggestive of an infection of Aino virus in Okayama Prefecture. *J Jpn Vet Med Assoc.* (1991) 44:17–9. doi: 10.12935/jvma1951.44.17
101. Kitano Y, Yamashita S, Furukawa M, Makinoda K. Congenital abnormality of calves suggesting Aino virus infection in Kagoshima Prefecture. *J Jpn Vet Med Assoc.* (1993) 46:469–71. doi: 10.12935/jvma1951.46.469
102. Ishibashi K, Tomishita Y, Shirakawa H, Takaya M, Watanabe A. Congenital scoliosis of calves suspected of Aino virus infection. *J Jpn Vet Med Assoc.* (1994) 47:87–90. doi: 10.12935/jvma1951.47.87
103. Noda Y, Uchinuno Y, Shirakawa H, Nagasue S, Nagano N, Ohe R, et al. Aino virus antigen in brain lesions of a naturally aborted bovine fetus. *Vet Pathol.* (1998) 35:409–11. doi: 10.1177/030098589803500511
104. Uchinuno Y, Noda Y, Ishibashi K, Nagasue S, Shirakawa H, Nagano M, et al. Isolation of Aino virus from an aborted bovine fetus. *J Vet Med Sci.* (1998) 60:1139–40. doi: 10.1292/jvms.60.1139
105. Tsuda T, Yoshida K, Ohashi S, Yanase T, Sueyoshi M, Kamimura S, et al. Arthrogryposis, hydranencephaly and cerebellar hypoplasia syndrome in neonatal calves resulting from intrauterine infection with Aino virus. *Vet Res.* (2004) 35:531–8. doi: 10.1051/vetres:2004029
106. Yamakawa M, Yanase T, Kato T, Tsuda T. Molecular epidemiological analyses of the teratogenic Aino virus based on the sequences of a small RNA segment. *Vet Microbiol.* (2008) 129:40–7. doi: 10.1016/j.vetmic.2007.11.001
107. Yanase T, Aizawa M, Kato T, Yamakawa M, Shirafuji H, Tsuda T. Genetic characterization of Aino and Peaton virus field isolates reveals a genetic reassortment between these viruses in nature. *Virus Res.* (2010) 153:1–7. doi: 10.1016/j.virusres.2010.06.020
108. Wang J, Firth C, Amos-Ritchie R, Davis SS, Yin H, Holmes EC, et al. Evolutionary history of Simbu serogroup orthobunyaviruses in the Australian epizootic. *Virology.* (2019) 535:32–44. doi: 10.1016/j.virol.2019.06.013
109. Tzeng HY, Wu HH, Ting LJ, Chang NT, Chou YC, Tu WC. Monitoring Taiwanese bovine arboviruses and non-arboviruses using a vector-based approach. *Med Vet Entomol.* (2019) 33:195–202. doi: 10.1111/mve.12345
110. Doherty RL, Carley JG, Standfast HA, Dyce AL, Snowdon WA. Virus strains isolated from arthropods during an epizootic of bovine ephemeral fever in Queensland. *Aust Vet J.* (1972) 48:81–6. doi: 10.1111/j.1751-0813.1972.tb02220.x
111. Miura Y, Inaba Y, Goto Y, Takahashi E, Kurogi H, Hayashi S, et al. Serological comparison of Aino and Samford viruses in Simbu group of bunyaviruses. *Microbiol Immunol.* (1978) 22:651–4. doi: 10.1111/j.1348-0421.1978.tb00417.x
112. Coverdale OR, Cybinski DH, St George TD. Congenital abnormalities in calves associated with Akabane virus and Aino virus. *Aust Vet J.* (1978) 54:151–2. doi: 10.1111/j.1751-0813.1978.tb05538.x
113. Golender N, Brenner J, Valdman M, Khinich Y, Bumarov V, Panshin A, et al. Malformations caused by Shuni virus in ruminants, Israel, 2014–2015. *Emerg Infect Dis.* (2015) 21:2267–8. doi: 10.3201/eid2112.150804
114. Golender N, Bumarov V, Assis I, Beer M, Khinich Y, Koren O, et al. Shuni virus in Israel: neurological disease and fatalities in cattle. *Transbound Emerg Dis.* (2019) 66:1126–31. doi: 10.1111/tbed.13167
115. Shin YK, Oem JK, Yoon S, Hyun BH, Cho IS, Yoon S, et al. Monitoring of five bovine arboviral diseases transmitted by arthropod vectors in Korea. *J Bacteriol Virol.* (2009) 39:353–62. doi: 10.4167/jbv.2009.39.4.353
116. Goto Y, Miura Y, Kono Y. Serologic evidence for the etiologic role of Chuzan virus in an epizootic of congenital abnormalities with hydranencephaly-cerebellar hypoplasia syndrome of calves in Japan. *Am J Vet Res.* (1988) 49:2026–9.
117. Miura Y, Goto Y, Kubo M, Kono Y. Isolation of Chuzan virus, a new member of the Palyam subgroup of the genus *Orbivirus*, from cattle and *Culicoides oxystoma* in Japan. *Am J Vet Res.* (1988) 49:2022–5.

118. Miura Y, Kubo M, Goto Y, Kono Y. Hydranencephaly-cerebellar hypoplasia in a newborn calf after infection of its dam with Chuzan virus. *Nihon Juigaku Zasshi*. (1990) 52:689–94. doi: 10.1292/jvms1939.52.689
119. Jusa ER, Inaba Y, Kadoi K, Kurogi H, Fonseca E, Shope RE. Identification of Kagoshima and Chuzan viruses of Japan as Kasba Virus, an orbivirus of the Palyam serogroup. *Aust Vet J*. (1994) 71:57. doi: 10.1111/j.1751-0813.1994.tb06155.x
120. Yamakawa M, Kubo M, Furuuchi S. Molecular analysis of the genome of Chuzan virus, a member of the Palyam serogroup viruses, and its phylogenetic relationships to other orbiviruses. *J Gen Virol*. (1999) 80:937–41. doi: 10.1099/0022-1317-80-4-937
121. Ebersohn K, Coetzee P, Snyman LP, Swanepoel R, Venter EH. Phylogenetic characterization of the Palyam serogroup orbiviruses. *Viruses*. (2019) 11:pii: E446. doi: 10.3390/v11050446
122. Liao YK, Lu YS, Hwang C, Chang CE. The study on the epidemiology of Akabane virus and Chuzan virus infection in cattle in Taiwan. *Exp Rep TPRIAH*. (1994) 30:47–53.
123. Yang H, Xiao L, Meng J, Xiong H, Gao L, Liao D, et al. Complete genome sequence of a Chuzan virus strain isolated for the first time in mainland China. *Arch Virol*. (2016) 161:1073–7. doi: 10.1007/s00705-015-2734-2
124. Wang F, Lin J, Chang J, Cao Y, Qin S, Wu J, et al. Isolation, complete genome sequencing, and phylogenetic analysis of the first Chuzan virus in China. *Virus Genes*. (2016) 52:138–41. doi: 10.1007/s11262-015-1282-x
125. Wang M, Wang Y, Baloch AR, Pan Y, Tian L, Xu F, et al. Chuzan virus in yaks, Qinghai-Tibetan Plateau, China. *Emerg Infect Dis*. (2018) 24:2371–3. doi: 10.3201/eid2412.171414
126. Miura Y, Kubo M, Goto Y, Kono Y. Chuzan disease as congenital hydranencephaly-cerebellar hypoplasia syndrome in calves. *JARQ*. (1991) 25:55–60. <https://dl.ndl.go.jp/info:ndljp/pid/9286080>
127. Goto Y, Miura Y, Kono Y. A preliminary study for development of an inactivated Chuzan virus vaccine. *Nihon Juigaku Zasshi*. (1988) 50:673–8. doi: 10.1292/jvms1939.50.673
128. Belbis G, Zientara S, Bréard E, Sailleau C, Caignard G, Vitour D, et al. Bluetongue virus: from BTV-1 to BTV-27. *Adv Virus Res*. (2017) 99:161–97. doi: 10.1016/bs.aivir.2017.08.003
129. Miura Y, Inaba Y, Hayashi S, Takahashi E, Matumoto M. A survey of antibodies to arthropod-borne viruses in Japanese cattle. *Vet Microbiol*. (1980) 5:277–82. doi: 10.1016/0378-1135(80)90026-7
130. Miura Y, Inaba Y, Tsuda T, Tokuhisa S, Sato K, Akashi H. Seroepizootiological survey on bluetongue virus infection in cattle in Japan. *Natl Inst Anim Health Q*. (1982) 22:154–8.
131. Goto Y, Yamaguchi O, Kubo M. Epidemiological observations on bluetongue in sheep and cattle in Japan. *Vet Ital*. (2004) 40:78–82. Available Online at: http://www.izs.it/vet_italiana/2004/40_3/17.pdf
132. Shirafuji H, Yanase T, Kato T, Yamakawa M. Genetic and phylogenetic characterization of genome segments 2 and 6 of bluetongue virus isolates in Japan from 1985 to 2008. *J Gen Virol*. (2012) 93:1465–73. doi: 10.1099/vir.0.040717-0
133. Zhang N, Li Z, Zhang K, Hu Y, Li G, Peng K, et al. Bluetongue history, serology and virus isolation in China. In: *Proceedings of the First Southeast Asia and Pacific Regional Bluetongue Symposium*. 1995 Aug. 22–24. Kunming (1996). p. 43–50.
134. Zhang N, MacLachlan NJ, Bonneau KR, Zhu J, Li Z, Zhang K, et al. Identification of seven serotypes of bluetongue virus from the people's Republic of China. *Vet Rec*. (1999) 145:427–9. doi: 10.1136/vr.145.15.427
135. Qin S, Yang H, Zhang Y, Li Z, Lin J, Gao L, et al. Full genome sequence of the first bluetongue virus serotype 21 (BTV-21) isolated from China: evidence for genetic reassortment between BTV-21 and bluetongue virus serotype 16 (BTV-16). *Arch Virol*. (2018) 163:1379–82. doi: 10.1007/s00705-018-3718-9
136. Sun EC, Huang LP, Xu QY, Wang HX, Xue XM, Lu P, et al. Emergence of a novel bluetongue virus serotype, China 2014. *Transbound Emerg Dis*. (2016) 63:585–9. doi: 10.1111/tbed.12560
137. Ma JG, Zhang XX, Zheng WB, Xu YT, Zhu XQ, Hu GX, et al. Seroprevalence and risk factors of bluetongue virus infection in Tibetan sheep and yaks in Tibetan Plateau, China. *Biomed Res Int*. (2017) 2017:5139703. doi: 10.1155/2017/5139703
138. Ma J, Gao X, Liu B, Xiao J, Chen H, Wang H. Spatial patterns and risk factors of bluetongue virus infection in Inner Mongolia, China. *Vector Borne Zoonotic Dis*. (2019) 19:525–32. doi: 10.1089/vbz.2018.2361
139. Ma J, Gao X, Liu B, Chen H, Xiao J, Wang H. Epidemiology and spatial distribution of bluetongue virus in Xinjiang, China. *Peer J*. (2019) 7:e6514. doi: 10.7717/peerj.6514
140. Ting LJ, Lee MS, Huang TS, Huang CC, Kuo ST, Lee F, et al. Identification of bluetongue virus in goats in Taiwan. *Vet Rec*. (2005) 156:52. doi: 10.1136/vr.156.2.52
141. Lee F, Ting LJ, Jong MH, Chang WM, Wang FI. Subclinical bluetongue virus infection in domestic ruminants in Taiwan. *Vet Microbiol*. (2010) 142:225–231. doi: 10.1016/j.vetmic.2009.10.005
142. Seo HJ, Park JY, Cho YS, Cho IS, Yeh JY. First report of bluetongue virus isolation in the Republic of Korea and analysis of the complete coding sequence of the segment 2 gene. *Virus Genes*. (2015) 50:156–9. doi: 10.1007/s11262-014-1140-2
143. Yeh JY, Kim JG, Choi J, Kim JK, Kim KW. Bluetongue virus antibodies in domestic goats: a countrywide and retrospective study in the Republic of Korea. *Vector Borne Zoonotic Dis*. (2018) 18:323–30. doi: 10.1089/vbz.2017.2181
144. Hwang JM, Kim JG, Yeh JY. Evidence of bluetongue virus circulation in farmed and free-ranging cervids from the Republic of Korea: a retrospective cross-sectional study. *Vet Microbiol*. (2019) 230:145–9. doi: 10.1016/j.vetmic.2019.02.009
145. Hwang JM, Kim JG, Yeh JY. Serological evidence of bluetongue virus infection and serotype distribution in dairy cattle in South Korea. *BMC Vet Res*. (2019) 15:255. doi: 10.1186/s12917-019-2000-z
146. Kim HJ. Complete genome sequence analysis of the bluetongue virus serotype 3 in South Korea. In: *Abstract Retrieved From Abstracts in 11th World Congress on Virology and Infectious Diseases*. 2018 May 17–18. Tokyo (2018). doi: 10.4172/2161-0517-C1-024
147. Lee F, Ting LJ, Lee MS, Chang WM, Wang FI. Genetic analysis of two Taiwanese bluetongue viruses. *Vet Microbiol*. (2011) 148:140–9. doi: 10.1016/j.vetmic.2010.08.016
148. Boyle DB, Bulach DM, Amos-Ritchie R, Adams MM, Walker PJ, Weir R. Genomic sequences of Australian bluetongue virus prototype serotypes reveal global relationships and possible routes of entry into Australia. *J Virol*. (2012) 86:6724–31. doi: 10.1128/JVI.00182-12
149. Zhu J, Yang H, Li H, Xiao L, Wang J, Li N, et al. Full-genome sequence of bluetongue virus serotype 1 (BTV-1) strain Y863, the first BTV-1 isolate of eastern origin found in China. *Genome Announc*. (2013) 1:e00403-13. doi: 10.1128/genomeA.00403-13
150. Liao D, Yang H, Xiao L, Zhu J, Wang J, Li Z. Complete genome sequence of a bluetongue virus serotype 15 strain isolated from China in 1996. *Genome Announc*. (2018) 6:e00557-18. doi: 10.1128/genomeA.00557-18
151. Yang H, Lv M, Sun M, Lin L, Kou M, Gao L, et al. Complete genome sequence of the first bluetongue virus serotype 7 isolate from China: evidence for entry of African-lineage strains and reassortment between the introduced and native strains. *Arch Virol*. (2016) 161:223–7. doi: 10.1007/s00705-015-2624-7
152. MacLachlan NJ, Mayo CE, Daniels PW, Savini G, Zientara S, Gibbs EP. Bluetongue. *Rev Sci Tech*. (2015) 34:329–40. doi: 10.20506/rst.34.2.2360
153. Shimizu S, Toyota I, Arishima T, Goto Y. Frequency of serological cross-reactions between Ibaraki and bluetongue viruses using the agar gel immunodiffusion test. *Vet Ital*. (2004) 40:583–6. http://www.izs.it/vet_italiana/2004/40_4/583.pdf
154. St George TD, Standfast HA, Cybinski DH, Filippich C, Carley JG. Peaton virus: a new Simbu group arbovirus isolated from cattle and *Culicoides brevitarsis* in Australia. *Aust J Biol Sci*. (1980) 33:235–43. doi: 10.1071/bi9800235
155. Matsumori Y, Inai K, Yanase T, Ohashi S, Kato T, Yoshida K, et al. Serological and genetic characterization of newly isolated Peaton virus in Japan. *Arch Virol*. (2002) 147:401–10. doi: 10.1007/s705-002-8328-4
156. Parsonson IM, McPhee DA. Bunyavirus pathogenesis. *Adv Virus Res*. (1985) 30:279–316. doi: 10.1016/S0065-3527(08)60453-4
157. Matsumori Y, Aizawa M, Sakai Y, Inoue D, Kodani M, Tsuha O, et al. Congenital abnormalities in calves associated with Peaton virus infection in Japan. *J Vet Diagn Invest*. (2018) 30:855–61. doi: 10.1177/1040638718796269

158. Brenner J, Yanase T, Kato T, Yaakobi S, Khinich E, Paz R, et al. Serological evidence suggests that several Simbu serogroup viruses circulated in Israel. *Vet Ital.* (2019) 55:81–9. doi: 10.12834/VetIt.1397.7622.2
159. Behar A, Leibovich BB, Edery N, Yanase T, Brenner J. First genomic detection of Peaton virus in a calf with hydranencephaly in Israel. *Vet Med Sci.* (2019) 5:87–92. doi: 10.1002/vms3.129
160. Dandawate CN, Pajagopalan PK, Pavri KM, Work TH. Virus isolations from mosquitoes collected in North Arcot district, Madras state, and Chittoor district, Andhra Pradesh between November 1955 and October 1957. *Indian J Med Res.* (1969) 57:1420–6.
161. Causey OR, Kemp GE, Causey CE, Lee VH. Isolations of Simbu-group viruses in Ibadan, Nigeria 1964–69, including the new types Sango, Shamonda, Sabo and Shuni. *Ann Trop Med Parasitol.* (1972) 66:357–62. doi: 10.1080/00034983.1972.11686835
162. Yanase T, Fukutomi T, Yoshida K, Kato T, Ohashi S, Yamakawa M, et al. The emergence in Japan of Sathuperi virus, a tropical Simbu serogroup virus of the genus *Orthobunyavirus*. *Arch Virol.* (2004) 149:1007–13. doi: 10.1007/s00705-003-0266-7
163. Yanase T, Kato T, Aizawa M, Shuto Y, Shirafuji H, Yamakawa M, et al. Genetic reassortment between Sathuperi and Shamonda viruses of the genus *Orthobunyavirus* in nature: implications for their genetic relationship to Schmallenberg virus. *Arch Virol.* (2012) 157:1611–16. doi: 10.1007/s00705-012-1341-8
164. Yanase T, Maeda K, Kato T, Nyuta S, Kamata H, Yamakawa M, et al. The resurgence of Shamonda virus, an African Simbu group virus of the genus *Orthobunyavirus*, in Japan. *Arch Virol.* (2005) 150:361–9. doi: 10.1007/s00705-004-0419-3
165. Hirashima Y, Sakaguchi Z, Okada I, Fujioka M, Kitahara S, Iwamoto J, et al. Retrospective survey of Shamonda virus infection in sentinel and breeding cattle and in malformed calves in Kagoshima Prefecture. *J Jpn Vet Med Assoc.* (2017) 70:729–34. doi: 10.12935/jvma.70.729
166. Hirashima Y, Kitahara S, Kato T, Shirafuji H, Tanaka S, Yanase T. Congenital malformations of calves infected with Shamonda virus, southern Japan. *Emerg Infect Dis.* (2017) 23:993–6. doi: 10.3201/eid2306.161946
167. Fukuda M, Horiuchi S, Maruta T, Setoyama H, Onitsuka Y, Yanase T. Malformed calves possibly associated with Shamonda virus in Miyazaki Prefecture. *J Vet Med.* (2018) 71:813–9.
168. Briese T, Calisher CH, Higgs S. Viruses of the family *Bunyaviridae*: are all available isolates reassortants? *Virology.* (2013) 446:207–16. doi: 10.1016/j.virol.2013.07.030
169. Goller KV, Höper D, Schirrmeyer H, Mettenleiter TC, Beer M. Schmallenberg virus as possible ancestor of Shamonda virus. *Emerg Infect Dis.* (2012) 18:1644–6. doi: 10.3201/eid1810.120835
170. Coupeau D, Bayrou C, Baillieux P, Marichal A, Lenaerts AC, Caty C, et al. Host-dependence of *in vitro* reassortment dynamics among the Sathuperi and Shamonda Simbuviruses. *Emerg Microbes Infect.* (2019) 8:381–95. doi: 10.1080/22221751.2019.1586410
171. Watanabe Y, Makiuchi H, Imafuji T, Yamasaki K, Onitsuka T, Ohashi S. An outbreak of Ibaraki disease in Kagoshima Prefecture in 1997 and properties of the virus isolated from bovine aborted and still birth fetus. *J Jpn Vet Med Assoc.* (2000) 53:302–6. doi: 10.12935/jvma1951.53.302
172. Ohashi S, Yoshida K, Watanabe Y, Tsuda T. Identification and PCR-restriction fragment length polymorphism analysis of a variant of the Ibaraki virus from naturally infected cattle and aborted fetuses in Japan. *J Clin Microbiol.* (1999) 37:3800–3. doi: 10.1128/JCM.37.12.3800-3803.1999
173. Ohashi S, Yoshida K, Yanase T, Tsuda T. Analysis of intratypic variation evident in an Ibaraki virus strain and its epizootic hemorrhagic disease virus serogroup. *J Clin Microbiol.* (2002) 40:3684–8. doi: 10.1128/JCM.40.10.3684-3688.2002
174. Shirafuji H, Kato T, Yamakawa M, Tanaka T, Minemori Y, Yanase T. Characterization of genome segments 2, 3 and 6 of epizootic hemorrhagic disease virus strains isolated in Japan in 1985–2013: Identification of their serotypes and geographical genetic types. *Infect Genet Evol.* (2017) 53:38–46. doi: 10.1016/j.meegid.2017.05.010
175. Qi Y, Wang F, Chang J, Zhang Y, Zhu J, Li H, et al. Identification and complete-genome phylogenetic analysis of an epizootic hemorrhagic disease virus serotype 7 strain isolated in China. *Arch Virol.* (2019) 164:3121–6. doi: 10.1007/s00705-019-04412-9
176. Kamomae Y, Kamomae M, Ohta Y, Nabe M, Kagawa Y, Ogura Y, et al. Epizootic hemorrhagic disease virus serotype 6 infection in cattle, Japan, 2015. *Emerg Infect Dis.* (2018) 24:902–5. doi: 10.3201/eid2405.171859
177. Miura Y, Miyazato S, Kubo M, Goto Y, Kono Y, Kawanabe virus, an isolate from a calf in Japan: a new virus belonging to the New Jersey serotype of the epizootic hemorrhagic disease serogroup of genus *Orbivirus*. *Nihon Juigaku Zasshi.* (1988) 50:942–5. doi: 10.1292/jvms1939.50.942
178. Ohashi S, Matsumori Y, Yanase T, Yamakawa M, Kato T, Tsuda T. Evidence of an antigenic shift among Palyam serogroup orbiviruses. *J Clin Microbiol.* (2004) 42:4610–14. doi: 10.1128/JCM.42.10.4610-4614.2004
179. Kato T, Shirafuji H, Tanaka S, Sato M, Yamakawa M, Tsuda T, et al. Bovine arboviruses in *Culicoides* biting midges and sentinel cattle in southern Japan from 2003 to 2013. *Transbound Emerg Dis.* (2016) 63:e160–e172. doi: 10.1111/tbed.12324
180. Kato T, Yanase T, Suzuki M, Katagiri Y, Ikemiyagi K, Takayoshi K, et al. Monitoring for bovine arboviruses in the most southwestern islands in Japan between 1994 and 2014. *BMC Vet Res.* (2016) 12:125. doi: 10.1186/s12917-016-0747-z
181. Cybinski DH, St George TD. Preliminary characterization of D'Aguilar virus and three Palyam group viruses new to Australia. *Aust J Biol Sci.* (1982) 35:343–51. doi: 10.1071/BI9820343
182. Attoui H, Mohd Jaafar F, Belhouche M, Aldrovandi N, Tao S, Chen B, et al. Yunnan orbivirus, a new orbivirus species isolated from *Culex tritaeniorhynchus* mosquitoes in China. *J Gen Virol.* (2005) 86:3409–17. doi: 10.1099/vir.0.81258-0
183. Cowled C, Melville L, Weir R, Walsh S, Hyatt A, Van Driel R, et al. Genetic and epidemiological characterization of middle point orbivirus, a novel virus isolated from sentinel cattle in northern Australia. *J Gen Virol.* (2007) 88:3413–22. doi: 10.1099/vir.0.83231-0
184. Attoui H, Mohd Jaafar F. Zoonotic and emerging orbivirus infections. *Rev Sci Tech.* (2015) 34:353–61. doi: 10.20506/rst.34.2.2362
185. Yang H, Li Z, Zhang Y, Gao L, Xie J, Liao D, et al. Isolation and genomic characterization of a novel orbivirus strain from the blood of cattle. *Bing Du Xue Bao.* (2019) 34:75–84.
186. Attoui H, Mendez-Lopez MR, Rao S, Hurtado-Alendes A, Lizaraso-Caparo F, Jaafar FM, et al. Peruvian horse sickness virus and Yunnan orbivirus, isolated from vertebrates and mosquitoes in Peru and Australia. *Virology.* (2009) 394:298–310. doi: 10.1016/j.virol.2009.08.032
187. Morita K, Nabeshima T, Buerano CC. Japanese encephalitis. *Rev Sci Tech.* (2015) 34:441–52. doi: 10.20506/rst.34.2.2370
188. Arai S, Matsunaga Y, Takasaki T, Tanaka-Taya K, Taniguchi K, Okabe N, et al. Japanese encephalitis: surveillance and elimination effort in Japan from 1982 to 2004. *Jpn J Infect Dis.* (2008) 61:333–8. <https://www.ncbi.nlm.nih.gov/pubmed/18806337>
189. Yamamoto S, Tsubara H, Yoshida T, Harada K. First observation on case of Japanese B encephalitis of cattle. *Bull Natl Inst Anim Hlth.* (1949) 22:197–203.
190. Shimizu T, Mochizuki H, Sugawa Y, Okazaki K, Matumoto M. Studies on Japanese encephalitis of cattle. 1. Bovine encephalitis caused by natural infection with Japanese encephalitis virus. *Natl Inst Anim Hlth Q.* (1951) 23:111–8.
191. Katayama K, Ikeda A, Saika K, Ishizaka M, Onodera M, Ito T. A case of Japanese encephalitis in a black Japanese heifer. *J Jpn Vet Med Assoc.* (2000) 53:293–6. doi: 10.12935/jvma1951.53.293
192. Katayama T, Saito S, Horiuchi S, Maruta T, Kato T, Yanase T, Yamakawa M, et al. Nonsuppurative encephalomyelitis in a calf in Japan and isolation of Japanese encephalitis virus genotype 1 from the affected calf. *J Clin Microbiol.* (2013) 51:3448–53. doi: 10.1128/JCM.00737-13
193. Kako N, Suzuki S, Sugie N, Kato T, Yanase T, Yamakawa M, et al. Japanese encephalitis in a 114-month-old cow: pathological investigation of the affected cow and genetic characterization of Japanese encephalitis virus isolate. *BMC Vet Res.* (2014) 10:63. doi: 10.1186/1746-6148-10-63
194. McVey DS, Wilson WC, Gay CG. West Nile virus. *Rev Sci Tech.* (2015) 34:431–9. doi: 10.20506/rst.34.2.2369
195. Lu Z, Fu SH, Cao L, Tang CJ, Zhang S, Li ZX, et al. Human infection with West Nile virus, Xinjiang, China, 2011. *Emerg Infect Dis.* (2014) 20:1421–3. doi: 10.3201/eid2008.131433

196. Kim CY, Oh H, Song J, Hur M, Suh JH, Jheong WH, et al. First detection of West Nile virus in domestic pigeon in Korea. *J Vet Sci.* (2016) 17:587–9. doi: 10.4142/jvs.2016.17.4.587
197. Rimoldi G, Mete A, Adaska JM, Anderson ML, Symmes KP, Diab S. West Nile virus infection in sheep. *Vet Pathol.* (2017) 54:155–8. doi: 10.1177/0300985816653796
198. Xu P, Wang Y, Zuo J, Lin J, Xu P. New orbiviruses isolated from patients with unknown fever and encephalitis in Yunnan Province. *Chin J Virol.* (1990) 6:27–33.
199. Attoui H, Billoir F, Biagini P, de Micco P, de Lamballerie X. Complete sequence determination and genetic analysis of Banna virus and Kadipiro virus: proposal for assignment to a new genus (*Seadornavirus*) within the family *Reoviridae*. *J Gen Virol.* (2000) 81:1507–15. doi: 10.1099/0022-1317-81-6-1507
200. Liu H, Li MH, Zhai YG, Meng WS, Sun XH, Cao YX, et al. Banna virus, China, 1987–2007. *Emerg Infect Dis.* (2010) 16:514–7. doi: 10.3201/eid1603.091160
201. Song S, Li Y, Fu S, Lei W, Guo X, Feng Y, et al. Genome sequencing and phylogenetic analysis of Banna virus (genus *Seadornavirus*, family *Reoviridae*) isolated from *Culicoides*. *Sci China Life Sci.* (2017) 60:1372–82. doi: 10.1007/s11427-017-9190-6
202. Yanase T, Kato T, Yamakawa M, Takayoshi K, Nakamura K, Kokuba T, et al. Genetic characterization of Batai virus indicates a genomic reassortment between orthobunyaviruses in nature. *Arch Virol.* (2006) 151:2253–60. doi: 10.1007/s00705-006-0808-x
203. Fu SH, Sun XH, Wang HY, Cao YX, Wang HQ, Liu WB, et al. Molecular biological identification of Batai virus isolated in China. *Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi.* (2005) 19:331–4.
204. Liu H, Shao XQ, Hu B, Zhao JJ, Zhang L, Zhang HL, et al. Isolation and complete nucleotide sequence of a Batai virus strain in Inner Mongolia, China. *Virol J.* (2014) 11:138. doi: 10.1186/1743-422X-11-138
205. Zhang L, Zhang Q, Wang J, An N, Cao Y, Fu G, et al. Characterization of Batai virus isolated from a domestic muscovy duck (*Cairina moschate*). *Virus Genes.* (2017) 53:121–5. doi: 10.1007/s11262-016-1400-4
206. Hubálek Z. Mosquito-borne viruses in Europe. *Parasitol Res.* (2008) 103(Suppl. 1):29–43. doi: 10.1007/s00436-008-1064-7
207. Ziegler U, Groschup MH, Wysocki P, Press F, Gehrmann B, Fast C, et al. Seroprevalence of Batai virus in ruminants from East Germany. *Vet Microbiol.* (2018) 227:97–102. doi: 10.1016/j.vetmic.2018.10.029
208. Gerrard SR, Li L, Barrett AD, Nichol ST. Ngari virus is a Bunyamwera virus reassortant that can be associated with large outbreaks of hemorrhagic fever in Africa. *J Virol.* (2004) 78:8922–26. doi: 10.1128/JVI.78.16.8922-8926.2004
209. Briese T, Bird B, Kapoor V, Nichol ST, Lipkin WI. Batai and Ngari viruses: M segment reassortment and association with severe febrile disease outbreaks in East Africa. *J Virol.* (2006) 80:5627–30. doi: 10.1128/JVI.02448-05
210. Liang G, Li X, Gao X, Fu S, Wang H, Li M, Lu Z, et al. Arboviruses and their related infections in China: a comprehensive field and laboratory investigation over the last 3 decades. *Rev Med Virol.* (2018) 28:e1959. doi: 10.1002/rmv.1959
211. Spengler JR, Bergeron É, Rollin PE. Seroepidemiological studies of Crimean-Congo hemorrhagic fever virus in domestic and wild animals. *PLoS Negl Trop Dis.* (2016) 10:e0004210. doi: 10.1371/journal.pntd.0004210
212. Yoshii K, Song JY, Park SB, Yang J, Schmitt HJ. Tick-borne encephalitis in Japan, Republic of Korea and China. *Emerg Microbes Infect.* (2017) 6:e82. doi: 10.1038/emi.2017.69
213. Paulsen KM, Stuen S, das Neves CG, Suhel F, Gurung D, Soleng A, et al. Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway. *Zoonoses Public Health.* (2019) 66:216–22. doi: 10.1111/zph.12554
214. Wang J, Zhang H, Fu S, Wang H, Ni D, Nasci R, et al. Isolation of Kyasanur Forest disease virus from febrile patient, Yunnan, China. *Emerg Infect Dis.* (2009) 15:326–8. doi: 10.3201/eid1502.080979
215. Boshell J. Kyasanur Forest disease: ecologic considerations. *Am J Trop Med Hyg.* (1969) 18:67–80. doi: 10.4269/ajtmh.1969.18.67
216. Yu XJ, Liang MF, Zhang SY, Liu Y, Li JD, Sun YL, et al. Fever with thrombocytopenia associated with a novel bunyavirus in China. *N Engl J Med.* (2011) 364:1523–32. doi: 10.1056/NEJMoa1010095
217. Kim KH, Yi J, Kim G, Choi SJ, Jun KI, Kim NH, et al. Severe fever with thrombocytopenia syndrome, South Korea, 2012. *Emerg Infect Dis.* (2013) 19:1892–4. doi: 10.3201/eid1911.130792
218. Takahashi T, Maeda K, Suzuki T, Ishido A, Shigeoka T, Tominaga T, et al. The first identification and retrospective study of severe fever with thrombocytopenia syndrome in Japan. *J Infect Dis.* (2014) 209:816–27. doi: 10.1093/infdis/jit603
219. Li Z, Hu J, Bao C, Li P, Qi X, Qin Y, et al. Seroprevalence of antibodies against SFTS virus infection in farmers and animals, Jiangsu, China. *J Clin Virol.* (2014) 60:185–9. doi: 10.1016/j.jcv.2014.03.020
220. Tian H, Yu P, Chowell G, Li S, Wei J, Tian H, et al. Severe fever with thrombocytopenia syndrome virus in humans, domesticated animals, ticks, and mosquitoes, Shaanxi Province, China. *Am J Trop Med Hyg.* (2017) 96:1346–9. doi: 10.4269/ajtmh.16-0333
221. Tabara K, Fujita H, Hirata A, Hayasaka D. Investigation of severe fever with thrombocytopenia syndrome virus antibody among domestic bovines transported to slaughterhouse in Shimane Prefecture, Japan. *Jpn J Infect Dis.* (2016) 69:445–7. doi: 10.7883/yoken.JJID.2015.624
222. Yu KM, Yu MA, Park SJ, Kim YI, Robles NJ, Kwon HI, et al. Seroprevalence and genetic characterization of severe fever with thrombocytopenia syndrome virus in domestic goats in South Korea. *Ticks Tick Borne Dis.* (2018) 9:1202–6. doi: 10.1016/j.ttbdis.2018.05.001
223. Robles NJC, Han HJ, Park SJ, Choi YK. Epidemiology of severe fever and thrombocytopenia syndrome virus infection and the need for therapeutics for the prevention. *Clin Exp Vaccine Res.* (2018) 7:43–50. doi: 10.7774/cevr.2018.7.1.43
224. Lindeborg M, Barboutis C, Ehrenborg C, Fransson T, Jaenson TG, Lindgren PE, et al. Migratory birds, ticks, and Crimean-Congo hemorrhagic fever virus. *Emerg Infect Dis.* (2012) 18:2095–7. doi: 10.3201/eid1812.120718
225. Yun Y, Heo ST, Kim G, Hewson R, Kim H, Park D, et al. Phylogenetic analysis of severe fever with thrombocytopenia syndrome virus in South Korea and migratory bird routes between China, South Korea, and Japan. *Am J Trop Med Hyg.* (2015) 93:468–74. doi: 10.4269/ajtmh.15-0047
226. Fukunaga Y, Kumanomido T, Kamada M. Getah virus as an equine pathogen. *Vet Clin North Am Equine Pract.* (2000) 16:605–17. doi: 10.1016/S0749-0739(17)30099-8
227. Yago K, Hagiwara S, Kawamura H, Narita M. A fatal case in newborn piglets with Getah virus infection: isolation of the virus. *Jpn J Vet Sci.* (1987) 49:989–94. doi: 10.1292/jvms1939.49.989
228. Izumida A, Takuma H, Inagaki S, Kubota M, Hirahara T, Kodama K, et al. Experimental infection of Getah virus in swine. *Jpn J Vet Sci.* (1988) 50:679–84. doi: 10.1292/jvms1939.50.679
229. Li YY, Liu H, Fu SH, Li XL, Guo XF, Li MH, et al. From discovery to spread: the evolution and phylogeny of Getah virus. *Infect Genet Evol.* (2017) 55:48–55. doi: 10.1016/j.meegid.2017.08.016
230. Chang CY, Huang CC, Huang TS, Deng MC, Jong MH, Wang FI. Isolation and characterization of a Sagiyama virus from domestic pigs. *J Vet Diagn Invest.* (2006) 18:156–61. doi: 10.1177/104063870601800203
231. Nemoto M, Bannai H, Tsujimura K, Kobayashi M, Kikuchi T, Yamanaka T, et al. Getah virus infection among racehorses, Japan, 2014. *Emerg Infect Dis.* (2015) 21:883–5. doi: 10.3201/eid2105.141975
232. Bannai H, Ochi A, Nemoto M, Tsujimura K, Yamanaka T, Kondo T. A 2015 outbreak of Getah virus infection occurring among Japanese racehorses sequentially to an outbreak in 2014 at the same site. *BMC Vet Res.* (2016) 12:98. doi: 10.1186/s12917-016-0741-5
233. Li Y, Fu S, Guo X, Li X, Li M, Wang L, et al. Serological survey of Getah virus in domestic animals in Yunnan Province, China. *Vector Borne Zoonotic Dis.* (2019) 19:59–61. doi: 10.1089/vbz.2018.2273
234. Lu G, Ou J, Ji J, Ren Z, Hu X, Wang C, et al. Emergence of Getah virus infection in horse with fever in China, 2018. *Front Microbiol.* (2019) 10:1416. doi: 10.3389/fmicb.2019.01416
235. Kaneko N, Inaba Y, Akashi H, Miura Y, Shorthose J, Kurashige K. Isolation of a new bovine ephemeral fever group virus. *Aust Vet J.* (1985) 62:388–9. doi: 10.1111/j.1751-0813.1985.tb14220.x
236. Kaneko N, Inaba Y, Akashi H, Kurashige K. Further studies on FUK-11 virus-withdrawal of a member of bovine ephemeral fever group viruses. *Aust Vet J.* (1987) 64:289. doi: 10.1111/j.1751-0813.1987.tb15968.x

237. Calisher CH, Karabatsos N, Zeller H, Digoutte JP, Tesh RB, Shope RE, et al. Antigenic relationships among rhabdoviruses from vertebrates and hematophagous arthropods. *Intervirology*. (1989) 30:241–57. doi: 10.1159/000150100
238. Noda M, Inaba Y, Banjo M, Kubo M. Isolation of Fukuoka virus, a member of the Kern Canyon serogroup viruses of the family *Rhabdoviridae*, from cattle. *Vet Microbiol*. (1992) 32:267–71. doi: 10.1016/0378-1135(92)90149-N
239. Blasdel KR, Guzman H, Widen SG, Firth C, Wood TG, Holmes EC, et al. *Ledantavirus*: a proposed new genus in the *Rhabdoviridae* has a strong ecological association with bats. *Am J Trop Med Hyg*. (2015) 92:405–10. doi: 10.4269/ajtmh.14-0606
240. Sakai K, Hagiwara K, Omatsu T, Hamasaki C, Kuwata R, Shimoda H, et al. Isolation and characterization of a novel Rhabdovirus from a wild boar (*Sus scrofa*) in Japan. *Vet Microbiol*. (2015) 179:197–203. doi: 10.1016/j.vetmic.2015.05.013
241. Kapoor A, Tesh RB, Duraisamy R, Popov VL, Travassos da Rosa AP, Lipkin WI. A novel mosquito-borne orbivirus species found in South-east Asia. *J Gen Virol*. (2013) 94:1051–7. doi: 10.1099/vir.0.046748-0
242. Sadeghi M, Popov V, Guzman H, Phan TG, Vasilakis N, Tesh R, et al. Genomes of viral isolates derived from different mosquito species. *Virus Res*. (2017) 242:49–57. doi: 10.1016/j.virusres.2017.08.012
243. Kato T, Aizawa M, Takayoshi K, Yanase T, Tanaka S, Shirafuji H, et al. Full genome sequence of a Sathuvachari virus strain isolated in the southwestern-most archipelago of Japan. *Virus Genes*. (2018) 54:729–32. doi: 10.1007/s11262-018-1592-x
244. Li M, Zheng Y, Zhao G, Fu S, Wang D, Wang Z, et al. Tibet orbivirus, a novel orbivirus species isolated from *Anopheles maculatus* mosquitoes in Tibet, China. *PLoS ONE*. (2014) 9:e88738. doi: 10.1371/journal.pone.0088738
245. Lei W, Guo X, Fu S, Feng Y, Nie K, Song J, et al. Isolation of Tibet orbivirus, TIBOV, from *Culicoides* collected in Yunnan, China. *PLoS ONE*. (2015) 10:e0136257. doi: 10.1371/journal.pone.0136257
246. Wang J, Li H, He Y, Zhou Y, Xin A, Liao D, et al. Isolation of Tibet orbivirus from *Culicoides* and associated infections in livestock in Yunnan, China. *Virol J*. (2017) 14:105. doi: 10.1186/s12985-017-0774-9
247. Qin XC, Shi M, Tian JH, Lin XD, Gao DY, He JR, et al. A tick-borne segmented RNA virus contains genome segments derived from unsegmented viral ancestors. *Proc Natl Acad Sci USA*. (2014) 111:6744–9. doi: 10.1073/pnas.1324194111
248. Shi M, Lin XD, Vasilakis N, Tian JH, Li CX, Chen LJ, et al. Divergent viruses discovered in arthropods and vertebrates revise the evolutionary history of the *Flaviviridae* and related viruses. *J Virol*. (2015) 90:659–9. doi: 10.1128/JVI.02036-15
249. Jia N, Liu HB, Ni XB, Bell-Sakyi L, Zheng YC, Song JL, et al. Emergence of human infection with Jingmen tick virus in China: a retrospective study. *EBioMedicine*. (2019) 43:317–24. doi: 10.1016/j.ebiom.2019.04.004
250. Carpenter S, Veronesi E, Mullens B, Venter G. Vector competence of *Culicoides* for arboviruses: three major periods of research, their influence on current studies and future directions. *Rev Sci Tech*. (2015) 34:97–112. doi: 10.20506/rst.34.1.2347
251. Sick F, Beer M, Kampen H, Wernike K. *Culicoides* biting midges—underestimated vectors for arboviruses of public health and veterinary importance. *Viruses*. (2019) 11:E376. doi: 10.3390/v11040376
252. Manley R, Harrup LE, Veronesi E, Stubbins F, Stoner J, Gubbins S, et al. Testing of UK populations of *Culex pipiens* L. for Schmallenberg virus vector competence and their colonization. *PLoS ONE*. (2015) 10:e0134453. doi: 10.1371/journal.pone.0134453
253. Purse BV, Carpenter S, Venter GJ, Bellis G, Mullens BA. Bionomics of temperate and tropical *Culicoides* midges: knowledge gaps and consequences for transmission of *Culicoides*-borne viruses. *Annu Rev Entomol*. (2015) 60:373–92. doi: 10.1146/annurev-ento-010814-020614
254. Muller MJ. Veterinary arbovirus vectors in Australia –a retrospective. *Vet Microbiol*. (1995) 46:101–6. doi: 10.1016/0378-1135(95)00076-M
255. Wirth WW, Hubert A. The *Culicoides* of Southeast Asia (Diptera: Ceratopogonidae). *Mem Am Entomol Inst*. (1989) 44:1–508.
256. Henna M, Shinjo K, Kokuba T. Identification of biting midges (*Culicoides*) and their distribution in Okinawa. *Bull Okinawa Prefect Inst Anim Health*. (1991) 27:43–9.
257. Yanase T, Hirata M, Matsumori Y, Matsumura M, Kato T, Shirafuji H, et al. Detection of *Culicoides brevitarsis* activity in Kyushu. *J Vet Med Sci*. (2011) 73:1649–52. doi: 10.1292/jvms.11-0231
258. Bellis G, Dyce A, Gopurenko D, Yanase T, Carros C, Labushagne K, et al. Revision of the *Culicoides* (Avaritia) Imicola complex Khamala & Kettle (Diptera: Ceratopogonidae) from the Australian region. *Zootaxa*. (2014) 3768:401–27. doi: 10.11646/zootaxa.3768.4.1
259. Gopurenko D, Bellis GA, Yanase T, Wardhana AH, Thepparat A, Wang J, et al. Integrative taxonomy to investigate species boundaries within *Culicoides* (Diptera: Ceratopogonidae): a case study using subgenus *Avaritia* from Australasia and Eastern Asia. *Vet Ital*. (2015) 51:345–78. doi: 10.12834/VetIt.515.2463.2
260. Yanase T, Kato T, Katagiri Y, Aizawa M, Nakamura K, Kokuba T, et al. Isolation and characterization of bluetongue virus from *Culicoides brevitarsis* (Diptera: Ceratopogonidae) in Okinawa. *Med Entomol Zool*. (2010) 61:85–91. doi: 10.7601/mez.61.85
261. Yanase T, Kato T, Hayama Y, Shirafuji H, Yamakawa M, Tanaka S. Oral susceptibility of Japanese *Culicoides* (Diptera: Ceratopogonidae) species to Akabane virus. *J Med Entomol*. (2019) 56:533–9. doi: 10.1093/jme/tfjy201
262. Bellis GA, Gibson DS, Polkinghorne IG, Johnson SJ, Flanagan M. Infection of *Culicoides brevitarsis* and *C. wadai* (Diptera: Ceratopogonidae) with four Australian serotypes of bluetongue virus. *J Med Entomol*. (1994) 31:382–7. doi: 10.1093/jmedent/31.3.382
263. Carpenter S, Lunt HL, Arav D, Venter GJ, Mellor PS. Oral susceptibility to bluetongue virus of *Culicoides* (Diptera: Ceratopogonidae) from the United Kingdom. *J Med Entomol*. (2006) 43:73–8. doi: 10.1093/jmedent/43.1.73
264. Venter GJ. *Culicoides* spp. (Diptera: Ceratopogonidae) as vectors of bluetongue virus in South Africa - a review. *Vet Ital*. (2015) 51:325–33. doi: 10.12834/VetIt.505.2436.2
265. Pagès N, Talavera S, Verdún M, Pujol N, Valle M, Bensaid A, et al. Schmallenberg virus detection in *Culicoides* biting midges in Spain: first laboratory evidence for highly efficient infection of *Culicoides* of the *Obsoletus* complex and *Culicoides imicola*. *Transbound Emerg Dis*. (2018) 65:e1–6. doi: 10.1111/tbed.12653
266. Barber J, Harrup LE, Silk R, Veronesi E, Gubbins S, Bachanek-Bankowska K, et al. Blood-feeding, susceptibility to infection with Schmallenberg virus and phylogenetics of *Culicoides* (Diptera: Ceratopogonidae) from the United Kingdom. *Parasit Vectors*. (2018) 11:116. doi: 10.1186/s13071-018-2650-x
267. Yanase T, Hayama Y, Shirafuji H, Tsutsui T, Terada Y. Surveillance of *Culicoides* biting midges in northern Honshu, Japan, during the period of Akabane virus spread. *J Vet Med Sci*. (2019) 81:1496–503. doi: 10.1292/jvms.19-0303
268. McDonald JL, Lu LC. Female *Culicoides* of Taiwan with descriptions of new species (Diptera, Ceratopogonidae). *J Med Entomol*. (1972) 9:396–417. doi: 10.1093/jmedent/9.5.396
269. Oem JK, Chung JY, Kwon MS, Kim TK, Lee TU, Bae YC. Abundance of biting midge species (Diptera: Ceratopogonidae, *Culicoides* spp.) on cattle farms in Korea. *J Vet Sci*. (2013) 14:91–4. doi: 10.4142/jvs.2013.14.1.91
270. Chang QQ, Jiang XH, Liu GP, Li XF, Hou XH. A species checklist of the subgenus *Culicoides* (Avaritia) in China, with a description of a new species (Diptera, Ceratopogonidae). *Zookeys*. (2017) 4:117–35. doi: 10.3897/zookeys.706.13535
271. Liu Y, Tao H, Yu Y, Yue L, Xia W, Zheng W, et al. Molecular differentiation and species composition of genus *Culicoides* biting midges (Diptera, Ceratopogonidae) in different habitats in southern China. *Vet Parasitol*. (2018) 254:49–57. doi: 10.1016/j.vetpar.2018.02.035
272. Venter GJ, Hamblin C, Paweska JT. Determination of the oral susceptibility of South African livestock-associated biting midges, *Culicoides* species, to bovine ephemeral fever virus. *Med Vet Entomol*. (2003) 17:133–7. doi: 10.1046/j.1365-2915.2003.00414.x
273. Carpenter S, Wilson A, Mellor PS. *Culicoides* and the emergence of bluetongue virus in northern Europe. *Trends Microbiol*. (2009) 17:172–8. doi: 10.1016/j.tim.2009.01.001
274. Balenghien T, Pagès N, Goffredo M, Carpenter S, Augot D, Jacquier E, et al. The emergence of Schmallenberg virus across *Culicoides*

- communities and ecosystems in Europe. *Prev Vet Med.* (2014) 116:360–9. doi: 10.1016/j.prevetmed.2014.03.007
275. Aziz-Boaron O, Gleser D, Yadin H, Gelman B, Kedmi M, Galon N, et al. The protective effectiveness of an inactivated bovine ephemeral fever virus vaccine. *Vet Microbiol.* (2014) 173:1–8. doi: 10.1016/j.vetmic.2014.06.021
 276. Aziz-Boaron O, Leibovitz K, Gelman B, Kedmi M, Klement E. Safety, immunogenicity and duration of immunity elicited by an inactivated bovine ephemeral fever vaccine. *PLoS ONE.* (2013) 8:e82217. doi: 10.1371/journal.pone.0082217
 277. Zheng FY, Chen QW, Li Z, Gong XW, Wang JD, Yin H. Experimental infection with bovine ephemeral fever virus and analysis of its antibody response cattle. *Res Vet Sci.* (2016) 104:146–51. doi: 10.1016/j.rvsc.2015.12.018
 278. Gyawali N, Bradbury RS, Aaskov JG, Taylor-Robinson AW. Neglected Australian arboviruses: quam gravis? *Microbes Infect.* (2017) 19:388–401. doi: 10.1016/j.micinf.2017.05.002
 279. Trinidad L, Blasdel KR, Joubert DA, Davis SS, Melville L, Kirkland PD, et al. Evolution of bovine ephemeral fever virus in the Australian epizootic. *J Virol.* (2014) 88:1525–35. doi: 10.1128/JVI.02797-13
 280. Xia H, Liu H, Zhao L, Atoni E, Wang Y, Yuan Z. First isolation and characterization of a group C Banna virus (BAV) from *Anopheles sinensis* mosquitoes in Hubei, China. *Viruses.* (2018) 10:555. doi: 10.3390/v10100555
 281. Chaisirirat T, Sangthong P, Arunvipas P, Petcharat N, Thangthamniyom N, Chumsing W, et al. Molecular characterization of bovine ephemeral fever virus in Thailand between 2013 and 2017. *Vet Microbiol.* (2019) 227:1–7. doi: 10.1016/j.vetmic.2018.10.013
 282. Duan YL, Miao HS, Liao F, Kou ML, Li ZH, Wang Z, et al. The serologic investigation and viral isolation of bluetongue virus in Shangri-La in Southwest China. *Transbound Emerg Dis.* (2019) 66:2353–61. doi: 10.1111/tbed.13292
 283. Schuh AJ, Ward MJ, Brown AJ, Barrett AD. Phylogeography of Japanese encephalitis virus: genotype is associated with climate. *PLoS Negl Trop Dis.* (2013) 7:e2411. doi: 10.1371/journal.pntd.0002411
 284. Miura Y, Inaba Y, Tsuda T, Tokuhisa S, Sato K, Akashi H, et al. A survey of antibodies to arthropod-borne viruses in Indonesian cattle. *Nihon Juigaku Zasshi.* (1982) 44:857–63. doi: 10.1292/jvms1939.44.857
 285. Daniels PW, Sendow I, Soleha E, Sukarsih, Hunt NT, Bahri S. Australian-Indonesian collaboration in veterinary arbovirology—a review. *Vet Microbiol.* (1995) 46:151–174. doi: 10.1016/0378-1135(95)00080-T
 286. Bryant JE, Crabtree MB, Nam VS, Yen NT, Duc HM, Miller BR. Isolation of arboviruses from mosquitoes collected in northern Vietnam. *Am J Trop Med Hyg.* (2005) 73:470–3. doi: 10.4269/ajtmh.2005.73.470
 287. Purnomo Edi S, Ibrahim A, Sukoco R, Bunali L, Taguchi M, Kato T, et al. Molecular characterization of an Akabane virus isolate from West Java, Indonesia. *J Vet Med Sci.* (2017) 79:774–9. doi: 10.1292/jvms.17-0009
 288. Elbers AR, Koenraadt CJ, Meiswinkel R. Mosquitoes and *Culicoides* biting midges: vector range and the influence of climate change. *Rev Sci Tech.* (2015) 34:123–37. doi: 10.20506/rst.34.1.2349
 289. Burgin LE, Gloster J, Sanders C, Mellor PS, Gubbins S, Carpenter S. Investigating incursions of bluetongue virus using a model of long-distance *Culicoides* biting midge dispersal. *Transbound Emerg Dis.* (2013) 60:263–72. doi: 10.1111/j.1865-1682.2012.01345.x
 290. Durr PA, Graham K, van Klinken RD. Sellers' revisited: a big data reassessment of historical outbreaks of bluetongue and African horse sickness due to the long-distance wind dispersion of *Culicoides* midges. *Front Vet Sci.* (2017) 4:98. doi: 10.3389/fvets.2017.00098
 291. Eagles D, Melville L, Weir R, Davis S, Bellis G, Zalucki MP. Long-distance aerial dispersal modelling of *Culicoides* biting midges: case studies of incursions into Australia. *BMC Vet Res.* (2014) 10:135. doi: 10.1186/1746-6148-10-135
 292. Thompson PN, Etter E. Epidemiological surveillance methods for vector-borne diseases. *Rev Sci Tech.* (2015) 34:235–47. doi: 10.20506/rst.34.1.2356
 293. Vasilakis N, Tesh RB, Popov VL, Widen SG, Wood TG, Forrester NL. Exploiting the legacy of the arbovirus hunters. *Viruses.* (2019) 11:471. doi: 10.3390/v11050471
 294. Hayama Y, Moriguchi S, Yanase T, Ishikura Y, Abe S, Higashi T, et al. Spatial epidemiological analysis of bovine encephalomyelitis outbreaks caused by Akabane virus infection in western Japan in 2011. *Trop Anim Health Prod.* (2016) 48:843–7. doi: 10.1007/s11250-016-1014-9
 295. Hayama Y, Yanase T, Suzuki M, Unten K, Tomochi H, Takehi M, et al. Meteorological factors affecting seroconversion of Akabane disease in sentinel calves in the subtropical Okinawa Islands of Japan. *Trop Anim Health Prod.* (2018) 50:209–15. doi: 10.1007/s11250-017-1404-7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Yanase, Murota and Hayama. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Ecological Dynamics Impacting Bluetongue Virus Transmission in North America

Christie Mayo^{1*}, Emily McDermott², Jennifer Kopanke³, Mark Stenglein¹, Justin Lee¹, Candace Mathiason¹, Molly Carpenter¹, Kirsten Reed¹ and T. Alex Perkins⁴

¹ Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO, United States,

² Entomology Branch, Walter Reed Army Institute of Research, Silver Spring, MD, United States, ³ Office of the Campus Veterinarian, Washington State University, Spokane, WA, United States, ⁴ Department of Biological Sciences, University of Notre Dame, Notre Dame, IN, United States

OPEN ACCESS

Edited by:

Stéphan Zientara,
Agence Nationale de Sécurité
Sanitaire de l'Alimentation, de
l'Environnement et du Travail
(ANSES), France

Reviewed by:

Nigel James MacLachlan,
University of California, Davis,
United States
David Stallknecht,
University of Georgia, United States

*Correspondence:

Christie Mayo
Christie.Mayo@colostate.edu

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 02 October 2019

Accepted: 20 March 2020

Published: 17 April 2020

Citation:

Mayo C, McDermott E, Kopanke J,
Stenglein M, Lee J, Mathiason C,
Carpenter M, Reed K and Perkins TA
(2020) Ecological Dynamics Impacting
Bluetongue Virus Transmission in
North America. *Front. Vet. Sci.* 7:186.
doi: 10.3389/fvets.2020.00186

Bluetongue virus (BTV) is an arbovirus transmitted to domestic and wild ruminants by certain species of *Culicoides* midges. The disease resulting from infection with BTV is economically important and can influence international trade and movement of livestock, the economics of livestock production, and animal welfare. Recent changes in the epidemiology of *Culicoides*-transmitted viruses, notably the emergence of exotic BTV genotypes in Europe, have demonstrated the devastating economic consequences of BTV epizootics and the complex nature of transmission across host-vector landscapes. Incursions of novel BTV serotypes into historically enzootic countries or regions, including the southeastern United States (US), Israel, Australia, and South America, have also occurred, suggesting diverse pathways for the transmission of these viruses. The abundance of BTV strains and multiple reassortant viruses circulating in Europe and the US in recent years demonstrates considerable genetic diversity of BTV strains and implies a history of reassortment events within the respective regions. While a great deal of emphasis is rightly placed on understanding the epidemiology and emergence of BTV beyond its natural ecosystem, the ecological contexts in which BTV maintains an enzootic cycle may also be of great significance. This review focuses on describing our current knowledge of ecological factors driving BTV transmission in North America. Information presented in this review can help inform future studies that may elucidate factors that are relevant to longstanding and emerging challenges associated with prevention of this disease.

Keywords: bluetongue virus, evolution, ecology, epidemiology, infectious disease dynamics

INTRODUCTION

Arthropod-borne viruses (arboviruses) constitute a significant group of emerging pathogens, many of which are increasing in global distribution as a result of climate change, urbanization, and changing of travel or trade (1–3). Bluetongue virus (BTV) is the etiologic agent of bluetongue (BT), an economically important arboviral disease of wild and domestic ruminants that is transmitted by various species of *Culicoides* midges (4–7). The expansion of *Culicoides*-transmitted arboviral diseases (BT, epizootic hemorrhagic disease [EHDV], Schmallenberg) on essentially every continent confirms that these diseases constitute a growing threat to ruminant communities (8–13). They also have substantial economic consequences.

During an outbreak of EHDV in Israel in 2006, losses caused by reduced milk production and increased mortality were estimated at ~\$2.5 million US dollars (USD), whereas the annual losses to the US livestock industry due to enzootic BTV infection were estimated at \$144 million USD ~15 years ago (14, 15). In 2006, an unprecedented, multi-year epizootic of highly virulent BTV-8 began in Western Europe, outside of the assumed range of the disease. Country-specific costs associated with lost production, trade restrictions, control, and vaccination are estimated to be as high as 207€ million (16), and the cost per country to vaccinate animals alone is estimated in the tens of millions of Euros (17). Still, other *Culicoides*-borne threats loom that could be even more economically devastating, such as African horse sickness, which as the potential to severely impact the \$122 billion USD US horse industry (18).

Novel BTV serotypes have also recently been identified in historically enzootic countries or regions including the southeastern United States (US), South America, Israel, and Australia, reflecting diverse means for spread of these viruses between regions (13, 19–22). Despite the link of climate change to recent incursions of BTV in Europe and expansion of geographic range in North America, there is limited information on how factors related to the ecology of BTV's vertebrate and invertebrate hosts might impact the evolution, distribution, and transmission dynamics of BTV (1, 23, 24). This is potentially important because a changing climate interacts with habitat and landscape variability to jointly determine opportunities for host-vector contact and the competence of vectors engaged in contact (25, 26). While transmission patterns of midge-borne viruses have been linked to heterogeneity in climate and land use worldwide, the role of host density and diversity in regulating the spread or viral evolution of these multi-host pathogens has been underappreciated (27–29). Identification and characterization of these ecological drivers could play a role in analyses to estimate the risk of BTV transmission and to inform appropriate strategies for control and prevention. In this article, we briefly summarize the current understanding of ecological factors driving BTV transmission within North America.

BLUETONGUE VIRUS

BTV is within the genus *Orbivirus*, family *Reoviridae* (30). The BTV genome consists of 10 segments of double-stranded RNA (dsRNA), and each gene segment encodes at least one protein (31, 32). The BTV virion includes seven proteins (VP 1–7), and at least five additional non-structural proteins (NS 1,2,3/3A,4) that are produced in virus-infected cells (32, 33). The structural protein VP7 expresses group antigens common to all BTV strains and serotypes, whereas segregation of BTV into serotypes is largely determined by VP2 outer capsid protein (34–37). At least 29 serotypes are recognized worldwide, but the virus strains of the same serotype may have markedly different virulence even to highly susceptible ruminants (38–42). Genetic diversity is generated among field strains of the virus by both genome segment reassortment and mutation (43). Intrasegment recombination also can occur between virus strains, either within

the vertebrate (ruminant) or invertebrate host (*Culicoides* midge) (43, 44). North American BTV isolates have been previously characterized by genotype based on segment 10 sequences (820bp region of the NS3 protein) (45). Although these analyses have provided key information into the relationships of BTV strains that circulate within the US and adjacent (such as the Caribbean Basin and Central America) and distant (such as Europe, Africa, Asia, and Australia) regions, there is a lack of comprehensive sequence data for all genomic segments. As a result, estimates of gene flow among field strains of BTV tend to be highly speculative. Similarly, the genetic determinants of viral phenotype that may impact spread and persistence, such as virulence, remain poorly characterized. Genome sequencing of field and laboratory strains of BTV has shown a high degree of segment reassortment resulting in the variety of currently circulating viral strains in the field, as compared to historic isolates, which could lead to amplification of viral transmission (46–49).

Emergence of a virulent virus (by reassortment or mutation) could stem from enzootic viruses that currently circulate in the US, or the translocation of a novel virus from an adjacent (Caribbean Basin, Latin South America) or distant (Asia, Europe, and Africa) region. In North America, BTV-2 was recently (2010) isolated in California, representing trans-continental dissemination of this virus serotype first described in the US in Florida in 1982 and that had previously been considered restricted to the southeastern US (21). The strain of BTV-2 isolated in California is a reassortant of BTV-2 and BTV-6, the latter a previously exotic serotype to North America (50). Similarly, strains of BTV-3 that have recently expanded their range beyond the southeastern US are able to readily reassort with BTV strains historically enzootic in the US (51). Recent studies based on BTV field isolates have shown reassortment is common and may drive phenotypic change resulting in a fitness advantage for the virus (46, 48, 49, 52).

Additionally, there is the issue of live attenuated vaccines being able to reassort with enzootic viruses contributing to the genetic backbone and potentially introducing novel biological properties of circulating viruses (53). Studies in both North America and Europe suggest that live-attenuated BTV vaccine viruses (or individual genome segments thereof) used to vaccinate livestock can be acquired and transmitted in the field by vector midges, thereby contributing to the gene pool of circulating viruses (54–57). Midge movement between vaccinated livestock populations and susceptible wild ruminant populations could drive viral evolution and reduce the efficacy of vaccination. Most of the major BTV vector species, including *C. sonorensis*, the primary US vector, feed opportunistically on large mammals (58), and are potential bridge vectors between livestock and wildlife populations.

Since the recent European BT epizootic, considerable focus has been placed on quantitatively defining aspects of *Culicoides* vector ecology and the genetic diversity of BTV strains (27, 59–61). Studies on the small-scale movement of *Culicoides* between farms and adjacent wildlife habitats, as well as on the frequency of contact between livestock and wildlife (e.g., deer and sheep sharing pasture) are needed to better understand BTV ecology.

With the advent of next-generation sequencing and other technologies, quantifying within-host pathogen evolution is happening increasingly (62, 63). Acquisition of such information is pivotal for the future prediction of emergence and impact of *Culicoides*-disseminated viruses in divergent ecosystem contexts with transmission models.

DISTRIBUTION

The global distribution of BTV infection corresponds with that of competent *Culicoides* vectors and suitable environmental ecosystems and the range historically has been between 40–50°N and 35–40°S (5, 39). The global distribution of BTV has altered recently, perhaps as a consequence of the impact of climate change on *Culicoides* midges that serve as the biological vectors of the virus (1, 6, 23). In particular, since 1998 multiple BTV serotypes spread throughout the Mediterranean Basin and, in 2006, additional virus serotypes invaded and spread throughout extensive portions of northern Europe to precipitate an economically devastating epizootic (64–66). This epizootic was ultimately controlled in 2010 with an extensive vaccination campaign and use of inactivated vaccines; however, the re-emergence of BTV-8 in France in 2015 has caused speculation with regards to source or mechanism of viral introduction (67). Additionally, novel serotypes of BTV have recently invaded historically-enzootic countries (Israel, South America, and Australia) and non-enzootic countries (China, Republic of South Korea) (1, 11–13, 19, 22, 68). The expansion of novel BTV serotypes into these regions demonstrates the wide distribution of competent *Culicoides* species and, with the impact of climate change, it can be anticipated that new BTV strains and serotypes will continue to be introduced on a regular basis (40, 69).

Coincident with this invasion of novel serotypes in Europe and elsewhere, 11 previously exotic serotypes (serotypes 1,3,5,6,9,12,14,18,19,22,24) have been isolated in the southeastern US since 1999 (39). Four serotypes (serotypes 10,11,13,17) have long been enzootic throughout much of the US (40, 70, 71). While BTV serotype 2 has been considered enzootic in the US since its identification in 1982, infection was thought to be confined to the southeastern US (Florida and adjacent states) until the isolation of this virus in California in 2010 (21, 72). Most recently, BTV serotype 3 has spread throughout much of the US exhibiting extensive reassortment with genes of traditionally enzootic serotypes (BTV-10, 11, 13, 17) (51). Historically, national surveys conducted by United States Department of Agriculture (USDA)/Animal and Plant Health Inspection Service (APHIS) have utilized a threshold of <2% seroprevalence to differentiate between BTV-free and BTV-enzootic areas (73). The latest survey conducted from 1991 to 2004 concluded that BTV was enzootic in all states excluding Alaska, Hawaii, Michigan, Minnesota, New York, and Wisconsin (74). There is regional variation in the prevalence of BTV infection of livestock throughout the US (75–78). Additional serologic surveys of wild ruminant species have confirmed that several species (white-tailed deer, black-tailed deer, mule deer, elk, pronghorn, and bighorn sheep) are infected in enzootic areas (79). Although the strains of BTV that currently circulate in the US typically cause

mild disease, epizootics of severe BT occur regularly amongst sheep and wildlife (white-tailed deer in particular).

Within North America, *Culicoides* midges were initially confirmed to be vectors of BTV by experimental infection of sheep using *Culicoides* midges collected during an outbreak of BT in Texas (80). At least two distinct and apparently stable BTV ecosystems have been identified in the Americas: one that includes Central and South America, the Caribbean Basin, and portions of the southeastern US, and a second area consisting of the remaining section of North America (72, 81, 82). *Culicoides sonorensis* is the predominant, if not exclusive, vector of BTV serotypes 10, 11, 13, and 17 across most of the US, south and west of the so-called “Sonorensis Line” which extends from approximately Washington State to Maryland (81). In the southeast US, *C. sonorensis* is rarely collected in areas with active BTV transmission (83), and so is not considered to be the primary vector in this area and the Caribbean. Although not conclusively proven to transmit BTV, several wildlife-associated species, including *C. stellifer* and *C. insignis*, are implicated in transmission in the southeast, as they are known to feed on livestock and wild ruminants and frequently test positive for BTV and/or EHDV (58, 83, 84). Many other *Culicoides* species known to feed on large ruminants are present within the US, but their contribution to the transmission of BTV remains uncertain (85). The absence of BTV in the northeast US appears to be due to a lack of a competent vector species. *Culicoides variipennis*, a sister species to *C. sonorensis*, is present in the northeast in livestock habitats, but either its vector competence or vectorial capacity are low enough that it apparently cannot support BTV transmission (81). Historical serological studies of ruminants in northern North America over many years have confirmed that climatic conditions prevent substantial virus transmission to ruminants (86).

C. sonorensis has also been recorded in parts of Canada, and BTV has periodically and transiently incurred in the Okanagan Valley, British Columbia, though these outbreaks appeared to be seasonal introductions without evidence of overwintering (87, 88). More recently, *C. sonorensis* was identified in Ontario on multiple farms during consecutive years (2013–14), suggesting established, overwintering populations (79). The discovery of *C. sonorensis* in Ontario was quickly followed in 2015 by the first recorded case of BTV in Canada outside of the Okanagan Valley, in an animal near where midges were collected (88). Although Canada was previously thought to be unable to support persistent *C. sonorensis* populations, the discovery of both virus and vector in Ontario suggests that a changing climate may be allowing a northward expansion of the disease.

TRANSMISSION

Inter-seasonality and Maintenance of Virus in Seasonally Enzootic Regions

The primary route of BTV transmission to its vertebrate (ruminant) host is through the bites of virus-infected hematophagous *Culicoides* midges which serve as biological vectors of the virus (5, 39, 58, 65). Although BTV is transmitted between ruminants in tropical regions throughout the year,

infection is distinctly seasonal in temperate areas where the vast majority of infections occur during the late summer and autumn months (3, 76, 89–91). The virus largely disappears from resident ruminants in much of the Northern Hemisphere between early winter until mid-summer (mid-November until at least late July) (39). The precise mechanism of this highly seasonal nature of annual BTV infection remains poorly defined, including the relative contributions of animals and insects to the process of “over-wintering” (92). Even in subtropical regions of the US, *C. sonorensis* population density is seasonal, with peaks in mid to late summer and low abundance during the winter (93, 94). In these areas, *Culicoides* populations may persist trans-seasonally as long-lived adults with potentially some continued reproduction. Recent studies in California have confirmed the presence in mid-winter of BTV-infected parous female *Culicoides* midges without concurrent infection of adjacent sentinel cattle, suggesting that vectors infected in the prior seasonal period of transmission might sustain BTV throughout the over-wintering period in seasonally enzootic areas (93). Adult midges could survive for long periods during the winter months in farm buildings, or other habitats such as tree holes and vegetation, sporadically re-surfacing to feed on hosts or nectars from plant sources (93, 95). In temperate enzootic zones, like Colorado, freezing winter temperatures preclude adult activity, and it is thought that in these areas, *C. sonorensis* populations persist as overwintering larvae (81). However, laboratory experiments have shown that eggs are the most cold-tolerant life stage (96), and are also highly desiccation tolerant (97), suggesting that they may be the true overwintering stage. Although eggs may be how the vector persists, they are unlikely to be how the virus persists. Despite a single report of BTV RNA being detected in field-collected *C. sonorensis* larvae in the north-central US, subsequent studies have been unable to recover either RNA or live virus from *Culicoides* larvae, and transovarial transmission of virus has not been described in *Culicoides* spp. (98, 99).

Although the bite of infected *Culicoides* remains the primary source of BTV infection, transmission of BTV can occur independent of the vector. Some of the novel, small ruminant BTV strains (BTV-25, BTV-26, BTV-27) may be transmitted by contact (horizontally) without the involvement of *Culicoides* midges (100–102). Oral BTV infection of both ruminant livestock and wild and zoo carnivores has been described, including infection of calves by infectious colostrum (103–106). Vertical transmission of BTV in animals has been described, in particular with live-attenuated BTV and European BTV-8 vaccine strains (107–109). Lastly, the movement of BTV-infected animals may be responsible for translocation of BTV; however, these events are only relevant if the local vector population is competent and capable of transmitting the virus.

ECOLOGICAL DRIVERS OF BTV TRANSMISSION

The dynamics of BTV transmission in multi-host ruminant systems are complex (110, 111), particularly with the additional

complication of one or more vector species with heterogeneous host feeding preferences and contact rates (112, 113). Vertebrate host communities are variable in space and dynamic in time, making it particularly difficult to generalize about the impacts of host community structure on pathogen transmission. There may also be potential to confuse effects of host density and diversity on transmission given inherent challenges associated with experiments that have been performed to address effects of host diversity on pathogen transmission (110, 111). This ecological context is especially rich with arboviruses, which are subject to selective pressures in multiple host species and experience an environmentally sensitive stage in ectothermic vectors.

Vertebrate Host

Culicoides feeding can cause physiologic and immunologic responses in mouse models resulting in the recruitment of leukocytic cells to bite sites (114). Recruitment of susceptible cell populations to the position of deposited virus occurs within hours of feeding and may explain a single infected midge's ability to transmit BTV to naive sheep with an efficiency of 80–100% (114, 115). BTV preferentially infects endothelial cells that line the walls of blood vessels, mononuclear phagocytic cells, and dendritic cells (114). After replication, BTV is released into the bloodstream where it interacts with blood cells (platelets, erythrocytes). Due to the intimate association of BTV with ruminant erythrocytes, viremia can be prolonged and is critical for transmission of the virus to susceptible *Culicoides* vectors via contaminated blood meals (116–118).

Virus-mediated damage to endothelial cells leads to vascular thrombosis, infarction of the tissue, necrosis, and hemorrhage (117, 119). The lesions of bluetongue are characterized by coronitis and laminitis, mucosal erosions, myonecrosis, subcutaneous and fascial edema, gastrointestinal ulceration, pulmonary edema, pericardial effusion, hemorrhage, ecchymoses and petechiae, and coagulopathy, among other features (38, 116). All ruminants are susceptible to BTV infection, but the most severely affected are sheep of European breeds (38). Bluetongue may also occur in other domestic and wild ruminant species (e.g., bighorn sheep, white-tailed deer, pronghorn, etc.), but severe clinical BT was rarely described in cattle prior to the 2008 BTV-8 epizootic in Europe (120, 121). Within enzootic regions such as the US, disease tends to be subclinical although sporadic epizootics have occurred. The most significant epizootic reported within the last two decades occurred throughout southern Montana and Wyoming during November of 2007 (122). Over three-hundred domestic sheep died as the result of BTV-17 infection that also affected wildlife populations of pronghorn antelope, white-tailed deer, and mule deer (122).

The ability for BTV to result in an epizootic requires the virus to overcome significant barriers. Aside from a susceptible host's physical presence, BTV needs to evade the ruminant host's adaptive and innate immune responses. Infected animals respond with interferon production to BTV infection, and both humoral and cell-mediated immune responses (123). The VP2 outer capsid protein induces serotype-specific neutralizing antibodies and provides protection against reinfection with homologous virus serotypes with minimal cross reactivity between serotypes

(124–126). Such antibodies are detected about 2 weeks after natural infection and can last for up to 4–6 years (106, 127). Once an animal has developed immunologic memory to BTV serotypes circulating within a region, herd immunity can provide a limitation for viral infection and should be considered during epidemiologic investigations and surveillance strategies.

BTV's intimate association with erythrocytes facilitates both sustained ruminant infection and infection of *Culicoides* vectors that feed on viremic ruminants (128). With regards to disease ecology, this unique feature inevitably determines its potential for enzootic stability and geographic spread. The duration of viremia is highly variable among different ruminant species. Although the duration of viremia in cattle ranges from 7 to 63 days based on virus isolation, the maximum duration in other wild ruminants has varied by species: 17 days in blesbok (*Damaliscus dorcas*); 41 days in bison (*Bison bison*); 22–28 days in white-tailed deer (*Odocoileus virginianus*); 10 days in North American elk (*Cervus elaphus*), and 35 days in mountain gazelle (*Gazella gazella*) (118, 129–131). The variability in duration of viremia reported in these species can depend on the virus serotype, blood fraction examined, and virus detection system used. Ultimately, the duration of viremia in BTV-infected ruminants that is infectious to vector insects is a prerequisite to understanding disease transmission and ecology.

Invertebrate Host

After BTV-infected ruminant blood is ingested by a competent female *Culicoides* midge, it passes with the blood meal into the lumen of the hind portion of the midgut. From there, the virus must pass several epithelial tissue infection and escape barriers to infect, disseminate within, and be transmitted by the insect. In the midgut, viral infection and multiplication occurs in the mesenteron cells, followed by release of progeny virus into the hemocoel (dissemination). Recent studies have identified a functional response to RNAi in KC cells derived from *C. sonorensis* which are successful in inhibiting BTV infection (132). While other studies have demonstrated that putative RNAi pathway members exist in *C. sonorensis*, it is unclear how these interactions limit viral replication within the invertebrate host (133). Successful release of BTV into the hemocoel allows for transit and subsequent multiplication in multiple tissues and organs. In a disseminated infection, BTV infects nearly every tissue in the insect's body, with the exception of the reproductive tissues, and this likely explains the lack of observed vertical transmission in *Culicoides* (99). Infection of the salivary glands, and escape into the salivary gland lumen, is required before the vector can transmit BTV to a susceptible vertebrate host during blood feeding. The time required to achieve this cycle is called the extrinsic incubation period (EIP), and lasts, on average, between 1 and 2 weeks, but the range can vary substantially depending on environmental conditions (primarily temperature) (99).

ENVIRONMENTAL INFLUENCES

As with all vector-borne diseases, the natural transmission cycle of BTV is dependent on relationships between the pathogen (BTV), vector (*Culicoides* spp.), and host (ruminant). Many

of these interactions can be influenced by environmental and anthropogenic factors (134). A mathematical quantity known as vectorial capacity has been established to better estimate the relative capability of a vector population to transmit a pathogen to a population of susceptible hosts (135–137). This quantity is defined as:

$$C = \frac{ma^2 V p^n}{-ln p},$$

where C = vectorial capacity, m = vector-host ratio, a = bites per vector per day, V = vector competence (suitability of the vector population for pathogen infection and transmission), p = the daily probability of survival of the vector, and n = the extrinsic incubation of the pathogen (136). An important limitation of this formulation is that, in reality, its components are altered by fluctuating environmental factors. Due to the complexity of the factors that influence vectorial capacity, few studies have attempted to calculate it based on field data for any pathogen. Gerry et al. (136) estimated the vectorial capacity of *C. sonorensis* on a southern California dairy farm by experimentally measuring all components of the equation in the field. Their model was predictive of sentinel calf seroconversions in 2 of 3 years. Different components of the vectorial capacity equation may be more or less influential in the outcome in different vector-pathogen systems. For BTV, host biting rate may have the greatest effect on vectorial capacity and is heavily influenced by temperature (136).

While a variety of factors (humidity, food quality, and adult body size) can influence seasonal activity of *C. sonorensis*, temperature remains one of the most predictable variables in determining fluctuations of the total population (87, 136, 138). Within temperate regions such as North America, the greatest abundance of adult *C. sonorensis* populations occurs with temperatures ranging from 28 to 30°C (77, 94, 139, 140). Temperature affects the daily survival probability, p , the biting rate, a , and the ratio of vectors to hosts, m , through shorter generation times. A four-degree increase in temperature (13–17°C) was associated with a 5 day decrease in egg development time. Fecundity in females held at 13°C was also significantly lower than in females held at temperatures of 17°C or higher (141). The increase in reproductive output associated with higher temperatures is somewhat offset by a decrease in daily survival at temperatures above 20°C (142, 143). However, it is important to note that the environmental air temperature measured in the field may not be equivalent to the actual temperature experienced by midges. *Culicoides* are crepuscular insects and likely rest in shaded or otherwise protected locations during the heat of the day, and the temperature in these microhabitats may be much lower than the overall air temperature. The number of adults generated per season is also dependent on the development and survival of immature midges, which are sensitive and directly related to temperature (144).

Vector Survivorship and Larval Development

As with adult midges, warmer temperatures reduce the time of development for the four larval instar stages allowing pupae to

emerge at a faster rate. Increasing the temperature from 20 to 30°C reduced *C. variipennis* larval development time from first instar to pupation by 9.0 days for a New York population (141) and 17.8 days for a Virginia population (145), but the speed of emergence during warmer conditions compromises the size and fecundity of newly emergent female *Culicoides* (141, 145). Small body size is also associated with a higher susceptibility to viral infection in mosquitoes (146).

Recent studies have identified that habitat suitability for *C. sonorensis* is associated with a number of biotic factors including temperature, land use, distribution of hosts, and Normalized Vegetation Index (23, 27, 29, 147). However, the relationship of these variables to *C. sonorensis* populations remains poorly defined in North America. Additional environmental factors that support larval populations of *C. sonorensis* include standing or slow-moving, sunlight-exposed aquatic environments, especially those contaminated with manure (148, 149). *C. sonorensis* larval habitats commonly have higher salinity concentrations than those of *C. variipennis* (148). Dramatic fluctuations in precipitation can indirectly affect the development of immature *C. sonorensis* by providing alterations in breeding habitat, but temperature remains the most influential factor affecting their development rates (150, 151). While *C. sonorensis* is typically associated with man-made livestock habitats, the putative BTV vectors in the southeast US (e.g., *C. stellifer* and *C. insignis*) are often found in sylvatic environments. Studies on the ecology of other BTV vectors in North America are far less numerous than those on *C. sonorensis*, but recent work indicates that standing water and stream margins support the development of species such as *C. hematopodus*, *C. stellifer*, and *C. venustus* (152).

Vector Competence to BTV

Vector competence of adult midges is genetically determined but environmentally influenced. Susceptibility to BTV infection is specific and may vary between *Culicoides* species, serotype of BTV ingested, or even geographical populations of *Culicoides* of the same species (153). The infection and dissemination process can be complicated by genetic (midgut infection and escape barriers) or temperature influences (148, 154). For example, optimal BTV transmission by *C. sonorensis* occurs at high temperatures 27–30°C, whereas the virus is unable to develop at temperatures below 14–15°C. As temperatures increase, virogenesis increases in a competent vector (153, 155). Due to increased virogenesis at higher temperatures, the extrinsic incubation period varies dramatically based on ambient temperature fluctuations. Studies have identified that the duration to reach peak virus titre is 4–8 days when *C. sonorensis* are held at 27–30°C, whereas it can take 16–22 days for titer to peak when held at 21°C (91, 153). Therefore, transmission of BTV is optimized during periods of warmer weather, at least above a threshold level of ~9–15°C, when the extrinsic incubation period has shortened sufficiently to permit transmission within the lifespan of *C. sonorensis* (91, 138, 153).

Temperature may have additional effects on *Culicoides* competence for BTV beyond increasing the speed of viral replication. *C. nubeculosis* is considered refractory to BTV infection under normal conditions; however, when larvae

are held at high temperatures during their development, a proportion (~10%) of those individuals can become infected with African horse sickness virus as adults (156). It has been suggested that high temperatures may damage the midgut epithelium such that viruses are able to bypass the midgut infection barrier and pass directly from the gut lumen into the hemocoel, thereby allowing non-vector species to transmit arboviruses, and that climate change may increase the frequency of these events (157).

ANTHROPOGENIC INFLUENCES

Beyond temperature, the ecology of BTV transmission is highly unpredictable, as many other factors can serve as drivers of transmission. Studies have quantified the effect of measurable parameters such as temperature, wind speed, or precipitation on BTV transmission in invertebrate and vertebrate hosts, but most of these studies are limited to work with a single vector species, or used *Culicoides* reared in laboratory conditions (64, 147, 158–160). While important, these studies cannot account for the ecological interactions occurring in the field due to anthropogenic confounders, like land use and animal husbandry practices. These anthropogenic influences are difficult to measure, both locally and globally, and it is challenging to assess the tangible effect humans have had or will have on the transmission cycle of BTV. On the largest scale, there is a great deal of uncertainty as to how climate volatility will be either associated or driven by anthropogenic influences altering the dispersion of arboviral diseases (147, 161).

Many authors have suggested that pathogen-vector-host relationships may be affected by landscape alterations that contribute to changes in the conditions of vector breeding. Although *C. sonorensis* is stereotypically considered to develop primarily in livestock wastewater ponds, these midges would have evolved to develop in more transient water sources associated with wild ruminant species. In the plains states, *C. sonorensis* larvae can be found in active bison wallows, which share features with artificial wastewater ponds that make them appropriate for development: gentle slopes, free from vegetation, and enriched with animal manure (162). These wallows are temporary puddles, and the transient nature of this resource would naturally limit midge population sizes. Wastewater ponds, on the other hand, are largely permanent water sources, providing excellent year-round development sites for *C. sonorensis*, and encouraging high levels of vector-host contact (140). On individual California dairy farms, these local management/land use practices are necessary to operate a successful and profitable business. However, these practices also increase the processes that result in the intersection of multiple natural and anthropogenic landscapes. Ecotones were originally identified as specialized wildlife habitat characterized by readily identifiable edges or transitions zones between major types of vegetation (134, 163). Recent definitions have described ecotones as a dynamic process where constituents of ecological systems influence biodiversity and ecosystem function (164). This expands the definition of ecotones as areas where biophysical factors, biological activity, and ecological evolution

are associated and may be intensified (164). Recent studies have suggested that the occurrence of BT and associated *Culicoides* populations could be related to either landscape features (forest, open pasture, and areas without vegetation) or host distribution. However, little information is provided as to a clear mechanism by which landscape indices could influence BTV circulation (23, 147, 165, 166).

By concentrating large populations of vectors near large populations of suitable hosts, BTV transmission is likely intensified in livestock systems compared to normal sylvatic cycles. This intensification provides suitable opportunities for BTV to infect multiple vertebrate and invertebrate host species, and genetically reassort genome segments which may lead to increased virulence. It can be concluded then that human activity has resulted in alterations of the spatial hierarchy of BTV infection leading to “ecotones within ecotones” from local habitat (enzootic California dairies) to the global biome (Northern Europe epizootic). Two principal points remain to be thoroughly characterized with regards to the influence of anthropogenic factors on BTV transmission: local management and landscape scale alterations (i.e., forest edge habitat, wastewater lagoons), and larger scale climate alterations from anthropogenic influences [i.e., climate change secondary to greenhouse gas emission; (167–170)].

EPIDEMIOLOGIC SURVEILLANCE STRATEGIES INFORMING PREDICTIVE MODELS

Better characterization of the environmental and anthropogenic drivers of emergence of BTV infections is clearly a prerequisite to predicting future occurrence and distribution of the disease, and to its control (10, 142, 171, 172). Surveillance in the veterinary community is described as surveying the occurrence of a disease and its status in the animal population. Entomological surveillance for vector-borne diseases involves collecting insects from the environment using mainly passive traps, and testing the species most likely to be infected with the pathogen of interest in pools of multiple individuals. Most often this is done to obtain basic information about a disease (incidence, prevalence, transmission, enzootic presence, and epizootic spread) required to inform statistical analyses and ultimately guide policy makers to make informed decisions for mitigation strategies or policy change. Therefore, it is critical to understand the questions driving the purpose of surveillance before designing strategies to achieve those goals (159, 173, 174). Many countries focus their surveillance efforts on diseases notifiable to the World Organization for Animal Health (the OIE); however, the collection of surveillance data, regardless of criteria for reporting, can be an invaluable first step and critical when developing accurate models to predict risk and assess mitigation strategies for the future (174, 175).

Vertebrate Host

Surveillance systems utilized to monitor BTV among ruminant hosts in North America have largely relied on disease reporting

or periodic cross sectional testing of sera from slaughter cattle or the monitoring of hunter-killed white-tailed deer (74, 75, 122). While additional surveillance has been conducted within individual states of the US, there are limited nationwide strategies to account for both the temporal and spatial variation of BTV infection among ruminants and vectors within a given season (77, 78, 160, 176, 177). Sentinel animal surveillance, as recommended by the OIE code, provides a useful tool in monitoring arthropod populations and viral infection rates of sentinel ruminant hosts in order to detect arboviral activity. The advantage to this targeted surveillance vs. other forms of random surveillance is the ability to act as an early warning system. Many countries experiencing both enzootic (e.g., Australia) and epizootic (e.g., Switzerland) cycles of infection utilize sentinel animal surveillance as a successful monitoring tool to advise their models for decision support and mitigation strategies [e.g., info-gap theory, scenario tree modeling; (173, 174, 178)].

Invertebrate Host

As with vertebrate host surveillance, one of the most critical points in invertebrate surveillance is assessing the goals of the program. There are many ways to categorize an insect population but optimal trapping systems have been developed to characterize species of insects present in a particular geographic location in order to: (1). examine host associations of animal-biting insects; (2). assess the seasonal activity or geographic distribution of insect species, (3). or measure parameters of pathogen transmission including host feeding preference, pathogen infection prevalence, or host biting rate (bites per host per time) (93, 154, 179–182). Animal-baited trapping provides the most useful tool when researchers are seeking to understand arboviral transmission in a natural ecosystem (136, 181, 183, 184). Host biting rate is of particular interest in the transmission cycle, as one would expect that an increase in biting rate would result in increased pathogen transmission to susceptible hosts (136, 185). Accurate measurement of biting rate can be difficult for hematophagous insects even when using animal-baited aspiration methods, but these methods provide the most accurate information in the field setting. Some rather important limitations include enclosures or aspirations that may trap biting insects in addition to those simply attracted to the vicinity of the host but not feeding (186–188). Assessing engorgement rate and parity of these species can provide additional information with regards to feeding status, but other complications include competition of the human collector who stands in close proximity to the target host.

Besides the biological considerations, use of bait animals is labor- and cost-intensive, and there are inherent risks of injury to the handlers or animals themselves. Therefore, other trapping devices are most often used in field surveillance. These traps [e.g., New Jersey light trap, CDC miniature light trap, encephalitis vector/vector surveillance (EVS) trap] are either baited with artificial semiochemicals (e.g., CO₂) or light of an appropriate wavelength (e.g., UV), and capture a diverse subset of the *Culicoides* population. However, these trap designs may reduce the overall capture of insects at individual locations compared to animal-baited aspiration, as observed in southern

California, where *C. sonorensis* abundance was 3.7 times greater when captured from “bait” cattle than from suction traps baited with CO₂ (136). Furthermore, subsequent studies demonstrated that BTV field infection rates in *C. sonorensis* were lower in insects collected by suction traps baited with both CO₂ and UV light vs. traps baited with CO₂ alone, suggesting that light actually repelled infected midges (184, 189). Further evaluation of viral dissemination within *C. sonorensis* demonstrated strong signal for viral deposition within the cornea and rhabdom (189). These structures within the compound eye are responsible for collecting and focusing light to form images; therefore, it was hypothesized that viral damage reduced *Culicoides* visual acuity, with subsequent changes in behavioral phenotypes (189). Pathogen manipulation of *C. sonorensis* behavior toward visual cues is supported by transcriptome analyses showing significant downregulation of genes known to be involved in sensory processes, particularly vision, after infection with EHDV, as well as downregulation of genes associated with memory and startle responses (190). It is unknown whether this effect is adaptive to BTV transmission, or whether it is a side effect of disseminated viral infection (189, 190). Therefore, the use of traps baited with UV light may provide a poor estimate of biting rate and lead to a misunderstanding of pathogen transmission, factors of critical importance when modeling risk of BTV transmission.

FUTURE RESEARCH NEEDS

Host-vector-virus transmission systems are dynamic and complex, with a variety of ecological drivers. Knowledge of the mechanisms driving the emergence or incursion of BTV from its natural maintenance cycle is still very limited. Studies have reported that maintenance and distribution of BTV is attributed to biotic factors, whereas patterns of vector behavior and abundance are likely related to abiotic factors (165). Understanding and defining these interactions is critical to predicting the occurrence of BTV infection of livestock through comprehensive determination of the impact of these drivers on vector abundance, competence,

and vectorial capacity (87, 138, 191). Individual species of *Culicoides* midges require distinct and specific conditions for breeding, which could explain in part the increased rate of BTV transmission within certain geographic areas (180, 192). However, few studies to date have attempted to specifically address how biotic and abiotic drivers of infection are related to abundance of vectors and virus transmission among ruminants in North America.

Many questions about bluetongue remain, in part because of a lack of data and in part because of the overwhelming complexity of the studies that are necessary to capture important features of the ecology and evolution. Although data collection and accessibility are developing, more precise data are necessary to uncover some of the most pressing mysteries of this particular arbovirus. Identifying mechanisms for defining interactions among ecological drivers, host diversity, and the emerging risk of vector-borne diseases within ruminant communities could offer new insights into understanding the ecology of this virus. The domestic animal-wildlife-human interface is becoming an increasingly greater concern as a result of habitat fragmentation and land-use change. The ultimate goal is to provide tangible outcomes for predicting risk and mitigating vector-borne disease, particularly in the face of climate variability. These questions establish context for developing innovative ecological studies linking processes across multiple scales and have the potential to inform cost-effective, science-driven approaches to the development of mitigation strategies.

AUTHOR CONTRIBUTIONS

CMay, EM, JK, MS, JL, CMat, MC, KR, and TP made equal contributions to this review article.

FUNDING

This work was funded by USDA-NIFA AFRI grant # 2019-67015-28982 as part of the joint USDA-NSF-NIH-BBSRC-BSF Ecology and Evolution of Infectious Diseases program.

REFERENCES

- Purse BV, Mellor PS, Rogers DJ, Samuel AR, Mertens PC, Baylis M. Climate change and the recent emergence of bluetongue in Europe. *Nat Rev Microbiol.* (2005) 3:171–81. doi: 10.1038/nrmicro1090
- Tabachnick WJ. Climate change and the arboviruses: lessons from the evolution of the dengue and yellow fever viruses. *Annu Rev Virol.* (2016) 3:125–45. doi: 10.1146/annurev-virology-110615-035630
- Gould EA, Higgs S. Impact of climate change and other factors on emerging arbovirus diseases. *Trans R Soc Trop Med Hyg.* (2009) 103:109–21. doi: 10.1016/j.trstmh.2008.07.025
- Mellor PS, Boorman J, Baylis M. *Culicoides* biting midges: their role as arbovirus vectors. *Annu Rev Entomol.* (2000) 45:307–40. doi: 10.1146/annurev.ento.45.1.307
- Gibbs EP, Greiner EC. The epidemiology of bluetongue. *Comp Immunol Microbiol Infect Dis.* (1994) 17:207–20. doi: 10.1016/0147-9571(94)90044-2
- Purse BV, Brown HE, Harrup L, Mertens PC, Rogers DJ. Invasion of bluetongue and other orbivirus infections into Europe: the role of biological and climatic processes. *Rev Sci Tech.* (2008) 27:427–42. doi: 10.20506/rst.27.2.1801
- Gibbs EPJ, Tabachnick WJ, Holt TJ, Stallknecht DE. U.S. concerns over bluetongue. *Science.* (2008) 320:872. doi: 10.1126/science.320.5878.872a
- Carpenter S, Groschup MH, Garros C, Felipe-Bauer ML, Purse BV. *Culicoides* biting midges, arboviruses and public health in Europe. *Antivir Res.* (2013) 100:102–13. doi: 10.1016/j.antiviral.2013.07.020
- Hateley G. Emergence and spread of Schmallenberg virus. *Vet Rec.* (2013) 173:139–40. doi: 10.1136/vr.f4923
- Maclachlan NJ, Mayo CE. Potential strategies for control of bluetongue, a globally emerging, *Culicoides*-transmitted viral disease of ruminant livestock and wildlife. *Antivir Res.* (2013) 99:79–90. doi: 10.1016/j.antiviral.2013.04.021
- Qin S, Yang H, Zhang Y, Li Z, Lin J, Gao L, et al. Full genome sequence of the first bluetongue virus serotype 21 (BTV-21) isolated from China: evidence for genetic reassortment between BTV-21 and bluetongue virus serotype 16 (BTV-16). *Arch Virol.* (2018) 163:1379–82. doi: 10.1007/s00705-018-3718-9

12. White JR, Williams DT, Wang J, Chen H, Melville LF, Davis SS, et al. Identification and genomic characterization of the first isolate of bluetongue virus serotype 5 detected in Australia. *Vet Med Sci.* (2019) 5:129–45. doi: 10.1002/vms3.156
13. Firth C, Blasdel KR, Amos-Ritchie R, Sendow I, Agnihotri K, Boyle DB, et al. Genomic analysis of bluetongue virus episystems in Australia and Indonesia. *Vet Res.* (2017) 48:82. doi: 10.1186/s13567-017-0488-4
14. Kedmi M, Van Straten M, Ezra E, Galon N, Klement E. Assessment of the productivity effects associated with epizootic hemorrhagic disease in dairy herds. *J Dairy Sci.* (2010) 93:2486–95. doi: 10.3168/jds.2009-2850
15. Hoar BR, Carpenter TE, Singer RS, Gardner IA. Regional risk of exporting cattle seropositive for bluetongue virus from the United States. *Am J Vet Res.* (2003) 64:520–9. doi: 10.2460/ajvr.2003.64.520
16. Velthuis AGJ, Saatkamp HW, Mourits MCM, de Koeijer AA, Elbers ARW. Financial consequences of the Dutch bluetongue serotype 8 epidemics of 2006 and 2007. *Prev Vet Med.* (2010) 93:294–304. doi: 10.1016/j.prevetmed.2009.11.007
17. Tago D, Hammitt JK, Thomas A, Raboisson D. Cost assessment of the movement restriction policy in France during the 2006 bluetongue virus episode (BTV-8). *Prev Vet Med.* (2014) 117:577–89. doi: 10.1016/j.prevetmed.2014.10.010
18. AHCF. *Economic Impact Study of the US Horse Industry*, Washington, DC: American Horse Council Foundation (2017).
19. Cameron AR. The impact of current and proposed changes to general guidelines on bluetongue surveillance of the Office International des Epizooties. *Vet Ital.* (2004) 40:693–6.
20. Brenner J, Oura C, Asis I, Maan S, Elad D, Maan N, et al. Multiple serotypes of bluetongue virus in sheep and cattle, Israel. *Emerg Infect Dis.* (2010) 16:2003–4. doi: 10.3201/eid1612.100239
21. MacLachlan NJ, Wilson WC, Crossley BM, Mayo CE, Jaspersen DC, Breitmeyer RE, et al. Novel serotype of bluetongue virus, western North America. *Emerg Infect Dis.* (2013) 19:665–6. doi: 10.3201/eid1904.120347
22. Verdezoto J, Breard E, Viarouge C, Quenault H, Lucas P, Sailleau C, et al. Novel serotype of bluetongue virus in South America and first report of epizootic haemorrhagic disease virus in Ecuador. *Transbound Emerg Dis.* (2018) 65:244–7. doi: 10.1111/tbed.12625
23. Guis H, Caminade C, Calvete C, Morse AP, Tran A, Baylis M. Modelling the effects of past and future climate on the risk of bluetongue emergence in Europe. *J R Soc Interface.* (2012) 9:339–50. doi: 10.1098/rsif.2011.0255
24. Purse BV, Nedelchev N, Georgiev G, Veleva E, Boorman J, Denison E, et al. Spatial and temporal distribution of bluetongue and its *Culicoides* vectors in Bulgaria. *Med Vet Entomol.* (2006) 20:335–44. doi: 10.1111/j.1365-2915.2006.00636.x
25. Landis WG, Durda JL, Brooks ML, Chapman PM, Menzie CA, Stahl RG Jr, et al. Ecological risk assessment in the context of global climate change. *Environ Toxicol Chem.* (2013) 32:79–92. doi: 10.1002/etc.2047
26. Kraemer MUG, Reiner RC Jr, Brady OJ, Messina JP, Gilbert M, Pigott DM, et al. Past and future spread of the arbovirus vectors *Aedes aegypti* and *Aedes albopictus*. *Nat Microbiol.* (2019) 4:854–63. doi: 10.1038/s41564-019-0376-y
27. Rigot T, Drubbel MV, Delécolle J-C, Gilbert M. Farms, pastures and woodlands: the fine-scale distribution of Palearctic *Culicoides* spp. biting midges along an agro-ecological gradient. *Med Vet Entomol.* (2013) 27:29–38. doi: 10.1111/j.1365-2915.2012.01032.x
28. Nicolas G, Tisseuil C, Conte A, Allepuz A, Pioz M, Lancelot R, et al. Environmental heterogeneity and variations in the velocity of bluetongue virus spread in six European epidemics. *Prev Vet Med.* (2018) 149:1–9. doi: 10.1016/j.prevetmed.2017.11.005
29. Chanda MM, Carpenter S, Prasad G, Sedda L, Henrys PA, Gajendragad MR, et al. Livestock host composition rather than land use or climate explains spatial patterns in bluetongue disease in South India. *Sci Rep.* (2019) 9:4229. doi: 10.1038/s41598-019-40450-8
30. Maan S, Maan NS, Nomikou K, Batten C, Antony F, Belaganahalli MN, et al. Novel bluetongue virus serotype from Kuwait. *Emerg Infect Dis.* (2011) 17:886–9. doi: 10.3201/eid1705.101742
31. Mertens PP, Brown F, Sangar DV. Assignment of the genome segments of bluetongue virus type 1 to the proteins which they encode. *Virology.* (1984) 135:207–17. doi: 10.1016/0042-6822(84)90131-4
32. Patel A, Roy P. The molecular biology of bluetongue virus replication. *Virus Res.* (2014) 182:5–20. doi: 10.1016/j.virusres.2013.12.017
33. Ratniner M, Caporale M, Golder M, Franzoni G, Allan K, Nunes SF, et al. Identification and characterization of a novel non-structural protein of bluetongue virus. *PLoS Pathog.* (2011) 7:e1002477. doi: 10.1371/journal.ppat.1002477
34. DeMaula CD, Bonneau KR, James MacLachlan N. Changes in the outer capsid proteins of bluetongue virus serotype ten that abrogate neutralization by monoclonal antibodies. *Virus Res.* (2000) 67:59–66. doi: 10.1016/S0168-1702(00)00130-1
35. Huismans H, Erasmus BJ. Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort J Vet Res.* (1981) 48:51–8.
36. Huismans H, van der Walt NT, Cloete M, Erasmus BJ. Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. *Virology.* (1987) 157:172–9. doi: 10.1016/0042-6822(87)90326-6
37. Maan NS, Maan S, Belaganahalli MN, Ostlund EN, Johnson DJ, Nomikou K, et al. Identification and differentiation of the twenty six bluetongue virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. *PLoS ONE.* (2012) 7:e32601. doi: 10.1371/journal.pone.0032601
38. Verwoerd DW, Erasmus BJ. Bluetongue. In: Coetzer JAW, Tustin RC, editors. *Diseases of Livestock*. 2nd ed. Cape Town, South Africa: Oxford University Press (2004). p. 1201–20.
39. MacLachlan NJ. Bluetongue: history, global epidemiology, and pathogenesis. *Prev Vet Med.* (2011) 102:107–111. doi: 10.1016/j.prevetmed.2011.04.005
40. MacLachlan NJ. Global implications of the recent emergence of bluetongue virus in Europe. *Vet Clin North Am Food Anim Pract.* (2010) 26:163–71. doi: 10.1016/j.cvfa.2009.10.012
41. Savini G, Puggioni G, Meloni G, Marcacci M, Di Domenico M, Rocchigiani AM, et al. Novel putative Bluetongue virus in healthy goats from Sardinia, Italy. *Infect Genet Evol.* (2017) 51:108–17. doi: 10.1016/j.meegid.2017.03.021
42. Schulz C, Bréard E, Sailleau C, Jenckel M, Viarouge C, Vitour D, et al. Bluetongue virus serotype 27: detection and characterization of two novel variants in Corsica, France. *J Gen Virol.* (2016) 97:2073–83. doi: 10.1099/jgv.0.000557
43. Bonneau KR, Mullens BA, MacLachlan NJ. Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of bluetongue virus upon passage between sheep, cattle, and *Culicoides sonorensis*. *J Virol.* (2001) 75:8298–305. doi: 10.1128/JVI.75.17.8298-8305.2001
44. He C-Q, Ding N-Z, He M, Li S-N, Wang X-M, He H-B, et al. Intragenic recombination as a mechanism of genetic diversity in bluetongue virus. *J Virol.* (2010) 84:11487–95. doi: 10.1128/JVI.00889-10
45. Balasuriya UBR, Nadler SA, Wilson WC, Pritchard LI, Smythe AB, Savini G, et al. The NS3 proteins of global strains of bluetongue virus evolve into regional topotypes through negative (purifying) selection. *Vet Microbiol.* (2008) 126:91–100. doi: 10.1016/j.vetmic.2007.07.006
46. Nomikou K, Hughes J, Wash R, Kellam P, Breard E, Zientara S, et al. Widespread reassortment shapes the evolution and epidemiology of Bluetongue virus following European invasion. *PLoS Pathog.* (2015) 11:e1005056. doi: 10.1371/journal.ppat.1005056
47. Caporale M, Di Galleonorado L, Janowicz A, Wilkie G, Shaw A, Savini G, et al. Virus and host factors affecting the clinical outcome of Bluetongue virus infection. *J Virol.* (2014) 88:10399–411. doi: 10.1128/JVI.01641-14
48. Shaw AE, Ratniner M, Nunes SF, Nomikou K, Caporale M, Golder M, et al. Reassortment between two serologically unrelated bluetongue virus strains is flexible and can involve any genome segment. *J Virol.* (2013) 87:543–57. doi: 10.1128/JVI.02266-12
49. Jacquot M, Rao PP, Yadav S, Nomikou K, Maan S, Jyothi YK, et al. Contrasting selective patterns across the segmented genome of bluetongue virus in a global reassortment hotspot. *Virus Evol.* (2019) 5:vez027. doi: 10.1093/ve/vez027
50. Gaudreault NN, Mayo CE, Jaspersen DC, Crossley BM, Breitmeyer RE, Johnson DJ, et al. Whole genome sequencing and phylogenetic analysis of Bluetongue virus serotype 2 strains isolated in the Americas including a novel strain from the western United States. *J Vet Diagn Invest.* (2014) 26:553–7. doi: 10.1177/1040638714536902

51. Schirtzinger EE, Jaspersen DC, Ostlund EN, Johnson DJ, Wilson WC. Recent US bluetongue virus serotype 3 isolates found outside of Florida indicate evidence of reassortment with co-circulating endemic serotypes. *J Gen Virol.* (2018) 99:157–68. doi: 10.1099/jgv.0.000965
52. McDonald SM, Patton JT. Assortment and packaging of the segmented rotavirus genome. *Trends Microbiol.* (2011) 19:136–44. doi: 10.1016/j.tim.2010.12.002
53. McVey DS, MacLachlan NJ. Vaccines for prevention of Bluetongue and epizootic hemorrhagic disease in livestock: a North American perspective. *Vector Borne Zoonotic Dis.* (2015) 15:385–96. doi: 10.1089/vbz.2014.1698
54. Osburn BI, de Mattos CA, de Mattos CC, MacLachlan NJ. Bluetongue disease and the molecular epidemiology of viruses from the Western United States. *Comp Immunol Microbiol Infect Dis.* (1996) 19:181–90. doi: 10.1016/0147-9571(96)00003-3
55. Ferrari G, De Liberato C, Scavia G, Lorenzetti R, Zini M, Farina F, et al. Active circulation of bluetongue vaccine virus serotype-2 among unvaccinated cattle in central Italy. *Prev Vet Med.* (2005) 68:103–13. doi: 10.1016/j.prevetmed.2004.11.011
56. Batten CA, Bachanek-Bankowska K, Bin-Tarif A, Kgosana L, Swain AJ, Corteyn M, et al. Bluetongue virus: European Community inter-laboratory comparison tests to evaluate ELISA and RT-PCR detection methods. *Vet Microbiol.* (2008) 129:80–8. doi: 10.1016/j.vetmic.2007.11.005
57. Van den Bergh C, Coetzee P, Venter EH. Reassortment of bluetongue virus vaccine serotypes in cattle. *J S Afr Vet Assoc.* (2018) 89:e1–e7. doi: 10.4102/jsava.v89i0.1649
58. Purse BV, Carpenter S, Venter GJ, Bellis G, Mullens BA. Bionomics of temperate and tropical Culicoides midges: knowledge gaps and consequences for transmission of Culicoides-borne viruses. *Annu Rev Entomol.* (2015) 60:373–92. doi: 10.1146/annurev-ento-010814-020614
59. Eagles D, Walker PJ, Zalucki MP, Durr PA. Modelling spatio-temporal patterns of long-distance Culicoides dispersal into northern Australia. *Prev Vet Med.* (2013) 110:312–22. doi: 10.1016/j.prevetmed.2013.02.022
60. González M, López S, Mullens BA, Baldet T, Goldarazena A. A survey of Culicoides developmental sites on a farm in northern Spain, with a brief review of immature habitats of European species. *Vet Parasitol.* (2013) 191:81–93. doi: 10.1016/j.vetpar.2012.08.025
61. Harrup LE, Purse BV, Golding N, Mellor PS, Carpenter S. Larval development and emergence sites of farm-associated Culicoides in the United Kingdom. *Med Vet Entomol.* (2013) 27:441–9. doi: 10.1111/mve.12006
62. Beerenwinkel N, Zagordi O. Ultra-deep sequencing for the analysis of viral populations. *Curr Opin Virol.* (2011) 1:413–8. doi: 10.1016/j.coviro.2011.07.008
63. Manske M, Miotto O, Campino S, Auburn S, Almagro-Garcia J, Maslen G, et al. Analysis of *Plasmodium falciparum* diversity in natural infections by deep sequencing. *Nature.* (2012) 487:375–9. doi: 10.1038/nature11174
64. Gloster J, Burgin L, Witham C, Athanassiadou M, Mellor PS. Bluetongue in the United Kingdom and northern Europe in 2007 and key issues for 2008. *Vet Rec.* (2008) 162:298–302. doi: 10.1136/vr.162.10.298
65. Saegerman C, Berkvens D, Mellor PS. Bluetongue epidemiology in the European Union. *Emerg Infect Dis.* (2008) 14:539–44. doi: 10.3201/eid1404.071441
66. Mellor PS, Carpenter S, Harrup L, Baylis M, Mertens PPC. Bluetongue in Europe and the Mediterranean Basin: history of occurrence prior to 2006. *Prev Vet Med.* (2008) 87:4–20. doi: 10.1016/j.prevetmed.2008.06.002
67. Pascall DJ, Nomikou K, Bréard E, Zientara S, da Silva Filipe A, Hoffmann B, et al. Evolutionary stasis of an RNA virus indicates arbovirus re-emergence triggered by accidental release. *bioRxiv [preprint].* (2019). doi: 10.1101/2019.12.11.872705
68. Seo H-J, Park J-Y, Cho YS, Cho I-S, Yeh J-Y. First report of Bluetongue virus isolation in the Republic of Korea and analysis of the complete coding sequence of the segment 2 gene. *Virus Genes.* (2015) 50:156–9. doi: 10.1007/s11262-014-1140-2
69. Coetzee P, Stokstad M, Venter EH, Myrmel M, Van Vuuren M. Bluetongue: a historical and epidemiological perspective with the emphasis on South Africa. *Virol J.* (2012) 9:198. doi: 10.1186/1743-422X-9-198
70. MacLachlan NJ, Osburn BI. Impact of bluetongue virus infection on the international movement and trade of ruminants. *J Am Vet Med Assoc.* (2006) 228:1346–9. doi: 10.2460/javma.228.9.1346
71. Barber TL. Temporal appearance, geographic distribution, and species of origin of bluetongue virus serotypes in the United States. *Am J Vet Res.* (1979) 40:1654–6.
72. Gibbs EP, Greiner EC, Taylor WP, Barber TL, House JA, Pearson JE. Isolation of bluetongue virus serotype 2 from cattle in Florida: serotype of bluetongue virus hitherto unrecognized in the Western Hemisphere. *Am J Vet Res.* (1983) 44:2226–8.
73. Dargatz D, Akin K, Green A, Herrero M, Holland S, Kane A, et al. Bluetongue surveillance methods in the United States of America. *Vet Ital.* (2004) 40:182–3.
74. Ostlund EN, Moser KM, Johnson DJ, Pearson JE, Schmitt BJ. Distribution of bluetongue in the United States of America, 1991–2002. *Vet Ital.* (2004) 40:83–8.
75. Metcalf HE, Pearson JE, Klingsporn AL. Bluetongue in cattle: a serologic survey of slaughter cattle in the United States. *Am J Vet Res.* (1981) 42:1057–61.
76. Osburn BI, McGowan B, Heron B, Loomis E, Bushnell R, Stott J, et al. Epizootologic study of bluetongue: virologic and serologic results. *Am J Vet Res.* (1981) 42:884–7.
77. Stott JL, Osburn BI, Bushnell R, Loomis EC, Squire KR. Epizootiological study of bluetongue virus infection in California livestock: an overview. *Prog Clin Biol Res.* (1985) 178:571–82.
78. Uhah IJ, Riemann HP, Thurmond MC, Franti CE. A seroepidemiological study on bluetongue virus in dairy cattle in the central valley of California. *Vet Res Commun.* (1990) 14:99–112. doi: 10.1007/BF00367058
79. Stallknecht DE, Lisa Kellogg M, Blue JL, Pearson JE. Antibodies to Bluetongue and epizootic hemorrhagic disease viruses in a barrier island white-tailed deer population. *J Wildl Dis.* (1991) 27:668–74. doi: 10.7589/0090-3558-27.4.668
80. Price DA, Hardy WT. Isolation of the bluetongue virus from Texas sheep-Culicoides shown to be a vector. *J Am Vet Med Assoc.* (1954) 124:255–8.
81. Tabachnick WJ. Culicoides variipennis and bluetongue-virus epidemiology in the United States. *Annu Rev Entomol.* (1996) 41:23–43. doi: 10.1146/annurev.en.41.010196.000323
82. Tabachnick WJ. Culicoides and the global epidemiology of bluetongue virus infection. *Vet Ital.* (2004) 40:144–50.
83. Smith KE, Stallknecht DE. Culicoides (Diptera: Ceratopogonidae) collected during epizootics of hemorrhagic disease among captive white-tailed deer. *J Med Entomol.* (1996) 33:507–10. doi: 10.1093/jmedent/33.3.507
84. McGregor BL, Sloyer KE, Sayler KA, Goodfriend O, Krauer JMC, Acevedo C, et al. Field data implicating *Culicoides stellifer* and *Culicoides venustus* (Diptera: Ceratopogonidae) as vectors of epizootic hemorrhagic disease virus. *Parasit Vectors.* (2019) 12:258. doi: 10.1186/s13071-019-3514-8
85. Mullens BA, Owen JP, Heft DE, Soback RV. Culicoides and other biting flies on the Palos Verdes Peninsula of Southern California, and their possible relationship to equine dermatitis. *J Am Mosq Control Assoc.* (2005) 21:90–5. doi: 10.2987/8756-971X(2005)21[90:CAOBFO]2.0.CO;2
86. Vangeneugden T, Laenen A, Geys H, Renard D, Molenberghs G. Applying linear mixed models to estimate reliability in clinical trial data with repeated measurements. *Control Clin Trials.* (2004) 25:13–30. doi: 10.1016/j.cct.2003.08.009
87. Lysyk TJ. Seasonal abundance, parity, and survival of adult *Culicoides sonorensis* (Diptera: Ceratopogonidae) in southern Alberta, Canada. *J Med Entomol.* (2007) 44:959–69. doi: 10.1603/0022-2585(2007)44[959:SAPASO]2.0.CO;2
88. Jewiss-Gaines A, Barelli L, Hunter FF. First records of *Culicoides sonorensis* (Diptera: Ceratopogonidae), a known vector of Bluetongue virus, in Southern Ontario. *J Med Entomol.* (2017) 54:757–62. doi: 10.1093/jme/tjw215
89. Spreull J. Malarial catarrhal fever (Bluetongue) of sheep in South Africa. *J Comp Pathol Ther.* (1905) 18:321–37. doi: 10.1016/S0368-1742(05)80073-6
90. Nevill EM. Cattle and Culicoides biting midges as possible overwintering hosts of bluetongue virus. *Onderstepoort J Vet Res.* (1971) 38:65–71.

91. Wittmann EJ, Mello PS, Baylis M. Effect of temperature on the transmission of orbiviruses by the biting midge, *Culicoides sonorensis*. *Med Vet Entomol.* (2002) 16:147–56. doi: 10.1046/j.1365-2915.2002.00357.x
92. Mayo C, Mullens B, Gibbs EP, MacLachlan NJ. Overwintering of Bluetongue virus in temperate zones. *Vet Ital.* (2016) 52:243–6. doi: 10.12834/VetIt.521.2473.3
93. Mayo CE, Mullens BA, Reisen WK, Osborne CJ, Gibbs EPJ, Gardner IA, et al. Seasonal and interseasonal dynamics of Bluetongue virus infection of dairy cattle and *Culicoides sonorensis* midges in Northern California - implications for virus overwintering in temperate zones. *PLoS ONE.* (2014) 9:e106975. doi: 10.1371/journal.pone.0106975
94. Gerry AC, Mullens BA. Seasonal abundance and survivorship of *Culicoides sonorensis* (Diptera: Ceratopogonidae) at a Southern California dairy, with reference to potential Bluetongue virus transmission and persistence. *J Med Entomol.* (2000) 37:675–88. doi: 10.1603/0022-2585-37.5.675
95. Kettle DS, Linley JR. The biting habits of some Jamaican *Culicoides*. II. *C. furens* (Poey). *B Entomol Res.* (1969) 59:1–20. doi: 10.1017/S0007485300002984
96. McDermott EG, Mayo CE, Mullens BA. Low temperature tolerance of *Culicoides sonorensis* (Diptera: Ceratopogonidae) eggs, larvae, and pupae from temperate and subtropical climates. *J Med Entomol.* (2016) 54:264–74. doi: 10.1093/jme/tjw190
97. McDermott EG, Mullens BA. Desiccation tolerance in the eggs of the primary North American Bluetongue virus vector, *Culicoides sonorensis* (Diptera: Ceratopogonidae), and implications for vector persistence. *J Med Entomol.* (2014) 51:1151–8. doi: 10.1603/MEI14049
98. White DM. Studies on overwintering of bluetongue viruses in insects. *J Gen Virol.* (2005) 86:453–62. doi: 10.1099/vir.0.80290-0
99. Osborne CJ, Mayo CE, Mullens BA, McDermott EG, Gerry AC, Reisen WK, et al. Lack of evidence for laboratory and natural vertical transmission of Bluetongue virus in *Culicoides sonorensis* (Diptera: Ceratopogonidae). *J Med Entomol.* (2015) 52:274–7. doi: 10.1093/jme/tju063
100. Chaignat V, Worwa G, Scherrer N, Hilbe M, Ehrensperger F, Batten C, et al. Toggenburg Orbivirus, a new bluetongue virus: initial detection, first observations in field and experimental infection of goats and sheep. *Vet Microbiol.* (2009) 138:11–9. doi: 10.1016/j.vetmic.2009.02.003
101. Batten CA, Henstock MR, Steedman HM, Waddington S, Edwards L, Oura CA. Bluetongue virus serotype 26: infection kinetics, pathogenesis and possible contact transmission in goats. *Vet Microbiol.* (2013) 162:62–7. doi: 10.1016/j.vetmic.2012.08.014
102. Bréard E, Schulz C, Sailleau C, Bernelin-Cottet C, Viarouge C, Vitour D, et al. Bluetongue virus serotype 27: experimental infection of goats, sheep and cattle with three BTV-27 variants reveal atypical characteristics and likely direct contact transmission BTV-27 between goats. *Transbound Emerg Dis.* (2018) 65:e251–e63. doi: 10.1111/tbed.12780
103. Alexander KA, Holekamp K, James MacLachlan N, Frank LG, Sawyer M, Osburn BI, et al. Evidence of natural bluetongue virus infection among African carnivores. *Am J Trop Med Hyg.* (1994) 51:568–76. doi: 10.4269/ajtmh.1994.51.568
104. Backx A, Heutink R, van Rooij E, van Rijn P. Transplacental and oral transmission of wild-type bluetongue virus serotype 8 in cattle after experimental infection. *Vet Microbiol.* (2009) 138:235–43. doi: 10.1016/j.vetmic.2009.04.003
105. Jauniaux TP, De Clercq KE, Cassart DE, Kennedy S, Vandenbussche FE, Vandemeulebroucke EL, et al. Bluetongue in Eurasian lynx. *Emerg Infect Dis.* (2008) 14:1496. doi: 10.3201/eid1409.080434
106. Mayo CE, Crossley BM, Hietala SK, Gardner IA, Breitmeyer RE, James MacLachlan N. Colostral transmission of Bluetongue virus nucleic acid among newborn dairy calves in California. *Transbound Emerg Dis.* (2010) 57:277–81. doi: 10.1111/j.1865-1682.2010.01149.x
107. Saegeman C, Bolkaerts B, Baricalla C, Raes M, Wiggers L, de Leeuw I, et al. The impact of naturally-occurring, trans-placental bluetongue virus serotype-8 infection on reproductive performance in sheep. *Vet J.* (2011) 187:72–80. doi: 10.1016/j.tvjl.2009.11.012
108. Vögtlin A, Hofmann MA, Nenniger C, Renzullo S, Steinrigl A, Loitsch A, et al. Long-term infection of goats with bluetongue virus serotype 25. *Vet Microbiol.* (2013) 166:165–73. doi: 10.1016/j.vetmic.2013.06.001
109. van der Sluijs MTW, de Smit AJ, Moormann RJM. Vector independent transmission of the vector-borne bluetongue virus. *Crit Rev Microbiol.* (2016) 42:57–64. doi: 10.3109/1040841X.2013.879850
110. Holt RD, Lawton JH. Apparent competition and enemy-free space in insect host-parasitoid communities. *Am Nat.* (1993) 142:623–45. doi: 10.1086/285561
111. Girard JM, Wagner DM, Vogler AJ, Keys C, Allender CJ, Drickamer LC, et al. Differential plague-transmission dynamics determine *Yersinia pestis* population genetic structure on local, regional, and global scales. *Proc Natl Acad Sci U S A.* (2004) 101:8408–13. doi: 10.1073/pnas.0401561101
112. Hamer GL, Chaves LF, Anderson TK, Kitron UD, Brawn JD, Ruiz MO, et al. Fine-scale variation in vector host use and force of infection drive localized patterns of west Nile virus transmission. *PLoS ONE.* (2011) 6:e23767. doi: 10.1371/journal.pone.0023767
113. Marm Kilpatrick A, Daszak P, Jones MJ, Marra PP, Kramer LD. Host heterogeneity dominates West Nile virus transmission. *Proc R Soc B Biol Sci.* (2006) 273:2327–33. doi: 10.1098/rspb.2006.3575
114. Lehiy CJ, Reister-Hendricks LM, Ruder MG, McVey DS, Drolet BS. Physiological and immunological responses to *Culicoides sonorensis* blood-feeding: a murine model. *Parasit Vectors.* (2018) 11:358. doi: 10.1186/s13071-018-2935-0
115. Baylis M, O'Connell L, Mellor PS. Rates of bluetongue virus transmission between *Culicoides sonorensis* and sheep. *Med Vet Entomol.* (2008) 22:228–37. doi: 10.1111/j.1365-2915.2008.00732.x
116. MacLachlan NJ, Drew CP, Darpel KE, Worwa G. The pathology and pathogenesis of bluetongue. *J Comp Pathol.* (2009) 141:1–6. doi: 10.1016/j.jcpa.2009.04.003
117. Barratt-Boyes SM, MacLachlan NJ. Pathogenesis of bluetongue virus infection of cattle. *J Am Vet Med Assoc.* (1995) 206:1322–9.
118. Bonneau KR, DeMaula CD, Mullens BA, MacLachlan NJ. Duration of viraemia infectious to *Culicoides sonorensis* in bluetongue virus-infected cattle and sheep. *Vet Microbiol.* (2002) 88:115–25. doi: 10.1016/S0378-1135(02)00106-2
119. DeMaula CD, Leutenegger CM, Bonneau KR, MacLachlan NJ. The role of endothelial cell-derived inflammatory and vasoactive mediators in the pathogenesis of bluetongue. *Virology.* (2002) 296:330–7. doi: 10.1006/viro.2002.1476
120. De Clercq K, De Leeuw I, Verheyden B, Vandemeulebroucke E, Vanbinst T, Herr C, et al. Transplacental infection and apparently immunotolerance induced by a wild-type bluetongue virus serotype 8 natural infection. *Transbound Emerg Dis.* (2008) 55:352–9. doi: 10.1111/j.1865-1682.2008.01044.x
121. Elbers ARW, Backx A, Meroc E, Gerbier G, Staubach C, Hendrickx G, et al. Field observations during the bluetongue serotype 8 epidemic in 2006: I. Detection of first outbreaks and clinical signs in sheep and cattle in Belgium, France and the Netherlands. *Prev Vet Med.* (2008) 87:21–30. doi: 10.1016/j.prevetmed.2008.06.004
122. Miller MM, Brown J, Cornish T, Johnson G, Mecham JO, Reeves WK, et al. Investigation of a bluetongue disease epizootic caused by bluetongue virus serotype 17 in sheep in Wyoming. *J Am Vet Med Assoc.* (2010) 237:955–9. doi: 10.2460/javma.237.8.955
123. MacLachlan NJ, James MacLachlan N, Henderson C, Schwartz-Cornil I, Zientara S. The immune response of ruminant livestock to bluetongue virus: from type I interferon to antibody. *Virus Res.* (2014) 182:71–7. doi: 10.1016/j.virusres.2013.09.040
124. Cowley JA, Gorman BM. Effects of proteolytic enzymes on the infectivity, haemagglutinating activity and protein composition of bluetongue virus type 20. *Vet Microbiol.* (1990) 22:137–52. doi: 10.1016/0378-1135(90)90101-Z
125. Dungu B, Gerdes T, Smit T. The use of vaccination in the control of bluetongue in southern Africa. *Vet Ital.* (2004) 40:616–22.
126. Rossitto PV, MacLachlan NJ. Neutralizing epitopes of the serotypes of bluetongue virus present in the United States. *J Gen Virol.* (1992) 73:1947–52. doi: 10.1099/0022-1317-73-8-1947
127. Eschbaumer M, Eschweiler J, Hoffmann B. Long-term persistence of neutralising antibodies against bluetongue virus serotype 8 in naturally infected cattle. *Vaccine.* (2012) 30:7142–3. doi: 10.1016/j.vaccine.2012.08.030

128. MacLachlan NJ. The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comp Immunol Microbiol Infect Dis.* (1994) 17:197–206. doi: 10.1016/0147-9571(94)90043-4
129. Hourrigan JL, Klingsporn AL. Certification of ruminants, semen, and ova for freedom from bluetongue virus. *Aust Vet J.* (1975) 51:211–2. doi: 10.1111/j.1751-0813.1975.tb00058.x
130. Richards RG, MacLachlan NJ, Heidner HW, Fuller FJ. Comparison of virologic and serologic responses of lambs and calves infected with bluetongue virus serotype 10. *Vet Microbiol.* (1988) 18:233–42. doi: 10.1016/0378-1135(88)90090-9
131. Tessaro SV, Clavijo A. Duration of bluetongue viremia in experimentally infected American bison. *J Wildl Dis.* (2001) 37:722–9. doi: 10.7589/0090-3558-37.4.722
132. Schnettler E, Ratniner M, Watson M, Shaw AE, McFarlane M, Varela M, et al. RNA interference targets arbovirus replication in *Culicoides* cells. *J Virol.* (2013) 87:2441–54. doi: 10.1128/JVI.02848-12
133. Mills MK, Michel K, Pfannenstiel RS, Ruder MG, Veronesi E, Nayduch D. *Culicoides*-virus interactions: infection barriers and possible factors underlying vector competence. *Curr Opin Insect Sci.* (2017) 22:7–15. doi: 10.1016/j.cois.2017.05.003
134. Chaney RW. Early tertiary ecotones in western north America. *Proc Natl Acad Sci U S A.* (1949) 35:356–9. doi: 10.1073/pnas.35.7.356
135. Durand B, Zanella G, Biteau-Coroller F, Locatelli C, Baurier F, Simon C, et al. Anatomy of bluetongue virus serotype 8 epizootic wave, France, 2007–2008. *Emerg Infect Dis.* (2010) 16:1861–8. doi: 10.3201/eid1612.100412
136. Gerry AC, Mullens BA, James MacLachlan N, Mecham JO. Seasonal transmission of bluetongue virus by *Culicoides sonorensis* (Diptera: Ceratopogonidae) at a Southern California dairy and evaluation of vectorial capacity as a predictor of bluetongue virus transmission. *J Med Entomol.* (2001) 38:197–209. doi: 10.1603/0022-2585-38.2.197
137. Reisen WK, Boreham PFL. Estimates of malaria vectorial capacity for anopheles culicifacies and anopheles stephensi in rural Punjab Province, Pakistan. *J Med Entomol.* (1982) 19:98–103. doi: 10.1093/jmedent/19.1.98
138. Mullens BA, Gerry AC, Lysyk TJ, Schmidtman ET. Environmental effects on vector competence and virogenesis of bluetongue virus in *Culicoides*: interpreting laboratory data in a field context. *Vet Ital.* (2004) 40:160–6.
139. Foster NM, Jones RH, McCrory BR. Preliminary investigations on insect transmission of bluetongue virus in sheep. *Am J Vet Res.* (1963) 24:1195–200.
140. Mullens BA. A Quantitative Survey of *Culicoides variipennis* (Diptera: Ceratopogonidae) in dairy waste water ponds in Southern California. *J Med Entomol.* (1989) 26:559–65. doi: 10.1093/jmedent/26.6.559
141. Mullens BA, Holbrook FR. Temperature effects on the gonotrophic cycle of *Culicoides variipennis* (Diptera: Ceratopogonidae). *J Am Mosq Control Assoc.* (1991) 7:588–91.
142. Lysyk TJ, Danyk T. Effect of temperature on life history parameters of adult *Culicoides sonorensis* (Diptera: Ceratopogonidae) in relation to geographic origin and vectorial capacity for bluetongue virus. *J Med Entomol.* (2007) 44:741–51. doi: 10.1603/0022-2585(2007)44[741:EOTOLH]2.0.CO;2
143. Wellby MP, Baylis M, Rawlings P, Mellor PS. Effect of temperature on survival and rate of virogenesis of African horse sickness virus in *Culicoides variipennis* sonorensis (Diptera: Ceratopogonidae) and its significance in relation to the epidemiology of the disease. *B Entomol Res.* (1996) 86:715–20. doi: 10.1017/S0007485300039237
144. Mullens BA, Rutz DA. Age structure and survivorship of *Culicoides Variipennis* (Diptera: Ceratopogonidae) in central New York State, USA. *J Med Entomol.* (1984) 21:194–203. doi: 10.1093/jmedent/21.2.194
145. Vaughan JA, Turner EC Jr. Development of immature *Culicoides variipennis* (Diptera: Ceratopogonidae) from Saltville, Virginia, at constant laboratory temperatures. *J Med Entomol.* (1987) 24:390–5. doi: 10.1093/jmedent/24.3.390
146. Alto BW, Reiskind MH, Lounibos LP. Size alters susceptibility of vectors to dengue virus infection and dissemination. *Am J Trop Med Hyg.* (2008) 79:688–95. doi: 10.4269/ajtmh.2008.79.688
147. Conte A, Goffredo M, Ippoliti C, Meiswinkel R. Influence of biotic and abiotic factors on the distribution and abundance of *Culicoides imicola* and the obsoletus complex in Italy. *Vet Parasitol.* (2007) 150:333–44. doi: 10.1016/j.vetpar.2007.09.021
148. Schmidtman ET, Bobian RJ, Belden RP. Soil chemistries define aquatic habitats with immature populations of the *Culicoides variipennis* complex (Diptera: Ceratopogonidae). *J Med Entomol.* (2000) 37:58–64. doi: 10.1603/0022-2585-37.1.58
149. Mullens BA, Lip K-S. Larval population dynamics of *Culicoides variipennis* (Diptera: Ceratopogonidae) in Southern California. *J Med Entomol.* (1987) 24:566–74. doi: 10.1093/jmedent/24.5.566
150. Amar A, Arroyo B, Meek E, Redpath S, Riley H. Influence of habitat on breeding performance of Hen Harriers *Circus cyaneus* in Orkney. *Ibis.* (2007) 150:400–4. doi: 10.1111/j.1474-919X.2007.00765.x
151. Mullen GR, Durden LA. *Medical Veterinary Entomology*. 2nd ed. London, UK: Academic Press (2009).
152. Erram D, Blosser EM, Burkett-Cadena N. Habitat associations of *Culicoides* species (Diptera: Ceratopogonidae) abundant on a commercial cervid farm in Florida, USA. *Parasit Vectors.* (2019) 12:367. doi: 10.1186/s13071-019-3626-1
153. Mullens BA, Tabachnick WJ, Holbrook FR, Thompson LH. Effects of temperature on virogenesis of bluetongue virus serotype 11 in *Culicoides variipennis* sonorensis. *Med Vet Entomol.* (1995) 9:71–6. doi: 10.1111/j.1365-2915.1995.tb00119.x
154. Loomis EC, Bushnell RB, Osburn BI. Epidemiologic study of bluetongue virus on the Tejon Ranch, California: vector, host, virus relationships. *Prog Clin Biol Res.* (1985) 178:583–8.
155. Paweska JT, Venter GJ, Mellor PS. Vector competence of South African *Culicoides* species for bluetongue virus serotype 1 (BTV-1) with special reference to the effect of temperature on the rate of virus replication in *C. imicola* and *C. bolitinos*. *Med Vet Entomol.* (2002) 16:10–21. doi: 10.1046/j.1365-2915.2002.00334.x
156. Mellor PS, Rawlings P, Baylis M, Wellby MP. Effect of temperature on African horse sickness virus infection in *Culicoides*. *Arch Virol Suppl.* (1998) 155–63. doi: 10.1007/978-3-7091-6823-3_15
157. Wittmann EJ, Baylis M. Climate change: effects on culicoides-transmitted viruses and implications for the UK. *Vet J.* (2000) 160:107–17. doi: 10.1053/tvj.2000.0470
158. Rokicki J. Ecology of ectoparasite vectors. *Wiad Parazytol.* (1999) 45:551.
159. Giovannini A, Paladini C, Calistri P, Conte A, Colangeli P, Santucci U, et al. Surveillance system of bluetongue in Italy. *Vet Ital.* (2004) 40:369–84.
160. Green AL, Dargatz DA, Schmidtman ET, Herrero MV, Seitzinger AH, Ostlund EN, et al. Risk factors associated with herd-level exposure of cattle in Nebraska, North Dakota, and South Dakota to bluetongue virus. *Am J Vet Res.* (2005) 66:853–60. doi: 10.2460/ajvr.2005.66.853
161. Foley DH, Klein TA, Kim HC, Sames WJ, Wilkerson RC, Rueda LM. Geographic distribution and ecology of potential malaria vectors in the Republic of Korea. *J Med Entomol.* (2009) 46:680–92. doi: 10.1603/033.046.0336
162. Pfannenstiel RS, Ruder MG. Colonization of bison (*Bison bison*) wallows in a tallgrass prairie by *Culicoides* spp (Diptera: Ceratopogonidae). *J Vector Ecol.* (2015) 40:187–90. doi: 10.1111/jvec.12150
163. Tuyá F, Vanderklift MA, Wernberg T, Thomsen MS. Gradients in the number of species at reef-seagrass ecotones explained by gradients in abundance. *PLoS ONE.* (2011) 6:e20190. doi: 10.1371/journal.pone.0020190
164. Despommier D, Ellis BR, Wilcox BA. The role of ecotones in emerging infectious diseases. *Ecohealth.* (2006) 3:281–89. doi: 10.1007/s10393-006-0063-3
165. Acevedo P, Ruiz-Fons F, Estrada R, Márquez AL, Miranda MA, Gortázar C, et al. A broad assessment of factors determining *Culicoides imicola* abundance: modelling the present and forecasting its future in climate change scenarios. *PLoS ONE.* (2010) 5:e14236. doi: 10.1371/journal.pone.0014236
166. Napp S, Allepuz A, García-Bocanegra I, Alba A, Vilar MJ, Casal J. Quantitative assessment of the probability of bluetongue virus transmission by bovine semen and effectiveness of preventive measures. *Theriogenology.* (2011) 75:920–32. doi: 10.1016/j.theriogenology.2010.10.035
167. Eisen L, Coleman M, Lozano-Fuentes S, McEachen N, Orlans M, Coleman M. Multi-disease data management system platform for vector-borne diseases. *PLoS Neglect Trop D.* (2011) 5:e1016. doi: 10.1371/journal.pntd.0001016

168. Lothrop HD, Lothrop B, Reisen WK. Nocturnal microhabitat distribution of adult *Culex tarsalis* (Diptera: Culicidae) impacts control effectiveness. *J Med Entomol.* (2002) 39:574–82. doi: 10.1603/0022-2585-39.4.574
169. Massad E, Coutinho FAB, Burattini MN, Amaku M. Estimation of R0 from the initial phase of an outbreak of a vector-borne infection. *Trop Med Int Health.* (2009) 15:120–6. doi: 10.1111/j.1365-3156.2009.02413.x
170. Massad E, Coutinho FAB, Lopez LF, da Silva DR. Modeling the impact of global warming on vector-borne infections. *Phys Life Rev.* (2011) 8:169–99. doi: 10.1016/j.plrev.2011.01.001
171. Mayo C, Shelley C, MacLachlan NJ, Gardner I, Hartley D, Barker C. A deterministic model to quantify risk and guide mitigation strategies to reduce bluetongue virus transmission in California dairy cattle. *PLoS ONE.* (2016) 11:e0165806. doi: 10.1371/journal.pone.0165806
172. Baylis M, Caminade C, Turner J, Jones AE. The role of climate change in a developing threat: the case of bluetongue in Europe. *Rev Sci Tech.* (2017) 36:467–78. doi: 10.20506/rst.36.2.2667
173. Kirkland PD. Bluetongue viruses, vectors and surveillance in Australia - the current situation and unique features. *Vet Ital.* (2004) 40:47–50.
174. Monteiro DMS, Souza Monteiro DM, Roman Carrasco L, Joe Moffitt L, Cook AJC. Robust surveillance of animal diseases: an application to the detection of bluetongue disease. *Prev Vet Med.* (2012) 105:17–24. doi: 10.1016/j.prevetmed.2012.01.011
175. Welfare EP on AHA, EFSA Panel on Animal Health and Welfare. Scientific opinion on bluetongue monitoring and surveillance. *EFSA J.* (2011) 9:2192. doi: 10.2903/j.efsa.2011.2192
176. Boyer TC, Ward MP, Wallace RL, Singer RS. Regional seroprevalence of bluetongue virus in cattle in Illinois and western Indiana. *Am J Vet Res.* (2007) 68:1212–9. doi: 10.2460/ajvr.68.11.1212
177. Reuter JD, Nelson SL. Hematologic parameters and viral status for zika, chikungunya, bluetongue, and epizootic hemorrhagic disease in white-tailed deer (*Odocoileus virginianus*) on St. John, US Virgin Islands. *J Wildl Dis.* (2018) 54:843–7. doi: 10.7589/2017-12-315
178. Racloz V, Straver R, Kuhn M, Thur B, Vanzetti T, Stärk KDC, et al. Establishment of an early warning system against Bluetongue virus in Switzerland. *Schweizer Archiv für Tierheilkunde.* (2006) 148:593–8. doi: 10.1024/0036-7281.148.11.593
179. Carpenter S. Culicoides and the spread of bluetongue: finding the needle in the haystack. *Vet Rec.* (2011) 168:238–9. doi: 10.1136/vr.d1385
180. Meiswinkel R, Goffredo M, Leijts P, Conte A. The Culicoides “snapshot”: a novel approach used to assess vector densities widely and rapidly during the 2006 outbreak of bluetongue (BT) in the Netherlands. *Prev Vet Med.* (2008) 87:98–118. doi: 10.1016/j.prevetmed.2008.06.013
181. Gerry AC, Sarto i Monteys V, Moreno Vidal JO, Francino O, Mullens BA. Biting rates of *Culicoides* midges (Diptera: Ceratopogonidae) on sheep in northeastern Spain in relation to midge capture using UV light and carbon dioxide-baited traps. *J Med Entomol.* (2009) 46:615–24. doi: 10.1603/033.046.0329
182. McDermott EG, Mayo CE, Gerry AC, Mullens BA. Trap placement and attractant choice affect capture and create sex and parity biases in collections of the biting midge, *Culicoides sonorensis*. *Med Vet Entomol.* (2016) 30:293–300. doi: 10.1111/mve.12177
183. Viennet E, Garros C, Lancelot R, Allène X, Gardès L, Rakotoarivony I, et al. Assessment of vector/host contact: comparison of animal-baited traps and UV-light/suction trap for collecting Culicoides biting midges (Diptera: Ceratopogonidae), vectors of Orbiviruses. *Parasit Vectors.* (2011) 4:119. doi: 10.1186/1756-3305-4-119
184. Mayo CE, Mullens BA, Gerry AC, Barker CM, Mertens PC, Maan S, et al. The combination of abundance and infection rates of *Culicoides sonorensis* estimates risk of subsequent bluetongue virus infection of sentinel cattle on California dairy farms. *Vet Parasitol.* (2012) 187:295–301. doi: 10.1016/j.vetpar.2012.01.004
185. McGregor BL, Stenn T, Saylor KA, Blosser EM, Blackburn JK, Wisely SM, et al. Host use patterns of Culicoides spp. biting midges at a big game preserve in Florida USA, and implications for the transmission of orbiviruses. *Med Vet Entomol.* (2019) 33:110–20. doi: 10.1111/mve.12331
186. Dyce AL, Marshall BD. An early record of Culicoides species (Diptera: Ceratopogonidae) developing in the dung of game animals in southern Africa. *Onderstepoort J Vet Res.* (1989) 56:85–6.
187. Akey DH, Luedke AJ, Jones RH. Salivary gland homogenates from the vector *Culicoides variipennis* may aid in detection of bluetongue virus in chronically infected cattle. *Prog Clin Biol Res.* (1985) 178:135–45.
188. Akey DH, Potter HW. Pigmentation associated with oogenesis in the biting fly *Culicoides variipennis* (Diptera: Ceratopogonidae): determination of parity. *J Med Entomol.* (1979) 16:67–70. doi: 10.1093/jmedent/16.1.67
189. McDermott EG, Mayo CE, Gerry AC, Laudier D, MacLachlan NJ, Mullens BA. Bluetongue virus infection creates light averse Culicoides vectors and serious errors in transmission risk estimates. *Parasit Vectors.* (2015) 8:460. doi: 10.1186/s13071-015-1062-4
190. Nayduch D, Shankar V, Mills MK, Robl T, Drolet BS, Ruder MG, et al. Transcriptome response of female *Culicoides sonorensis* biting midges (Diptera: Ceratopogonidae) to early infection with epizootic hemorrhagic disease virus (EHDV-2). *Viruses.* (2019) 11:473. doi: 10.3390/v11050473
191. Wilson AJ, Mellor PS. Bluetongue in Europe: past, present and future. *Philos Trans R Soc Lond B Biol Sci.* (2009) 364:2669–81. doi: 10.1098/rstb.2009.0091
192. Sebastiani F, Meiswinkel R, Gomulski LM, Guglielmino CR, Mellor PS, Malacrida AR, et al. Molecular differentiation of the Old World *Culicoides imicola* species complex (Diptera, Ceratopogonidae), inferred using random amplified polymorphic DNA markers. *Mol Ecol.* (2001) 10:1773–86. doi: 10.1046/j.0962-1083.2001.01319.x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Mayo, McDermott, Kopanke, Stenglein, Lee, Mathiason, Carpenter, Reed and Perkins. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



A Duplex Quantitative Real-Time Reverse Transcription-PCR for Simultaneous Detection and Differentiation of Flaviviruses of the Japanese Encephalitis and Ntaya Serocomplexes in Birds

OPEN ACCESS

Edited by:

Michael Kogut,

United States Department of
Agriculture, United States

Reviewed by:

Venkatramana D. Krishna,
University of Minnesota Twin Cities,
United States

Santosh Dhakal,
Johns Hopkins University,
United States

*Correspondence:

Jovita Fernández-Pinero
jpinero@inia.es

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 15 January 2020

Accepted: 27 March 2020

Published: 21 April 2020

Citation:

Elizalde M, Cano-Gómez C, Llorente F, Pérez-Ramírez E, Casades-Martí L, Aguilera-Sepúlveda P, Ruiz-Fons F, Jiménez-Clavero M^A and Fernández-Pinero J (2020) A Duplex Quantitative Real-Time Reverse Transcription-PCR for Simultaneous Detection and Differentiation of Flaviviruses of the Japanese Encephalitis and Ntaya Serocomplexes in Birds. *Front. Vet. Sci.* 7:203. doi: 10.3389/fvets.2020.00203

Maia Elizalde¹, Cristina Cano-Gómez¹, Francisco Llorente¹, Elisa Pérez-Ramírez¹, Laia Casades-Martí², Pilar Aguilera-Sepúlveda¹, Francisco Ruiz-Fons², Miguel Ángel Jiménez-Clavero^{1,3} and Jovita Fernández-Pinero^{1*}

¹ Centro de Investigación en Sanidad Animal (CISA), Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Valdeolmos-Alalpardo, Spain, ² Instituto de Investigación de Recursos Cinegéticos IREC (CSIC-UCLM-JCCM), SaBio Group, Ciudad Real, Spain, ³ Epidemiología y Salud Pública, CIBERESP, Madrid, Spain

High impact, mosquito-borne flaviviruses such as West Nile virus (WNV), Usutu virus (USUV), Japanese encephalitis virus (JEV), Tembusu virus (TMUV), and Bagaza/Israel turkey meningoencephalomyelitis virus (BAGV/ITV) are emerging in different areas of the world. These viruses belong to the Japanese encephalitis (JE) serocomplex (JEV, WNV, and USUV) and the Ntaya serocomplex (TMUV and BAGV/ITV). Notably, they share transmission route (mosquito bite) and reservoir host type (wild birds), and some of them co-circulate in the same areas, infecting overlapping mosquito and avian population. This may simplify epidemiological surveillance, since it allows the detection of different infections targeting the same population, but also represents a challenge, as the diagnostic tools applied need to detect the whole range of flaviviruses surveyed, and correctly differentiate between these closely related pathogens. To this aim, a duplex real-time RT-PCR (dRRT-PCR) method has been developed for the simultaneous and differential detection of JE and Ntaya flavivirus serocomplexes. The method has been standardized and evaluated by analyzing a panel of 49 flaviviral and non-flaviviral isolates, and clinical samples of different bird species obtained from experimental infections or from the field, proving its value for virus detection in apparently healthy or suspicious animals. This new dRRT-PCR technique is a reliable, specific and highly sensitive tool for rapid detection and differentiation of JE and Ntaya flavivirus groups in either domestic or wild animals. This novel method can be implemented in animal virology diagnostic laboratories as screening tool in routine surveillance and in the event of bird encephalitis emergence.

Keywords: flavivirus, Japanese encephalitis serocomplex, Ntaya serocomplex, West Nile virus, diagnostics, duplex real-time RT-PCR

INTRODUCTION

The genus *Flavivirus*, within the family *Flaviviridae*, comprises more than 70 different viruses, many of which represent relevant pathogens for humans and animals (1, 2). Serological affinities allowed their classification into eight antigenic groups or serocomplexes (3). Two of these, namely Japanese encephalitis (JE) and Ntaya groups, comprise a number of pathogenic viruses associated with neurological diseases in different vertebrate species, including domestic species and human beings, being most of them potentially or factually zoonotic (4–7). Clinically relevant viruses within the JE group are, for instance, West Nile virus (WNV), Saint Louis encephalitis virus (SLEV), Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV), and Usutu virus (USUV), while the Ntaya serocomplex includes Bagaza virus (BAGV), its synonymous Turkey meningoencephalomyelitis virus (ITV) (8), and Tembusu virus (TMUV). All these viruses are maintained in nature in a cycle involving avian reservoir hosts and *Culex* spp. mosquitoes. Also, all of them have caused an increasing number of outbreaks over the last years (9–11). In fact, the incidence and geographic spread of these flaviviral infections has risen dramatically worldwide and should be regarded as a threat to animal and human health (12). In Europe and the Mediterranean region, increasing flavivirus activity has been observed in recent times (13). The number of WNV outbreaks has intensely risen since late 1990's (14–16) and USUV has spread widely since its first detection in Austria in 2001 (17–19). In 2010, BAGV emerged in Southern Spain (20) in an area where WNV and USUV were co-circulating in the same avian population (21). Its synonymous virus, ITV, also re-emerged in Israel in the same time period (22). Likewise, in other areas of the world, similar patterns of flavivirus emergence are being observed, particularly involving those belonging to the JE and Ntaya groups (7, 23, 24). Also, the risk of emergence of any of those viruses in distant territories should not be disregarded, as some members of these groups have demonstrated their capacity to undergo transcontinental displacements. Notably, WNV was able to reach the Americas in 1999, probably introduced from the Mediterranean area (25). Similarly, USUV and BAGV were able to reach Europe likely from Sub-Saharan Africa (12, 20, 26).

As the number of flaviviruses circulating in given geographic areas (such as those already mentioned) grows, molecular diagnosis of flaviviral infections relies more and more on generic RT-PCR approaches, which may be particularly advantageous in bird disease diagnostics and surveillance. However, pan-flavivirus PCR methods described so far are focused essentially on public health application or entomological surveillance (27–33), and no PCR-based system is currently available for avian monitoring. Most important bird-pathogenic flaviviruses belong to the above-mentioned JE and Ntaya serocomplexes. The generic detection of viral species of both serocomplexes in a single test would potentially provide more accurate and rapid diagnostic results in monitoring programs, where high-sensitive methods are demanded for large screening. This study describes the development and standardization of a quantitative duplex real-time RT-PCR (dRRT-PCR) method for the simultaneous

detection and differentiation of flaviviruses from the JE and Ntaya serocomplexes, to be used as a screening tool in routine avian surveillance and in the event of bird encephalitis outbreaks.

MATERIALS AND METHODS

Viruses

A collection of 49 different viral isolates was used for the development and standardization of the dRRT-PCR assay (Table 1). Briefly, a flavivirus panel composed of 27 isolates from JE serocomplex, 7 isolates from Ntaya serocomplex and 5 reference strains of other flavivirus species was employed. When needed, viral isolates were propagated and titrated by cell culture standard techniques. All flavivirus isolates used in the study belong to the virus collection held in reserve at INIA-CISA, Valdeolmos, Spain, and were originally obtained from different providers or collaborators as described in Table 1.

Additionally, a set of 10 avian or equine non-flavivirus isolates were analyzed in the specificity studies, namely: Avian influenza virus (AIV, subtypes H5N2, H7N1, H1N1), Newcastle disease virus (NDV), African horse sickness virus (AHSV, serotype 4), Western equine encephalitis virus (WEEV), Equine herpesvirus-1 (EHV-1), Venezuelan equine encephalitis virus (VEEV), Equine influenza virus (EIV, subtype H3N8), and Vesicular stomatitis virus (VSV, Indiana serotype). AIV isolates were provided by IZSLER (Brescia, Italy); WEEV, EHV-1 and VEEV were obtained from ANSES (Maisons-Alfort, France); NDV, AHSV, EIV, and VSV belong to the virus collection maintained at INIA-CISA (Valdeolmos, Spain).

Experimental and Field Samples

Clinical samples collected from *in vivo* experiments carried out with different bird species (house sparrow, red-legged partridge and gray partridge) in the BSL-3 animal facilities at INIA-CISA (36–38) were used for this particular study. Specifically, a panel of 20 immature feathers, 20 blood samples and 24 tissues (heart, liver, brain, spleen, and kidney) obtained from non-infected control birds, and 2 blood, 2 immature feathers and 20 tissue samples collected at different times post-infection from birds experimentally inoculated with WNV or BAGV were analyzed (Table 2A).

On the other hand, a set of 9 WNV-positive (lineage 1) field samples (5 feathers, 2 swabs, and 2 brain samples) collected from different avian species were included in this study. The samples were obtained from WNV cases occurred in wild birds in Southern Spain, and were provided as WNV PCR positive by the National Reference Laboratory (NRL) for avian diseases, Laboratorio Central de Veterinaria (LCV, Algete, Spain). Seven samples (feather and tissues) obtained from a red-legged partridge found dead during the BAGV outbreaks occurred in Southern Spain in 2010, which was submitted by Estación Biológica de Doñana (EBD-CSIC, Seville, Spain) for diagnostic confirmation, were incorporated to this study (Table 2B).

In addition, a panel of 25 feathers and tissue samples (heart, liver, brain, lung, spleen and kidney), obtained from 4 individuals (2 little owls, 1 goshawk, and 1 crow) showing neurological

TABLE 1 | Flavivirus isolates used in this study and results obtained by the dRRT-PCR and the RT-PCR methods used as reference.

Flavivirus sero complex	Virus	Isolate (lineage)	Geographical origin	Species of origin	Year of isolation	Source	Duplex RRT-PCR		Reference PCR
							Fam (JE)	Joe (Ntaya)	
Japanese encephalitis (JE)	WNV	E-101 (1a)	Egypt	Human	1951	Institut Pasteur Lyon (France)	21.33	No Ct	22.8 ^a
		IS-98 STD (1a)	Israel	White stork	1998	ANSES (France)	29.55	No Ct	31.2 ^a
		NY99 034EDV (1a)	New York	American crow	1999	Diagnostic Virology Laboratory USDA Ames (USA)	17.34	No Ct	17.02 ^a
		04.05 (1a)	Morocco	Horse	2003	Biopharma Rabat (Morocco)	18.26	No Ct	19.57 ^a
		GE-1b/B (1a)	Spain	Golden eagle	2007	INIA-CISA Madrid (Spain)	18	No Ct	19.73 ^a
		15803 (1a)	Italy	Magpie	2008	IZSLER Brescia (Italy)	23.48	No Ct	26.61 ^a
		225677/2009 (1a)	Italy	Jay	2009	IZSLER Brescia (Italy)	24.55	No Ct	25.46 ^a
		233184/4/2009 (1a)	Italy	Black headed gull	2009	IZSLER Brescia (Italy)	24.19	No Ct	26.87 ^a
		Spain/2010/H-1b (1a)	Spain	Horse	2010	INIA-CISA Madrid (Spain)	20.74	No Ct	20.98 ^a
		Spain/2012 (1a)	Spain	Horse	2012	LCV Algete (Spain)	19.35	No Ct	20.57 ^a
		Kunjin KJ359-11 (1b)	Australia	Horse	1984	ISCIII Madrid (Spain)	29.4	No Ct	33.67 ^a
		B956 (2)	Uganda	Human	1937	ATCC (USA)	16.53	No Ct	17.07 ^a
		Austria/2008 (2)	Austria	Goshawk	2008	VetMedUni Vienna (Austria)	26.48	No Ct	31.8 ^a
		WN/Horse/RSA/1/08 (2)	South Africa	Horse	2008	Onderstepoort Veterinary Institute Pretoria (South Africa)	31.48	No Ct	35.22 ^a
		178907/2013 (2)	Italy	<i>Culex pipiens</i>	2013	IZSLER Brescia (Italy)	16.72	No Ct	19.32 ^a
		Rabensburg 97-103 (3)	Czech Republic	<i>Culex pipiens</i>	1997	ISCIII Madrid (Spain)	18.78	No Ct	Pos ^c
		MP502-66 (putative 6)	Malaysia	<i>Culex pseudovishnui</i>	1966	ISCIII Madrid (Spain)	25.86	No Ct	Pos ^c
		Koutango ArD96655 (putative 7)	Senegal	<i>Rhipicephalus guihoni</i>	1993	Institute Pasteur Dakar (Senegal)	16.42	No Ct	Pos ^c
	USUV	SAAR 1776/1958	South Africa	<i>Culex neavei</i>	1958	ANSES (France)	24.77	No Ct	28.31 ^a
		939	Austria	Blackbird	2001	ISCIII Madrid (Spain)	19.9	No Ct	22.25 ^a

(Continued)

TABLE 1 | Continued

Flavivirus sero complex	Virus	Isolate (lineage)	Geographical origin	Species of origin	Year of isolation	Source	Duplex RRT-PCR		Reference PCR
							Fam (JE)	Joe (Ntaya)	
Ntaya	JEV	231247/2011	Italy	Blackbird	2011	IZSLER Brescia (Italy)	22.19	No Ct	25.37 ^a
		206795-3/2012	Italy	<i>Culex pipiens</i>	2012	IZSLER Brescia (Italy)	24.77	No Ct	28.31 ^a
		229615-1/2013	Italy	<i>Culex pipiens</i>	2013	IZSLER Brescia (Italy)	23.3	No Ct	28.11 ^a
		HautRhin7315/France/2015	France	Blackbird	2015	ANSES (France)	25.39	No Ct	28.27 ^a
		Nakayama	Japan	–	1935	ISCIII Madrid (Spain)	23.9	No Ct	Pos ^c
		MVEV	–	–	1951	ISCIII Madrid (Spain)	26.91	No Ct	Pos ^c
		SLEV	–	–	–	ISCIII Madrid (Spain)	38	No Ct	Neg ^c
		BAGV	Spain H/2010	Spain	2010	INIA-CISA Madrid (Spain)	No Ct	25.46	24.71 ^b
		ITV	Vaccine strain	Israel	1985	Kimron Veterinary Institute (Israel)	No Ct	27.73	26.74 ^b
		618	Israel	Domestic turkey	1995	Kimron Veterinary Institute (Israel)	No Ct	26.34	24.53 ^b
		107458	Israel	Domestic turkey	2010	Kimron Veterinary Institute (Israel)	No Ct	19.19	17.61 ^b
		106819	Israel	Domestic turkey	2010	Kimron Veterinary Institute (Israel)	No Ct	29.11	27.68 ^b
		105520	Israel	Domestic turkey	2010	Kimron Veterinary Institute (Israel)	No Ct	30.69	27.96 ^b
Other flaviviruses	TMUV	UVE/TMUV/1955/MY/MM1775	Malaysia	<i>Culex tritaeniorhynchus</i>	1955	European Virus Archive (EVAg)	No Ct	19.85	Pos ^c
	TBEV	Neudorfl	–	–	–	ISCIII Madrid (Spain)	No Ct	No Ct	Pos ^c
		Hypv	–	–	–	ANSES (France)	No Ct	No Ct	Pos ^c
	LIV	–	–	–	–	ISCIII Madrid (Spain)	No Ct	No Ct	Pos ^c
	ZIKV	–	–	–	–	ISCIII Madrid (Spain)	No Ct	No Ct	Pos ^c
	DENV	Hawaii	–	–	–	ISCIII Madrid (Spain)	No Ct	No Ct	Pos ^c

TBEV, Tick-borne encephalitis virus; LIV, Louping-ill virus; ZIKV, Zika virus; DENV, Dengue virus.

^aDel Amo et al. (34). ^bBuitrago et al. (35). ^cScaramozzino et al. (27).

TABLE 2A | Clinical samples used in this study and results obtained by the dRRT-PCR and the RT-PCR methods used as reference; experimental samples from *in vivo* experiments performed at INIA-CISA.

Virus	Species	Animal Id	Type of Sample	DPI*	DUPLIX RRT-PCR		WNV-1/WNV-2/USUV RRT-PCR ^a			BAGV RRT-PCR ^b (Ct)
					JE (Ct)	NTAYA (Ct)	WNV-1 (Ct)	WNV-2 (Ct)	USUV (Ct)	
WNV L1 (GE-1b/B Spain 2007)	House sparrow	11	Brain	7	28.44	No Ct	31.25	No Ct	No Ct	
			Kidney		27.37	No Ct	29.15	No Ct	No Ct	
			Spleen		24.01	No Ct	25.68	No Ct	No Ct	
			Heart		26.85	No Ct	28.53	No Ct	No Ct	
			Liver		24.78	No Ct	27.15	No Ct	No Ct	
WNV L2 (Austria/2008)	Red-legged partridge	7	Brain	7	28.41	No Ct	No Ct	32.45	No Ct	
			Kidney		26.43	No Ct	No Ct	31.20	No Ct	
			Spleen		27.6	No Ct	No Ct	35.19	No Ct	
			Heart		24.48	No Ct	No Ct	30.89	No Ct	
			Liver		36.75	No Ct	No Ct	39.03	No Ct	
			Blood		35.84	No Ct	No Ct	No Ct	No Ct	
			Feather		23.38	No Ct	No Ct	30.01	No Ct	
BAGV (Spain H/2010)	Red-legged partridge	4	Brain	6	No Ct	31.19				29.54
			Kidney		No Ct	26.17				25.81
			Spleen		No Ct	24.31				25.69
			Heart		No Ct	23.47				25.81
			Liver		No Ct	26.05				26.84
	Grey partridge	4	Brain	4	No Ct	35.83				27.11
			Kidney		No Ct	22.97				21.19
			Spleen		No Ct	24.27				19.66
			Heart		No Ct	29.16				20.5
			Liver		No Ct	26.37				22.45
			Blood		No Ct	24.95				20.81
			Feather		No Ct	23.28				19.98

*DPI: days-post-infection. ^aDel Amo et al. (34). ^bBuitrago et al. (35).

disorders were also incorporated in the study. These samples remained undiagnosed and were submitted by the Veterinary Faculty of Universidad de Extremadura (UEX, Cáceres, Spain) for diagnostic investigation (**Table 2B**).

Finally, the dRRT-PCR technique was transferred to IREC (Ciudad Real, Spain) and applied in a surveillance study of wild birds in Castilla-La Mancha (Spain). Briefly, a total of 237 birds were sampled, belonging to the following families: *Alcedinidae* ($n = 1$), *Certhiidae* ($n = 6$), *Corvidae* ($n = 20$), *Emberizidae* ($n = 15$), *Fringillidae* ($n = 10$), *Hirundinidae* ($n = 13$), *Laniidae* ($n = 8$), *Muscicapidae* ($n = 1$), *Oriolidae* ($n = 1$), *Paridae* ($n = 61$), *Passeridae* ($n = 36$), *Prunellidae* ($n = 1$), *Sylviidae* ($n = 27$), and *Turdidae* ($n = 36$). As far as possible, blood, immature feathers and oral and cloacal swabs were collected; the feather being the sample tested, when available, in the PCR screening.

Nucleic Acid Extraction

Total RNA was extracted at INIA-CISA (Valdeolmos, Spain) from 200 μ l of sample (virus suspension, blood, feathers, swabs and tissue homogenates 10% in PBS) using the automated BioSprint 15 workstation and the BioSprint DNA blood kit (Qiagen, Valencia, CA), according to manufacturer's instructions with small modifications (carrier RNA was added to AL buffer for

a final concentration of 5 μ g/ml). Finally, RNA was recovered in 100 μ l of nuclease-free water.

The Macherey-Nagel NucleoSpin TriPrep kit (Fisher Scientific, Leicestershire, UK) was used for RNA extraction of field samples at IREC (Ciudad Real, Spain), following the protocol recommended by the manufacturer, using 200 μ l of sample and recovering RNA in 50 μ l of nuclease-free water.

Duplex Real-Time RT-PCR: Design and Methodology

A comprehensive selection of Ntaya and JE serocomplex viruses' full-length genome sequences available from GenBank was individually aligned using Clustal Omega software (European Bioinformatic Institute, Hinxton, Cambridge CB10 1SD, UK). Two primers and TaqMan probe set, each specific for one serogroup covering all representative virus species were designed, targeting non-structural protein 2A (NS2A) gene and 3' end non-coding region (3'NCR) for JE and Ntaya groups, respectively. Properties of the designed primers and probes were analyzed *in silico* with Primer Express 2.0 (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Probes were labeled with different reporter dyes, FAM for JE serocomplex and JOE for Ntaya serocomplex, to allow a simultaneous, but differential detection of both groups in a single run (**Table 3**).

TABLE 2B | Clinical samples used in this study and results obtained by the dRRT-PCR and the RT-PCR methods used as reference; field samples from wild birds collected in Spain.

Virus	Species	Source/ Animal Id	Type of Sample	DUPLEX RRT-PCR		WNV-1/WNV-2/USUV RRT-PCR ^a			BAGV RRT-PCR ^b (Ct)
				JE (Ct)	NTAYA (Ct)	WNV-1 (Ct)	WNV-2 (Ct)	USUV (Ct)	
WNV L1	Red-legged partridge	LCV/30/11	Brain	28.54	No Ct	31.36	No Ct	No Ct	
		LCV/2496/15	Cloacal swab	38.10	No Ct	33.71	No Ct	No Ct	
		LCV/3179/13	Oral swab	31.60	No Ct	34.83	No Ct	No Ct	
	White stork	LCV/2439/16-1	Feather	36.89	No Ct	No Ct	No Ct	No Ct	
	Black vulture	LCV/2442/16-5	Brain	24.34	No Ct	28.17	No Ct	No Ct	
		LCV/2442/16-4	Feather	28.28	No Ct	30.75	No Ct	No Ct	
	Cinereous vulture	LCV/2442/16-3	Feather	31.66	No Ct	31.49	No Ct	No Ct	
	Duck	LCV/2439/16-11	Feather	27.58	No Ct	30.43	No Ct	No Ct	
	Little owl	LCV/2858/16	Feather	34.10	No Ct	39.15	No Ct	No Ct	
		UEX/10B	Feather	22.34	No Ct	26.36	No Ct	No Ct	
			Brain	24.65	No Ct	25.69	No Ct	No Ct	
			Liver	24.67	No Ct	27.64	No Ct	No Ct	
			Lung	18.79	No Ct	21.54	No Ct	No Ct	
			Heart	23.21	No Ct	26.09	No Ct	No Ct	
			Spleen	34.38	No Ct	38.32	No Ct	No Ct	
		UEX/A	Feather	19.38	No Ct	22.26	No Ct	No Ct	
			Brain	27.62	No Ct	29.25	No Ct	No Ct	
			Liver	25.26	No Ct	28.48	No Ct	No Ct	
			Lung	18.85	No Ct	21.89	No Ct	No Ct	
			Heart	19.70	No Ct	22.96	No Ct	No Ct	
			Spleen	26.77	No Ct	30.64	No Ct	No Ct	
			Kidney	24.21	No Ct	25.51	No Ct	No Ct	
USUV	Common blackbird	IREC/A18/177	Oral swab	35.99	No Ct	No Ct	No Ct	39.37	
			Cloacal swab	No Ct	No Ct	No Ct	No Ct	No Ct	
BAGV	Red-legged partridge	EBD/2010A	Feather	No Ct	24.98				23.26
			Brain	No Ct	28.98				28.62
			Kidney	No Ct	28.86				28.3
			Eye	No Ct	22.72				22.47
			Heart	No Ct	32.93				33.13
			Muscle	No Ct	32				32.08
			Skin	No Ct	29.98				28.34

Only the results of the individuals giving positive result to any JE or Ntaya flavivirus serogroup are shown. ^aDel Amo et al. (34), ^bBuitrago et al. (35).

A BLASTn search of the selected primers and probes sequences against the GenBank database confirmed the specificity to the corresponding JE or Ntaya flavivirus serogroup.

Initially, RRT-PCR assays were optimized individually for JE or Ntaya serocomplex detection under the same reaction conditions, to be afterwards modified into a duplex format using the commercial AgPath-ID one-step RT-PCR kit (Life Technologies, Thermo Fisher Scientific, USA). For the dRRT-PCR, a reaction mix was prepared containing (per vial) 3 µl of RNA template, 0.75 µM of each primer and 0.2 µM of each probe (Table 3), 0.8 µl of 25X RT-PCR enzyme

mix (containing ArrayScript™ Reverse Transcriptase and AmpliTaq Gold® DNA Polymerase) and 10 µl of 2X RT-PCR Buffer (includes ROX™ passive reference dye for quantitative fluorescent signal normalization), and nuclease-free water to reach a reaction volume of 20 µl. Reaction mixes for individual RRT-PCR assays were prepared with same reagents, volumes and concentrations described for the dRRT-PCR, except only primers/probe for the target flaviviral serocomplex were added. All reactions were carried out using Mx3005P equipment and software (Stratagene Inc., La Jolla, CA, USA) using the following thermal profile: reverse transcription at 48°C

TABLE 3 | List of primers and probes designed for the detection of JE and Ntaya serocomplexes by dRRT-PCR assay.

Primer/Probe name	Sequence and labeling (5'→ 3') ^a	Nucleotide positions	Product size	Target genome region
Japanese encephalitis serocomplex				
JE-3533F	TCAGYTGGGCCTTCTGGT	3,533–3,550 ^b	65 bp	NS2A
JE-3597R	TGGCCGTCCACCTCTTSC	3,597–3,580 ^b		
JE-FAM-3553	6FAM-TGTTYTTGGCCACCCAGGAGGT -BHQ1	3,553–3,574 ^b		
Ntaya Serocomplex				
NT-10510F	GTTGGATGACGGTGCWGYCTG	10,510–10,530 ^c	171 bp	3' NTR
NT-10680R	GCTGCACTGCATGCTARTGGC	10,680–10,660 ^c		
NT-JOE-10594	JOE-TACCGWCTCGGAGARCTCCCTGGC-BHQ1	10,594–10,617 ^c		

Primers and probes were purchased from Roche Applied Science (Spain).

^aCode for mixed bases positions: R:A/G, Y:C/T, S:G/C, and W:A/T.

^bNucleotide positions regarding to the WNV L1 Spain/2010/H-1b strain. GenBank accession no. JF719069.

^cNucleotide positions regarding to the BAGV Spain H/2010 strain. GenBank accession no. HQ644143.

for 20 min, initial PCR activation step at 95°C for 10 min, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. The fluorescence signal emitted by FAM and JOE reporter dyes was measured simultaneously and independently at the end of each cycle. A threshold cycle value (Ct) >40 was set as negative result.

Construction of *in vitro*-Transcribed RNA Standards

Two ssRNA standard controls were produced for analytical sensitivity estimation of the dRRT-PCR. Specifically, WNV-L1 GE-1b/B and BAGV Spain H/2010 strains were used as templates in a PCR using outer primers of the duplex assay. The resulting products were sequenced to ensure the specificity of the amplification. Likewise, the cDNAs were gel-purified with Wizard SV Gel and PCR Clean-Up System (Promega, USA) and quantified by spectrophotometry. Each cDNA was cloned into pGEM-T easy vector system (Promega, USA) and DNA plasmids were further purified using Wizard Plus SV Miniprep DNA purification system (Promega, USA) and finally quantified by spectrophotometry. *In vitro* transcription was performed with Riboprobe[®] *in vitro* Transcription System (Promega, USA) over linearized DNA plasmid, following the manufacturer's instructions. RNA transcripts were then purified using MEGAclear Transcription Clean-Up Kit (Ambion, Thermo Fisher Scientific, USA), treated with 2U of Turbo[™] DNase (Ambion, Thermo Fisher Scientific, USA) and re-purified with TriPure Isolation Reagent (Roche Applied Science, Germany). The produced RNA standards were quantified by ND-1000 NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Mean concentration values were 1.57 E⁺¹¹ and 4.72 E⁺¹¹ RNA copies/μl for WNV-L1 and BAGV *in vitro*-transcribed RNA control preparations, respectively. A series of dilutions were generated for each quantified WNV and BAGV RNA and stored as standard RNA samples for further use in sensitivity assays.

Best-fit lines (standard curves) were calculated by the least squares regression method from the Ct values obtained for the serial dilutions of the two RNA standards produced to determine the dynamic range and detection limit of the dRRT-PCR method.

Reference RT-PCR Methods

Two previously validated techniques, namely a triplex RRT-PCR for WNV (lineages 1 and 2) and USUV simultaneous detection (34) and a single RRT-PCR for BAGV detection (35), were employed as reference methods in comparative assays. For detection of other flaviviruses, a widely used conventional RT-PCR (27) was performed with minor modifications, and amplification products were further sequenced to confirm the virus identity.

RESULTS

Analytical Performance: Dynamic Range, Linearity, Efficiency, and Detection Limit

Initially, the analytical sensitivity of the dRRT-PCR was studied by analyzing duplicates of 10-fold serial dilutions of viral suspensions of WNV-L1 (Spain/2010/H-1b), WNV-L2 (B956), USUV (SAAR 1776/1958), and BAGV (Spain H/2010). All experiments were run in parallel with the equivalent reference RRT-PCR method (34, 35), obtaining a similar (BAGV) or at least 10 times higher (WNV-L1, WNV-L2, USUV) sensitivity with the dRRT-PCR (Supplementary Table 1).

The analytical performance of the dRRT-PCR method was assessed by analyzing the *in vitro*-transcribed RNA standards produced for WNV and BAGV, as above described. Specifically, the dilutions containing a range from 1.57 E⁺⁸ to 1.57 E⁺⁰ RNA copies/μl for WNV-L1 and 1.32 E⁺⁷ to 1.32 E⁺⁰ RNA copies/μl for BAGV were tested in triplicates to construct the standard curves for both JE and Ntaya serocomplexes, respectively. The assays reacted in a dose-dependent manner with each reference RNA standard along a dynamic range of eight and seven 10-fold dilutions for both WNV-L1 and BAGV, respectively. The detection limit of the dRRT-PCR was estimated to be below 50 RNA copies for both JE and Ntaya serogroups. The standard curve produced with the WNV-L1 RNA standard showed a value of correlation coefficient (R) of 0.996 and efficiency rate (E) of 105.1%. For BAGV RNA standard, R was 0.999 and the efficiency of the dRRT-PCR was 99.7% (Figure 1, Supplementary Table 2).

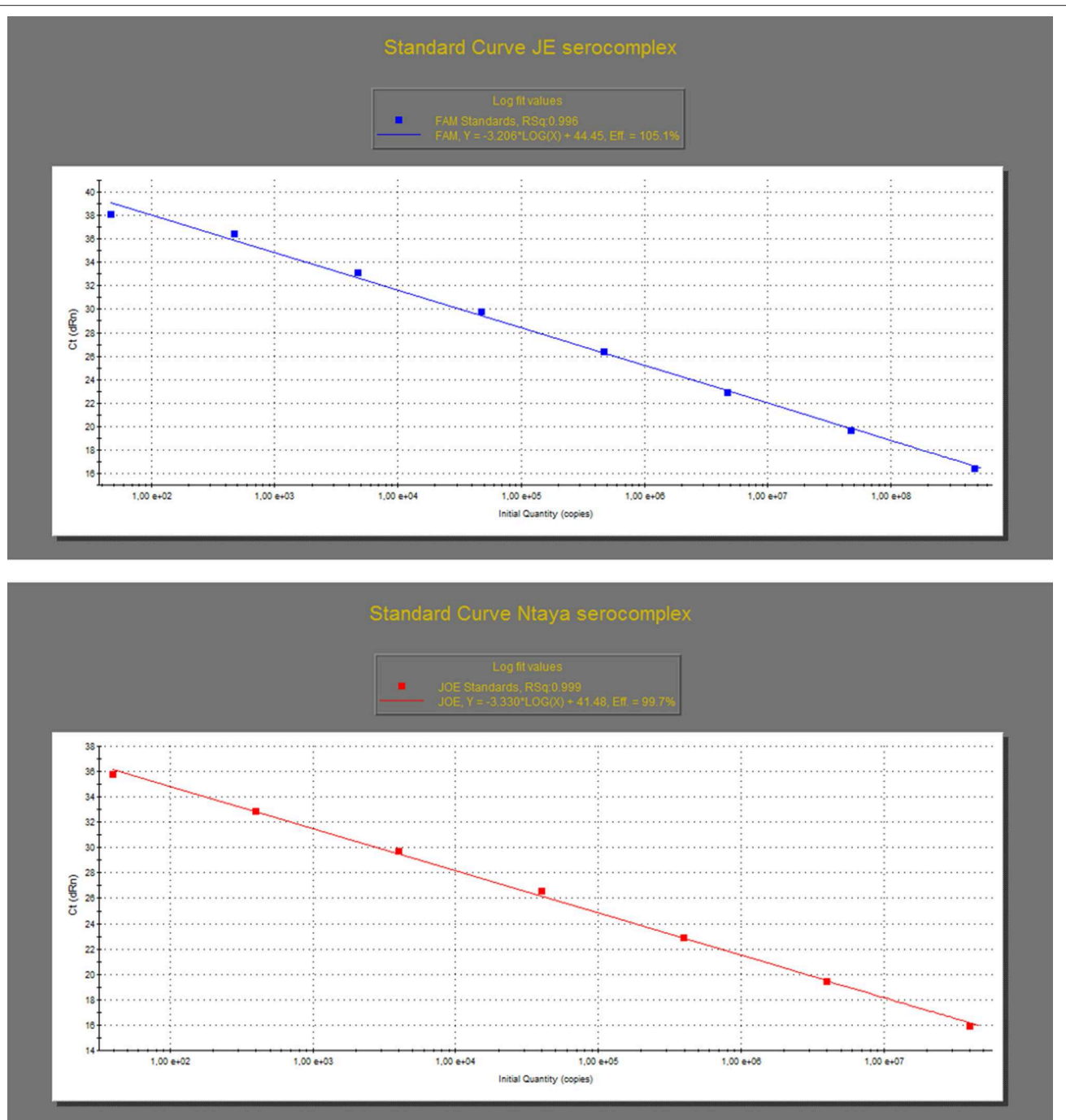


FIGURE 1 | Standard curves of the dRRT-PCR for the detection of JE (top graph) and Ntaya flavivirus serocomplexes (bottom graph). The curves were generated by analysis of triplicates of 10-fold serial dilutions of the quantified *in vitro*-transcribed WNV (top graph) and BAGV (bottom graph) RNA standards produced as synthetic positive controls. Each • corresponds to the mean value of three replicates.

Finally, the capacity of the dRRT-PCR to detect the presence of two target viruses in a single sample was evaluated with a set of WNV+BAGV RNA standards combinations. Specifically, dilutions of both RNA standards representing strong ($Ct < 20$) and weak ($Ct > 33$)

positive samples were mixed in equivalent and disparate proportions. The panel of artificial “co-infected samples” were analyzed in triplicates and in parallel with triplicates of the corresponding “single infected samples.” All preparations were correctly scored and Ct values were

similar for each dilution analyzed pooled or individually (**Supplementary Table 3**).

Specificity Assays and Detection Range

To assess the analytical specificity of the dRRT-PCR, a panel of RNAs from 39 different flavivirus isolates and 10 avian and equine non-flavivirus isolates were analyzed. Fluorescence signals were obtained correctly for the range of JE and Ntaya serocomplex isolates analyzed, being able to clearly differentiate between them without any cross-reactions. The reference RRT-PCR techniques were carried out in parallel for comparison, the dRRT-PCR reporting similar or lower Ct values for the range of WNV-L1, WNV-L2, and USUV isolates tested, while some slightly higher Ct values were obtained for BAGV/ITV isolates (**Table 1**). The specificity of the assay was further proved since no fluorescence signal was reported with any heterologous flavivirus (**Table 1**) and non-flavivirus isolates (data not shown).

Performance With Clinical Samples

The performance of the developed dRRT-PCR method for practical use in diagnosis was assessed initially by analyzing a panel of samples ($n = 24$), including blood, feathers, heart, spleen, liver, kidney, and brain, obtained from house sparrows, red-legged partridges and gray partridges experimentally infected with GE-1b/B Spain 2007 strain of WNV-L1, Austria/2008 strain of WNV-L2, or Spain H/2010 strain of BAGV, at INIA-CISA BSL-3 animal facilities (36–38). All experimental samples were correctly reported by the duplex assay, overall showing similar or lower Ct values for those samples infected with WNV-L1 or WNV-L2 than those obtained using the corresponding reference technique. For samples infected with BAGV, Ct values scored by the dRRT-PCR were generally higher than by the reference RRT-PCR (**Table 2A**). No fluorescence signal was obtained when experimental samples ($n = 64$) from non-infected control birds were tested, confirming the diagnostic specificity of the dRRT-PCR (data not shown).

A first collection of WNV-L1 positive field samples ($n = 9$) provided by the Spanish NRL was subjected to analysis to evaluate the diagnostic sensitivity of the dRRT-PCR. All feathers, swabs and tissues were scored correctly in the JE serocomplex. Running in parallel the reference RRT-PCR, most WNV-L1 samples reported lower Ct values in the dRRT-PCR, and even one feather was missed by the triplex reference method (**Table 2B**). In addition, all samples ($n = 7$) of a red-legged partridge (*Alectoris rufa*) found dead during the outbreaks of BAGV in 2010 were reported as positive for the Ntaya serocomplex by the dRRT-PCR, and were confirmed as BAGV positive by the reference technique. Similar Ct values were obtained for most samples by the two techniques (**Table 2B**).

To evaluate the competence of the dRRT-PCR with undiagnosed field material, a panel of feathers and tissue samples ($n = 25$) of different wild birds (2 little owls, 1 goshawk, and 1 crow), that had died with neurological signs, were examined in parallel by the dRRT-PCR and the two reference RRT-PCR techniques. An infection due to JE serocomplex virus was detected in all samples of the two little owls (*Athene noctua*) by the dRRT-PCR and WNV-L1 was identified by the triplex

reference RRT-PCR, reporting the dRRT-PCR lower Ct values in all positive samples (**Table 2B**). Samples from the other two individuals remained negative by all assays.

Finally, the developed dRRT-PCR technique was implemented as screening tool in a surveillance study carried out in Castilla-La Mancha (Spain) by IREC. Of 237 sampled wild birds, 175 feathers, 58 oral swabs, and 4 cloacal swabs were analyzed. One feather from a common blackbird (*Turdus merula*) was found positive (Ct = 15) for the JE serocomplex. Further sequencing confirmed the infection due to USUV in this bird. Unfortunately, this sample could not be analyzed by the triplex reference RRT-PCR. Alternatively, oral and cloacal swabs of the same blackbird were examined at INIA-CISA by the dRRT-PCR and the WNV-L1/WNV-L2/USUV triplex reference RRT-PCR, obtaining a weak positive signal in the oral swab for JE serocomplex and USUV, respectively (**Table 2B**).

Repeatability Assessment

Positive extraction controls employed throughout this study were used to assess the intra- and inter-assay repeatability of the dRRT-PCR. These were prepared diluting two viral suspensions of WNV-L1 (as positive control for JE serocomplex) and BAGV (as positive control for Ntaya serocomplex) until getting the dilutions to give an expected Ct value of 30 ± 2 for each target. Aliquots of the two WNV and BAGV positive controls were stored at -20°C and further included in each RNA extraction run.

The analysis of 10 WNV and 10 BAGV positive extraction controls in 10 duplex RRT-PCR runs proved the inter-assay repeatability, obtaining mean Ct values of 30.39 and 30.04, with a standard deviation (SD) of 0.59 and 0.43, respectively. Finally, RNAs (stored at -20°C) from the same batch of WNV and BAGV positive extraction controls ($n = 10+10$) were tested in one single dRRT-PCR run to evaluate the intra-assay repeatability, giving a mean Ct value of 30.84 (SD = 0.74) for WNV and of 30.57 (SD = 0.59) for BAGV.

DISCUSSION

The recent emergence of different flaviviruses in wide regions of the world makes co-circulation of these pathogens in same geographic areas more likely (12, 13, 39). Furthermore, there is a range of flavivirus species sharing vectors and hosts, producing similar disease pattern in susceptible animals. This is of current relevance for the Japanese encephalitis (JE) and Ntaya serogroups, which include *Culex*-borne viral species infecting the same bird population, in which they can produce a similar encephalitic disease. The increasing spread and incidence of the viruses belonging to these two groups requires that avian surveillance plans, in countries where they can potentially emerge and circulate, implement methods capable of detecting any of them. These methods could be of particular interest in certain geographic regions such as Southern Spain, Israel and several African countries (e.g., South Africa or Senegal) where WNV, USUV, and BAGV/ITV are present and infect bird and mosquito population (21, 40–42).

Although some pan-flavivirus PCR methods have been described so far, most are focused on public health application or entomological surveillance (27–33), while none of them has been developed for birds. This is significant in the current epidemiological situation, where the recent and potential emergence of bird-pathogenic flaviviruses in different territories poses a complex challenge for the diagnostic laboratories and for the veterinary authorities. On the other hand, the combination of the relevant bird-pathogenic flaviviruses species (those belonging to the JE and Ntaya serocomplexes) in a single assay makes very difficult to develop a PCR test with the high sensitivity level demanded for a screening tool. This was solved, in this study, by designing a duplex RRT-PCR (dRRT-PCR) method for the generic and differential detection of these two target flavivirus serogroups. It is well-known that the design of generic molecular tools may be challenging to cover the range of target pathogens and may limit their sensitivity (43), especially when clinical material is analyzed. However, the new dRRT-PCR has demonstrated to be a specific and highly sensitive tool, capable of detecting the wide range of JE and Ntaya flaviviral species analyzed, and posing a performance similar to the RRT-PCR methods used as reference (34, 35). On the other hand, most PCR methods described for flaviviruses detection have been verified with human or mosquito samples, when available (28, 31–33). In this study, WNV, USUV and BAGV positive samples from a variety of wild bird species were available for the evaluation of the dRRT-PCR. The results analyzing an extensive panel of clinical material from experimental studies and from the field have proved its diagnostic capacity. In this regards, it deserves to point out the usefulness showed by the dRRT-PCR in the detection of viral infection in undiagnosed field samples (WNV in little owls) and in outbreak investigations (BAGV in red-legged partridge). Furthermore, the implementation of the dRRT-PCR technique in a wild bird monitoring study allowed the identification of a common blackbird infected with a JE-serogroup virus that was further confirmed as USUV. This is a relevant finding to support the potential value of this method for its use as a screening tool in routine diagnosis. Therefore, by combining two methods, one for JE and one for Ntaya in one duplex assay it is possible to detect a wide range of bird flaviviruses, including most important ones. In addition, by differentiating these two groups in one single analysis, we obtain a useful information to narrow the range of suspicious agents to analyse in a second, more specific method.

This quantitative method can also be used to estimate viral loads in blood and/or other organs or samples. Albeit not mandatory for diagnostic or surveillance studies, this ability can be used to monitor the clinical course of the infection and to determine if a given species develops enough viremia to act as a competent host (able to transmit the virus to the mosquito vector), which is essential to understand its role in the epidemiology of these flaviviruses.

Finally, the new dRRT-PCR was developed bearing in mind the current situation in some Mediterranean countries, where different flaviviruses of the two target serogroups have emerged and co-circulate in the avian population. However, this method can also be very useful in other geographic regions such as South-East Asia, where viruses belonging to the Ntaya serocomplex, e.g.,

Tembusu and Tembusu-related viruses, are spreading into areas where Japanese encephalitis virus is historically present (23, 44–47). As well, according to the epidemiological scenario, the RRT-PCR can be turned into individual tests for the only detection of JE or Ntaya serogroup providing equal diagnostic performance than the duplex format.

In conclusion, the duplex quantitative real-time RT-PCR described in this study provides a novel tool for the diagnostic and epidemiological surveillance of JE and Ntaya serocomplex flaviviruses, comprising a wide range of arboviral pathogens threatening animal and public health worldwide. This new method allows for the rapid detection and differentiation of these two serocomplexes being especially helpful as screening tool in bird flavivirus surveillance and in the diagnosis of avian encephalitis cases.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by INIA animal experimentation ethics committee and by the authorized organ of the Department of Environment and Land Management of the Community of Madrid.

AUTHOR CONTRIBUTIONS

JF-P conceived and designed the study, analyzed the data, and amended the article. ME participated in the design and development of the method and supervised the technical work. CC-G contributed to the design and development of the method and in the laboratory tests. CC-G, FL, and EP-R participated in sample selection and analysis and contributed to draft the article. PA-S contributed to sample selection and analysis. FR-F and LC-M collected and analyzed most of the field samples. MJ-C participated in the study design, coordination, and drafted the article. All authors read and approved the final manuscript.

FUNDING

This work has been funded by the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA) under the INIA-MAGRAMA agreements EG13-020 and EG17-141, and by the Spanish Ministry for Science, Innovation and Universities (MCIU) under the project E-RTA2015-00002-C02. PA-S and LC-M acknowledge funding from the Spanish Ministry for Science, Innovation and Universities (MCIU) by contract FPI-SGIT-2016-05 and PEJ2018-003155-A, respectively.

ACKNOWLEDGMENTS

The authors acknowledge Ana Robles, Amalia Villalba, and Maria del Carmen Barbero Ameller for their excellent technical

assistance during this work, and Encarnación Madueño for helping during the preparation of *in vitro*-transcribed RNA standards. Montserrat Agüero and Rubén Villalba from the Laboratorio Central de Veterinaria (LCV, Algete, Spain), Eva Frontera and Daniel Bravo from the Veterinary Faculty of the Universidad de Extremadura (UEX, Cáceres, Spain), and Jordi Figuerola from Estación Biológica de Doñana (EBD-CSIC, Seville, Spain) are acknowledged for providing field samples.

An abstract with preliminary results of this study was presented at the 10th ESVV/9th EPIZONE congress in

Montpellier 2015, entitled Development of a duplex real-time qRT-PCR method for detection of flaviviruses belonging to Japanese encephalitis and Ntaya serogroups, and authored by ME, FL, EP-R, A. Robles, A., Villalba, M. C. Barbero, MJ-C; J F-P.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2020.00203/full#supplementary-material>

REFERENCES

- Calisher CH, Gould EA. Taxonomy of the virus family Flaviviridae. *Adv Virus Res.* (2003) 59:1–19. doi: 10.1016/S0065-3527(03)59001-7
- Kuno G, Chang GJ, Tsuchiya KR, Cropp CB, Karabatsos N. Phylogeny of the genus flavivirus. *J Virol.* (1998) 72:73–83. doi: 10.1128/JVI.72.1.73-83.1998
- Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, et al. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol.* (1989) 70:37–43. doi: 10.1099/0022-1317-70-1-37
- Gould EA, Solomon T. Pathogenic flaviviruses. *Lancet.* (2008) 371:500–9. doi: 10.1016/S0140-6736(08)60238-X
- Tang Y, Gao X, Diao Y, Feng Q, Chen H, Liu X, et al. Tembusu virus in human, China. *Transbound Emerg Dis.* (2013) 60:193–96. doi: 10.1111/tbed.12085
- Bondre VP, Sapkal GN, Yergolkar PN, Fulmali P V, Sankaraman V, Ayachit VM, et al. Genetic characterization of Bagaza virus (BAGV) isolated in India and evidence of anti-BAGV antibodies in sera collected from encephalitis patients. *J Gen Virol.* (2009) 90:2644–9. doi: 10.1099/vir.0.012336-0
- Benzarti E, Linden A, Desmecht D, Garigliany M. Mosquito-borne epornitic flaviviruses: an update and review. *J Gen Virol.* (2019) 100:119–32. doi: 10.1099/jgv.0.001203
- Fernandez-Pinero J, Davidson I, Elizalde M, Perk S, Khinich Y, Jimenez-Clavero MA. Bagaza virus and Israel turkey meningoencephalomyelitis virus are a single virus species. *J Gen Virol.* (2014) 95:883–7. doi: 10.1099/vir.0.061465-0
- Kurane I, Takasaki T, Yamada KI. Trends in flavivirus infections in Japan. *Emerg Infect Dis.* (2000) 6:569–71. doi: 10.3201/eid0606.000603
- Daep CA, Muñoz-Jordán JL, Eugenin EA. Flaviviruses, an expanding threat in public health: focus on dengue, West Nile, and Japanese encephalitis virus. *J Neurovirol.* (2014) 20:539–60. doi: 10.1007/s13365-014-0285-z
- Holbrook MR. Historical perspectives on flavivirus research. *Viruses.* (2017) 9:97. doi: 10.3390/v9050097
- Weissenböck H, Hubálek Z, Bakonyi T, Nowotny N. Zoonotic mosquito-borne flaviviruses: worldwide presence of agents with proven pathogenicity and potential candidates of future emerging diseases. *Vet Microbiol.* (2010) 140:271–80. doi: 10.1016/j.vetmic.2009.08.025
- Beck C, Jimenez-Clavero MA, Leblond A, Durand B, Nowotny N, Leparc-Goffart I, et al. Flaviviruses in Europe: complex circulation patterns and their consequences for the diagnosis and control of west Nile disease. *Int J Environ Res Public Health.* (2013) 10:6049–83. doi: 10.3390/ijerph10116049
- Rizzoli A, Jimenez-Clavero M, Barzon L, Cordioli P, Figuerola J, Koraka P, et al. The challenge of West Nile virus in Europe: knowledge gaps and research priorities. *Euro Surveill.* (2015) 20:21135. doi: 10.2807/1560-7917.ES2015.20.20.21135
- Busquets N, Laranjo-González M, Soler M, Nicolás O, Rivas R, Talavera S, et al. Detection of West Nile virus lineage 2 in North-Eastern Spain (Catalonia). *Transbound Emerg Dis.* (2019) 66:617–21. doi: 10.1111/tbed.13086
- de Heus P, Kolodziejek J, Camp J V., Dimmel K, Bagó Z, Hubálek Z, et al. Emergence of West Nile virus lineage 2 in Europe: characteristics of the first seven cases of West Nile neuroinvasive disease in horses in Austria. *Transbound Emerg Dis.* (2019) 1–9. doi: 10.1111/tbed.13452
- Weissenböck H, Kolodziejek J, Url A, Lussy H, Rebel-Bauder B, Nowotny N. Emergence of usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe. *Emerg Infect Dis.* (2002) 8:652–6. doi: 10.3201/eid0807.020094
- Vazquez A, Jimenez-Clavero MA, Franco L, Donoso-Mantke O, Sambri V, Niedrig M, et al. Usutu virus: potential risk of human disease in Europe. *Euro Surveill.* (2011) 16:19935. doi: 10.2807/ese.16.31.19935-en
- Roesch F, Moratorio G, Moratorio G, Vignuzzi M. Usutu virus: an arbovirus on the rise. *Viruses.* (2019) 11:640. doi: 10.3390/v11070640
- Agüero M, Fernandez-Pinero J, Buitrago D, Sanchez A, Elizalde M, San Miguel E, et al. Bagaza virus in partridges and pheasants, Spain, 2010. *Emerg Infect Dis.* (2011) 17:1498–501. doi: 10.3201/eid1708.110077
- Llorente F, Pérez-Ramírez E, Fernández-Pinero J, Soriguer R, Figuerola J, Jiménez-Clavero MA. Flaviviruses in game birds, southern Spain, 2011–2012. *Emerg Infect Dis.* (2013) 19:1023–5. doi: 10.3201/eid1906.130122
- Davidson I, Raibstein I, Al-Tori A, Khinich Y, Simanov M, Yuval C, et al. Development of a reliable dual-gene amplification RT-PCR assay for the detection of Turkey Meningoencephalitis virus in Turkey brain tissues. *J Virol Methods.* (2012) 185:239–43. doi: 10.1016/j.jviromet.2012.06.001
- Mackenzie JS, Williams DT. The zoonotic flaviviruses of southern, south-eastern and eastern Asia, and Australasia: the potential for emergent viruses. *Zoonoses Public Health.* (2009) 56:338–56. doi: 10.1111/j.1863-2378.2008.01208.x
- Gill CM, Kapadia RK, Beckham JD, Piquet AL, Tyler KL, Pastula DM. Usutu virus disease: a potential problem for North America? *J Neurovirol.* (2019) doi: 10.1007/s13365-019-00818-y. [Epub ahead of print].
- Lanciotti R, Roehrig J, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science.* (1999) 286:2333–37. doi: 10.1126/science.286.5448.2333
- Weissenböck H, Bakonyi T, Rossi G, Mani P, Nowotny N. Usutu Virus, Italy. 1996. *Emerg Infect Dis.* (2013) 19:274–7. doi: 10.3201/eid1902.121191
- Scaramozzino N, Crance J, Jouan A, Briel D, DAE. Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. *J Clin Microbiol.* (2001) 39:1922–7. doi: 10.1128/JCM.39.5.1922-1927.2001
- Sánchez-Seco MP, Rosario D, Domingo C, Hernández L, Valdés K, Guzmán MG, et al. Generic RT-nested-PCR for detection of flaviviruses using degenerated primers and internal control followed by sequencing for specific identification. *J Virol Methods.* (2005) 126:101–9. doi: 10.1016/j.jviromet.2005.01.025
- Patel P, Landt O, Kaiser M, Faye O, Koppe T, Lass U, et al. Development of one-step quantitative reverse transcription PCR for the rapid detection of flaviviruses. *Virol J.* (2013) 10:58. doi: 10.1186/1743-422X-10-58
- Johnson N, Wakeley PR, Mansfield KL, McCracken F, Haxton B, Phipps LP, et al. Assessment of a novel real-time pan-flavivirus RT-polymerase chain reaction. *Vector-Borne Zoonot Dis.* (2010) 10:665–71. doi: 10.1089/vbz.2009.0210
- Moureaux G, Temmam S, Gonzalez JP, Charrel RN, Grard G, De Lamballerie X. A real-time RT-PCR method for the universal detection and identification of flaviviruses. *Vector-Borne Zoonot Dis.* (2007) 7:467–77. doi: 10.1089/vbz.2007.0206

32. Vina-Rodríguez A, Sachse K, Ziegler U, Chaintoutis SC, Keller M, Groschup MH, et al. a novel pan- flavivirus detection and identification assay based on RT-qPCR and microarray. *Biomed Res Int.* (2017) 2017:4248756. doi: 10.1155/2017/4248756
33. Dyer J, Chisenhall DM, Mores CN. A multiplexed TaqMan assay for the detection of arthropod-borne flaviviruses. *J Virol Methods.* (2007) 145:9–13. doi: 10.1016/j.jviromet.2007.05.001
34. Del Amo J, Sotelo E, Fernández-Pinero J, Gallardo C, Llorente F, Agüero M, et al. A novel quantitative multiplex real-time RT-PCR for the simultaneous detection and differentiation of West Nile virus lineages 1 and 2, and of Usutu virus. *J Virol Methods.* (2013) 189:321–7. doi: 10.1016/j.jviromet.2013.02.019
35. Buitrago D, Rocha A, Tena-Tomás C, Vigo M, Agüero M, Jiménez-Clavero MA. Real-time fluorogenic reverse transcription polymerase chain reaction assay for the specific detection of Bagaza virus. *J Vet Diagn Investig.* (2012) 24:959–63. doi: 10.1177/1040638712452723
36. Sotelo E, Gutierrez-Guzmán AV, Del Amo J, Llorente F, El-Harrak M, Pérez-Ramírez E, et al. Pathogenicity of two recent Western Mediterranean West Nile virus isolates in a wild bird species indigenous to Southern Europe: the red-legged partridge. *Vet Res.* (2011) 42:11. doi: 10.1186/1297-9716-42-11
37. Del Amo J, Llorente F, Figuerola J, Soriguer RC, Moreno AM, Cordioli P, et al. Experimental infection of house sparrows (*Passer domesticus*) with West Nile virus isolates of Euro-Mediterranean and North American origins. *Vet Res.* (2014) 45:33. doi: 10.1186/1297-9716-45-33
38. Cano-Gómez C, Llorente F, Pérez-Ramírez E, Soriguer RC, Sarasa M, Jiménez-Clavero MÁ. Experimental infection of grey partridges with Bagaza virus: pathogenicity evaluation and potential role as a competent host. *Vet Res.* (2018) 49:44. doi: 10.1186/s13567-018-0536-8
39. Bournez L, Umhang G, Faure E, Boucher J-M, Boué F, Jourdain E, et al. Exposure of wild ungulates to the usutu and tick-borne encephalitis viruses in France in 2009 – 2014 : evidence of undetected flavivirus circulation a decade ago. *Viruses.* (2020) 12:10. doi: 10.3390/v12010010
40. Nikolay B, Diallo M, Boye CSB, Sall AA. Usutu virus in Africa. *Vector Borne Zoonot Dis.* (2011) 11:1417–23. doi: 10.1089/vbz.2011.0631
41. Mannasse B, Mendelson E, Orshan L, Mor O, Shalom U, Yeger T, et al. Usutu virus RNA in mosquitoes, Israel, 2014–2015. *Emerg Infect Dis.* (2017) 23:1699–702. doi: 10.3201/eid2310.171017
42. Steyn J, Botha EM, Lourens C, Coetzer JAW, Venter M. Bagaza virus in Himalayan monal pheasants, South Africa, 2016–2017. *Emerg Infect Dis.* (2019) 25:2299–302. doi: 10.3201/eid2512.190756
43. Johnson N, Voller K, Phipps LP, Mansfield K, Fooks AR. Rapid molecular detection methods for arboviruses of livestock of importance to northern Europe. *J Biomed Biotechnol.* (2012) 2012:719402. doi: 10.1155/2012/719402
44. Yan P, Zhao Y, Zhang X, Xu D, Dai X, Teng Q, et al. An infectious disease of ducks caused by a newly emerged Tembusu virus strain in mainland China. *Virology.* (2011) 417:1–8. doi: 10.1016/j.virol.2011.06.003
45. Su J, Li S, Hu X, Yu X, Wang Y, Liu P, et al. Duck egg-drop syndrome caused by BYD virus, a new Tembusu-related flavivirus. *PLoS ONE.* (2011) 6:e18106. doi: 10.1371/journal.pone.0018106
46. Thontiravong A, Ninvilai P, Tunterak W, Nonthabenjawan N, Chaiyavong S, Angkabkingkaew K, et al. Tembusu-Related Flavivirus in Ducks, Thailand. *Emerg Infect Dis.* (2015) 21:2164–7. doi: 10.3201/eid2112.150600
47. Liu P, Lu H, Li S, Moureau G, Deng Y-Q, Wang Y, et al. Genomic and antigenic characterization of the newly emerging Chinese duck egg-drop syndrome flavivirus : genomic comparison with tembusu and sitiawan viruses. *J Gen Virol.* (2012) 93:2158–70. doi: 10.1099/vir.0.043554-0

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Elizalde, Cano-Gómez, Llorente, Pérez-Ramírez, Casades-Martí, Aguilera-Sepúlveda, Ruiz-Fons, Jiménez-Clavero and Fernández-Pinero. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Development of a Digital RT-PCR Method for Absolute Quantification of Bluetongue Virus in Field Samples

Angela M. Rocchigiani¹, Maria G. Tilocca^{1*}, Ottavio Portanti², Bruna Vodret¹, Roberto Bechere¹, Marco Di Domenico², Giovanni Savini², Alessio Lorusso² and Giontonella Puggioni¹

¹ Department of Sanità Animale, Istituto Zooprofilattico Sperimentale Della Sardegna, Sassari, Italy, ² OIE Reference Laboratory for Bluetongue, Istituto Zooprofilattico Sperimentale Abruzzo e Molise, Teramo, Italy

OPEN ACCESS

Edited by:

Jesus Hernandez,
Centro de Investigación en
Alimentación y Desarrollo
(CIAD), Mexico

Reviewed by:

Faten Abdelaal Okda,
St. Jude Children's Research Hospital,
United States
Semmannan Kalaiyarasu,
ICAR-National Institute of High
Security Animal Diseases
(ICAR-NIHSAD), India

*Correspondence:

Maria G. Tilocca
mgtilocca@gmail.com

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 29 November 2019

Accepted: 11 March 2020

Published: 21 April 2020

Citation:

Rocchigiani AM, Tilocca MG,
Portanti O, Vodret B, Bechere R, Di
Domenico M, Savini G, Lorusso A and
Puggioni G (2020) Development of a
Digital RT-PCR Method for Absolute
Quantification of Bluetongue Virus in
Field Samples. *Front. Vet. Sci.* 7:170.
doi: 10.3389/fvets.2020.00170

Bluetongue (BT) is a major Office International des Epizooties (OIE)-listed disease of wild and domestic ruminants caused by several serotypes of Bluetongue virus (BTV), a virus with a segmented dsRNA genome belonging to the family *Reoviridae*, genus *Orbivirus*. BTV is transmitted through the bites of *Culicoides* midges. The aim of this study was to develop a new method for quantification of BTV Seg-10 by droplet digital RT-PCR (RTdd-PCR), using nucleic acids purified from complex matrices such as blood, tissues, and midges, that notoriously contain strong PCR inhibitors. First, RTdd-PCR was optimized by using RNAs purified from serially 10-fold dilutions of a BTV-1 isolate ($10^{5.43}$ TCID₅₀/ml up to $10^{-0.57}$ TCID₅₀/ml) and from the same dilutions spiked into fresh ovine EDTA-blood and spleen homogenate. The method showed a good degree of linearity ($R^2 \geq 0.995$). The limit of detection (LoD) and the limit of quantification (LoQ) established were $10^{-0.67}$ TCID₅₀/ml (0.72 copies/ μ l) and $10^{0.03}$ TCID₅₀/ml (3.05 copies/ μ l) of BTV-1, respectively. Second, the newly developed test was compared, using the same set of biological samples, to the quantitative RT-PCR (RT-qPCR) detecting Seg-10 assay widely used for the molecular diagnosis of BTV from field samples. Results showed a difference mean of 0.30 log between the two assays with these samples ($p < 0.05$). Anyway, the analysis of correlation demonstrated that both assays provided similar measurements with a very close agreement between the systems.

Keywords: Bluetongue, Reoviridae, RNA quantification, droplet digital RT-PCR, Quantitative Real Time -RT-PCR

INTRODUCTION

Digital polymerase chain reaction (dPCR) is a recent technology enables an accurate absolute quantification of target nucleic acids. The principle of dPCR was first described in the 1990s (1, 2). The dPCR approach combines limiting dilutions, PCR, and Poisson distribution to quantitate the total number of amplifiable targets within a sample (1).

Digital technology is based on end-point PCR that provides the direct measure of nucleic acids without relying on a standard curve (3). In a dPCR assay, the sample is randomly partitioned into individual reactions, such that some contain no nucleic acid template and others contain one or more template copies (4). The very high number of partitions of the sample allows a significant precision on results (5).

After end-point PCR amplification, each partition is analyzed and distinct as positive (presence of PCR products) or negative (absence of PCR products). The fraction of amplification positive partitions is used to estimate the concentration of the initial target sequence using binomial Poisson statistics (4, 6). Nowadays, different dPCR commercial platforms are available as a useful tool for precise quantification of nucleic acids in a variety of basic research and clinical applications (4, 7–9).

Despite the fact that quantitative PCR or real-time PCR (qPCR) has been widely utilized to quantify nucleic acid in many areas of research and diagnostics tests, it has some disadvantages such as the necessity for a standard curve, the lack of universal standards of known quantity, and also the efficiency can be influenced by many factors including inhibitors. The qPCR is the gold standard for molecular quantitation in viral diagnostics; dPCR offers several potential advantages over qPCR (10). Digital PCR uses an amplification reaction system similar to a system of standard qPCR, but does not require the same level of calibration or controls as traditionally used in qPCR (5).

Digital PCR overcomes the need for a standard curve, and it is increasingly used for DNA/RNA viral quantification, in human and animal health (11–17). Bluetongue (BT) is an Office International des Epizooties (OIE)-listed infectious disease of domestic and wild ruminants (18), transmitted mainly through the bites of *Culicoides* midges. Bluetongue virus (BTV), which belongs to the genus Orbivirus of the family Reoviridae, causes significant economic losses due to mortality, decline in production, and restrictions on trade in animals from infected areas (19). BTV genome consists of 10 linear double strand segments (Seg-1 to Seg-10), encoding seven structural proteins (VP1–VP7) and five non-structural proteins (NS1 to NS4 and S10-ORF2) involved in viral replication, morphogenesis, and assembly processes (20).

Currently, there are 24 classic serotypes of the virus, all capable of causing BT, plus a series of new serotypes, defined as atypical because infected animals are asymptomatic (21–27).

For molecular diagnostic laboratories, OIE recommends the use of a real-time RT-PCR (RT-PCR_{NS3}) assay in order to confirm clinical cases, to establish uninfected animals before handling, to check the prevalence of infection, and for surveillance purposes.

The method real-time RT-PCR_{NS3} (21) allows to detect all circulating known BTV serotypes, by retro-transcription and amplification of a region of segment 10 of the viral RNA, coding for a non-structural NS3 protein, purified by blood-EDTA, biological liquid, and organ tissues taken from susceptible species and by hematophagous insects. RT-PCR targeting in Seg-2 coding for a least conserved virion outer capsid protein (VP2) identifies the specific BTV serotype (28, 29).

Because of its accuracy and precision, real-time quantitative RT-qPCR is the method of choice when quantitative analysis is required. However, there are no available reference certificated material (standard) for the quantification of bluetongue virus. Toussaint et al. (30) used recombinant plasmid obtained by inserting BTV PCR product into PCRII-TOPO vector by TA-cloning and RNA synthesized *in vitro* with Riboprobe system

T7 (Promega). (31) employed RiboMax Large scale RNA production System (Promega) for transcription of standard RNA by bluetongue recombinant plasmid PGEM –T Easy Vector RNA. Maan et al. (32) transcribed from recombinant pGEMT plasmid RNA BT using the mMessage mMachine T7 Ultra Kit (Life Technologies). Overall, the use of different calibration standards in different performing assays can lead to non-reproducible results between laboratories, even when testing the same material (12, 33).

To overcome these limitations, we aimed to develop a new method for quantification of BTV Seg-10 by droplet digital RT-PCR (RT-dd-PCR), using nucleic acids purified from complex matrices such as blood, tissues, and midges, that notoriously contain strong PCR inhibitors. The RT-PCR_{NS3} method recommended by OIE was transferred to the digital platform, optimized by using RNAs purified from serially 10-fold dilutions of a BTV-1 isolate, spiked into fresh ovine EDTA-blood and spleen homogenate. The limit of detection (LoD) and the limit of quantification (LoQ) were established by using serial dilutions of BTV-1 RNA purified. Using RNAs purified from field samples, the newly developed assay was compared, with the RT-qPCR_{NS3} detecting the same target Seg-10.

MATERIALS AND METHODS

Virus Strain, Spiked-In Samples, and Field Samples Collection

BTV-1/2006 strain, isolated from the spleen of an infected sheep that succumbed during the BTV-1 outbreak in Sardinia (Italy) during 2006, was employed for the study. The BTV-1/2006 strain was titrated by end-point onto VERO cells by Sperman/Karber method ($10^{5.43}$ /TCID₅₀/ml). Four 10-fold serial dilutions of BTV-1 suspensions (from $10^{2.43}$ to $10^{-0.57}$ TCID₅₀/ml) spiked into ovine blood samples and in ovine spleen homogenates (10% w/v) were used to evaluate possible inhibition caused by matrices. The optimized droplet digital RT-PCR (RT-ddPCR) was finally evaluated on a total of 44 field samples tested positive for real-time RT-PCR_{NS3}, including 16 of *Culicoides imicola* and 28 of ovine blood EDTA. *Culicoides* samples were collected in farms of southern Sardinia, during entomological surveillance, by the national surveillance plan, in the years 2017 and 2018. The blood samples were collected from farms located in the same part of the region. Four negative blood samples were used as negative controls. The blood samples were refrigerated at $5^\circ \pm 3^\circ\text{C}$. *C. imicola* samples were stored at $-70 \pm 10^\circ\text{C}$ until the time of processing and analysis. The results were compared with those obtained by the RT-qPCR_{NS3}.

Nucleic Acid Purification

RNA purification from viral suspensions, spiked-in samples, and field samples, mosquitos included, was performed by MagMax Core Nucleic Acid Purification Kit (Applied Biosystems-ThermoFisher Scientific-USA) in automated sample preparation workstation MagMAX Express 96 (Applied Biosystems) according to the manufacturer's instructions. Primers and probe were the same published by OIEb (34), which amplify a portion of Seg-10. This RT-PCR_{NS3} assay is widely used for molecular

diagnosis of BTV. In order to optimize RT-ddPCR assay, the same set of primers and probe were used in all experiments.

Droplet Digital RT-PCR (RT-ddPCR) Optimization

Purified nucleic acids were quantified using QX200™ Droplet Digital PCR System (BioRad Laboratories, USA). The assay was performed in 20 μ l using the One-step RT-ddPCR Advanced Kit for Probe (Bio-Rad) consisting of: 5 μ l supermix 4 X, 2 μ l of reverse transcriptase (20 U/ μ l), 1 μ l of 300 mM DTT, 2 μ l of RNA template, and the primers and probe at final concentrations of 0.9 and 0.25 μ M, respectively, according to the manufacturer's instructions. In order to optimize RT-ddPCR assay, primers and probe were further tested at different concentrations within the range of 0.4–0.9 μ M and 0.15–0.25 μ M by using RNA purified from different BTV-1 suspensions (titer $10^{2.43}$, $10^{1.43}$, $10^{0.43}$, $10^{-0.57}$ TCID₅₀/ml). RNA was denatured with primers for 5 min at 95°C, stabilized for 3 min at 4°C, and then added to the RT-ddPCR mixture reaction as indicated above. No template controls (NTC) were used for monitoring primer-dimer formation and contaminations. Twenty microliters RT-ddPCR mixture/sample were placed in each well of droplet generator DG8 cartridge (BioRad Laboratories, USA) with 70 μ l of droplet generator oil (BioRad Laboratories, USA) and emulsified in QX-200 Droplet Generator (BioRad Laboratories, USA) partitioning in 20,000 water in oil nanoliter-size droplet. Then, a volume of 40 μ l of emulsion/sample was transferred to a 96-well reaction plate (Eppendorf, Hauppauge, NY), heat-sealed with pierceable foil sheets by the PX1™ PCR Plate Sealer (BioRad Laboratories, USA), and amplified in C1000 Touch™ Thermal Cycler (BioRad Laboratories, USA). So as to allow an optimal distinction between positive and negative droplets, PCR annealing temperature was optimized by thermal gradient from 55 to 65°C. Cycling conditions were the following: 48°C for 30 min, 95°C for 2 min, followed by 50 cycles of 95°C for 15 s, 55C–65°C for 30 s, and 72°C for 30 s. At the end of amplification, the PCR plates were read by the QuantaSoft Droplet Reader (BioRad Laboratories, USA) that measures the fluorescence intensity of each droplet and detects the size and shape as droplets pass detector. The absolute concentration of each sample was automatically reported as copy number of Seg-10 of BTV/ μ l by the ddPCR QuantaSoft Software V.1.7.4.0917 (BioRad Laboratories, USA) by calculating the ratio of the positive droplets over the total droplets combined with Poisson distribution with 95% confidence interval.

Performance of RT-ddPCR Assay

Linearity of the assay was defined by using 10-fold dilutions of BTV1-RNA purified (ranging from $10^{2.43}$ TCID₅₀/ml up to $10^{-0.57}$ TCID₅₀/ml), analyzed in seven replicates, with optimized conditions of primers, probe, and amplification program. The range of linearity was defined by plotting the log value of titers (TCID₅₀/ml) BTV-1 dilution against the absolute measured value (copies/ μ l). The LoD and LoQ were evaluated by using 20 replicates of 5-fold serial dilutions of BTV-1 RNA purified (ranging from $10^{1.43}$ to $10^{-1.37}$ TCID₅₀/ml). The LoD of RT-ddPCR was determined as the last serial dilution detected in

95% of replicates, whereas the LoQ was set at the lowest dilution showing a coefficient of variation percentage below the threshold (CV% = 25) for acceptance criteria of quantitative methods (35, 36). Furthermore, to evaluate the intra-assay and inter-assay repeatability, three different dilutions of BTV-1 ($10^{2.43}$, $10^{1.43}$, $10^{0.43}$ TCID₅₀/ml) were tested in seven replicates in two different days; the CV% was then considered. pGEM T-easy vector (Promega, Milan-Italy) carrying Seg-10 of a BTV-1 strain in serial dilutions, from 2×10^4 to 2×10^1 copies, was also used to evaluate accuracy of the RT ddPCR for quantification purposes. Finally, matrix effect was evaluated comparing R^2 values of RNA isolated from four BTV-1 dilutions (from $10^{2.43}$ to $10^{-0.57}$ TCID₅₀/ml), and RNA isolated from four blood and four spleen homogenates spiked (10% w/v) with the same BTV-1 TCID₅₀/ml.

Quantitative Real Time Assay (RT-qPCR_{NS3})

RT-qPCR_{NS3} assay, as described above, was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems) and SuperScript™ III Platinum™ One-Step qRT-PCR Kit (ThermoFisher- Life Technologies). RNA was denatured with 0.4 μ M primers for 5 min at 95°C, stabilized for 3 min at 4°C, and then added to the RT-qPCR mixture reaction as indicated above. The one step RT-qPCR mixture was prepared in 25 μ l reaction volume, 12.5 μ l 2X Reaction Mix, 0.5 μ l of 50X ROX Reference Dye, 1 μ l Mg₂SO₄ 50 mM, 0.5 μ l SSIII RT-Platinum™ Taq Mix, 0.2 μ M probe, and 2 μ l of RNA. Cycling conditions were the following: 48°C for 30 min, 95°C for 2 min, followed by 50 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. In order to assess the standard curve the pGEM T-easy vector (Promega, Milan-Italy) carrying Seg-10 of a BTV-1 strain was employed, available at IZSAM (pGEM-BTV-NS3, 10^8 copies/ μ l). The standard curve was constructed placing Cq values of seven serial 10-fold dilutions of pGEM-BTV-NS3 (1×10^7 copies/ μ l up to 1×10^1 copies/ μ l), performed in triplicate wells, against the log value of the number of copies of BTV-1 Seg-10. BTV-1 Seg-10 copy number in each sample was determined by Cq value to the standard curve. Cq value was generated by 7900 Software SDS 2.4.1 (Applied Biosystems). Amplification Efficiency and R^2 of the calibration curve were calculated.

Comparison of RT-ddPCR and RT-qPCR_{NS3} Assays for Quantitation of BTV-1 Seg-10 in Field Samples

To evaluate the performance of RT-ddPCR against the RT-qPCR_{NS3}, 44 field samples were tested in triplicate wells with both assays and the difference of log of quantification was evaluated to verify the agreement between two assays.

Statistical Analysis

Data were converted into a logarithmic format. Linear regression analyses were conducted using Microsoft Excel 2010. In order to compare quantification by RT-ddPCR and RT-qPCR_{NS3} statistical analysis was conducted by Statgraphics 18 Centurion Software (Version 18.1.06).

RESULTS

Optimization of RT-ddPCR

The primers and probe concentrations were optimized by using RNA purified from dilution of BTV-1 from $10^{2.43}$ to $10^{-0.57}$. As shown in **Figure 1**, the optimal primers and probe concentrations were 0.9 and $0.25 \mu\text{M}$ per reaction, respectively, i.e., the highest concentrations, among those tested, as recommended by the manufacturer (Biorad). PCR annealing temperature was optimized by thermal gradient from 55 to 65°C . The optimum annealing temperature was at 58.8°C , which resulted in the greatest difference of fluorescence amplitude between positive and negative droplets. The optimal cycling conditions were: 48°C for 30 min, 95°C for 2 min, followed by 50 cycles of 95°C for 15 s, 58.8°C for 30 s, and 72°C for 30 s, 1 cycle of 98°C for 10 min essential for droplet stabilization and infinite 12°C hold. It was used a 2.5°C/s ramp rate to ensure each droplet reached the correct temperature for each step during the cycling. The parameters above were used in following RT-ddPCR experiments of our study.

Performance of RT-ddPCR Assay

The trend line of detection BTV-1 concentration by RT-ddPCR exhibited a good degree of linearity ($R^2 \geq 0.995$) in the range from $10^{2.43}$ TCID₅₀/ml to $10^{-0.57}$ TCID₅₀/ml (**Figure 2**). According to the definition, the LoD established was $10^{-0.67}$ TCID₅₀/ml BTV1 corresponding to 0.72 copies/ μl (**Table 1**). Conversely, the LoQ determined was $10^{0.03}$ TCID₅₀/ml of BTV-1 (3.05 copies/ μl) with a CV% value 21 (**Table 1**). Results of the copy number obtained by RT-ddPCR relative to the four pGEM 10-fold dilutions give a good degree of linearity ($R^2 = 0.998$), especially in the range 20–2000 copies as reported in **Table 2** and **Figure 3**. The CV% values were also considered to assess repeatability. The analysis of the seven replicates for the three

dilutions gave back CV% lower than the threshold (CV% = 25) in all cases for both intra- and inter-assay (**Table 3**).

Evaluation Matrix-Effect of RT-ddPCR

BTV-1 suspensions from $10^{2.43}$ to $10^{-0.57}$ TCID₅₀/ml spiked in blood and in spleen tissue showed good resilience to inhibitor blood and tissue factors compared to same viral suspensions without matrix, in linear range of the RT-ddPCR assay. As indicated in **Figure 4** quantitative linearity analysis in matrices showed a good linearity with R^2 close to 1.

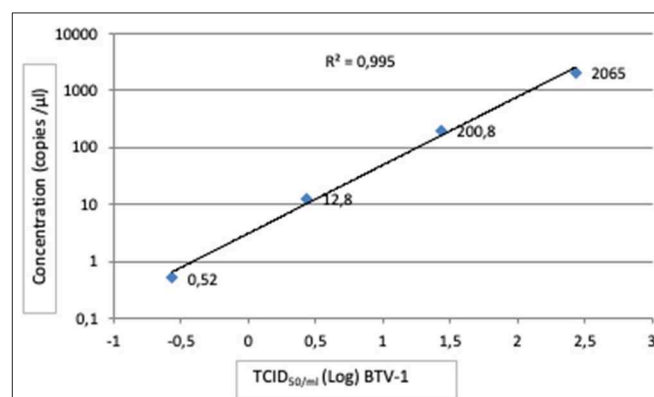


FIGURE 2 | Linear regression of RT-ddPCR assay using RNA extracted from 10-fold dilution of BTV-1/2006 from TCID₅₀ $10^{2.43}$ to $10^{-0.57}$, analyzed in seven replicates, at the final optimized conditions.

TABLE 1 | Limit of detection (LoD) and limit of quantification (LoQ) of droplet digital RT-PCR (RT-ddPCR).

BTV-1 Log ₁₀ TCID ₅₀ /ml	Mean values (copies/ μl) $\pm$ SD ^a	CV (%) ^b
1.43	195 $\pm$ 4.35	2.23
0.73	29 $\pm$ 1.17	4.01
0.03	3.05 $\pm$ 0.64 ^c	21
-0.67	0.72 $\pm$ 0.40 ^d	25
-1.37	0.09 $\pm$ 0.09	91

^aMean values of copies number BTV-1 in μl detected by RT-ddPCR and standard deviation.

^bCoefficient of variation.

^cLoQ.

^dLoD.

TABLE 2 | pGEM detection of the RT-ddPCR.

Concentration of pGEM (copies/2 μl)	Concentration of pGEM (log)	Detected concentration copies	Detected concentration (log)
20000	4.30	14220	4.15
2000	3.30	1997	3.30
200	2.30	169	2.23
20	1.30	22	1.34

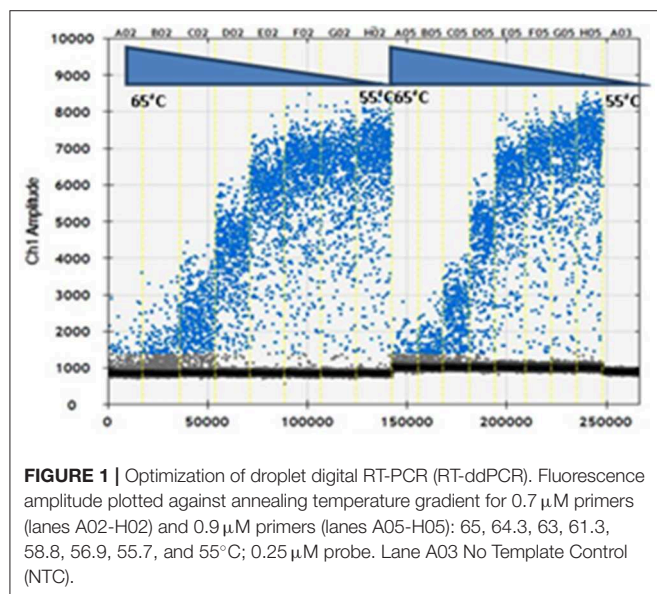


FIGURE 1 | Optimization of droplet digital RT-PCR (RT-ddPCR). Fluorescence amplitude plotted against annealing temperature gradient for 0.7 μM primers (lanes A02-H02) and 0.9 μM primers (lanes A05-H05): 65, 64.3, 63, 61.3, 58.8, 56.9, 55.7, and 55°C ; 0.25 μM probe. Lane A03 No Template Control (NTC).

Comparison of RTdd-PCR and RT-qPCRNS3 Assays for Quantitation of Seg-10 of BTV in Field Samples

The two assays were compared calculating the difference between the logarithm of RT-qPCR_{NS3} quantification, i.e., copies of Seg-10 of BTV / μ l of sample and the RT-ddPCR correspondent data. In field samples, the log difference average was 0.30 ($p =$

0.04968), in detail of 0.20 and 0.40, respectively, for blood EDTA samples and *Culicoides* midge as shown in Table 4 and Table S1. Figure 5 shows the correlations between the log copies of Seg-10 of BTV/ μ l of sample in RT-ddPCR with those in RT-qPCR_{NS3}.

DISCUSSION

BTV is responsible for an important disease of ruminant that induces variable clinical signs, its pathogenicity depends on the host species. Seasonal incursions of the disease in parts of Europe (Mediterranean basin) during the summer cause economic losses due to direct impact on livestock and trade restriction. In the last few years, new serotypes have been identified, probably, originated under evolutionary dynamics and selection pressure (37); new potential vectors have been identified (38, 39); some field strains/serotypes proved to be able to transmit vertically or horizontally, to reassort their RNA, and to alter their pathogenicity, specificity, and spread capacity (37). Nowadays, the molecular diagnosis of BTV (real time RT-PCR_{NS3}) by Hofmann allows to identify all circulating serotypes. These knowledges designate a complex contest in which it is necessary to have more sensitive and accurate methods, not only to assess the presence/absence of the virus but also to evaluate the RNA viral load in natural and/or experimental infected samples. In this study, we established a novel RT-ddPCR assay for the quantification of RNA BTV at

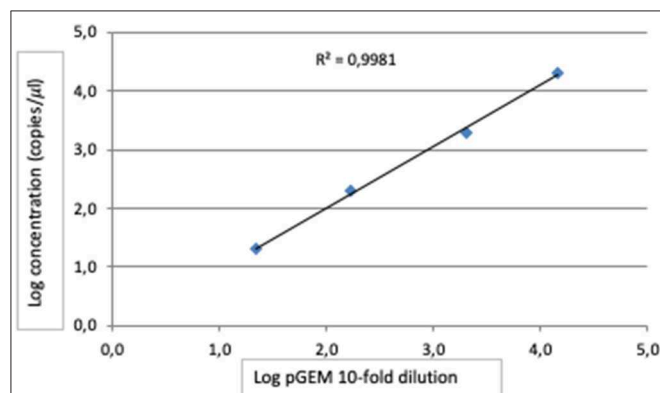


FIGURE 3 | Linear regression of RT-ddPCR assay using four 10-fold dilution of pGEM-BTV-NS3 from 2×10^4 to 2×10^1 , analyzed in three replicates, at the final optimized conditions.

TABLE 3 | Repeatability of RT-ddPCR assay.

Concentration of BTV-1 (TCID ₅₀ /ml)	Intra-assay	CV (%) ^b	Inter-assay	CV (%) ^b
	Mean values of seven replicates (copies / μ l) $\pm$ SD ^a		Mean values of seven replicates (copies / μ l) $\pm$ SD ^a	
$1 \times 10^{2.43}$	2066.43 $\pm$ 53.05	2.56	2017.43 $\pm$ 41.35	2.05
$1 \times 10^{1.43}$	200.86 $\pm$ 5.61	2.79	200.71 $\pm$ 8.47	4.22
$1 \times 10^{0.43}$	12.84 $\pm$ 1.62	12.62	12.07 $\pm$ 1.26	10.51

^aMean values of copies number BTV-1 in μ l of seven replicates detected by RT-ddPCR and standard deviation.

^bCoefficient of variation.

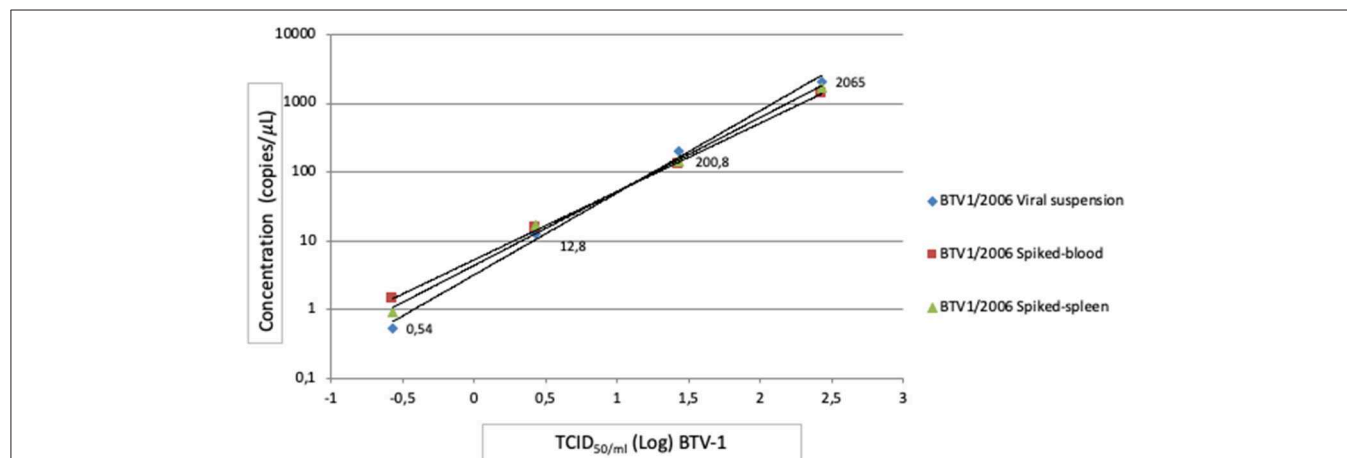
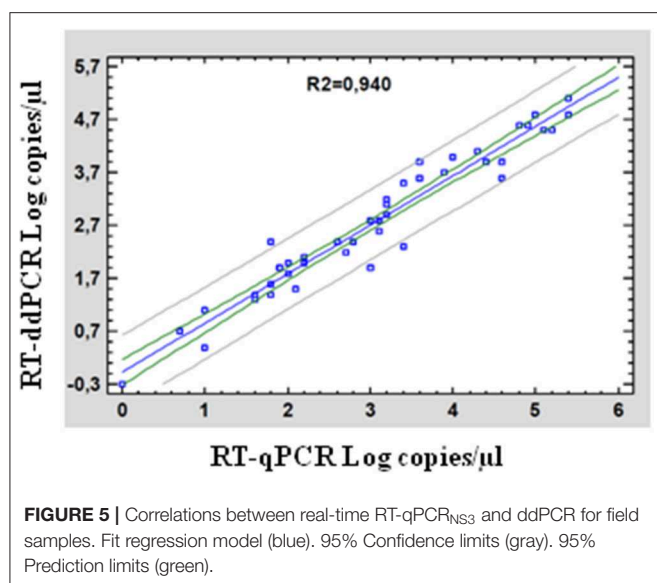


FIGURE 4 | RT-ddPCR Matrix effect. Linear regression analysis of 10-fold serial dilutions of BTV-1/2006, blood and spleen spiked with the same BTV-1/2006 TCID₅₀/ml. Blue rhombus BTV-1/2006 viral suspension $R^2 = 0.9954$; Green triangle BTV-1/2006 spiked-spleen $R^2 = 0.9964$; Red square BTV-1/2006 spiked-blood $R^2 = 0.9996$.

TABLE 4 | Comparison of RT-qPCRNS3 and RT-ddPCR assays for quantitative detection of BTV in field samples.

Field sample ^a	Real time RT-qPCRNS ₃		RT-ddPCR		
	Copies BTV/ μ l sample ^b	Log (copies/ μ l) ^c	Copies BTV/ μ l sample ^d	Log (copies/ μ l) ^e	Log difference ^f
Culicoides 79633/1	9701.38	4.0	9787.50	4.0	0.0
Culicoides 79633/3	7277.90	3.9	5400.00	3.7	0.2
Culicoides 79633/5	91433.73	5.0	60356.25	4.8	0.2
Culicoides 79633/8	75950.56	4.9	38812.00	4.6	0.3
Culicoides 79633/9	242211.14	5.4	116325.00	5.1	0.3
Culicoides 79633/10	1226.22	3.1	387.00	2.6	0.5
Culicoides 79633/11	70.02	1.8	38.50	1.6	0.2
Culicoides 71189/1	68638.03	4.8	97.20	4.6	0.2
Culicoides 71189/6	44221.17	4.6	48.15	3.9	0.7
Culicoides 71189/7	44530.87	4.6	22.95	3.6	1.0
Culicoides 71820/6	257642.51	5.4	96.08	4.8	0.6
Culicoides 71820/8	1090.00	3.0	5.18	1.9	1.1
Culicoides 72678/1	3625.14	3.6	8140.05	3.9	-0.3
Culicoides 72678/4	119916.18	5.1	72.90	4.5	0.6
Culicoides 72678/5	3642.75	3.6	4250.00	3.6	0.0
Culicoides 72678/7	144674.11	5.2	48.83	4.5	0.7
Blood 79384/1	1625.47	3.2	1433.25	3.2	0.0
Blood 79384/2	36.93	1.6	26.30	1.4	0.2
Blood 79384/3	160.06	2.2	117.90	2.1	0.1
Blood 79386/1	1187.62	3.1	695.25	2.8	0.0
Blood 79386/2	546.29	2.7	155.70	2.2	0.5
Blood 79386/3	1291.15	3.1	409.50	2.6	0.5
Blood 79390/1	168.73	2.2	101.70	2.0	0.2
Blood 79390/2	2712.23	3.4	191.25	2.3	1.1
Blood 79390/3	935.12	3.0	621.00	2.8	0.2
Blood 79364/1	36.02	1.6	18.00	1.3	0.3
Blood 79364/2	136.48	2.1	30.15	1.5	0.6
Blood 79364/3	100.49	2.0	57.82	1.8	0.2
Blood 127	2571.00	3.4	2944.35	3.5	-0.1
Blood 236	9.00	1.0	2.70	0.4	0.5
Blood 341	88.00	1.9	73.35	1.9	0.1
Blood 449	1550.00	3.2	806.85	2.9	0.3
Blood 695	390.00	2.6	226.35	2.4	0.2
Blood 8123	5.00	0.7	4.50	0.7	0.0
Blood 9124	1599.00	3.2	1134.00	3.1	0.1
Blood 11173	106.00	2.0	108.45	2.0	0.0
Blood 13247	577.00	2.8	264.15	2.4	0.3
Blood 14262	60.00	1.8	24.75	1.4	0.4
Blood 15278	11.00	1.0	11.70	1.1	0.0
Blood 16279	24750.00	4.4	8892.00	3.9	0.4
Blood 17290	20904.00	4.3	12326.85	4.1	0.2
Blood 18296	63.00	1.8	44.55	1.6	0.2
Blood 19301	66.00	1.8	252.00	2.4	-0.6
Blood 20307	1.00	0.0	0.45	-0.3	0.3

^aField samples analyzed in RT-qPCRNS₃ and RT-ddPCR.^bcopies BTV/ μ l of sample in RT-qPCRNS₃.^cLog of copies number BTV in μ l/sample in RT-qPCRNS₃ assay.^dcopies BTV/ μ l of sample in RT-ddPCR.^eLog of copies number BTV in μ l/sample in RT-ddPCR assay.^fLog (RT- qPCRNS₃ quantification) – Log (RT-ddPCR quantification).



low concentration of virus and in spiked and field samples including whole blood, tissues, and midges, showing the power and the potential of RT-ddPCR assay. This assay has been enabled to detect absolute target copy number in field samples without employing recombinant DNA or RNA plasmid to construct the standard curve. As a consequence, the amplification efficiency bias observed with quantitative RT-qPCR is minimized. A certified reference material (CRM) for BTV quantification is not available; therefore, further independent quantification methods are needed in order to quantify the BT virus as number of copies/ μl sample, in the greatest way possible. NS3 gene primers and probe sequences used in RT-ddPCR assay were from the previously published BT reverse transcriptase real-time PCR (RT-PCR_{NS3}) by Hofmann et al. (21) and OIE (34), mentioned above for diagnostic purposes. RT-qPCR_{NS3} is performed using degenerate primer and probe set that detect all serotypes of BTV; RT-ddPCRs were done with some specific modifications in order to optimize all parameters. RT-ddPCR assay exhibited a good degree of linearity ($R^2 \geq 0.995$) in the range from $10^{2.43}$ TCID₅₀/ml to $10^{-0.57}$ TCID₅₀/ml, overcoming the dependence on the availability of references or standards. BTV-1 $10^{2.43}$ TCID₅₀/ml was considered the first point of dynamic range. Evaluating the dynamic range of the digital assay, the $10^{3.43}$ TCID₅₀/ml viral dilution (corresponding to C_q 22.5 in RT-qPCR_{NS3}) was excessively concentrated to be detectable because all of the droplets were positive (saturation of the reaction). In contrast, the last point of the range (BTV-1 $10^{-1.57}$ TCID₅₀/ml) was excessively diluted (data not shown); then the last point was $10^{-0.57}$ TCID₅₀/ml viral dilution. As expected, the dynamic ranges of newly RT-ddPCR were lower (four orders of magnitude) than those obtained for the corresponding qPCRs for all samples. Several studies reported that digital PCR shows higher tolerance to inhibitors; as an end-point measurement, it can reduce the biases linked to matrix type often observed with qPCR (40), especially in clinical specimen (stool, sputum, tissue) (16, 17, 41). Pavšič et al. (42) suggested that it may be possible to perform ddPCR on samples without extraction

of nucleic acid. Quantitative RT-ddPCR data obtained from blood and spleen showed a good level of tolerance to inhibitors when compared to the same TCID₅₀/ml BTV-1 dilutions with a good linearity close to 1. Despite pGEM plasmid not being a properly standard reference, the use of serial dilutions of the plasmid allowed us to evaluate the precision of the RT-ddPCR for quantification purposes. These results suggest that RT-ddPCR assay provides an accurate quantification of BTV, unevenly distributed in different matrices, without precluding the quantitation efficiency due to impurities in the sample. The LoD and the LoQ were established at $10^{-0.67}$ TCID₅₀/ml and $10^{0.03}$ TCID₅₀/ml of BTV-1 corresponding to 0.72 and 3.05 copies/ μl , respectively. Data of intra- and inter-assay repeatability of the RT-ddPCR showed a good repeatability with a variability below the threshold (CV 25%) for acceptance criteria of quantitative methods (35, 36) and were assessed in two different days with <13% variability between the results. The good precision of RT-ddPCR is linked to the intrinsic characteristics of the method, enabling absolute quantification of the viral target at different work conditions. In the last part of this study, we compared the RT-ddPCR against RT-qPCR_{NS3} using 44 field samples that resulted positive to real-time RT-PCR_{NS3}. Applicability of the technology has been tested on characteristic matrices of field and on the range of viral load generally distributed in routine samples. The collected whole blood samples, tested as positive with the official method, stored at 4°C, and selected for comparative study with both assays, showed the same C_q found in RT-qPCR_{NS3} during the diagnosis (data not shown). These results support a previous work in which the persistence of BTV in stored blood samples were observed (43). The main limitation of RT-ddPCR respect to RT-qPCR_{NS3} was that the higher concentration of template resulted in saturation of positive droplets, confirming the saturation at high concentration of RNA. Thus, the concentrated samples were diluted for viral load quantification in RT-ddPCR. The results showed a log difference average of 0.30 in field samples. A higher Seg-10 BTV-1 quantification by RT-qPCR_{NS3} could be addressed to the C_q values that are established on transcription and amplification efficiency. This could affect the RT-qPCR, but does not have any effects on digital PCR. Anyway, the analysis of correlation demonstrated that both assays provided similar measurements with a very close agreement between the systems. On the other hand, the main advantage of the RT-ddPCR_{NS3} is the ability to quantify any BTV serotype without the use of standard curves properly constructed for each specific group. Moreover, the implementation of the serotype-specific multiplexing system should be suitable to detect and quantify simultaneously different BTV serotypes in case of viral coinfection in areas that are circulating different BTV serotypes. Despite its general advantages, compared to qPCR, dPCR is more time consuming and labor intensive, but will certainly give further, in terms of applicability and throughput.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study did not involve any animal experiment. Samples were collected from sheep with standard procedures by the Sardinian Veterinary Services A.T.S. and submitted to the Experimental Zooprophyllactic Institute of Sardinia for BTV testing. Special authorization for sampling activities was not necessary; these actions are regulated by the Italian Ministry of Health and performed in case of BT outbreaks.

AUTHOR CONTRIBUTIONS

MT, AR, and RB carried out experimental work. AR and MT wrote the first draft of the manuscript with support from GP and MD. All authors contributed to

conception and design of the study, data analysis and interpretation, manuscript revision, and read and approved the submitted version.

FUNDING

This study was funded by the Italian Ministry of Health (grant # RC IZS SA 02/16), and by Experimental Zooprophyllactic Institute of Sardinia—Sassari—Italy (grant #FNS 2016.11).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2020.00170/full#supplementary-material>

REFERENCES

- Sykes PJ, Neoh SH, Brisco MJ, Hugues E, Condon J, Morley AA. Quantitation of targets for PCR by use of limiting dilution. *Biotechniques*. (1992) 13:444–9.
- Vogelstein B, Kinzler KW. Digital PCR. *Proc Natl Acad Sci USA*. (1999) 16:9236–41. doi: 10.1073/pnas.96.16.9236
- Huggett JF, Cowen S, Foy CA. Considerations for digital PCR as an accurate molecular diagnostic tool. *Clin Chem*. (2015) 61:79–88. doi: 10.1373/clinchem.2014.221366
- Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, et al. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Analyt Chem*. (2012) 84:1003–11. doi: 10.1021/ac202578x
- Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal Chem*. (2011) 83:8604–10. doi: 10.1021/ac202028g
- Whale AS, Cowen S, Foy CA, Huggett JF. Methods for applying accurate digital PCR analysis on low copy DNA samples. *PLoS ONE*. (2013) 8:e58177. doi: 10.1371/journal.pone.0058177
- Quan PL, Sauzade M, Brouzes E. dPCR: a technology review. *Sensors*. (2018) 18:1271. doi: 10.3390/s18041271
- Sedlak RH, Jerome KR. Viral diagnostics in the era of digital polymerase chain reaction. *Diagn Microbiol Infect. Dis*. (2013) 75:1–4. doi: 10.1016/j.diagmicrobio.2012.10.009
- Doyle T, Smith C, Vitiello P, Cambiano V, Johnson M, Owen A, et al. Plasma HIV-1 RNA detection below 50 copies/ml and risk of virologic rebound in patients receiving highly active antiretroviral therapy. *Clin Infect Dis*. (2012) 54:724–32. doi: 10.1093/cid/cir936
- Sedlak RH, Jerome KR. The potential advantages of digital PCR for clinical virology diagnostics. *Expert Rev Mol. Diagn*. (2014) 14:501–7. doi: 10.1586/14737159.2014.910456
- Varela ME, Monteiro S, Rivadulla E, Santos R, Romalde JL. Development of a novel digital RT-PCR method for detection of human sapovirus in different matrices. *J Virol Methods*. (2018) 254:21–4. doi: 10.1016/j.jviromet.2018.01.005
- Jia P, Purcell MK, Pan G, Wang J, Kan S, Liu Y, et al. Analytical validation of a reverse transcriptase droplet digital PCR (RT-ddPCR) for quantitative detection of infectious hematopoietic necrosis virus. *J Virol Methods*. (2017) 245:73–80. doi: 10.1016/j.jviromet.2017.03.010
- Abachin E, Convers S, Falque S, Esson R, Mallet L, Nougarede N. Comparison of reverse-transcriptase qPCR and droplet digital PCR for the quantification of dengue virus nucleic acid. *Biologicals*. (2018) 52:49–54. doi: 10.1016/j.biologicals.2018.01.001
- Wu X, Lin H, Chen S, Xiao L, Yang M, An W, et al. Development of a reverse transcriptase droplet digital PCR (RT-ddPCR) for sensitive and rapid detection of Japanese encephalitis virus. *J Virol Methods*. (2017) 248:166–71. doi: 10.1016/j.jviromet.2017.06.015
- Fraisse A, Coudray-Meunier C, Martin-Latin S, Hennechart-Collette C, Delannoy S, Fach P, et al. Digital RT-PCR method for hepatitis A virus and norovirus quantification in soft berries. *Int J Food Microbiol*. (2017) 243:36–45. doi: 10.1016/j.ijfoodmicro.2016.11.022
- Sedlak RH, Nguyen T, Palileo I, Jerome R, Kuypers J. Superiority of digital Transcription-PCR(RT-PCR) over RealTime RT-PCR for quantitation of highly divergent human rhinoviruses. *J Clin Microbiol*. (2017) 55:442–9. doi: 10.1128/JCM.01970-16
- Strain M, Lada S, Luong T, Rought SE, Gianella S, Terry VH, et al. Highly precise measurement of HIV DNA by droplet digital PCR. *PLoS ONE*. (2013) 8:e55943. doi: 10.1371/journal.pone.0055943
- OIEa. Chapter 8.3: Infection with bluetongue virus. In: *OIE Terrestrial Animal Health Code*. Paris: OIE (2019).
- Rushton J, Lyons, N. Economic impact of Bluetongue: a review of the effects on production. *Vet Ital*. (2015) 51, 401–6. doi: 10.12834/VetIt.646.3183.1
- Patel A, Roy P. The molecular biology of Bluetongue virus replication. *Virus Res*. (2014) 182:5–20. doi: 10.1016/j.virusres.2013.12.017
- Hofmann M, Griot C, Chaigat V, Perler L, Thür B. Bluetongue disease reaches Switzerland. *Schweiz Arch. Tierheilk*. (2008) 150:49–56. doi: 10.1024/0036-7281.150.2.49
- Maan S, Maan NS, Nomikou K, Batten C, Antony F, Belaganahalli MN, et al. Novel bluetongue virus serotype from Kuwait. *Emerg Infect Dis*. (2011) 17:886–9. doi: 10.3201/eid1705.101742
- Zientara S, Sailleau C, Viarouge C, Höper D, Beer M, Jenckel M, et al. Novel bluetongue virus in goats, Corsica, France, 2014. *Emerg Infect Dis*. (2014) 20:2123–5. doi: 10.3201/eid2012.140924
- Schulz C, Breard E, Sailleau C, Jenckel M, Viarouge C, Vitour D, et al. Bluetongue virus serotype 27: detection and characterization of two novel variants in Corsica, France. *J Gen Virol*. (2016) 97:2073–83. doi: 10.1099/jgv.0.000557
- Sun EC, Huang LP, Xu QY, Wang HX, Xue XM, Lu P, et al. Emergence of a novel bluetongue virus serotype, China 2014. *Transbound Emerg Dis*. (2016) 63:585–9. doi: 10.1111/tbed.12560
- Savini G, Puggioni G, Meloni G, Marcaccia M, Di Domenico M, Rocchigiani AM, et al. Novel putative Bluetongue virus in healthy goats from Sardinia, Italy. *Infect Genet Evol*. (2017) 51:108–17. doi: 10.1016/j.meegid.2017.03.021
- Marcacci M, Sant S, Mangone I, Gorla M, Donato A, Zoppi S, et al. One after the others: a novel Bluetongue virus strain related to Toggenburg virus detected in the Piedmont region (North-western Italy), extends the panel of novel atypical BTV strains. *Transbound Emerg Dis*. (2018) 65:370–4. doi: 10.1111/tbed.12822

28. Huismans H, Erasmus BJ. Identification of the serotype-specific and group-specific. *Onderstepoort J Vet Res.* (1981) 48:51–8.
29. Shaw AE, Ratnien M, Nunes SF, Nomikou K, Caporale M, Golder M, et al. Reassortment between two serologically unrelated bluetongue virus strains is flexible and can involve any genome segment. *J Virol.* (2013) 87:543–57. doi: 10.1128/JVI.02266-12
30. Toussaint JF, Sailleau C, Breard E, Zientara S, De Clercq K. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *J Virol Methods.* (2007) 140:115–23. doi: 10.1016/j.jviromet.2006.11.007
31. Yin HQ, Zhang H, Shi LJ, Yang S, Zhang GP, Wang SQ, et al. Detection and quantitation of bluetongue virus serotypes by a TaqMan probe-based real-time RT-PCR and differentiation from epizootic hemorrhagic disease virus. *J Virol Methods.* (2010) 168:237–41. doi: 10.1016/j.jviromet.2010.04.018
32. Maan S, Maan NS, Belaganahalli MN, Rao PP, Singh KP, Hemadri D, et al. Full-genome sequencing as a basis for molecular epidemiology studies of blue-tongue virus in India. *PLoS ONE.* (2015) 10:e0131257. doi: 10.1371/journal.pone.0131257
33. Pavšič J, Devonshire A, Blejč A, Foy CA, Van Heuverswyn F, Jones GM, et al. Inter-laboratory assessment of different digital PCR platforms for quantification of human cytomegalovirus DNA. *Analyt Bioanal Chem.* (2017) 409:2601–14. doi: 10.1007/s00216-017-0206-0
34. OIEB. Chapter 3.1.3: Bluetongue. In: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (2019). Available online at: www.oie.int
35. George D, Czech J, John B, Yu M, Jennings LJ. Detection and quantification of chimerism by droplet digital PCR. *Chimerism.* (2013) 4:102–8. doi: 10.4161/chim.25400
36. EURL-GMFF (European Union Reference Laboratory for GM Food and Feed). *Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing.* (2015). Available online at: <https://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm> (accessed October 21, 2015).
37. Savini G. Bluetongue: a disease that does not speak ‘one tongue,’ only. *Vet Ital.* (2015) 51:247–8.
38. Foxi C, Meloni G, Puggioni G, Manunta D, Rocchigiani A, Vento L, et al. Bluetongue virus detection in new *Culicoides* species in Sardinia, Italy. *Vet Rec.* (2019) 184:621. doi: 10.1136/vr.105118
39. Paslaru AI, Mathis A, Torgerson P, Veronesi E. Vector competence of pre-alpine *Culicoides* (Diptera: Ceratopogonidae) for bluetongue virus serotypes 1, 4 and 8. *Parasites Vectors.* (2018) 11:466. doi: 10.1186/s13071-018-3050-y
40. Burns MJ, Burrell AM, Foy C. The applicability of digital PCR for the assessment of detection limits in GMO analysis. *Eur Food Res Technol.* (2010) 231:353–62. doi: 10.1007/s00217-010-1277-8
41. Vynck M, Trypsteen W, Thas O, Vandekerckhove L, De Spiegelaere W. The future of the polymerase chain reaction in virology. *Mol Diagn Ther.* (2016) 20:437–47. doi: 10.1007/s40291-016-0224-1
42. Pavšič J, Zel J, Milavec M. Digital PCR for direct quantification of viruses without DNA extraction. *Anal Bioanal Chem.* (2016) 408:67–75. doi: 10.1007/s00216-015-9109-0
43. Puggioni G, Pintus D, Meloni G, Rocchigiani AM, Manunta D, Savini G, et al. Persistence of Bluetongue virus serotype 1 virulence in sheep blood refrigerated for 10 years. *Vet Ital.* (2018) 54:349–53. doi: 10.12834/VetIt.1344.7401.3

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Rocchigiani, Tilocca, Portanti, Vodret, Bechere, Di Domenico, Savini, Lorusso and Puggioni. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Increased Susceptibility of Cattle to Intranasal RVFV Infection

Andrea L. Kroeker¹, Valerie Smid¹, Carissa Embury-Hyatt¹, Brad Collignon¹,
Mathieu Pinette¹, Shawn Babiuk^{1,2*} and Bradley Pickering^{1,3,4}

¹ Canadian Food Inspection Agency, National Centre for Foreign Animal Disease, Winnipeg, MB, Canada, ² Department of Immunology, University of Manitoba, Winnipeg, MB, Canada, ³ Department of Medical Microbiology, University of Manitoba, Winnipeg, MB, Canada, ⁴ College of Veterinary Medicine, Iowa State University, Ames, IA, United States

OPEN ACCESS

Edited by:

Dana Mitzel,
Arthropod-Borne Animal Diseases
Research Unit (USDA-ARS),
United States

Reviewed by:

Amy Hartman,
University of Pittsburgh, United States
Tetsuro Ikegami,
University of Texas Medical Branch at
Galveston, United States
Darci R. Smith,
Naval Medical Research Center,
United States

*Correspondence:

Shawn Babiuk
shawn.babiuk@canada.ca

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 03 November 2019

Accepted: 24 February 2020

Published: 29 April 2020

Citation:

Kroeker AL, Smid V, Embury-Hyatt C,
Collignon B, Pinette M, Babiuk S and
Pickering B (2020) Increased
Susceptibility of Cattle to Intranasal
RVFV Infection. *Front. Vet. Sci.* 7:137.
doi: 10.3389/fvets.2020.00137

Rift Valley Fever virus (RVFV) is a zoonotic mosquito-borne virus that belongs to the Phenuiviridae family. Infections in animal herds cause abortion storms, high mortality rates in neonates, and mild to severe symptoms. Infected animals can also transmit the virus to people, particularly people who live or work in close contact with livestock. There is currently an ongoing effort to produce safe and efficacious veterinary vaccines against RVFV in livestock to protect against both primary infection in animals and zoonotic infections in people. To test the efficacy of these vaccines it is essential to have a reliable challenge model in relevant target species, including ruminants. In this study we evaluated three routes of inoculation (intranasal, intradermal and a combination of routes) in Holstein cattle using an infectious dose of 10^7 pfu/ml and a virus strain from the 2006–2007 outbreak in Kenya and Sudan. Our results demonstrated that all routes of inoculation were effective at producing viremia in all animals; however, the intranasal route induced the highest levels and longest duration of viremia, the most noticeable clinical signs, and the most widespread infection of tissues. We therefore recommend using the intranasal inoculation for future vaccine and challenge studies.

Keywords: virus, Rift Valley Fever, cattle, animal model, Phenuiviridae

INTRODUCTION

Rift Valley Fever virus (RVFV) is a single-stranded RNA virus that belongs to the Phenuiviridae family. It was first described in Eastern Africa in the early 1900s (1) and initially drew attention during animal outbreaks that resulted in high rates of abortion. Since it was first detected, RVFV has spread to new regions and continues to circulate widely throughout much of Africa (2, 3). Serosurveys have demonstrated the presence of antibodies against RVFV in a variety of animal species including domestic ruminants such as sheep, goats, cattle, alpacas and camels in addition to a variety of wildlife such as the African buffalo (2, 4–8). Interestingly, these serosurveys have shown that RVFV circulates not only during outbreaks but also during inter-epidemic periods including areas where outbreaks have never occurred (9, 10). Although the number of seropositive animals varies widely based on timing and region, where seropositivity ranges from 0 to 100% in sheep and cattle, to 0–50% in goats and 0–30% in camels and humans (2, 3), these studies clearly highlight the important role that animals play in the evolution and spread of RVFV.

Despite its widespread presence in Africa, RVFV outbreaks only occur sporadically and do not necessarily occur in every area with seropositive animals. Outbreaks typically occur during

periods of increased rain which are associated with an increase in mosquito populations (11). In ruminants, outbreaks are characterized by abortion storms and high rates of mortality, especially in neonates. Although mortality rates can vary significantly between different outbreaks, during the South African outbreak in 2010–2011, adult cattle, sheep and goats had an estimated 50–62% mortality rate while camels, buffaloes and other wildlife species experienced 100% mortality (12). Other studies have also reported high rates of abortions such as 70% in sheep and goats during outbreak in Mauritania in 2003 (13).

RVFV outbreaks also pose significant risks to human populations. Susceptible animals such as ruminants amplify the virus to titers that are high enough to transmit to humans and are one of the primary reservoirs for human infections. The major risk factors associated with RVFV infections in humans are related to close proximity with livestock, including animal husbandry, animal slaughtering and exposure to raw milk (14–18). In addition to health risks, the loss of fetuses and newborn livestock to RVFV infections can have a severe socio-economic impact on farmers (19). Together this data suggests that vaccinating livestock against RVFV may be highly beneficial not only in protecting livestock but also to the people who are in direct contact with them (20, 21). Since the risk of human infections increases as the seropositivity increases in animal populations (22), surveillance systems in countries where RVFV circulates are extremely important. Although RVFV surveillance data for many African or other at-risk countries is currently sparse, the development of international surveillance networks (23–26) will make it much easier to monitor and share data regarding the presence of RVFV. Surveillance data will also be useful for informing vaccination programs about areas requiring preferential targeting. These regions should also be studied to identify potential barriers against uptake of the vaccine; for example, limited health education and cost of the vaccine have prevented vaccination of livestock in the past (27).

There are currently several RVFV veterinary vaccine options available to African farmers such as formalin-inactivated vaccines and the Smithburn vaccine. However, the formalin-inactivated vaccine is inadequate at preventing viremia (28) and safety issues have been identified with the live attenuated Smithburn vaccine (29), which have stimulated the development of several new RVFV vaccines (30). Some of the new RVFV vaccines have already undergone safety and immunogenicity testing in sheep such as a four-segmented RVFV vaccine (31), a Gn subunit vaccine (32), a DNA vaccine containing either GP and NP genes (33), a non-spreading (NSR) RVFV vaccine (34) and an equine herpesvirus type 1 vector (35); others have been tested for safety and immunogenicity in other natural host species including a Gn-based vaccine with a paramyxovirus vector in sheep and calves (36, 37), a Gn-based vaccine with a modified vaccinia Ankara vector in sheep and baboons (38, 39), a Gn-based vaccine with a Chimpanzee adenovirus vector in sheep, calves and camels (40), a Gn-based vaccine with capripox vector (33, 41, 42), MP12 in sheep, goats and cattle (43), and Clone 13 in sheep, goats, calves and camels (44–50). In terms of efficacy, many novel RVFV vaccines have proven efficacious in mouse models; however,

as of yet, only a few efficacy challenges have been performed in ruminants: a Gn subunit vaccine (51), R566 (52) and non-spreading vaccine were 100% efficacious in sheep (52), and Clone 13 was 100% efficacious in sheep (45) and cattle (50).

Recently, in partnership with Kansas State University, we sought to develop optimal RVFV infections in ruminants to provide tools for evaluation of vaccines. These include sheep (53, 54), goats (54–56), and cattle (57) which were tested using a variety of factors such as different virus doses, viral strains, routes of inoculation, and animal breeds. While sheep and goats had consistent viremia, cattle proved to be more resistant to infection as only 2 out of 5 animals developed robust viremia (57). Therefore, the aim of this study was to develop a robust RVFV infection with an increased proportion of cattle with viremia.

The experimental design of this study was based on the cattle model developed at Kansas State University (57) as well as previous sheep and goat model development at the NCFAD (54–56), but with several adaptations. Holstein calves were used instead of the Angus breed, although the age range of the animals was similar and at an appropriate age for vaccination (4–6 months). A unique virus isolate from the Kenya/Sudan 2006–2007 outbreak, previously characterized in goats [RVFV-UAP (55), at a slightly higher inoculation titer (10^7 pfu instead of 10^6 pfu)]. The RVFV-UAP virus isolate was chosen for the goat study because the Wilson group had had good success with the Ken06-128b isolate in terms of inducing viremia and systemic spread to the tissues, including liver lesions and detection of virus in the brain (57). However, due to the complexities in shipping live viruses between countries, the RVFV-UAP isolate was evaluated instead. Since the RVFV-UAP isolate and the 10^7 pfu dose proved to be robust in goats (55), it was used in the current study. The inoculation titer of 10^7 pfu was chosen based on previous sheep and goat model data from NCFAD (54, 55). Different routes of inoculation were evaluated. Whereas, Wilson et al. had used subcutaneous inoculation which is widely utilized in the literature, it was previously demonstrated that the intranasal route could induce higher levels of viremia in goats than the subcutaneous route (55) and therefore it was hypothesized that the intranasal route may work well in cattle as well. Although most of the literature utilizes subcutaneous injections, it is possible that a different subset or a greater number of dendritic cells could be infected by intradermal injection. For example, dendritic cells have been shown to infiltrate the dermis upon infection and play a role in presenting antigens from skin vaccinations and infections (58, 59). In addition, a combination of routes was used (10^7 pfu subcutaneous, 10^7 pfu intradermal and 10^7 pfu intranasal) in the anticipation that cattle could be fairly resistant to infection and may require more than just a single injection. It was previously demonstrated that intravenous injection was not any better than a subcutaneous injection at inducing viremia (54), and similarly, inoculating twice (once on day 1 and a second inoculation on day 2) did not increase viremia titers over and above a single inoculation (54). By modifying the parameters from the previous RVFV challenge models, we sought to increase the robustness of the cattle challenge model to more effectively test RVFV vaccines

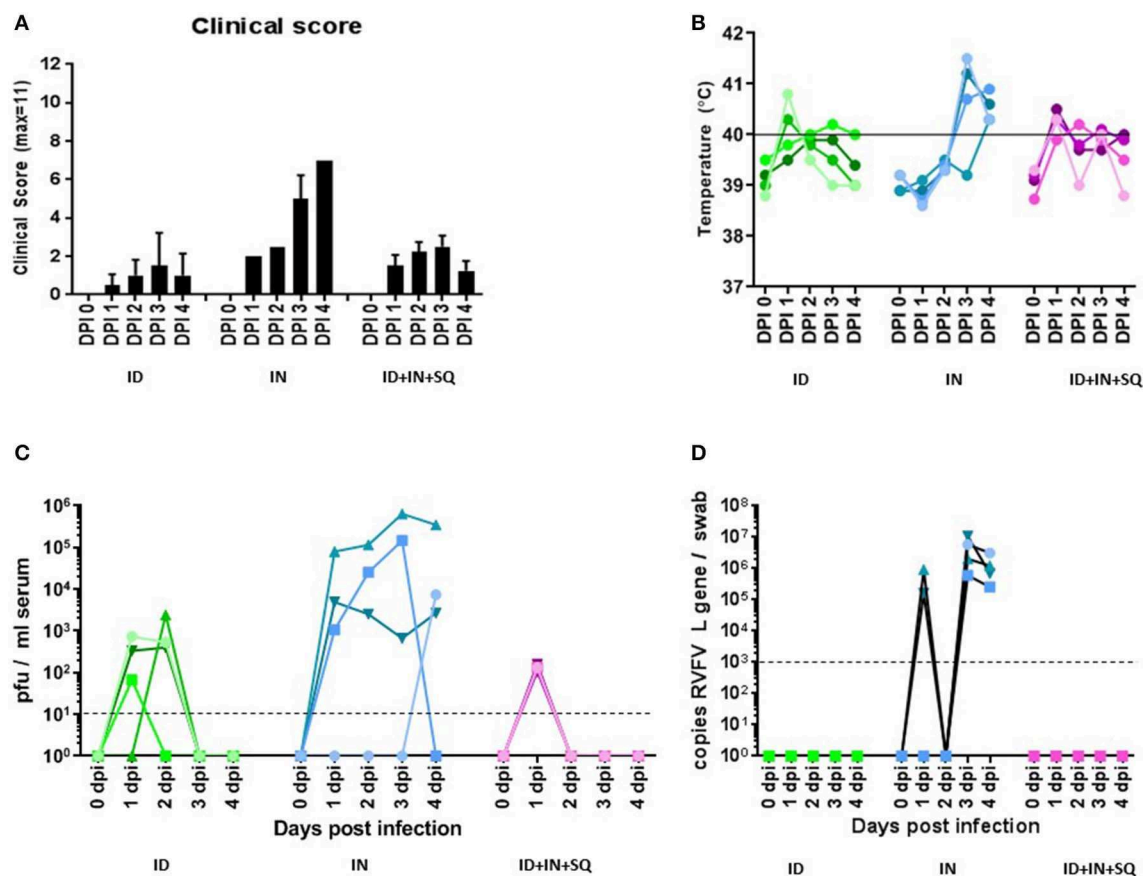


FIGURE 1 | Clinical signs, viremia, and shedding. **(A)** All animals were assessed daily for signs of disease, rectal temperature, eating and drinking habits, disposition and stool consistency and given a clinical score. The average clinical score per group of animals ($n = 4$) is shown. **(B)** Rectal temperatures for each animal on a daily basis; each value represents an individual animal. **(C)** Infectious virus was measured in the blood by plaque assay on a daily basis; each value represents an individual animal. The horizontal dashed line indicates the detection limit of the plaque assay. **(D)** Viral RNA was measured in nasal swabs by RT-PCR on a daily basis; each value represents an individual animal. The horizontal dashed line indicates the diagnostic detection limit of the RT-PCR assay.

and to minimize the number of animals needed to produce statistically relevant vaccine efficacy data.

RESULTS

Clinical Signs, Temperature, Clinical Chemistry, and Viremia

Throughout the experiment, animals were evaluated for clinical signs of disease on a daily basis. The clinical score was a sum of the animals' general appearance, rectal temperature, alertness, eating and drinking habits, and stool consistency. The endpoint was defined as reaching a clinical score of 11, not eating or drinking for more than 24 h or for any other unforeseen cause identified by the institutional veterinarian. For the intradermal and combination groups, the signs of disease were very mild with clinical scores of 1.3 to 2.5 after inoculation (Figure 1A); with the exception of a mild fever around 39.5–40°C (Figure 1B), the animals were generally asymptomatic. In contrast, the intranasal inoculation produced mild but noticeable clinical signs with a clinical score of 6 and 7 on days 3 and 4 (Figure 1A), which

was accompanied by a more pronounced fever between 40–41°C (Figure 1B). A summary of the individual scoring data can be found in **Supplementary Table 1**.

Viremia was measured daily using plaque assays. All animals in all groups became viremic, although the duration and level of viremia varied. In the intradermal group, infectious virus was detected on days 1 and 2 with peak levels of 10³ pfu/ml serum (Figure 1C). The combination group generated 10² pfu/ml of infectious virus only on day 1 (Figure 1C). The intranasal group developed the highest levels of virus, showing viremia on all 4 days with infectious virus ranging from 7 × 10² to 6 × 10⁵ pfu/ml serum (Figure 1C) with peak levels on day 3 or 4.

In addition, a clinical biochemistry panel was performed on the serum to evaluate the impact of RVFV infection on organ function. Mild increases were observed in ALB, TP, ALP, CA in all groups and additional increases in BUN were seen in the intranasal group. The average values for each group are listed in **Tables 1–3**, and the individual data can be viewed in **Supplementary Table 2**.

TABLE 1 | Average serum clinical chemistry values for the intradermal inoculation group.

	Normal range	DPI 0	DPI 1	DPI 2	DPI 3	DPI 4
ALB	2.5–3.8	3.40	4.68	5.18	3.95	3.90
ALP	23–135	127.50	201.25	236.75	160.25	145.50
AST	66–211	70.25	108.50	129.00	75.75	67.75
CA	7.9–9.6	10.75	13.73	15.33	11.88	11.70
GGT	12–48	16.50	22.75	28.00	18.50	20.00
TP	6.6–9.3	7.60	9.20	11.13	7.63	7.50
GLOB	4.4–5.5	3.50	4.50	5.45	3.68	3.60
BUN	6–20	14.25	12.50	15.25	10.75	9.25
CK	83–688	320.00	437.25	482.50	363.00	252.50
PHOS	4.1–9.2	7.00	9.00	10.40	7.48	7.60
MG	1.7–2.9	2.38	2.73	3.25	2.30	2.28

TABLE 2 | Average serum clinical chemistry values for the intranasal inoculation group.

	Normal range	DPI 0	DPI 1	DPI 2	DPI 3	DPI 4
ALB	2.5–3.8	3.68	4.80	3.98	3.78	4.38
ALP	23–135	136.00	181.00	138.00	137.25	183.00
AST	66–211	63.50	99.25	78.50	142.25	189.25
CA	7.9–9.6	11.28	13.97	12.00	11.00	12.55
GGT	12–48	89.00	100.00	75.75	68.25	79.75
TP	6.6–9.3	7.33	10.40	7.75	7.35	9.00
GLOB	4.4–5.5	3.65	5.63	3.78	3.55	4.60
BUN	6–20	16.50	22.75	16.75	18.75	24.75
CK	83–688	281.50	444.50	554.50	312.25	393.25
PHOS	4.1–9.2	6.73	8.23	7.30	5.70	8.33
MG	1.7–2.9	2.28	3.13	2.33	1.98	2.50

TABLE 3 | Average serum clinical chemistry values for the combination inoculation group.

	Normal range	DPI 0	DPI 1	DPI 2	DPI 3	DPI 4
ALB	2.5–3.8	3.68	4.49	5.28	4.63	4.37
ALP	23–135	136	143.38	234.92	174.08	159.25
AST	66–211	63.5	87.00	105.42	86.25	81.08
CA	7.9–9.6	10.01	12.15	11.30	7.21	12.80
GGT	12–48	16.5	20.94	22.17	18.08	17.83
TP	6.6–9.3	7.33	9.17	11.01	9.19	8.56
GLOB	4.4–5.5	3.65	4.67	5.71	4.54	4.19
BUN	6–20	16.5	15.88	23.17	18.75	18.83
CK	83–688	281.5	306.50	328.08	291.50	284.58
PHOS	4.1–9.2	6.73	9.93	10.84	9.02	9.28
MG	1.7–2.9	2.28	2.74	3.43	2.54	2.63

Shedding and Mucosal Immunity

No infectious virus was isolated in the nasal and oral swabs at any time or in any group, and no viral RNA was detected in any of the oral swabs. However, viral RNA was observed in nasal swabs by qRT-PCR in the intranasal group with levels between 10^5 and 10^7 copies/swab at days 1, 3, and 4 post infection (**Figure 1D**).

Due to the lack of infectious virus despite high levels of viral RNA in the nasal swabs, it was hypothesized that any virus in the nasal cavity had been inhibited directly, for example through the antiviral action of interferons on cells in the nasal cavity. Therefore, ELISAs were performed to monitor the levels of interferons alpha (IFN- α), beta (IFN- β) and gamma (IFN- γ) in the swabs. In the intranasal group, IFN- α , IFN- β , and IFN- γ were all detected in the nasal swabs starting at 1 or 2 dpi and peaked at 40–60 ng/swab at 3 or 4 dpi (**Figures 2A–C**); in comparison, the oral swabs from the intranasal group contained similar amounts of IFN- α but did not contain significant amounts of IFN- β or IFN- γ , except in one animal (**Figures 2D–F**). The intradermal inoculation also contained levels of IFN- β and IFN- γ in the nasal swabs, with peak levels at 20–50 ng/swab at 4 dpi, but not IFN- α (**Figures 2A–C**); in comparison, the oral swabs from two animals in the intradermal group had increased IFN- β after infection, while IFN- α and IFN- γ did not change from baseline (**Figures 2D–F**). We did not detect INFs in the nasal or oral swabs from the combined inoculation route group except for a low level of INF- α at 3–4 dpi (**Figure 2E**).

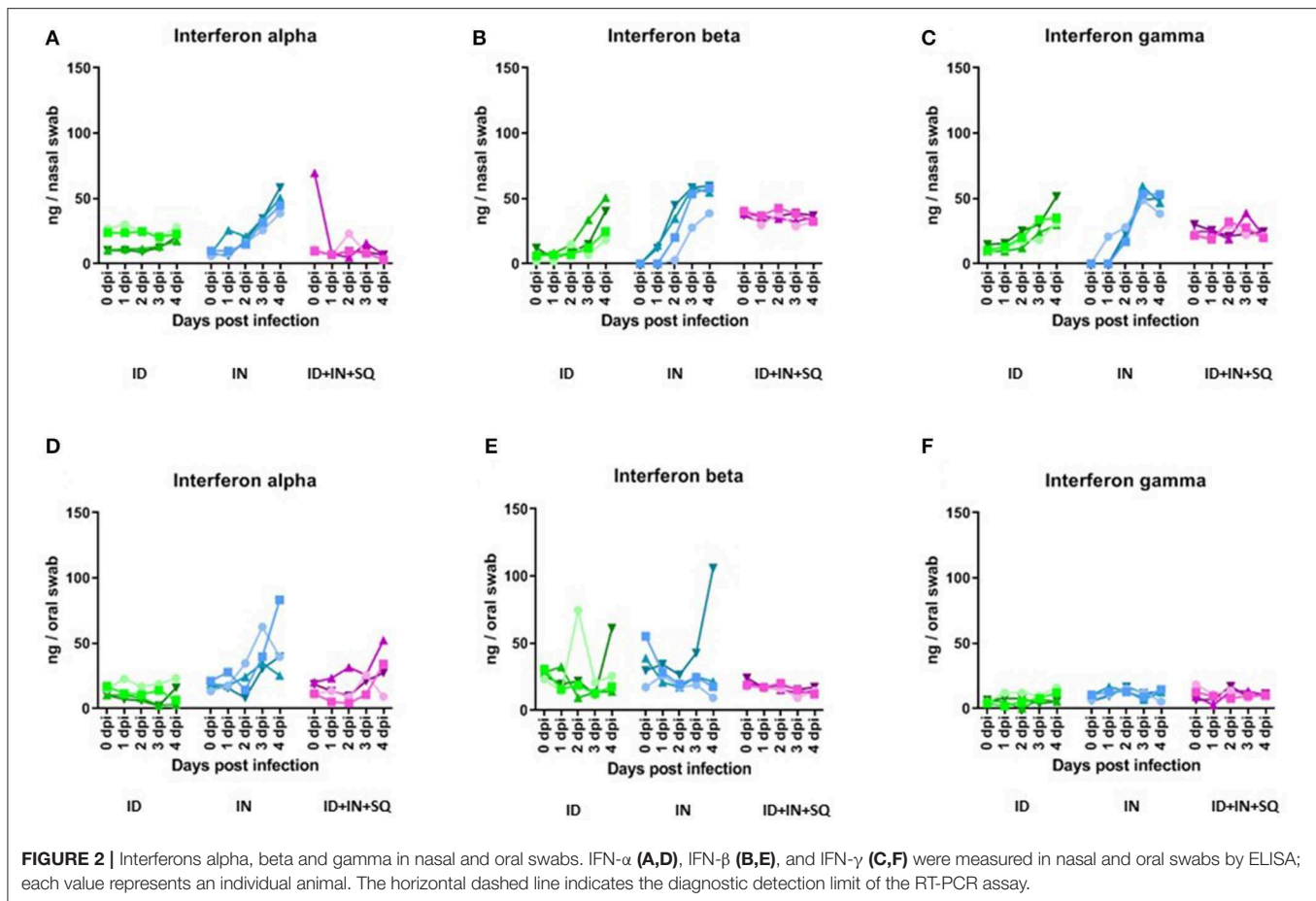
Infection of Tissues and Pathology

The endpoint was determined by the parameters chosen to compare future vaccinated and non-vaccinated groups including: viremia, virus isolation from tissues, changes in blood chemistry, viral shedding in swabs, clinical signs and liver pathology. If present, all of these parameters should be detectable throughout the acute phase of infection (usually within the first week after infection); however, based on data from Wilson's cattle model, infectious virus is only present in the tissues at days 3 and 4 post infection (57); therefore 4 dpi was chosen as the endpoint. Inspection of the animals at necropsy did not indicate any gross pathology, except for animal #1835 in which we noted significant

fibrosis in the liver. Tissues were collected fresh to evaluate viral loads using virus isolation or placed into formalin for sectioning to identify lesions if present.

The intradermal and combination groups presented the fewest number of tissues infected by the virus; the intradermal group harbored virus in the spleen, turbinates, prescapular lymph nodes, and retropharyngeal lymph node (**Figure 3**, ID group) and the combination group contained virus in the liver, turbinates, olfactory bulb and trigeminal nerve (**Figure 3**, ID-IN-SQ group). In contrast, the intranasal group had the greatest number of tissues infected by RVFV; infectious virus was isolated from mesenteric and retropharyngeal lymph nodes, spleen, liver, lung, trachea, turbinate, ileum, heart, brainstem, cerebellum, midbrain, and cerebral spinal fluid (**Figure 3**, IN group). Some of the tissues were consistently infected in all four animals within a group; however, some tissues were only infected in one or two animals within a group, in line with the variability between animals that is commonly seen in livestock.

Livers from all 3 groups of inoculated animals had lesions that were consistent with RVFV infection; however, they differed slightly in severity and stage of pathogenesis. Livers from



animals in the ID-IN-SQ combined inoculation group had small numbers of lesions (**Figure 4A**, arrow, calf 1818) which on higher magnification (**Figure 4B**) were characterized by areas of hepatocyte necrosis (arrows) and loss with replacement by a mixed inflammatory infiltrate (*); the presence of RVFV in the lesions was confirmed using *in situ* hybridization (**Figure 4C**, calf 1818). Most livers from the intranasally inoculated animals had numerous lesions (**Figure 4D**, arrows, calf 1836) which on higher magnification (**Figure 4E**) were characterized by replacement of normal hepatocytes (*) with large areas of necrosis (delineated by arrows); the presence of RVFV lesions was confirmed using *in situ* hybridization (**Figure 4F**, calf 1836). Numerous lesions were also observed in several livers from the intradermally inoculated group (**Figure 4G**, arrows, calf 1912). In this group there was significant hemorrhage associated with the areas of hepatocyte loss (**Figure 4H**); the presence of RVFV in the lesions was confirmed using *in situ* hybridization (**Figure 4I**, calf 1912). In contrast, no lesions were found in the spleen of any animal.

METHODS

Ethics Statement

All animal experiments were carried out in the enhanced biosafety level 3 (BSL3) facility at the National Centre for Foreign

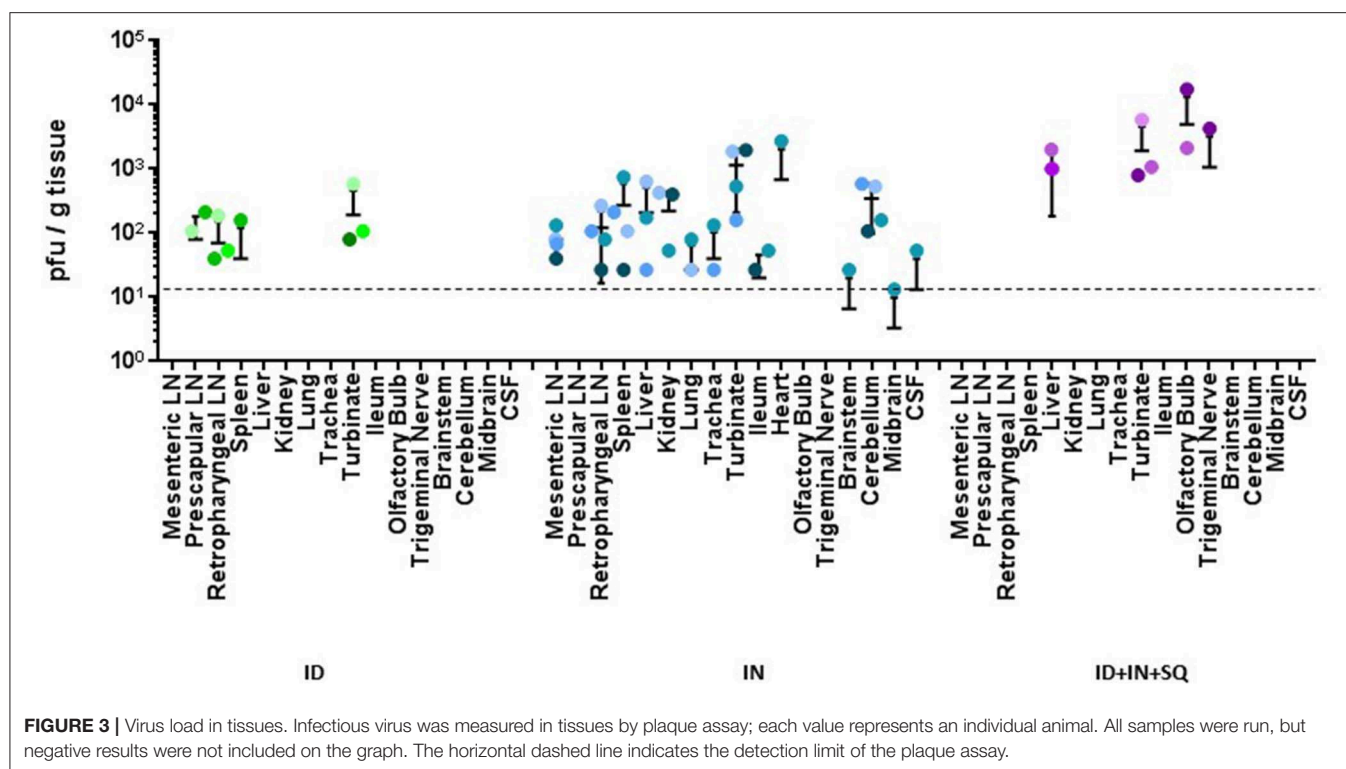
Animal Disease (NCFAD) in Winnipeg, Manitoba. All protocols for animal use were approved under the animal document use number C-17-002 at the Canadian Science Centre for Human and Animal Health (CSCAH) in Winnipeg, Manitoba by the Animal Care Committee. Care was taken to minimize animal suffering and to follow the Canadian Council on Animal Care guidelines for animal manipulations.

Cells

Mosquito C6/36 cells (ATCC, USA) were grown and infected in 1:1 EMEM and ESF-921 (Expression Systems, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% L-glutamine and maintained at 28°C without CO₂. Mammalian Vero E6 (VE6) cells were grown and infected in DMEM (Gibco) supplemented with 10% FBS and maintained at 37°C with 95% relative humidity and 5% CO₂.

Virus Production and Titration

VE6 cells were infected with a virus isolate from the 2006–2007 Kenyan outbreak (RVFV-UAP; Genbank #MH175203, MH175204, MH175205) (55) at an MOI 0.1 and maintained in DMEM with 10% FBS. Thereafter, virus was alternatively propagated between VE6 and C636 cells twice. All passages were titrated on VE6 cells with a plaque assay to determine virus



concentration. The calves were then infected using passage 6 C6/36-derived virus.

RVFV Inoculation of Cattle

Twelve Holstein calves (3–4 months) were inoculated with RVFV-UAP that was grown in mosquito cell culture. Group 1 ($n = 4$) received intradermal (ID) inoculation; group 2 ($n = 4$) received intranasal (IN) inoculation; and group 3 ($n = 4$) received a combination (SQ/ID/IN) of all three routes. The subcutaneous injections consisted of 1×10^7 pfu in 100 μ l PBS in the left flank; the intradermal inoculations consisted of five injections of 2×10^6 pfu in 100 μ l PBS each in the left lumbosacral region; and the intranasal inoculations consisted of 1×10^7 pfu in 1 ml PBS with half in each nostril.

Sampling

All calves were carefully monitored for signs of illness and rectal temperature on a daily basis. We also collected serum on a daily basis and stored at -70°C . Nasal and oral swabs were collected on a daily basis, placed into 2 ml sterile PBS containing antibiotics and an antifungal and stored at -70°C .

Clinical Chemistry

Serum biochemistry was evaluated daily with the VetScan VS2 blood analyzer (Abaxis, USA) and Large Animal Profile rotors (Abaxis, USA). All assays were run as per manufacturer's instructions and the bovine reference ranges were provided by Abaxis.

Post-mortem Tissue Collection

At 4 days post infection we examined the calves for changes in gross pathology and collected fifteen tissues including liver, spleen, kidney, lung, ileum, retropharyngeal lymph node, prescapular lymph node, mesenteric lymph node, cerebral spinal fluid, brainstem, midbrain, cerebellum, olfactory bulb and trigeminal nerve. Separate pieces of each tissue were collected fresh and subsequently frozen at -80°C or placed in 10% formalin.

Tissue Homogenization

We made 10% homogenates of each tissue by placing 5 g of tissue in a 7 ml PreCellys tube and adding 5 ml with PBS. This sample was then homogenized for 30 s at maximum speed using the Personal Homogenizer. A single homogenate was used for both downstream qRT-PCR and plaque assays without any freeze/thaw cycles.

Virus Isolation From Oral and Nasal Swabs

Virus was isolated from oral and nasal swabs using two blind passages in 95% confluent monolayers of Vero E6 cells: 200 μ l of each swab was adsorbed to cells in 24-well plates for 1 h at 37°C with gentle rocking, then overlaid with 1 ml serum-free DMEM, incubated for 7 days, and were visually checked for cytopathic effects. The entire contents from each well were then transferred to cells in T25 flasks, adsorbed to cell for 1 h at 37°C with gentle rocking, then overlaid with 4 ml serum-free DMEM, incubated for a further 7 days, and were visually checked for cytopathic effects.

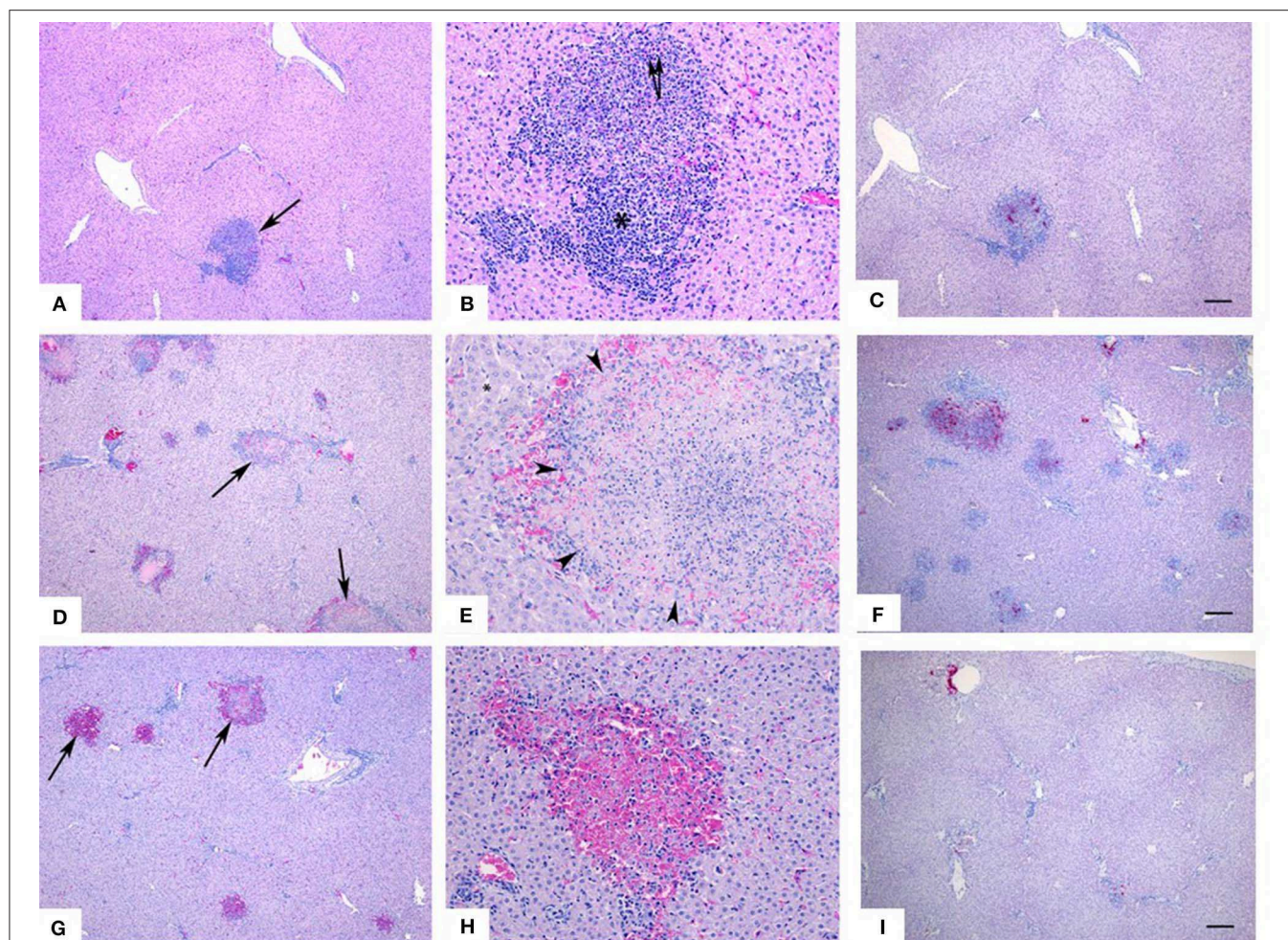


FIGURE 4 | Liver histopathology and *in situ* hybridization. Livers from animals in the ID-IN-SQ combined inoculation group had small numbers of lesions (A, arrow) which on higher magnification (B) were characterized by areas of hepatocyte necrosis (arrows) and loss with replacement by a mixed inflammatory infiltrate (*). Most livers from intranasally inoculated animals had numerous lesions (D, arrows) which on higher magnification (E) were characterized by replacement of normal hepatocytes (*) with large areas of necrosis (delineated by arrows). Numerous lesions were also observed in several livers from the intradermally inoculated group (G, arrows). In this group there was significant hemorrhage associated with the areas of hepatocyte loss (H). The presence of RVFV in the lesions was confirmed using *in situ* hybridization, in bright pink (C,F,I).

Virus Quantitation by qRT-PCR

RVFV RNA was extracted from serum using the TriPure Isolation Reagent (Roche) according to the manufacturer's instructions. Purified RNA was stored at -70°C . Viral RNA was detected using the TaqMan Fast Virus 1-Step RT-PCR master mix as per manufacturer's instructions and ran the samples on the ABI 7500 thermocycler with the following conditions: 5 min at 50°C , 2 min at 95°C and 40 cycles of 3 s at 95°C and 30 s at 60°C . Primers (Invitrogen) and probe (Biosearch) targeted nucleotides 2912 to 3001 for the RVFV L gene segment. All Ct values were plotted on a standard curve using a DNA plasmid containing the targeted RVFV L gene segment (GenScript) and quantified.

Virus Quantitation by Plaque Assay

Serial dilutions of serum, nasal swabs, oral swabs and 10% tissues homogenates were used to infect confluent monolayers of VE6 cells in 48-well plates. Seventy five microliter of inoculum was

added to the cells in triplicate for 1 h at 37°C with rocking. The inoculum was then removed and the cells were overlaid with 2 ml 1.75% carboxymethylcellulose (CMC). After 4 days the cells were formalin-fixed and stained with 0.5% crystal violet (Sigma) to visualize and count plaques.

ELISA for Interferons

We used bovine interferon alpha (IFN- α A), beta and gamma ELISA kits (Kingfisher Biotech Inc., USA) to detect protein in nasal and oral swabs. 96-well MaxiSorp ELISA plates (Nunc) were coated with 2.5 ng/ml capture antibody diluted in DPBS and incubated at room temperature for 24 h. The plates were blocked with DPBS+4% bovine serum albumin at room temperature for 1 h. Oral and nasal samples were diluted 1:2 in DPBS before plating, standards were diluted in DPBS+4% bovine serum albumin and plates were incubated at room temperature for 1 h. Detection antibody was diluted in DPBS +4% bovine

serum albumin at room temperature for 1 h, followed by 5 washes in TBS-Tween20 (0.05%). Plates were then incubated with Streptavidin-HRP at room temperature for 1 h, followed by 5 washes in TBS-Tween 20 (0.05%). TMB was added for colorimetric development, followed by 2N sulfuric acid as a stop solution. Plates were read on an Epoch (Biotek) plate reader at 450 nm.

Tissue Sectioning and Staining

Five-micron paraffin-embedded formalin fixed tissue sections were cut, air-dried, and melted onto charged slides in a 60°C oven. The slides were then cleared and hydrated in xylene and 100% ethanol, and then air-dried. The sections were stained with hematoxylin and eosin (H&E) and imaged with a Zeiss microscope at 40X and 200X.

In situ Hybridization

For the ISH technique, 5 µm paraffin-embedded formalin fixed tissue sections were cut, air dried then melted on to the charged slides in a 60°C oven. Then the slides were cleared and hydrated in xylene and 100% ethanol then air dried. The sections were quenched for 10 min in aqueous H₂O₂, boiled in target retrieval solution for 15 min, rinsed in 100% ethanol and air dry again. Then a final pre-treatment of protease plus enzyme for 15 min at 40°C was applied. The probe (V-RV-FV-ZH501-NP, from Advanced Cell Diagnostics) was applied and incubated at 40°C for 2 h. Then the Hybridization amplification steps (AMP 1-6) are applied to the slides for the recommended times and temps as per the manual for the RNAscope® 2.5HD Detection Reagent – Red kit (ACD). The signal is then visualized by the chromogen Fast Red. The sections were then counter stained with Gill's 1 hematoxylin, dried, cleared and cover-slipped.

DISCUSSION

Robust and reliable models are essential for efficient vaccine evaluation. For RVFV, there are currently a variety of effective small animal, NHP, sheep and goat models, although cattle have proven more difficult to reliably infect. In this study, successful infection of 3–6 month old Holstein calves with three different inoculation routes was demonstrated: intradermal (ID), intranasal (IN) and a combination of intradermal, intranasal and subcutaneous (SQ/ID/IN). All three routes reliably elicited viremia, with the combination and ID routes producing similar viral titers while the IN route generated much higher viral titers. The clinical scores for each group correlated strongly with the intensity of viremia, where higher clinical scores and rectal temperatures were seen in the IN group whereas mild to asymptomatic clinical scores were observed in the ID and combination groups.

Clinical biochemistry markers were evaluated to monitor organ function throughout infection which indicated mild increases in albumin (ALB), total protein (TP), alkaline phosphatase (ALP) and calcium (CA) in all groups and elevated blood urea nitrogen (BUN) levels in the intranasal group. Interestingly, a more prominent elevated level of ALP was

detected in Wilson's cattle model in both SA01 and Ken06 infected groups, but not in the uninfected control group (57). The mild increases in our current model could potentially be due to bone growth as our cattle are still growing; however, in light of Wilson's data it is also possible that the increase could be due to the infection. More data is needed to evaluate this further. Other clinical chemistry values that were elevated in this study include ALB, TP, and CA, which might indicate mild dehydration; the same mild elevations were not found in Wilson's study (57). Another change that was specific to the intranasal group was an increase in BUN levels. One cause of elevated BUN levels could be dehydration; however since only the intranasal group was affected and only the intranasal group had infectious virus isolated from the kidney, this may again be RVFV specific. Future experiments could include a urinalysis to confirm this. During the necropsy, it was noted that calf 1,835 had significant areas of portal fibrosis and bile duct hyperplasia in the liver which likely caused impairment of liver function prior to arriving at our facility and explains the high GGT values in that specific animal. Liver necrosis was also detected in all animals; however, as liver enzyme levels such as AST were not considered clinically abnormal, the extent of liver damage was likely not extensive enough to compromise organ function.

Interestingly, infectious virus was found in the turbinates of all three groups, suggesting that the nasal swabs could contain virus. Yet, only the intranasal group had detectable viral RNA in the nasal swabs and none of them contained infectious virus. These results were consistent with previous reports in Nubian goats (55); however, one other study has reported infectious virus in nasal swabs (57). While assaying potential virus neutralizing components in swabs, we found both ID and IN infections induced IFN-β and IFN-γ secretion, but only the IN infection contained IFN-α in nasal swabs. Many cell types can secrete interferons, which then act on the same or nearby cells to induce an intracellular antiviral state. Therefore, the presence of interferons in the swabs could indicate that the nasal and oral mucosal environment may be able to prevent active replication of RVFV through activation of an antiviral state via interferon and copies of viral RNA detected may represent incomplete virus found in the cytoplasm of cells. Alternatively, other components that were not measured may also be present in the swabs that are able to neutralize infectious virus, such as antibodies.

The only tissue to be consistently infected in all three experimental groups was the turbinate. The IN infection was much more widespread than the other groups and had a higher number of infected tissues, which may have been due to the increased titers and duration of viremia in this group. Perhaps most strikingly, some brain tissues and the cerebral spinal fluid (CSF) in one animal produced infectious virus in the IN group but not in the other groups, at least as measured at 4 days post infection. Other studies have identified RVFV in brain tissues as well, although it is unusual to find data on different areas of the brain. For example, RVFV was isolated from the brains of 21 day old calves infected subcutaneously with RVFV (60), 4 month old calves infected subcutaneously with RVFV (57) or 4 month old goats infected subcutaneously with RVFV (55). In addition, clinical neurological manifestations could be readily

seen in young 21 day old calves (60), but not in the 4 month old animals.

In comparison to Wilson's previous cattle model study, it was hypothesized that the success in infecting all animals was due to differences in virus dose, isolate, or cattle breed. Unfortunately there is no information about how the pathogenicity compares between the Kenya-128b isolate used in Wilson's study and the RVFV-UAP isolate used in the current study. The two isolates were shown to be phylogenetically distinct but still very similar in sequence (55). Any sequence or amino acid changes did not fall within critical sites that have been characterized such as the RNA polymerase active site or phosphorylation sites, although point mutations are not well-characterized in RVFV and their effects are unknown. It is also difficult to speculate whether the Holstein cattle breed was more susceptible to RVFV than the Angus breed as we did not directly compare the two with the same parameters. While more groups and more comparisons would have been scientifically interesting, we could not justify all of them due to the number of animals required. Since the focus was on developing an effective challenge model, parameters were chosen that would most likely produce a reliable challenge model with viremia.

Age is an important factor in RVFV cattle infections as both disease severity and the ability to mount an immune response to a vaccine are age dependent. For example, young ruminant neonates (1–2 months) are highly susceptible to RVFV with mortality rates of up to 100% and would demonstrate a severe RVFV infection. However, the goal was to create a RVFV infection for testing vaccines at an age with a mature immune system. It was previously demonstrated that 3–6 month-old sheep, goats and cattle all mount robust immune responses against RVFV infection (54–57). Perhaps because of these strong immune responses, the overall disease severity in our model was quite mild, especially considering the fact that RVFV can be lethal to adult ruminants during outbreaks. In this respect, it is also worth considering the fact that our animals were all of high health status, well-fed, in temperature-controlled housing, free of any obvious underlying disease, and free of many stressors.

CONCLUSION

This study was conducted to determine an optimal RVFV infection in cattle for vaccine efficacy studies. Overall, it was demonstrated that RVFV infection could be achieved via three different routes of infection in vaccine-aged cattle using an endpoint at 4 days post infection. This day coincides with the peak of infection and is ideal to compare vaccinated to non-vaccinated animals. Interestingly, all three routes were effective at inducing viremia and producing liver lesions, which are two

major hallmarks of RVFV infection. However, a major difference between the groups consisted of increased systemic spread of the virus to tissues in the intranasal group by 4 dpi, which was much less pronounced in the other groups. As the intranasal route is not thought to be a natural route of infection for livestock, the intradermal or subcutaneous models may mimic a natural infection more closely. However, the intranasal route generated the most severe clinical disease and most robust virus replication, making it an excellent challenge model to use to evaluate the ability of RVFV vaccines to decrease viremia in cattle.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by CACC.

AUTHOR CONTRIBUTIONS

Conceptualization, data curation, writing—original draft preparation, visualization, and project administration: AK. Methodology: MP. Formal analysis: AK and CE-H. Investigation: AK, VS, and BC. Writing—review and editing and supervision: AK, BP, and SB. Funding acquisition: BP and SB.

FUNDING

This study received funding through MCI Sante Animale from the International Development Research Centre (IDRC) Canada. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

ACKNOWLEDGMENTS

The authors would like to thank Nicole Fernandez from the University of Saskatchewan for her expert interpretation of the clinical chemistry data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2020.00137/full#supplementary-material>

Supplementary Figure 1 | Viral loads in serum and tissues.

REFERENCES

1. Daubney R, Hudson JR, Garnham, PC. Enzootic hepatitis or Rift Valley Fever. An undescribed virus disease of sheep, cattle and man from East Africa. *J Pathol Bacteriol.* (1931) 34:545–79. doi: 10.1002/path.1700340418
2. Clark MHA, Warimwe GM, Di Nardo A, Lyons NA, Gubbins S. Systematic literature review of Rift Valley Fever virus seroprevalence in livestock, wildlife and humans in Africa from 1968 to 2016. *PLoS Negl Trop Dis.* (2018) 12:e0006627. doi: 10.1371/journal.pntd.0006627

3. Nanyingi MO, Munyua P, Kiama SG, Muchemi GM, Thumbi SM, Bitek AO, et al. A systematic review of Rift Valley Fever epidemiology 1931–2014. *Infect Ecol Epidemiol.* (2015) 5:28024. doi: 10.3402/iee.v5.28024
4. Beechler BR, Bengis R, Swanepoel R, Paweska JT, Kemp A, van Vuren PJ, et al. Rift Valley Fever in Kruger national park: do buffalo play a role in the inter-epidemic circulation of virus? *Transbound Emerg Dis.* (2015) 62:24–32. doi: 10.1111/tbed.12197
5. Capobianco Dondona A, Aschenborn O, Pinoni C, Di Gialleonardo L, Maseke A, Bortone G, et al. Rift Valley Fever virus among wild ruminants, Etosha National Park, Namibia, 2011. *Emerg Infect Dis.* (2016) 22:128–30. doi: 10.3201/eid2201.150725
6. Olive MM, Goodman SM, Reynes JM. The role of wild mammals in the maintenance of Rift Valley Fever virus. *J Wildl Dis.* (2012) 48:241–66. doi: 10.7589/0090-3558-48.2.241
7. van Vuren PJ, Paweska JT. Comparison of enzyme-linked immunosorbent assay-based techniques for the detection of antibody to Rift Valley Fever virus in thermochemically inactivated sheep sera. *Vector Borne Zoonotic Dis.* (2010) 10:697–9. doi: 10.1089/vbz.2009.0213
8. Walsh MG, Mor SM. Interspecific network centrality, host range and early-life development are associated with wildlife hosts of Rift Valley Fever virus. *Transbound Emerg Dis.* (2018) 65:1568–75. doi: 10.1111/tbed.12903
9. Lichoti JK, Kihara A, Oriko AA, Okutoyi LA, Wauna JO, Tchouassi DP, et al. Detection of Rift Valley Fever virus interepidemic activity in some hotspot areas of Kenya by sentinel animal surveillance. 2009–2012. *Vet Med Int.* (2014) 2014:379010. doi: 10.1155/2014/379010
10. van den Bergh C, Venter EH, Swanepoel R, Thompson PN. High seroconversion rate to Rift Valley Fever virus in cattle and goats in far northern KwaZulu-Natal, South Africa, in the absence of reported outbreaks. *PLoS Negl Trop Dis.* (2019) 13:e0007296. doi: 10.1371/journal.pntd.0007296
11. WHO (World Health Organization). *Rift Valley Fever*, Fact sheet no. 207 (2010). Available online at: <https://www.who.int/news-room/fact-sheets/detail/rift-valley-fever>
12. Brand RF, Rostal MK, Kemp A, Anyamba A, Zwieggers H, Van Huyssteen CW, et al. A phytosociological analysis and description of wetland vegetation and ecological factors associated with locations of high mortality for the 2010–11 Rift Valley fever outbreak in South Africa. *PLoS One.* (2018) 13:e0191585. doi: 10.1371/journal.pone.0191585
13. Faye O, Diallo M, Diop D, Bezeid OE, Ba H, Niang M, et al. Rift Valley fever outbreak with East-Central African virus lineage in Mauritania. *Emerg Infect Dis.* (2007) 13:1016–23. doi: 10.3201/eid1307.061487
14. Chambers PG, Swanepoel R. Rift Valley Fever in abattoir workers. *Cent Afr J Med.* (1980) 26:122–6. Available online at: <https://www.who.int/news-room/fact-sheets/detail/rift-valley-fever>
15. Msimang V, Thompson PN, Jansen van Vuren P, Tempia S, Cordel C, Kgaladi J, et al. Rift Valley Fever virus exposure amongst farmers, farm workers, and veterinary professionals in central South Africa. *Viruses.* (2019) 11:E140. doi: 10.3390/v11020140
16. Nicholas DE, Jacobsen KH, Waters NM. Risk factors associated with human Rift Valley Fever infection: systematic review and meta-analysis. *Trop Med Int Health.* (2014) 19:1420–9. doi: 10.1111/tmi.12385
17. Nyakarahuka L, de St Maurice A, Purpura L, Ervin E, Balinandi S, Tumusiime A, et al. Prevalence and risk factors of Rift Valley Fever in humans and animals from Kabale district in Southwestern Uganda, 2016. *PLoS Negl Trop Dis.* (2018) 12:e0006412. doi: 10.1371/journal.pntd.0006412
18. Wilson ML, Chapman LE, Hall DB, Dykstra EA, Ba K, Zeller HG, et al. Rift Valley Fever in rural northern Senegal: human risk factors and potential vectors. *Am J Trop Med Hyg.* (1994) 50:663–75. doi: 10.4269/ajtmh.1994.50.663
19. Peyre M, Chevalier V, Abdo-Salem S, Velthuis A, Antoine-Moussiaux N, Thiry E, et al. A systematic scoping study of the socio-economic impact of Rift Valley Fever: research gaps and needs. *Zoonoses Public Health.* (2015) 62:309–25. doi: 10.1111/zph.12153
20. Grossi-Soyster EN, Lee J, King CH, LaBeaud AD. The influence of raw milk exposures on Rift Valley Fever virus transmission. *PLoS Negl Trop Dis.* (2019) 13:e0007258. doi: 10.1371/journal.pntd.0007258
21. LaBeaud AD, Ochiai Y, Peters CJ, Muchiri EM, King CH. Spectrum of Rift Valley Fever virus transmission in Kenya: insights from three distinct regions. *Am J Trop Med Hyg.* (2007) 76:795–800. doi: 10.4269/ajtmh.2007.76.795
22. Bett B, Lindahl J, Sang R, Wainaina M, Kairu-Wanyoike S, Bukachi S, et al. Association between Rift Valley Fever virus seroprevalences in livestock and humans and their respective intra-cluster correlation coefficients, Tana River County, Kenya. *Epidemiol Infect.* (2018) 147:e67. doi: 10.1017/S0950268818003242
23. Buliva E, Elhakim M, Tran Minh NN, Elkholy A, Mala P, Abubakar A, et al. Emerging and reemerging diseases in the World Health Organization (WHO) Eastern Mediterranean region—progress, challenges, and WHO initiatives. *Front Public Health.* (2017) 5:276. doi: 10.3389/fpubh.2017.00276
24. Di Lorenzo A, Di Sabatino D, Blanda V, Cioci D, Conte A, Bruno R, et al. The Arbozoonet information system. *Vet Ital.* (2016) 52:161–8. doi: 10.12834/VetIt.522.2484.1
25. Failloux AB, Bouattour A, Faraj C, Gunay F, Haddad N, Harrat Z, et al. Surveillance of Arthropod-Borne viruses and their vectors in the mediterranean and Black Sea regions within the medilabsecure network. *Curr Trop Med Rep.* (2017) 4:27–39. doi: 10.1007/s40475-017-0101-y
26. Monaco F, Cosseddu GM, Doumbia B, Madani H, El Mellouli F, Jimenez-Clavero MA, et al. First external quality assessment of molecular and serological detection of Rift Valley Fever in the western mediterranean region. *PLoS ONE.* (2015) 10:e0142129. doi: 10.1371/journal.pone.0142129
27. Mutua E, de Haan N, Tumusiime D, Jost C, Bett B. A qualitative study on gendered barriers to livestock vaccine uptake in Kenya and Uganda and their implications on Rift Valley Fever control. *Vaccines.* (2019) 7:E86. doi: 10.3390/vaccines7030086
28. Barnard BJ, Botha MJ. An inactivated Rift Valley Fever vaccine. *J S Afr Vet Assoc.* (1977) 48:45–8.
29. Alhaj M. Safety and efficacy profile of commercial veterinary vaccines against Rift Valley Fever: a review study. *J Immunol Res.* (2016) 2016:7346294. doi: 10.1155/2016/7346294
30. Faburay B, LaBeaud AD, McVey DS, Wilson WC, Richt JA. Current status of Rift Valley Fever vaccine development. *Vaccines.* (2017) 5:E29. doi: 10.3390/vaccines5030029
31. Wichgers Schreur PJ, Kant J, van Keulen L, Moormann RJ, Kortekaas J. Four-segmented Rift Valley Fever virus induces sterile immunity in sheep after a single vaccination. *Vaccine.* (2015) 33:1459–64. doi: 10.1016/j.vaccine.2015.01.077
32. Faburay B, Lebedev M, McVey DS, Wilson W, Morozov I, Young A, et al. A glycoprotein subunit vaccine elicits a strong Rift Valley Fever virus neutralizing antibody response in sheep. *Vector Borne Zoonot Dis.* (2014) 14:746–56. doi: 10.1089/vbz.2014.1650
33. Wallace DB, Ellis CE, Espach A, Smith SJ, Greyling RR, Viljoen GJ. Protective immune responses induced by different recombinant vaccine regimes to Rift Valley Fever. *Vaccine.* (2006) 24:7181–9. doi: 10.1016/j.vaccine.2006.06.041
34. Oreshkova N, van Keulen L, Kant J, Moormann RJ, Kortekaas J. A single vaccination with an improved nonspreading Rift Valley Fever virus vaccine provides sterile immunity in lambs. *PLoS ONE.* (2013) 8:e77461. doi: 10.1371/journal.pone.0077461
35. Said A, Elmanzalawy M, Ma G, Damiani AM, Osterrieder N. An equine herpesvirus type 1 (EHV-1) vector expressing Rift Valley Fever virus (RVFV) Gn and Gc induces neutralizing antibodies in sheep. *Virol J.* (2017) 14:154. doi: 10.1186/s12985-017-0811-8
36. Kortekaas J, de Boer SM, Kant J, Vloet RP, Antonis AF, Moormann RJ. Rift Valley Fever virus immunity provided by a paramyxovirus vaccine vector. *Vaccine.* (2010) 28:4394–401. doi: 10.1016/j.vaccine.2010.04.048
37. Kortekaas J, Dekker A, de Boer SM, Weerdmeester K, Vloet RP, de Wit AA, et al. Intramuscular inoculation of calves with an experimental Newcastle disease virus-based vector vaccine elicits neutralizing antibodies against Rift Valley Fever virus. *Vaccine.* (2010) 28:2271–6. doi: 10.1016/j.vaccine.2010.01.001
38. Busquets N, Lorenzo G, Lopez-Gil E, Rivas R, Solanes D, Galindo-Cardiel I, et al. Efficacy assessment of an MVA vectored Rift Valley Fever vaccine in lambs. *Antiviral Res.* (2014) 108:165–72. doi: 10.1016/j.antiviral.2014.05.020
39. Papin JF, Verardi PH, Jones LA, Monge-Navarro F, Brault AC, Holbrook MR, et al. Recombinant Rift Valley Fever vaccines induce protective levels of antibody in baboons and resistance to lethal challenge in mice. *Proc Natl Acad Sci USA.* (2011) 108:14926–31. doi: 10.1073/pnas.1112149108

40. Warimwe GM, Gesharisha J, Carr BV, Otieno S, Otingah K, Wright D, et al. Chimpanzee adenovirus vaccine provides multispecies protection against Rift Valley Fever. *Sci Rep.* (2016) 6:20617. doi: 10.1038/srep20617
41. Ayari-Fakhfakh E, Ghram A, Albina E, Cetre-Sossah C. Expression of cytokines following vaccination of goats with a recombinant capripoxvirus vaccine expressing Rift Valley Fever virus proteins. *Vet Immunol Immunopathol.* (2018) 197:15–20. doi: 10.1016/j.vetimm.2018.01.001
42. Soi RK, Rurangirwa FR, McGuire TC, Rwambo PM, DeMartini JC, Crawford TB. Protection of sheep against Rift Valley Fever virus and sheep poxvirus with a recombinant capripoxvirus vaccine. *Clin Vaccine Immunol.* (2010) 17:1842–9. doi: 10.1128/CVI.00220-10
43. Boumart Z, Daouam S, Bamouh Z, Jazouli M, Tadlaoui KO, Dungu B, et al. Safety and immunogenicity of a live attenuated Rift Valley Fever recombinant arMP-12DeltaNSm21/384 vaccine candidate for sheep, goats and calves. *Vaccine.* (2019) 37:1642–50. doi: 10.1016/j.vaccine.2019.01.067
44. Daouam S, Ghzal F, Naouli Y, Tadlaoui KO, Ennaji MM, Oura C, et al. Safety and immunogenicity of a live attenuated Rift Valley Fever vaccine (CL13T) in camels. *BMC Vet Res.* (2016) 12:154. doi: 10.1186/s12917-016-0775-8
45. Dungu B, Louw I, Lubisi A, Hunter P, von Teichman BF, Bouloy M. Evaluation of the efficacy and safety of the Rift Valley Fever clone 13 vaccine in sheep. *Vaccine.* (2010) 28:4581–7. doi: 10.1016/j.vaccine.2010.04.085
46. Lo MM, Mbao V, Sierra P, Thiongane Y, Diop M, Donadeu M, et al. Safety and immunogenicity of Onderstepoort biological products' Rift Valley Fever clone 13 vaccine in sheep and goats under field conditions in Senegal. *Onderstepoort J Vet Res.* (2015) 82:857. doi: 10.4102/ojvr.v82i1.857
47. Makoschey B, van Kilsdonk E, Hubers WR, Vrijenhoek MP, Smit M, Wichgers Schreur PJ, et al. Rift Valley Fever vaccine virus clone 13 is able to cross the ovine placental barrier associated with foetal infections, malformations, and stillbirths. *PLoS Negl Trop Dis.* (2016) 10:e0004550. doi: 10.1371/journal.pntd.0004550
48. Njenga MK, Njagi L, Thumbi SM, Kahariri S, Githinji J, Omondi E, et al. Randomized controlled field trial to assess the immunogenicity and safety of Rift Valley Fever clone 13 vaccine in livestock. *PLoS Negl Trop Dis.* (2015) 9:e0003550. doi: 10.1371/journal.pntd.0003550
49. Nyundo S, Adamson E, Rowland J, Palermo PM, Matiko M, Bettinger GE, et al. Safety and immunogenicity of Rift Valley Fever MP-12 and arMP-12DeltaNSm21/384 vaccine candidates in goats (*Capra aegagrus hircus*) from Tanzania. *Onderstepoort J Vet Res.* (2019) 86:e1–8. doi: 10.4102/ojvr.v86i1.1683
50. von Teichman B, Engelbrecht A, Zulu G, Dungu B, Pardini A, Bouloy M. Safety and efficacy of Rift Valley Fever Smithburn and Clone 13 vaccines in calves. *Vaccine.* (2011) 29:5771–7. doi: 10.1016/j.vaccine.2011.05.055
51. Faburay B, Wilson WC, Gaudreault NN, Davis AS, Shivanna V, Bawa B, et al. A recombinant Rift Valley Fever virus glycoprotein subunit vaccine confers full protection against Rift Valley Fever challenge in sheep. *Sci Rep.* (2016) 6:27719. doi: 10.1038/srep27719
52. Kortekaas J, Oreshkova N, van Keulen L, Kant J, Bosch BJ, Bouloy M, et al. Comparative efficacy of two next-generation Rift Valley Fever vaccines. *Vaccine.* (2014) 32:4901–8. doi: 10.1016/j.vaccine.2014.07.037
53. Faburay B, Gaudreault NN, Liu Q, Davis AS, Shivanna V, Sunwoo SY, et al. Development of a sheep challenge model for Rift Valley Fever. *Virology.* (2016) 489:128–40. doi: 10.1016/j.virol.2015.12.003
54. Weingartl HM, Miller M, Nfon C, Wilson WC. Development of a Rift Valley Fever virus viremia challenge model in sheep and goats. *Vaccine.* (2014) 32:2337–44. doi: 10.1016/j.vaccine.2014.02.066
55. Kroeker AL, Smid V, Embury-Hyatt C, Moffat E, Collignon B, Lung O, et al. RVFV Infection in goats by different routes of inoculation. *Viruses.* (2018) 10:E709. doi: 10.20944/preprints201811.0022.v1
56. Nfon CK, Marszal P, Zhang S, Weingartl HM. Innate immune response to Rift Valley Fever virus in goats. *PLoS Negl Trop Dis.* (2012) 6:e1623. doi: 10.1371/journal.pntd.0001623
57. Wilson WC, Davis AS, Gaudreault NN, Faburay B, Trujillo JD, Shivanna V, et al. Experimental infection of calves by two genetically-distinct strains of Rift Valley Fever virus. *Viruses.* (2016) 8:E145. doi: 10.3390/v8050145
58. Bond E, Liang F, Sandgren KJ, Smed-Sorensen A, Bergman P, Brighenti S, et al. Plasmacytoid dendritic cells infiltrate the skin in positive tuberculin skin test indurations. *J Invest Dermatol.* (2012) 132:114–23. doi: 10.1038/jid.2011.246
59. Clausen BE, Stoitzner P. Functional specialization of skin dendritic cell subsets in regulating T cell responses. *Front Immunol.* (2015) 6:534. doi: 10.3389/fimmu.2015.00534
60. Rippey MK, Topper MJ, Mebus CA, Morrill JC. Rift Valley fever virus-induced encephalomyelitis and hepatitis in calves. *Vet Pathol.* (1992) 29:495–502. doi: 10.1177/030098589202900602

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Kroeker, Smid, Embury-Hyatt, Collignon, Pinette, Babiuk and Pickering. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Enhanced West Nile Virus Circulation in the Emilia-Romagna and Lombardy Regions (Northern Italy) in 2018 Detected by Entomological Surveillance

Mattia Calzolari^{1*}, Paola Angelini², Luca Bolzoni¹, Paolo Bonilauri¹, Roberto Cagarelli², Sabrina Canziani¹, Danilo Cereda³, Monica Pierangela Cerioli¹, Mario Chiari³, Giorgio Galletti¹, Giovenale Moirano⁴, Marco Tamba¹, Deborah Torri¹, Tiziana Trogu¹, Alessandro Albieri⁵, Romeo Bellini⁵ and Davide Lelli¹

OPEN ACCESS

Edited by:

Michael Kogut,
United States Department of
Agriculture, United States

Reviewed by:

Francisco Rivera-Benitez,
Agriculturas y Pecuarias (INIFAP), Mexico
Dana Mitzel,
United States Department of
Agriculture, United States

*Correspondence:

Mattia Calzolari
mattia.calzolari@izsler.it

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 25 October 2019

Accepted: 09 April 2020

Published: 05 May 2020

Citation:

Calzolari M, Angelini P, Bolzoni L,
Bonilauri P, Cagarelli R, Canziani S,
Cereda D, Cerioli MP, Chiari M,
Galletti G, Moirano G, Tamba M,
Torri D, Trogu T, Albieri A, Bellini R and
Lelli D (2020) Enhanced West Nile
Virus Circulation in the
Emilia-Romagna and Lombardy
Regions (Northern Italy) in 2018
Detected by Entomological
Surveillance. *Front. Vet. Sci.* 7:243.
doi: 10.3389/fvets.2020.00243

¹ Istituto Zooprofilattico Sperimentale Della Lombardia e Dell'Emilia-Romagna, B. Ubertini, Brescia, Italy, ² Regional Health Authority of Emilia-Romagna, Bologna, Italy, ³ Regional Health Authority of Lombardy, Milan, Italy, ⁴ Cancer Epidemiology Unit-CERMS, Department of Medical Sciences, University of Turin and CPO-Piemonte, Turin, Italy, ⁵ Centro Agricoltura Ambiente "G. Nicoli", Crevalcore, Italy

With several human cases reported annually since 2008 and the unapparent risk of infection of blood donors, the West Nile virus (WNV) is emerging as an important health issue in Europe. Italy, as well as other European countries, experienced a recrudescence of the virus circulation in 2018, which led to an increased number of human cases. An integrated surveillance plan was activated in the Emilia-Romagna and Lombardy regions (Northern Italy) since 2008 in order to monitor the intensity and timing of WNV circulation. A fundamental part of this plan consists in entomological surveillance. In 2018, the surveillance plan made it possible to collect 385,293 mosquitoes in 163 stations in the two Regions. In total 269,147 *Culex* mosquitoes were grouped into 2,337 pools and tested for WNV, which was detected in 232 pools. Circulation started in the central part of the Emilia-Romagna region in the middle of June, about one month before the previous seasons. Circulation suddenly expanded to the rest of the region and reached the Lombardy region in the middle of July. WNV circulated more intensively in the eastern part of the surveyed area, as confirmed by the highest number of human cases. A relationship between the number of mosquitoes collected and the virus incidence emerged, but the data obtained highlighted that the probability of detecting the virus in a given site was less than expected with a higher number of collected mosquitoes. A significant relationship was observed between the temperature recorded one week before the sampling and the number of collected mosquitoes, as well as between the estimated number of WNV-positive mosquitoes and the temperature recorded two weeks before the sampling. The two weeks delay in the influence of temperature on the positive mosquitoes is in line with the time of the virus extrinsic incubation in the mosquito. This finding confirms that

temperature is one of the principal drivers in WNV mosquito infection. The surveillance system demonstrated the ability to detect the virus circulation early, particularly in areas where circulation was more intense. This allowed evaluating the effect of mosquito abundance and weather factors on virus circulation.

Keywords: West Nile virus, *Culex pipiens*, surveillance, infection rate, temperature, one-health

INTRODUCTION

The West Nile Virus (WNV) is a flavivirus that circulates in the environment among birds and mosquitoes, but can infect other animals such as equids and humans as dead-end hosts. In humans, virus infection is generally, asymptomatic, but about 20% of infected subjects can develop a febrile illness, which can evolve into a neuro-invasive disease (WNND) in a minority of cases (less than 1% of infected subjects). In addition to symptomatic human cases, the presence of infected, but asymptomatic, blood and organ donors is a primary health issue (1).

After sporadic reports in the '90s in Romania (2) and in Italy (3), as from 2008, the circulation of the virus was recorded at a different extent every season in Europe. In 2018, an alarming recrudescence of the virus circulation was recorded in Europe (4). This recrudescence was also noticed in Northern Italy, including the Emilia-Romagna and Lombardy regions, where an interdisciplinary surveillance plan targeting WNV was set up in 2008 and implemented over the years (5–10).

The plan focuses on the early detection and the pinpointing of the virus circulation area, including those areas where the virus did not circulate the previous season. Over time, the system has shown that it is capable of WNV circulation's early detection at provincial scale (6) and it is economically sustainable (11). Moreover, the plan was able to detect other arboviruses circulating in the surveyed area (12).

The plan has a one-health approach in order to maximize early detection, involving searching for the virus in humans, horses, birds, and mosquitoes. Entomological surveillance is a fundamental part of the plan, characterized by regular scheduling of samplings, and the precise geographic characterization of obtained samples. This permits a fine characterization of the virus circulation on the surveyed area. In this work, we characterize the extraordinary WNV circulation observed in 2018, utilizing data from entomological surveillance obtained in the Emilia-Romagna and Lombardy regions. In particular, we describe the relationship between WNV detection and the abundance of collected mosquitoes, as well as the influence of weather conditions, such as weekly maximum temperatures and number of wet days, on the virus circulation in mosquitoes.

MATERIALS AND METHODS

Surveyed Area

The surveyed area included the plan area of the Emilia-Romagna and Lombardy regions. This is a large portion of Italy's main plain, the Pianura Padana (or Pianura Padano-Veneta), which includes about 24,000 km² (51.3%) of the total area of 44,700

km². This area encompasses about 10 million inhabitants, with many urban areas, including two of the biggest Italian cities (Milan and Bologna), as well as several important industrial districts. This area has a relevant livestock patrimony and is one of the more important agricultural areas in Italy; distinctive cultivations such as rice fields, vineyards and orchards are highly represented in specific areas.

In general, a strong anthropic modification, with the abundant presence of industrial and urban settlements, characterizes the surveyed area. The rural part of the territory is connoted by an intensive agriculture and animal husbandry, with few hedges, rare scattered trees, and a dense irrigation network. Natural areas are rare, mainly represented by river borders, characterized by riparian vegetation, or re-naturalized areas. The eastern part of the surveyed area ends in the Adriatic Sea, where some of the largest wetlands in Europe are present (Valli di Comacchio and the Po River Delta); pinewood and typical Mediterranean vegetation can be found in this zone.

Trapping sites, with different densities, were selected to cover the entire plain area of the two regions: 95 traps in Emilia-Romagna (surveillance grid of 110 km²) and 39 traps in Lombardy (surveillance grid of 400 km²) (Table 1, Figure 1). These traps worked regularly throughout the surveillance season (seasonal traps). Surveillance was focused on rural areas, semi-natural, rural, or peri-urban locations in Emilia-Romagna, and farms in Lombardy. A supplementary effort was carried out by activating 23 specific traps along the surveillance season in Lombardy, and 9 supplementary traps, which worked once (on August 10th), among selected urban areas in Emilia-Romagna.

Mosquito Analysis

We collected mosquitoes in fixed geo-referenced stations using attractive traps baited by carbon dioxide, working overnight, roughly from 5:00 p.m. to 9:00 a.m. Every site regularly included in the surveillance was sampled every fortnight. Mosquitoes

TABLE 1 | Features of surveillance in the two surveyed regions.

	Emilia-Romagna	Lombardy
Number of seasonal traps	95	39
Number of extra traps	9	23
Maximum number of mosquitoes per pool	200	100
Maximum number of mosquitoes tested per sampling	all sampled	1000
Start date	June 12	June 4
End date	October 16	October 25

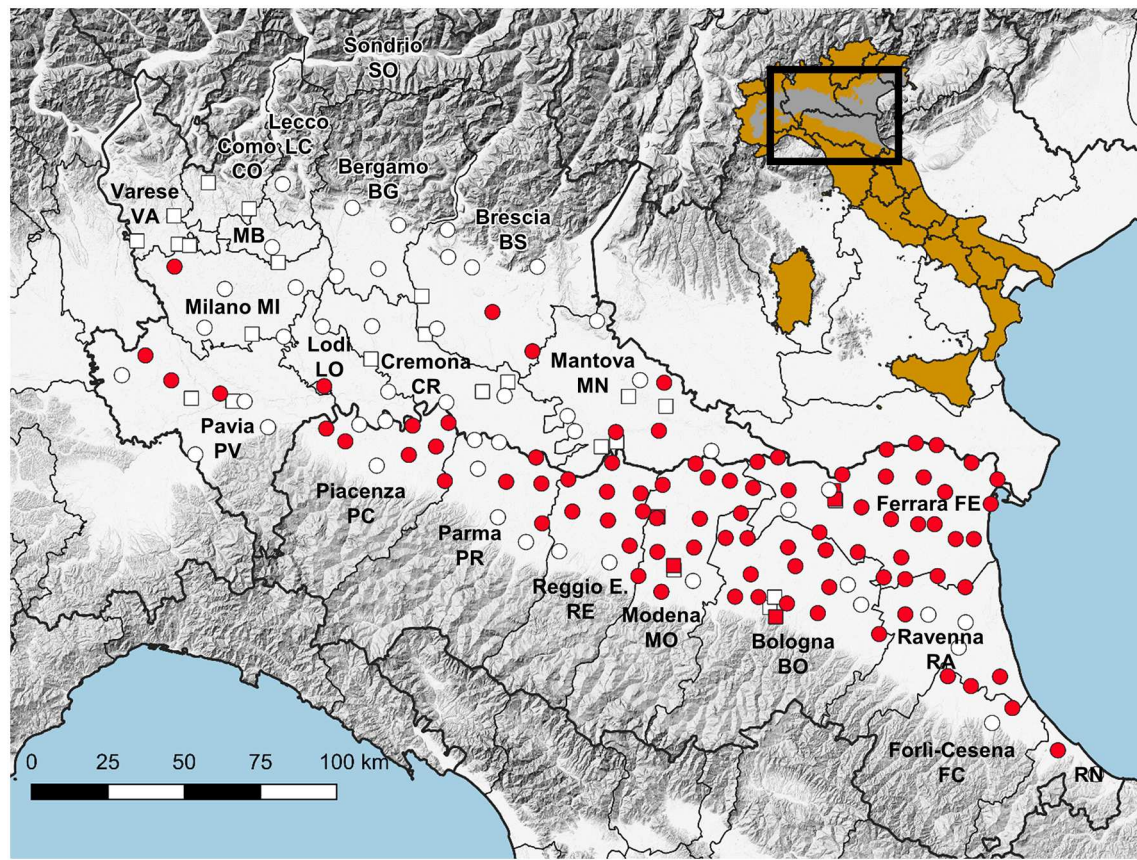


FIGURE 1 | Map showing the location of traps working throughout the season (circles) and for part of the season (squares), with reference to WNV detections (red), and the reference of the surveyed area on a map of Italy in which the Pianura Padano-Veneta is depicted in gray.

were identified using morphological characteristics according to the classification key of Severini et al. (13). Due to the used trap model, baited with carbon dioxide, we collected almost exclusively host seeking females, the catching of males was very rare. Identified mosquitoes were counted and pooled according to date, location, and species. The maximum number of specimens per pool was 200 in Emilia-Romagna and 100 in Lombardy. A maximum of 1,000 specimens per species was tested for every sample in Lombardy. Pooled mosquitoes belonging to the genus *Culex*, and selected pools of other species, were prepared and submitted to biomolecular tests as described elsewhere (14).

In brief, pooled mosquitoes were ground by a vortex mixer with 2–3 4.5-mm-diameter copper-plated round balls (H&N Sport, Münden, Germany) in 2 ml of PBS. After centrifugation, 200 μ L aliquots were collected and submitted to biomolecular analysis, for extracting and retrotranscribing RNA. We tested samples by a WNV real-time RT-PCR, according to the method described by Tang et al. (15). This was confirmed by the protocol of Del Amo et al. (16) and by a universal PCR protocol for the identification of flaviviruses (17).

We estimated the number of positive mosquitoes for every site and the day of sampling utilizing the Maximal Likelihood

Estimation (MLE) of the PooledInfRate 4.0 excel add-in (18), multiplying the MLE of the infectious rate for the number of collected mosquitoes, and rounding up the obtained result. MLE cannot be estimated when all sample pools test positive. In this case, we approximated the number of infected mosquitoes using the Minimum Infection Rate (MIR), assuming that only one positive mosquito was present in each pool.

We tested the relationship between the fraction of infected mosquitoes collected in each province, with at least two working seasonal traps, and the incidence of WNV cases in the same province through a linear model. Additionally, we checked the hypothesis of normality in the distribution of model residuals through the Shapiro-Wilk test.

Mosquito Abundance and WNV Circulation

We tested the effect of mosquito abundance in a given area (using the catches per trap as a proxy of abundance) on the probability of WNV circulation in the same area in 2018. To guarantee comparable catching efforts, considering the start/end of trapping seasons differed among traps, we used the total number of mosquitoes caught in each trap in July and August (for a total of four sampling sessions) as a measure of mosquito abundance. Traps with less than four sampling sessions in the

July–August time window were excluded from the analyses. We built a generalized linear model (GLM) with binomial error distribution and logit link function to estimate the probability of observing a WNV-positive trap in 2018, using the observed number of mosquitoes caught in each trap and the region where the trap was deployed as explanatory variables. In this model, the effect on the GLM of caught mosquitoes (namely, M_{OBS}) represents the marginal increase in the probability of observing a WNV-positive trap due to an additional mosquito caught (in the logit link).

To test the effect of mosquito abundance on WNV circulation, we built a null model where the probability of each mosquito to test WNV-positive only depends on the region where the mosquito was caught and not on the abundance of mosquitoes in the trap area. These probabilities, namely p_{NULL_ER} and p_{NULL_LO} , were estimated by maximizing the likelihood of obtaining the observed pattern of positive traps in 2018, given the number of mosquitoes caught in each trap in Emilia-Romagna and Lombardy, respectively. We then randomly generated 10,000 synthetic samples where we assigned a WNV-positive/negative status to each mosquito caught in Emilia-Romagna [resp. Lombardy] with probabilities p_{NULL_ER} [resp. p_{NULL_LO}]. Analogously to the observed sample, we estimated for each synthetic sample the effect of the number of mosquitoes caught on the probability of observing a WNV-positive trap (namely, M_{NULL}) through GLMs. Thus, we generated a distribution of the estimated M_{NULL} which represents the null hypothesis where mosquito abundance in a given area does not affect the probability of each single mosquito testing WNV-positive. We then tested the observed effect, M_{OBS} , against the distribution of M_{NULL} . If M_{OBS} is significantly higher [resp. smaller] than M_{NULL} (i.e., it falls outside the 95% range of the M_{NULL} distribution), then mosquito abundance provides an amplification [resp. dilution] effect on WNV circulation. Analyses were performed with R software.

In order to spatially characterize mosquito abundance, we interpolated the number of mosquitoes sampled in each seasonal trap between July–August (expressed as a natural logarithm) on a map, using the inverse distance weighting method (IDW). The virus circulation's spatial pattern was obtained using positive trap location with the kernel density estimation (KDE) method, with the bandwidth size of 15 km. The area of more intense WNV circulation was estimated through the 50% volume contour of the obtained WNV KDE surface. We used QGIS 3 software (<http://www.qgis.org>) to perform these analyses.

Meteorological Factors, Mosquito Abundance and Infection Rate

We retrieved daily maximum temperatures and precipitations data from the ECA&D dataset (19) and extracted the data for every surveyed trap site in 2018. We aimed to estimate the association of meteorological parameters with the number of sampled *Culex* mosquitoes and with the infection rate (namely the proportion of WNV-positive mosquitoes). Since the effect of meteorological parameters on the outcomes of interest might be delayed (lag-effect), we focused on the meteorological parameters

TABLE 2 | Mosquitoes sampled during the 2018 surveillance season.

Species	Emilia-Romagna (%)	Lombardy (%)	Extra traps	Total (%)
<i>Ae. albopictus</i>	4557 (1.6)	1743 (1.7)	665 (19.3)	6965 (1.8)
<i>Ae. berlandi</i>	1 (<0.5)			1 (<0.5)
<i>Ae. caspius</i>	43526 (15.7)	45299 (43.4)	118 (3.4)	88943 (23.1)
<i>Ae. cinereus</i>	3 (<0.5)			3 (<0.5)
<i>Ae. geniculatus</i>	15 (<0.5)			15 (<0.5)
<i>Ae. vexans</i>	7634 (2.8)	606 (0.6)	412 (11.9)	8652 (2.2)
<i>An. maculipennis s.l.</i>	753 (<0.5)	7007 (6.7)	1 (<0.5)	7761 (2.0)
<i>An. plumbeus</i>	10 (<0.5)	4 (<0.5)		14 (<0.5)
<i>Cq. richiardii</i>	658 (0.2)			658 (0.2)
<i>Cs. annulate</i>	3 (<0.5)	32 (<0.5)		35 (<0.5)
<i>Cs. longiareolata</i>	1 (<0.5)			1 (<0.5)
<i>Cx. modestus</i>	35 (<0.5)			35 (<0.5)
<i>Cx. pipiens</i>	220255 (79.4)	49697 (47.6)	2258 (65.4)	272210 (70.7)
Total	277451	104338	3454	385293

recorded up to 4 weeks (lag 1–4) before the night of sampling. Starting from the day before the sampling, we computed the weekly average of maximum temperatures and the weekly number of wet days (number of days in a week with precipitation > 0.5 mm).

Analyses were performed in the framework of the generalized linear mixed models (GLMMs) (20). Specifically, we applied a GLMM including a site-specific and week-specific random effect to account for site and temporal heterogeneity. The abundance of mosquitoes sampled per trap and night was analyzed by applying a linear mixed model with a normal distribution and identity link function. We used the natural logarithm of the count of sampled mosquitoes as the dependent variable and the meteorological parameters recorded up to 4 weeks before the sampling as predictors (lag 1–4). The per trap and night infection rate was analyzed by applying a generalized linear mixed model with Poisson distribution and log link function. We used the estimate of infected mosquitoes as the dependent variable, the number of sampled mosquitoes as offset and the meteorological parameters recorded up to 4 weeks before the sampling as predictors (lag 1–4). The association between temperatures and outcomes was assessed by estimating the coefficient for a unit increase (1°C) in the weekly average of maximum temperatures. The association between precipitations and outcomes was assessed by estimating the coefficient for a unit increase (1 day) in the weekly number of wet days. Analyses were performed with R software (*lme4* package).

RESULTS

Mosquito Results

385,293 mosquitoes belonging to 13 species were sampled between June 4 and October 25, (Table 2). Mosquitoes of the *Culex* genus were grouped into 2,337 pools, of which 232

TABLE 3 | Tested and WNV-positive mosquito pools for the 2018 surveillance season.

Species	E-R			Lom			Extra-traps			Total		
	N	Pools	pos	N	pools	pos	N	pools	pos	N	pools	pos
<i>Ae. albopictus</i>				16	2		177	11		193	13	
<i>Ae. caspius</i>	8211	160		5	1		107	5		8323	166	
<i>Cx. modestus</i>	35	6								35	6	
<i>Cx. pipiens</i>	220255	1554	194	46599	700	32	2258	77	6	269112	2331	232
Total	228501	1720	194	46620	703	32	2542	93	6	277663	2516	232

TABLE 4 | Details of entomological surveillance at provincial level during the 2018 surveillance season.

Region	Prov.	First WNV/+ pool	Last WNV/+ pool	Days	Average sampled*	CI	Total sampled	Est. positive mosq.	WNND cases
Emilia-Romagna	BO	19/6	7/9	80	227	180-273	30603	49	41
	FC	5/7	2/8	28	103	38-168	2780	2	2
	FE	19/6	6/9	79	248	201-295	61730	89	14
	MO	3/7	14/8	42	243	182-303	25864	39	23
	PC	10/7	2/10	84	380	292-468	37619	8	2
	PR	17/7	28/8	42	303	202-404	24257	14	1
	RA	24/7	21/8	28	124	88-160	6574	5	13
	RE	15/6	24/8	70	334	241-428	30067	25	5
	RN	31/7	31/7		88	22-154	1584	1	0
Lombardy	BS	26/7	23/8	28	185	115-255	13811	3	1
	LO	2/8	2/8	0	152	70-234	4103	1	1
	MI	7/8	6/9	30	62	38-85	2895	2	6
	MN	17/7	20/8	34	164	98-230	14047	14	4
	PV	30/7	20/8	21	175	120-230	11919	17	1
	BG				40	16-63	1472	0	0
	CO				—		20	0	1
	CR				92	38-147	2444	0	2
	LC				57	12-102	341	0	0
	MB				6	2-10	48	0	0
	VA				—		32	0	0

* In traps which work throughout the season.

tested positive for lineage 2 of the WNV. The vast majority of tested pools and all pools which tested positive were composed of *Culex (Cx.) pipiens* (Table 3). The 134 seasonal traps collected 269,952 *Cx. pipiens* specimens; of these, 266,854 were tested in 2,254 pools, and 226 were WNV-positive. From among the 134 seasonal traps, 85 collected at least one WNV-positive pool (Figure 1). A total of 1,101 mosquitoes were sampled in the 9 extra traps activated in cities (Bologna, Modena, and Ferrara) in August. All mosquitoes from these samples (823 *Cx. pipiens*, 171 *Aedes (Ae.) albopictus*, and 107 *Ae. caspius*) were tested, and 6 out of the 14 *Cx. pipiens* pools resulted positive. No positive pools were detected in the other extra traps only activated for part of the season, mainly in northern Lombardy. In order to evaluate the possible involvement of *Ae. caspius* in virus circulation, we also tested 160 selected pools of this species (for a total of 8,211 specimens) collected between July 17 and August 14. All gave negative results.

The average of collected mosquitoes per sample at provincial level showed the highest values in the western part of Emilia-Romagna, in Piacenza, Reggio Emilia, and Parma provinces (Table 4). A significant difference in the number of collected mosquitoes was recorded between Lombardy and Emilia-Romagna traps (considering only seasonal traps: $\mu_{E-R} = 258$, CI 233-283, $\mu_{LOM} = 135$, CI 110-159, $t = 5.9016$, $p = 4.6e-9$).

Characterization of Virus Circulation

In 2018, WNV circulation was first detected on June 15 in Emilia-Romagna and on July 17 in Lombardy. We collected the last positive pools on September 6 and October 2 in Lombardy and Emilia-Romagna, respectively (Table 4). The epidemic curve of WNND human cases was postponed with respect to the detection of positive mosquitoes (Figure 2).

Only taking seasonal trap data into account, we evaluated the intensity of circulation at the provincial level considering the number of estimated WNV positive mosquitoes on total

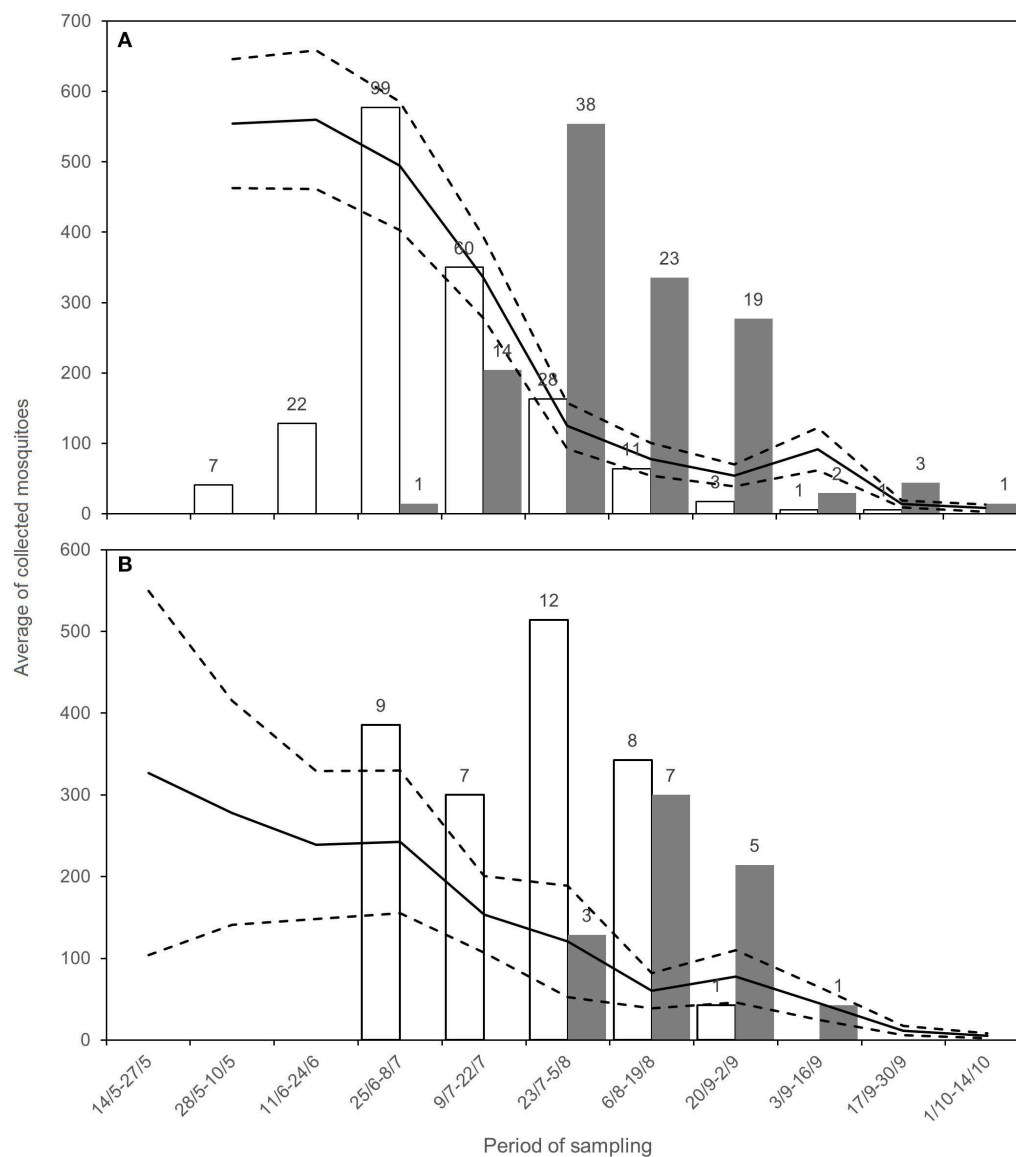


FIGURE 2 | Average of sampled *Cx. pipiens* mosquitoes (black line) with CI (dashed lines), positive estimated mosquitoes (white, number above the bar) and number of WNND cases (gray, number above the bar) for Emilia-Romagna (A) and Lombardy (B), during the surveillance.

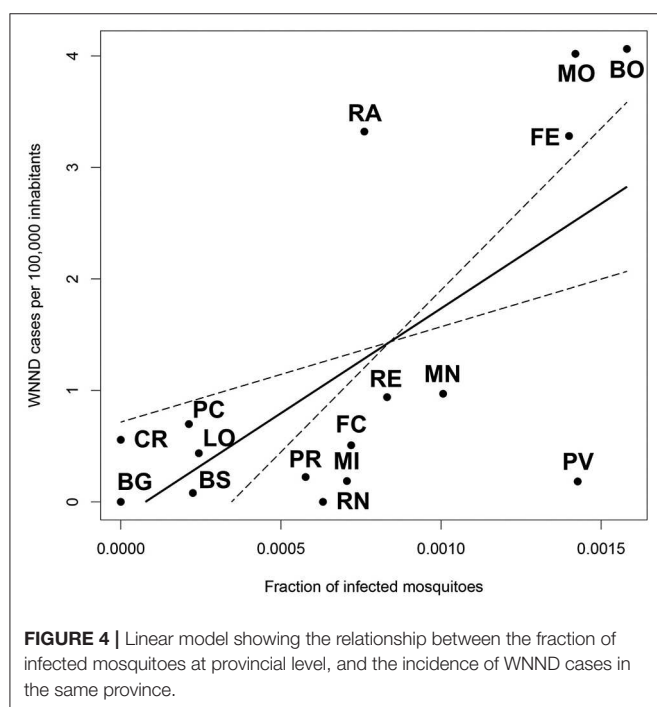
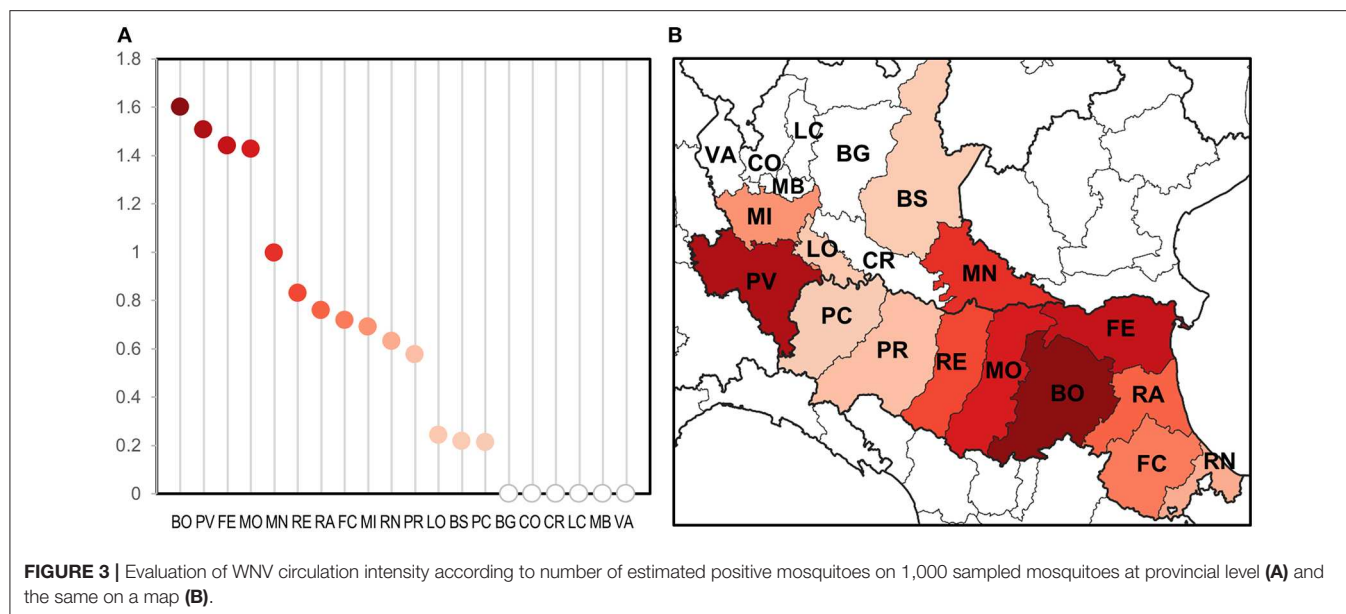
mosquitoes tested (Figure 3). This highlighted groups of provinces with a different circulation intensity: (i) a group of provinces with a more intense circulation (including central Emilia-Romagna and Pavia); (ii) a group of provinces with an intermediate circulation intensity (in the central and eastern surveillance zones); (iii) a group with a low circulation intensity (Lodi, Brescia, and Piacenza); and (iv) a group in which the virus was undetected (northern Lombardy). Provinces with a more intense circulation often recorded an early and more prolonged virus circulation (Table 4), and a major number of WNND cases.

We found the existence of a significant relationship between the rate of infected mosquitoes collected in each province and the incidence of WNND cases in the same province ($p = 0.0013$). Specifically, we estimated that an increase of 1% in the rate of

WNV positive pools leads to an additional 1.88 WNND cases per 100,000 inhabitants (Figure 4).

Relationship Between the Number of Collected Mosquitoes and WNV Incidence

We found a significantly higher probability of finding WNV-positive traps in Emilia-Romagna with respect to Lombardy (p -value = 8.7×10^{-5}). We also estimated the marginal increase in the probability of observing a WNV-positive trap due to an additional mosquito caught as $M_{OBS} = 0.00102$ (p -value = 0.0021). Figure 5B shows the distribution of the marginal increase in the probability of observing a WNV-positive trap due to an additional mosquito caught obtained with the null model (M_{NULL} , gray bars) compared to M_{OBS} (black line). We



found that M_{OBS} is significantly lower than M_{NULL} (p -value = 0.0232), this means that real number of captured mosquitoes affects less than in the simulations the probability to detect WNV in a trap, suggesting that mosquito abundance in a given site provides a dilution effect on WNV circulation. **Figure 5** displays the probabilities of observing a WNV-positive trap as a function of the number of mosquitoes per trap in Lombardy (panel A) and Emilia-Romagna (panel C), estimated through the observed data (black solid lines: best fit, dashed lines: 95% confidence interval) and the null model (white lines: median, gray areas: 95%

interval). Panels A and C in **Figure 5** show that the probability of finding WNV-positive traps is higher than expected when the abundance of caught mosquitoes is low (i.e., black lines are higher than the white lines), while the opposite is true when the abundance of caught mosquitoes is high (i.e., black lines are lower than the white lines).

Despite the probability of finding that WNV increases with the number of mosquitoes (i.e., $M_{OBS} > 0$), the probability of a single mosquito being infected decreases as the number of collected mosquitoes increases. Thus, the estimated probability of a single mosquito collected in one site being infected decreased with the increase in the number of collected mosquitoes in the same site.

Fifty percent of the positive traps' KDE revealed a single hot spot in Emilia-Romagna where WNV circulation is more intense (in orange in **Figure 6**). Overlaying this map onto the map of sampled mosquitoes, directly related to mosquito abundance, highlighted that areas where mosquitoes are most abundant are not necessarily included in this WNV hot spot (**Figure 6**).

Association Between Weather Data and WNV Circulation

Table 5 and **Figure 7** report the β coefficients and 95% CI for the fixed effects of lagged meteorological parameters. Fixed effect coefficients are average estimates from among all sampled traps of the association between a unit increase in the meteorological parameters and the outcomes. β coefficients are estimated under the null hypothesis of absence of association ($\beta = 0$), thus a positive estimate of β coefficient suggests that an increase in the meteorological parameter is associated with an increase in the outcome under study (number of sampled mosquitoes or infection rate).

As shown in **Table 5** and **Figure 7**, we found an association between the logarithm of the number of sampled mosquitoes and the weekly average of maximum temperatures recorded the week

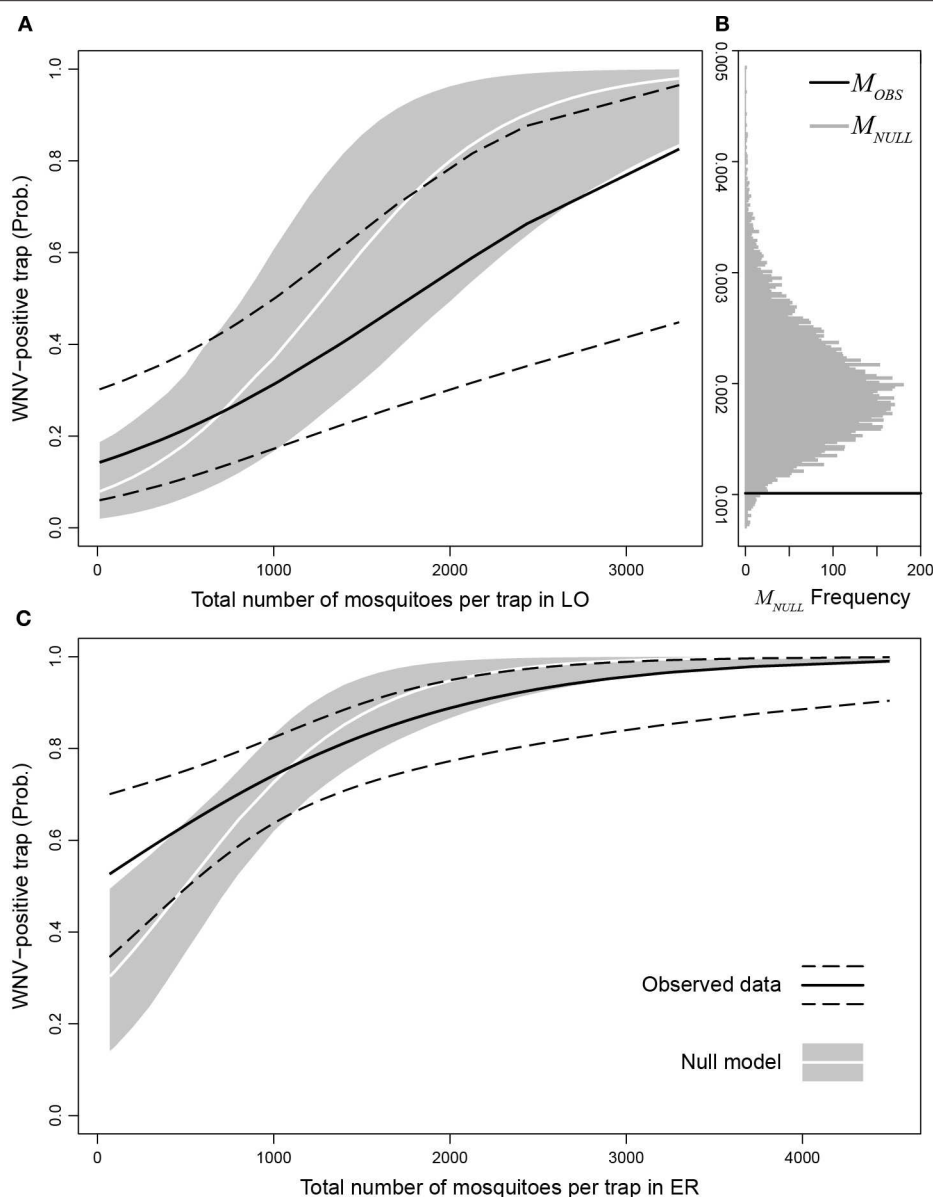


FIGURE 5 | Probability of observing a WNV-positive trap as a function of the number of mosquitoes per trap in Lombardy **(A)** and Emilia-Romagna **(C)** estimated through observed data (black solid lines: best fit, dashed lines: 95% confidence interval) compared with the null model (white lines: median, gray areas: 95% interval). **(B)** distribution of the marginal increase in the probability to observe a WNV-positive trap due to an additionally caught mosquito obtained with the null model (M_{NULL} , gray bars) compared to M_{OBS} (black line).

before sampling (β at lag1 : 0.24, 95% CI 0.16–0.31). In contrast, we did not find evidence of association between the logarithm of the count of sampled mosquitoes and the number of wet days at any lag (Table 5).

When analyzing the proportion of WNV positive mosquitoes, we observed a positive association between the infection rate and the weekly average of maximum temperatures recorded two weeks before the sampling (β lag 2: 0.22; 95% CI: 0.02–0.42) (Table 5). No evidence of association was detected between the infection rate and the weekly number of wet days observed in the previous 4 weeks (Table 6).

DISCUSSION

In 2018, in Italy as in other European countries (4), the WNV transmission season began earlier than in previous years. In fact, in Emilia-Romagna, the season started earlier by about one month with respect to previous transmission seasons and the Lombardy region. Circulation intensity and the number of human cases were also the highest observed since the first appearance of WNV in Italy. Starting from the central Provinces of Emilia-Romagna (Modena, Bologna, and Ferrara), WNV circulation progressively extended to both

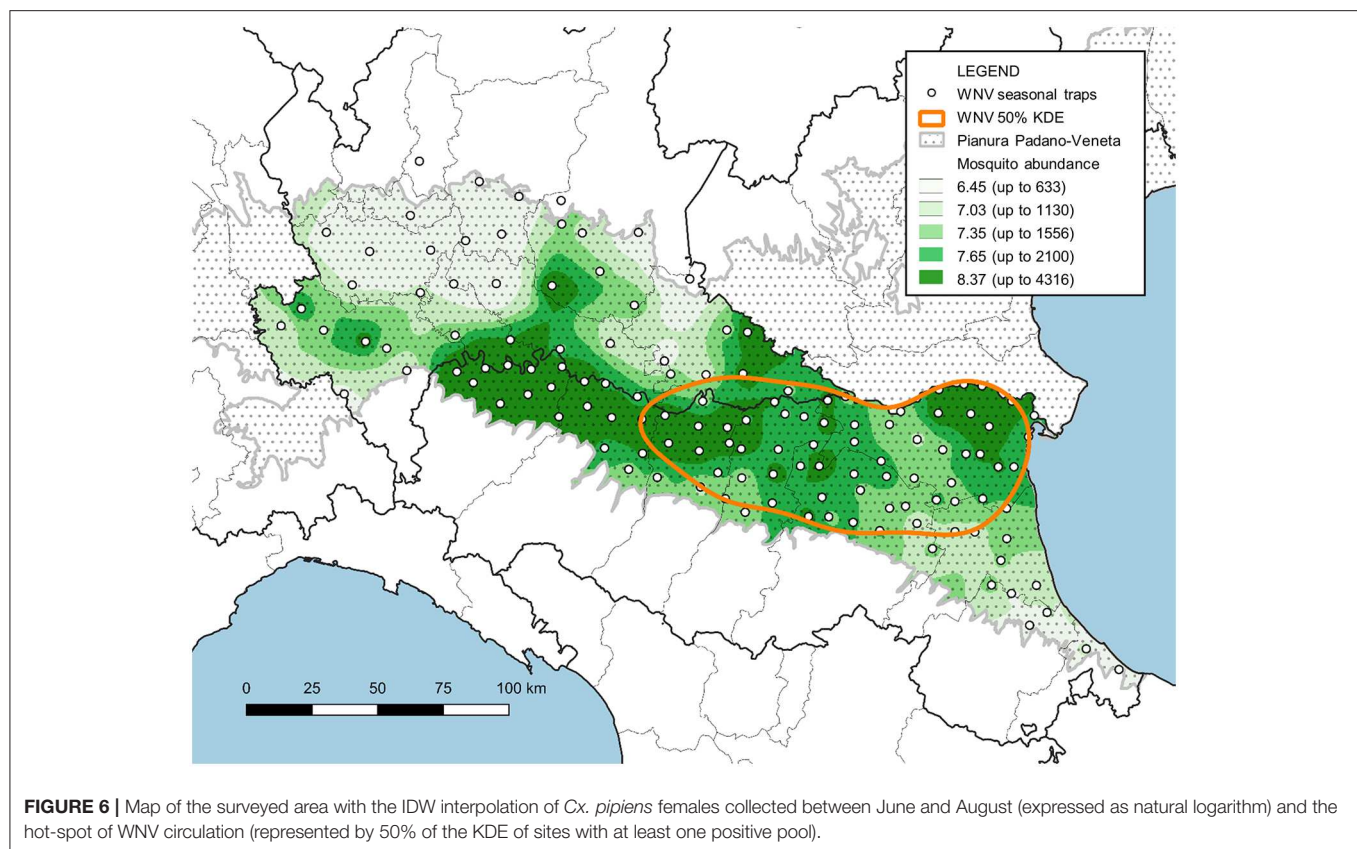


TABLE 5 | Linear regression coefficient (β) and 95% Confidence Interval (CI) for the logarithm of the number of *Culex pipiens* Sampled and Lagged Meteorological Parameters.

Parameter	β^1	95% IC
1°C Increase in weekly average maximum temperatures		
Lag1	0.24	0.16 – 0.31
Lag2	0.05	–0.03 – 0.12
Lag3	–0.01	–0.09 – 0.07
Lag4	–0.01	–0.09 – 0.08
1 Day increase in the weekly number of wet days		
Lag1	0	–0.10 – 0.10
Lag2	–0.06	–0.13 – 0.01
Lag3	–0.01	–0.08 – 0.06
Lag4	–0.04	–0.11 – 0.03

¹Linear regression coefficient (β): it indicates the average change in the logarithm of *Cx. pipiens* sampled associated with a 1°C increase in weekly average maximum temperatures and 1 day increase in weekly number of wet days. *p* value < 0.05 in bold.

the western and eastern part of the surveyed area covering all the Emilia-Romagna region right up to Lombardy (Pavia, Brescia, Milano, Lodi, and Mantua). However, the virus was undetected in mosquitoes collected in Cremona, Bergamo, Monza Brianza, Varese, and Lecco. The early detection of the virus circulation at provincial level makes it possible

to start test blood bags when an area is affected, avoiding infected transfusions.

Reported data confirmed the *Cx. pipiens* mosquito as the main vector of WNV in the surveyed area. To evaluate the involvement of other species, pools of *Ae. caspius* and *Ae. albopictus* were also tested during this survey, without any positive results. The possible involvement of *Ae. albopictus* as a bridge vector in the WNV cycle was deduced due to WNV positive pools collected in the field in the U.S. (21) and Turkey (22), and the vectorial competence demonstrated in experimental studies (23, 24). The possible vector role of *Ae. caspius*, since WNV-positive pools of this mosquito were collected in the field in the surveyed area (25), still remains to be demonstrated as we have no clues as to these two mosquitoes' involvement in the WNV cycle in our epidemiological scenario. Independently of intrinsic competence, this was likely due to the host preference of these mosquitoes which, contrary to *Cx. pipiens* (strictly ornithophilic), are mammophilic feeders. Also, *Cx. modestus*, a species more competent for WNV than *Cx. pipiens* in a laboratory study (26), had a marginal role in the monitored area's WNV cycle. This is due to the scarcity of this species, which is strictly linked to the natural environment (13). Results of extra-plan samples in some cities highlighted the intensive viral circulation in 2018, also involving urban areas which are usually less suitable than rural areas for WNV circulation. It is likely that virus circulation in urban areas, also sustained by *Cx. pipiens*, had an important role in causing the large amount of human cases recorded in 2018.

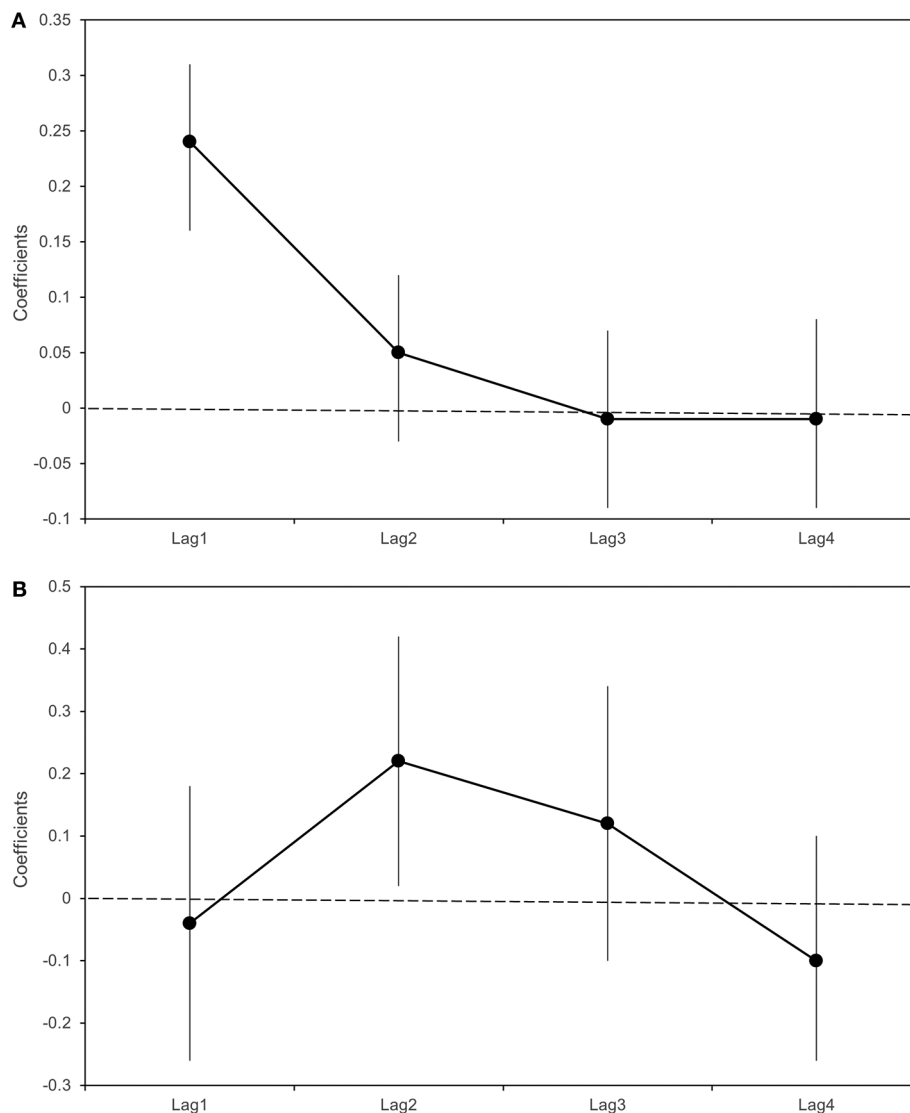


FIGURE 7 | Beta coefficients (black dots) and 95% Confidence intervals (bars) with respect to a 1°C increase in weekly average maximum temperatures in the previous 4 weeks (lag 1–4). Linear regression estimates for the logarithm of the number of sampled *Culex pipiens* (A); Poisson regression estimates for Infection Rate (B).

The surveillance system showed a relevant difference in WNV circulation intensity between the two regions, which was also confirmed by the difference in the number of human cases. Nevertheless, different mosquito sampling efforts between the two regions, and the difference recorded in the GLM, was largely ascribable to the different circulation extent between the two areas. The applied models demonstrate the final number of infected mosquitoes increases when the number of mosquitoes also increases. However, the probability of a single mosquito being infected decreased with mosquito abundance, revealing a non-linear relationship between mosquito abundance and virus circulation. This result agreed with the absence of a direct correlation between mosquito abundance and virus circulation in years with a lower WNV circulation (7). The geographic interpolation of the data utilized to characterize this relationship

demonstrated that the rate of infected mosquitoes was not higher where mosquito density was highest. This confirms a less than linear correlation between virus presence and mosquito abundance, temporally and spatially.

The data obtained demonstrated the obvious importance of mosquito abundance but highlighted that this is not the limiting factor for virus circulation in the considered area, which we identified as likely in the number of infected hosts. If we postulate the finite number of infected hosts as a limiting factor, and then the finite capacity to attract mosquitoes, we can hypothesize a dilution effect exerted by the mosquitoes not attracted to them, above a certain number of mosquitoes. In this theoretical scenario, the synchronization of mosquito population peaks and WNV-susceptible birds is fundamental in determining WNV dynamics and the environmental viral load for the season. The

TABLE 6 | Poisson regression coefficient for Infection Rate and Lagged Meteorological Parameters.

Parameter	β^1	95% IC
1°C Increase in weekly average maximum temperatures		
Lag1	−0.04	−0.26–0.18
Lag2	0.22	0.02–0.42
Lag3	0.12	−0.10–0.34
Lag4	−0.1	−0.26–0.10
1 Day increase in number of wet days		
Lag1	0.07	−0.17–0.32
Lag2	0.13	−0.06–0.32
Lag3	−0.18	−0.37–0.02
Lag4	0	−0.16–0.16

¹Poisson regression coefficient (β): the exponential of β indicates the rate ratio, namely the change in the infection rate (multiplicative term) associated with a 1°C increase in weekly average maximum temperatures and 1 day increase in weekly number of wet days. *p* value < 0.05 in bold.

observed pattern may also support the hypothesis that increased mosquito density leads to increased avian defensive behaviors, leading to a shift in mosquito feedings toward less defensive hosts, such as mammals which are not WNV-susceptible (27, 28).

A complex interaction between mosquito abundance and susceptible hosts determines the level of virus circulation, which differed in direct relation (namely more mosquitoes, more virus) and is usually retained in vector-borne diseases. We recommend that this result is considered in the construction of epidemiological models and in the cost-efficacy analysis of vector control methods.

In this study, temperature was confirmed as one of the most important factors in determining the virus circulation. We detected an association between temperatures recorded one week and two weeks before mosquito sampling and the mosquito infection rate. Temperature influenced the bionomics of mosquitoes in several ways, for example, by increasing vectorial capacity or shortening the extrinsic incubation period and development times (29). These results are consistent with previous studies that observed a positive association between temperatures and WNV-positive mosquitoes and human cases observed in the following weeks (30–32). This association, already confirmed by several research studies in different ecological settings, is alarming in a global warming scenario, since it implies that increasing temperatures will increase the risk of WNV circulation. The same association can partially explain the recent emergence of the virus, which could be linked to the increase in temperatures recorded in recent years in the surveyed area (33). Interestingly, we observed the difference of one or two weeks in the temperature's ability to influence the number of mosquitoes and the virus infection rate. This observed delay could be explained with the virus' extrinsic incubation period, which consistently lasts about one week at temperatures recorded in the surveyed area (34, 35).

As recorded in another study (31), we were unable to find a consistent link between rainfall (expressed as wet days), mosquito abundance and the mosquito infection rate. An issue related to

this process, which can hide the effects of rain, could be the background noise linked to the fall in temperature caused by rainfall. However, water availability under ordinary conditions was not a limiting factor in the study area (due to the widespread presence of irrigating networks, rivers, and water basins).

A human case of WNND is of striking relevance, due to the severity of the symptoms associated with the infection. However, occurrence is rare and not relevant for the environmental persistence of the virus. Despite this, occurrence of WNND was the best available indicator of circulation intensity in people, since there was no organic and standardized system for WNV fever diagnosis. The timing and location of WNND cases are random due to the low rate of WNND cases in infected persons (<1%), the complexity in defining a certain site of patient infection and has human density as one of the main determinants (36), in a particular area. Despite this, we were able to find a clear relationship between the rate of positive mosquitoes and human cases, highlighting the surveillance ability to assess the intensity of virus circulation in a given area at an early stage.

The entomological monitoring described in this work is part of a multidisciplinary surveillance integrating other monitoring systems (wild bird testing and syndromic surveillance of horses). Entomological surveillance often provided the first sign of WNV circulation and, due to the sampling program's standardized regularity, allowed for a fine characterization of the period, area and intensity of circulation. This provides essential data for modulating actions to limit the health risks associated with the circulation of WNV.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

MCa, PA, RC, DC, MPC, MCh, GG, MT, RB, and DL conceived and designed the surveillance system. MCa, SC, and TT conducted the sampling and mosquito identification. PB and DT performed the molecular analysis. MCa, GM, LB, and AA processed the data and performed statistical analyses. MCa wrote the manuscript. All authors reviewed and approved the final manuscript.

FUNDING

Data were obtained within the framework of the Regional Plan for Surveillance and Control of Arboviruses—2018 (Emilia-Romagna Region and the Lombardy Region), and the West Nile and Usutu Viruses Integrated National Surveillance and Response plan—2018. Scientific work was partially financed by IZSLER.

REFERENCES

- Sambri V, Capobianchi M, Charrel R, Fyodorova M, Gaibani P, Gould E, et al. West Nile virus in Europe: emergence, epidemiology, diagnosis, treatment, and prevention. *Clin Microbiol Infect.* (2013) 19:699–704. doi: 10.1111/1469-0691.12211
- Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. *Lancet.* (1998) 352:767–771. doi: 10.1016/S0140-6736(98)03538-7
- Autorino GL, Battisti A, Deubel V, Ferrari G, Forletta R, Giovannini A, et al. West Nile virus epidemic in horses, Tuscany region, Italy. *Emerg Infect Dis.* (2002) 8:1372–1378. doi: 10.3201/eid0812.02034
- ECDC. *Epidemiological update: West Nile virus transmission season in Europe, 2018.* Available online at: <https://ecdc.europa.eu/en/news-events/epidemiological-update-west-nile-virus-transmission-season-europe-2018>
- Angelini P, Tamba M, Finarelli AC, Bellini R, Albieri A, Bonilauri P, et al. West Nile virus circulation in Emilia-Romagna, Italy: the integrated surveillance system 2009. *Euro Surveill.* (2010) 15:19547.
- Bellini R, Calzolari M, Mattivi A, Tamba M, Angelini P, Bonilauri P, et al. The experience of West Nile virus integrated surveillance system in the Emilia-Romagna region: five years of implementation, Italy, 2009 to 2013. *Euro Surveill.* (2014) 19:20953. doi: 10.2807/1560-7917.ES2014.19.44.20953
- Calzolari M, Pautasso A, Montarsi F, Albieri A, Bellini R, Bonilauri P, et al. West Nile virus surveillance in 2013 via mosquito screening in northern Italy and the influence of weather on virus circulation. *PLoS ONE.* (2015) 10:e0140915. doi: 10.1371/journal.pone.0140915
- Chiari M, Prosperi A, Faccin F, Avisani D, Cerioli M, Zanoni M, et al. West Nile virus surveillance in the Lombardy Region, Northern Italy. *Transbound Emerg Dis.* (2015) 62:343–349. doi: 10.1111/tbed.12375
- Chiari M, Calzolari M, Prosperi A, Perulli S, Faccin F, Avisani D, et al. Surveillance of mosquitoes and selected arthropod-borne viruses in the context of Milan EXPO 2015. *Int J Environ Res Public Health.* (2016) 13:E689. doi: 10.3390/ijerph13070689
- Rovida F, Sarasini A, Campanini G, Percivalle E, Gorini G, Mariani B, et al. West Nile virus outbreak in the Lombardy region, northern Italy, summer 2013. *Vector Borne Zoonotic Dis.* (2015) 15:278–283. doi: 10.1089/vbz.2014.1711
- Paternoster G, Babo Martins S, Mattivi A, Cagarelli R, Angelini P, Bellini R, et al. Economics of one health: costs and benefits of integrated West Nile virus surveillance in Emilia-Romagna. *PLoS ONE.* (2017) 12:e0188156. doi: 10.1371/journal.pone.0188156
- Calzolari M, Bonilauri P, Bellini R, Albieri A, Defilippo F, Maioli G, et al. Evidence of simultaneous circulation of West Nile and Usutu viruses in mosquitoes sampled in Emilia-Romagna region (Italy) in 2009. *PLoS ONE.* (2010) 5:e14324. doi: 10.1371/journal.pone.0014324
- Severini F, Toma L, Di Luca M, Romi R. Le zanzare italiane: generalità e identificazione degli adulti (Diptera, Culicidae). *Frag Ent.* (2009) 41:213–372. doi: 10.4081/fe.2009.92
- Calzolari M, Bonilauri P, Bellini R, Caimi M, Defilippo F, Maioli G, et al. Arboviral survey of mosquitoes in two northern Italian regions in 2007 and 2008. *Vector Borne Zoonotic Dis.* (2010) 10:875–84. doi: 10.1089/vbz.2009.0176
- Tang Y, Anne Hapip C, Liu B, Fang CT. Highly sensitive TaqMan RT-PCR assay for detection and quantification of both lineages of West Nile virus RNA. *J Clin Virol.* (2006) 36:177–82. doi: 10.1016/j.jcv.2006.02.008
- Del Amo J, Sotelo E, Fernández-Pinero J, Gallardo C, Llorente F, Agüero M, et al. A novel quantitative multiplex real-time RT-PCR for the simultaneous detection and differentiation of West Nile virus lineages 1 and 2, and of Usutu virus. *J Virol Methods.* (2013) 189:321–7. doi: 10.1016/j.jviromet.2013.02.019
- Scaramozzino N, Crance JM, Jouan A, DeBriel DA, Stoll F, Garin D. Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. *J Clin Microbiol.* (2001) 39:1922–7. doi: 10.1128/JCM.39.5.1922-1927.2001
- Biggerstaff J. *PooledInfRate, Version 4.0: a Microsoft® Office Excel® Add-In to compute prevalence estimates from pooled samples.* Fort Collins, CO: Centers for Disease Control and Prevention (2009).
- Klein Tank AMG, Wijngaard JB, Können GP, Böhm R, Demarée G, Gocheva A, et al. Daily dataset of 20th-century surface air temperature and precipitation series for the European Climate Assessment. *Int J of Climatol.* (2002) 22:1441–53. doi: 10.1002/joc.773
- Wolfinger R & O'Connell M. Generalized linear mixed models a pseudo-likelihood approach. *J Stat Comp Simul.* (1993) 48:233–43. doi: 10.1080/00949659308811554
- Holick J, Kyle A, Ferraro W, Delaney RR, Iwaszczko M. Discovery of Aedes albopictus infected with West Nile virus in southeastern Pennsylvania. *J Am Mosq Control Assoc.* (2002) 18:131.
- Akiner MM, Öztürk M, Başer AB, Günay F, Hacıoglu S, Brinkmann A, et al. Arboviral screening of invasive Aedes species in northeastern Turkey: West Nile virus circulation and detection of insect-only viruses. *PLoS Negl Trop Dis.* (2019) 13:e0007334. doi: 10.1371/journal.pntd.007334
- Sardelis MR, Turell MJ, O'Guinn ML, Andre RG, Roberts DR. Vector competence of three North American strains of Aedes albopictus for West Nile virus. *J Am Mosq Control Assoc.* (2002) 18:284–289.
- Tiawsirisup S, Platt KB, Evans RB, Rowley WA. Susceptibility of *Ochlerotatus trivittatus* (Coq.), *Aedes albopictus* (Skuse), and *Culex pipiens* (L.) to West Nile virus infection. *Vector Borne Zoonotic Dis.* (2004) 4:190–7. doi: 10.1089/1530366042162524
- Mancini G, Montarsi F, Calzolari M, Capelli G, Dottori M, Ravagnan S, et al. Mosquito species involved in the circulation of West Nile and Usutu viruses in Italy. *Vet Ital.* (2017) 53:97–110. doi: 10.12834/VetIt.114.933.4764.2
- Balenghien T, Vazeille M, Grandadam M, Schaffner F, Zeller H, Reiter P, et al. Vector competence of some French Culex and Aedes mosquitoes for West Nile virus. *Vector Borne Zoonotic Dis.* (2008) 8:589–95. doi: 10.1089/vbz.2007.0266
- Edman JD, Taylor DJ. Culex nigripalpus: seasonal shift in the bird: mammal feeding rates in a mosquito vector of human encephalitis. *Science.* (1968) 161:67–8. doi: 10.1126/science.161.3836.67
- Thiemann TC, Wheeler SS, Barker CM, Reisen WK. Mosquito host selection varies seasonally with host availability and mosquito density. *PLoS Negl Trop Dis.* (2011) 5:e1452. doi: 10.1371/journal.pntd.001452
- Kramer LD. Complexity of virus-vector interactions. *Curr Opin Virol.* (2016) 21:81–6. doi: 10.1016/j.coviro.2016.08.008
- Moirano G, Gasparrini A, Acquafatta F, Fratianni S, Merletti F, Maule M, et al. West Nile Virus infection in Northern Italy: Case-crossover study on the short-term effect of climatic parameters. *Environ Res.* (2018) 167:544–9. doi: 10.1016/j.envres.2018.08.016
- Cotar AI, Falcuta E, Prioteasa LE, Dinu S, Ceianu CS, Paz S, et al. Transmission dynamics of the West Nile virus in mosquito vector populations under the influence of weather factors in the Danube Delta, Romania. *EcoHealth.* (2016) 13:796–807. doi: 10.1007/s10393-016-1176-y
- Paz S, Malkinson D, Green MS, Tsioni G, Papa A, Danis K, et al. Permissive summer temperatures of the 2010 European West Nile fever upsurge. *PLoS ONE.* (2013) 8:e56398. doi: 10.1371/journal.pone.0056398
- Antolini G, Pavan V, Tomozeiu R, Marletto V. *Atlante Climatico dell'Emilia-Romagna 1961-2015. Edizione 2017.* Bologna: Arpa Emilia-Romagna. (2017) pp 32.

34. Danforth ME, Reisen WK, Barker CM. Extrinsic incubation rate is not accelerated in recent california strains of West Nile Virus in culex tarsalis (Diptera: Culicidae). *J Med Entomol.* (2015) 52:1083–9. doi: 10.1093/jme/tjv082
35. Reisen WK, Fang Y, Martinez VM. Effects of temperature on the transmission of West Nile Virus by Culex tarsalis (Diptera: Culicidae). *J Med Entomol.* (2006) 43:309–17. doi: 10.1093/jmedent/43.2.309
36. Talbot B, Ardis M, Kulkarni MA. Influence of demography, land use, and urban form on West Nile Virus risk and human west Nile Virus Incidence in Ottawa, Canada. *Vector Borne Zoonotic Dis.* (2019) 19:533–9. doi: 10.1089/vbz.2018.2366

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Calzolari, Angelini, Bolzoni, Bonilauri, Cagarelli, Canziani, Cereda, Cerioli, Chiari, Galletti, Moirano, Tamba, Torri, Trogu, Albieri, Bellini and Lelli. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



African Swine Fever Virus: An Emerging DNA Arbovirus

Natasha N. Gaudreault^{1*}, Daniel W. Madden¹, William C. Wilson², Jessie D. Trujillo¹ and Juergen A. Richt^{1*}

¹ Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS, United States, ² Arthropod Borne Animal Diseases Research Unit, Agricultural Research Service, United States Department of Agriculture, Manhattan, KS, United States

OPEN ACCESS

Edited by:

Dirk Werling,
Royal Veterinary College (RVC),
United Kingdom

Reviewed by:

Marie Rene Culhane,
University of Minnesota, United States
Faten Abdelal Okda,
St. Jude Children's Research Hospital,
United States
Armanda Bastos,
University of Pretoria, South Africa

*Correspondence:

Natasha N. Gaudreault
nng5757@vet.k-state.edu
Juergen A. Richt
jricht@ksu.edu

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 30 November 2019

Accepted: 31 March 2020

Published: 13 May 2020

Citation:

Gaudreault NN, Madden DW,
Wilson WC, Trujillo JD and Richt JA
(2020) African Swine Fever Virus: An
Emerging DNA Arbovirus.
Front. Vet. Sci. 7:215.
doi: 10.3389/fvets.2020.00215

African swine fever virus (ASFV) is the sole member of the family *Asfarviridae*, and the only known DNA arbovirus. Since its identification in Kenya in 1921, ASFV has remained endemic in Africa, maintained in a sylvatic cycle between *Ornithodoros* soft ticks and warthogs (*Phacochoerus africanus*) which do not develop clinical disease with ASFV infection. However, ASFV causes a devastating and economically significant disease of domestic (*Sus scrofa domesticus*) and feral (*Sus scrofa ferus*) swine. There is no ASFV vaccine available, and current control measures consist of strict animal quarantine and culling procedures. The virus is highly stable and easily spreads by infected swine, contaminated pork products and fomites, or via transmission by the *Ornithodoros* vector. Competent *Ornithodoros* argasid soft tick vectors are known to exist not only in Africa, but also in parts of Europe and the Americas. Once ASFV is established in the argasid soft tick vector, eradication can be difficult due to the long lifespan of *Ornithodoros* ticks and their proclivity to inhabit the burrows of warthogs or pens and shelters of domestic pigs. Establishment of endemic ASFV infections in wild boar populations further complicates the control of ASF. Between the late 1950s and early 1980s, ASFV emerged in Europe, Russia and South America, but was mostly eradicated by the mid-1990s. In 2007, a highly virulent genotype II ASFV strain emerged in the Caucasus region and subsequently spread into the Russian Federation and Europe, where it has continued to circulate and spread. Most recently, ASFV emerged in China and has now spread to several neighboring countries in Southeast Asia. The high morbidity and mortality associated with ASFV, the lack of an efficacious vaccine, and the complex makeup of the ASFV virion and genome as well as its lifecycle, make this pathogen a serious threat to the global swine industry and national economies. Topics covered by this review include factors important for ASFV infection, replication, maintenance, and transmission, with attention to the role of the argasid tick vector and the sylvatic transmission cycle, current and future control strategies for ASF, and knowledge gaps regarding the virus itself, its vector and host species.

Keywords: African swine fever virus, DNA arbovirus, soft tick, domestic swine, wild boar, transmission, virus replication

ASFV EMERGENCE AND RE-EMERGENCE

Since its identification in Kenya in 1921 (1, 2), African swine fever virus (ASFV) has remained endemic in Africa, affecting up to 35 African countries (3). Between the late 1950s and early 1980s, ASFV genotype I emerged in Europe, Russia, the Caribbean and South America. ASFV was first identified in Europe in 1957 in Portugal, then was re-introduced in 1960 from which it quickly spread into Spain, Italy, France, Sardinia, Malta, Belgium, and The Netherlands (1, 4, 5). ASFV was first reported in Russia in 1977 (4), and in the late 1970s it emerged in Brazil, Cuba and the Caribbean Islands, with the last outbreaks in the Americas occurring between 1980 and 1984 (6). By the mid-1990s, ASFV had been eradicated outside of Africa, with the exceptions of an isolated outbreak in Portugal in 1999 and the island of Sardinia where it has remained endemic (7, 8).

In 2007, ASFV genotype II emerged in the Republic of Georgia and continued to spread through the Caucasus region and subsequently into the Russian Federation and Eastern Europe, where it has continued to circulate and spread as illustrated in **Figure 1A** (1, 4, 9). ASFV re-emerged in north-western Europe in Belgium in 2018 in wild boar (10). More recently it was detected in carcasses of wild boar in western Poland near the German boarder (<https://www.vettimes.co.uk>). In August 2018, ASFV was reported for the first time in the People's Republic of China, and by the end of September of 2019, ASFV was detected in neighboring countries including Mongolia, Vietnam, Cambodia, Democratic People's Republic of Korea (North Korea), Lao People's Democratic Republic, Myanmar, Timor-Leste, the Philippines, the Republic of Korea (South Korea), and Indonesia as shown in **Figure 1B** (FAO situation update, www.fao.org). African countries which have

notified the OIE of the presence of ASF from 2018 through September 2019 include Benin, Burkina Faso, Burundi, Cabo Verde, Central African Republic, Democratic Republic of the Congo, Republic of the Congo, The Gambia, Ghana, Guinea-Bissau, Madagascar, Malawi, Mozambique, Namibia, Nigeria, Rwanda, Senegal, Sierra Leone, South Africa, Tanzania, Togo, Uganda, Zambia, and Zimbabwe, indicated in **Figure 1C** [OIE WAHIS African Swine Fever (ASF) Report: September 13–26, 2019; www.oie.int].

Impact of Recent ASFV Emergence as of February 2020

ASFV does not cause disease in humans, is highly contagious and causes high mortality in domestic and feral swine, and has a significant economic impact on the global swine industry. While the situation remains ever-changing due to continued outbreaks and spread of ASFV globally, information from peer-reviewed manuscripts, situation reports, and press releases provide some indication of the impact of ASFV emergence on animal health and economics of effected countries. **Table 1** summarizes the ASF affected countries of Europe and Asia from 2007 to February 2020 including reported estimates of number of animals lost. ASF has especially affected China, which is the world's largest pork producer and consumer, producing about 50 percent of the world's pork supply (ChinaDaily.com.cn, 9/11/2019, "Swine fever may affect pork for several years," global.chinadaily.com.cn). Since the first reported outbreak in China in August 2018, ASF has been detected in at least 8 other countries in Asia and has resulted in the death or culling of more than 5 million pigs, with losses accounting for more than 10 percent of the total pig population in China, Mongolia and Vietnam [(11, 12); FAO situation update, www.fao.org; FAO press release, 09/08/2019, "One year on, close to 5 million pigs lost to Asia's swine fever

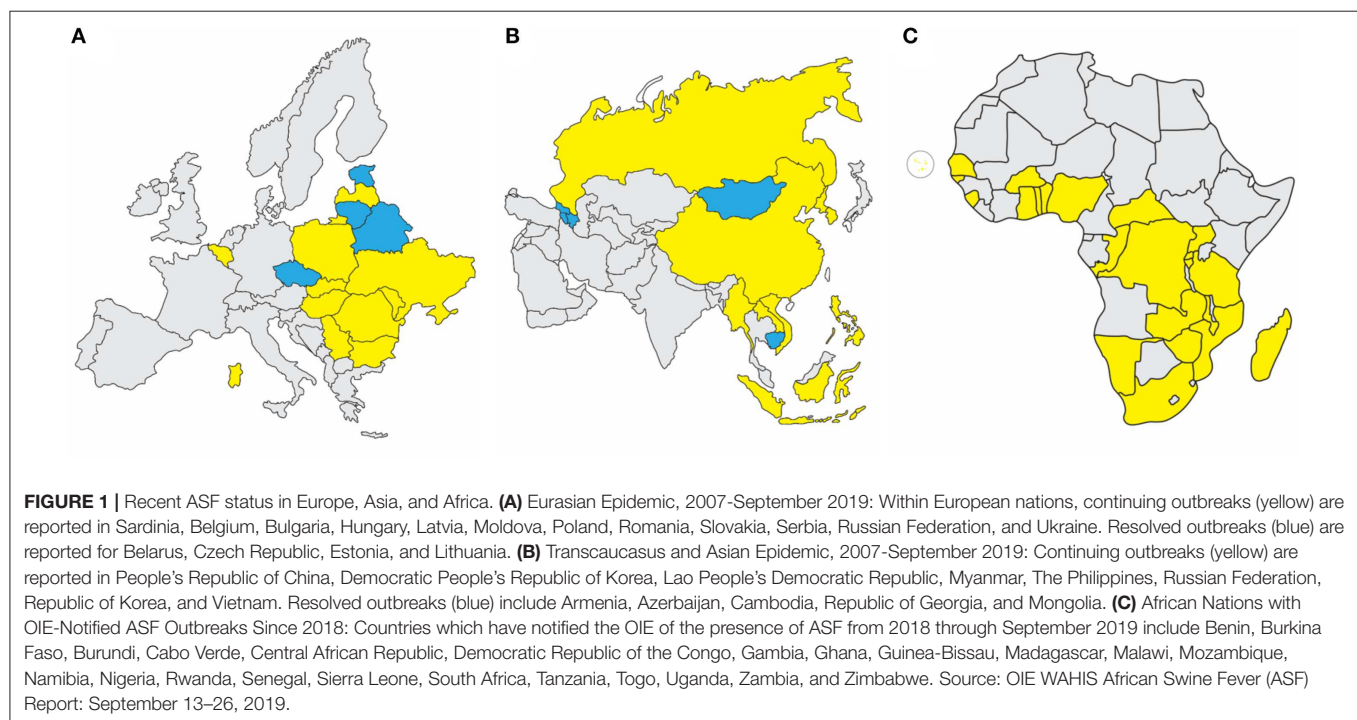


TABLE 1 | ASFV in Eurasia from January 2007 to February 2020.

Country	Year or date reported	Status	Estimated animal losses	Species
Georgia	2007–2008	Resolved	87,412	Swine
Armenia	2007–2008, 2010–2011	Resolved	2,483	Swine
Azerbaijan	2008	Resolved	4,832	Swine
Russian Federation	2007–2019	Continuing	79,632	Swine, wild boar
Ukraine	2012, 2014–2019	Continuing	20,166	Swine, wild boar
Belarus	2013	Resolved	20,627	Swine
Lithuania	2014–2019	Resolved	23,735	Swine
Latvia	2014–2019	Continuing	294	Swine, wild boar
Estonia	2014–2019	Resolved	26	Wild boar
Poland	2014–2019	Continuing	37,396	Swine, wild boar
Czech Republic	2017, 2018	Resolved	202	Wild boar
Romania	2017–2019	Continuing	90,698	Swine, wild boar
Hungary	2018–2019	Continuing	1,536	Wild boar
Bulgaria	2018–2019	Continuing	137,973	Swine, wild boar
Moldova	2016–2019	Continuing	348	Swine, wild boar
Belgium	2018–2019	Continuing	540	Wild boar
Slovakia	2019	Continuing	70	Swine, wild boar
Serbia	2019	Continuing	290	Swine
People's Republic of China/32 provinces	August 3, 2018	Continuing	1,193,000	Swine, wild boar
Mongolia/6 provinces	January 15, 2019	Resolved	3,115	Swine
Vietnam/19 provinces	February 19, 2019	Continuing	5,960,000	Swine
Cambodia/5 provinces	April 2, 2019	Resolved	3,673	Swine
Democratic People's Republic of Korea	May 23, 2019	Continuing	124	Swine, wild boar
Lao People's Democratic Republic /15 provinces	June 20, 2019	Continuing	40,130	Swine
The Philippines	July 25, 2019	Continuing	70,000	Swine
Myanmar	August 1, 2019	Continuing	128	Swine
Republic of Korea	September 17, 2019	Continuing	10,000	Swine, wild boar
Timor-Leste	September 9, 2019	Continuing	1,600	Swine
Indonesia	September 2019	Continuing	42,000	Swine

FAO situation update, www.fao.org, 02/19/2020; OIE WAHIS Interface, Disease information, Immediate notifications and Follow-ups, www.oie.int, 09/21/2019; OIE WAHIS Interface, Disease information, Disease Timelines, www.oie.int, 10/23/2019.

outbreak”], and industry insiders predict a 30–60% loss of pig stocks due to ASF (ChinaDaily.com.cn, 9/11/2019, “Swine fever may affect pork for several years,” global.chinadaily.com.cn). This has put other countries on high alert, including Thailand which culled 200 pigs in response to mysterious pig deaths although no confirmed cases of ASF had been reported, as of September 2019 (Reuters Health News, 09/18/2019, “Thailand culls 200 pigs amid heightened fears over African swine fever,” www.reuters.com). Since its identification in 1921, outbreaks of ASFV have been reported in more than 60 countries around the world, and global ASF outbreaks since late 2018 have increased 25 percent according to media reports (Global Times, 09/18/2019, “A global battle against African swine fever,” www.globaltimes.cn).

ASFV INFECTION, MAINTENANCE, AND TRANSMISSION

Ornithodoros Soft Ticks

The *Ornithodoros* genus of soft ticks in the family *Argasidae* serve as biological vectors and reservoir hosts for ASFV. To date, eight *Ornithodoros* species have been demonstrated as vector

competent for ASFV (13). ASFV-infected *Ornithodoros porcinus* soft ticks (often referred to as *O. moubata porcinus* or *O. moubata*) in Africa have been well-documented (14–19) and have also been found in Madagascar (20). Additionally, competent *Ornithodoros* vectors are also known to exist in parts of Europe and the Americas (13, 18). *Ornithodoros erraticus* (also known as *O. maroccanus* and renamed *Carios erraticus*) soft ticks inhabit the Iberian Peninsula and Mediterranean areas of Africa and Asia, and were an important vector and reservoir for ASFV in Portugal and Spain during the ASF epidemic in the twentieth century (7, 21–23).

Ornithodoros ticks have long lifespans, and ASFV can replicate to high titers and be maintained for long periods of time in the vector with minimal cytopathological effects or increased tick mortality (7, 14–18, 20, 24, 25); although increased mortality rates have also been reported (26–31). A study following ASFV infection in *O. porcinus porcinus* ticks after feeding on viremic pigs showed ASFV titers of 6 log₁₀ HAD₅₀/tick, which were maintained at that level for at least 290 days and declined only 2 log₁₀ HAD₅₀/tick or less after 3 years (18, 25). ASFV was isolated from *O. moubata* ticks from a farm in Madagascar 4 years

after the culling of all pigs (20). ASFV transmission to pigs by infected the Iberian soft tick has been demonstrated up to 588 days after infection (29) and ASFV persistence has been shown for at least 5 years in *O. erraticus* ticks collected from infected farms in Portugal (7). However, viral clearance after one year has also been observed (28, 32). Nonetheless, virus-tick adaptation is likely necessary to achieve high virus titers since significantly lower infection rates and viral titers, and increased mortality have been observed in studies using ASFV isolates not derived from ticks, or *Ornithodoros* species not native to Africa (18, 25, 33).

Multiple ASFV genetic elements have been identified as being associated with infectivity, replication, and generalized dissemination of ASFV in *Ornithodoros* ticks. Deletion of three multigene family (MGF) 360 genes (3H1, 3IL, and 3LL) from the tick-derived pathogenic ASFV Pr4 isolate resulted in reduced infectivity and a 2–3 log₁₀ decrease in viral titer within *O. porcinus porcinus* ticks compared to the parental virus (34). CD2v, the protein responsible for viral hemadsorption (HAD) in ASFV strains displaying the HAD phenotype, has also been demonstrated to possess an important function in virus-tick interaction. Restoration of the HAD phenotype to the non-hemadsorbing NH/P68 strain carrying a CD2v gene interrupted by frameshift mutations results in an ~1,000-fold increase in viral titer within *O. erraticus* ticks after feeding on infectious whole blood, most likely due to effects on virus uptake and replication in the tick midgut epithelium (35).

Studies of ASFV infection and replication in soft ticks show that ASFV infection takes 15–21 days to reach the midgut epithelium where viral replication is initiated, with peak virus titers achieved by 28 days post-infection (25). Restricted replication within midgut epithelial cells reduces the infectivity of the Malawi Li 20/1 strain for soft ticks orally exposed to the virus (36). For successful transmission, ASFV replication in the coxal and salivary glands is required, which is usually achieved by 48 days post-infection (25).

Within the tick life cycle, ASFV can be transmitted sexually from infected male to female (17, 32), transovarially from infected female to offspring (15, 27, 37), and maintained transstadially through the various life stages [(28, 29, 38, 39); see Figure 2]. An increase in mortality rates in ASFV-infected ticks has been reported during the first three ovipositions (18, 32). The number of infected ticks observed under field conditions is typically lower than infection rates observed after experimental infections (18, 40).

ASFV Sylvatic Cycle

In Africa, ASFV is mainly maintained in a sylvatic cycle between *Ornithodoros* soft ticks and warthogs (*Phacochoerus africanus*); warthogs become viremic but do not develop clinical disease after ASFV infection (3, 19, 22, 41). The sylvatic cycle has been documented primarily for countries in southern and eastern Africa (3). Juvenile warthogs dwelling in burrows are infected by soft ticks carrying the virus, and transmission to naive ticks occurs when the ticks take a blood meal from viremic young warthogs [(14, 41); Figure 2]. ASFV warthog blood titers of at least 10³ HAD₅₀/mL are required to infect feeding ticks, which is typically only achieved in young warthogs compared to adults

which rarely have ASFV titers above 10² HAD₅₀/mL (19, 41). Other wild suids in Africa such as bush pigs (*Potamochoerus larvatus*) can also become infected and transmit ASFV, but are generally considered to play a minor role compared to warthogs in the sylvatic cycle since their behaviors are less conducive for interactions with soft ticks; only one incidence of infection in a giant forest hog (*Hylochoerus meinertzhageni*) has been reported (3, 19, 22, 42, 43).

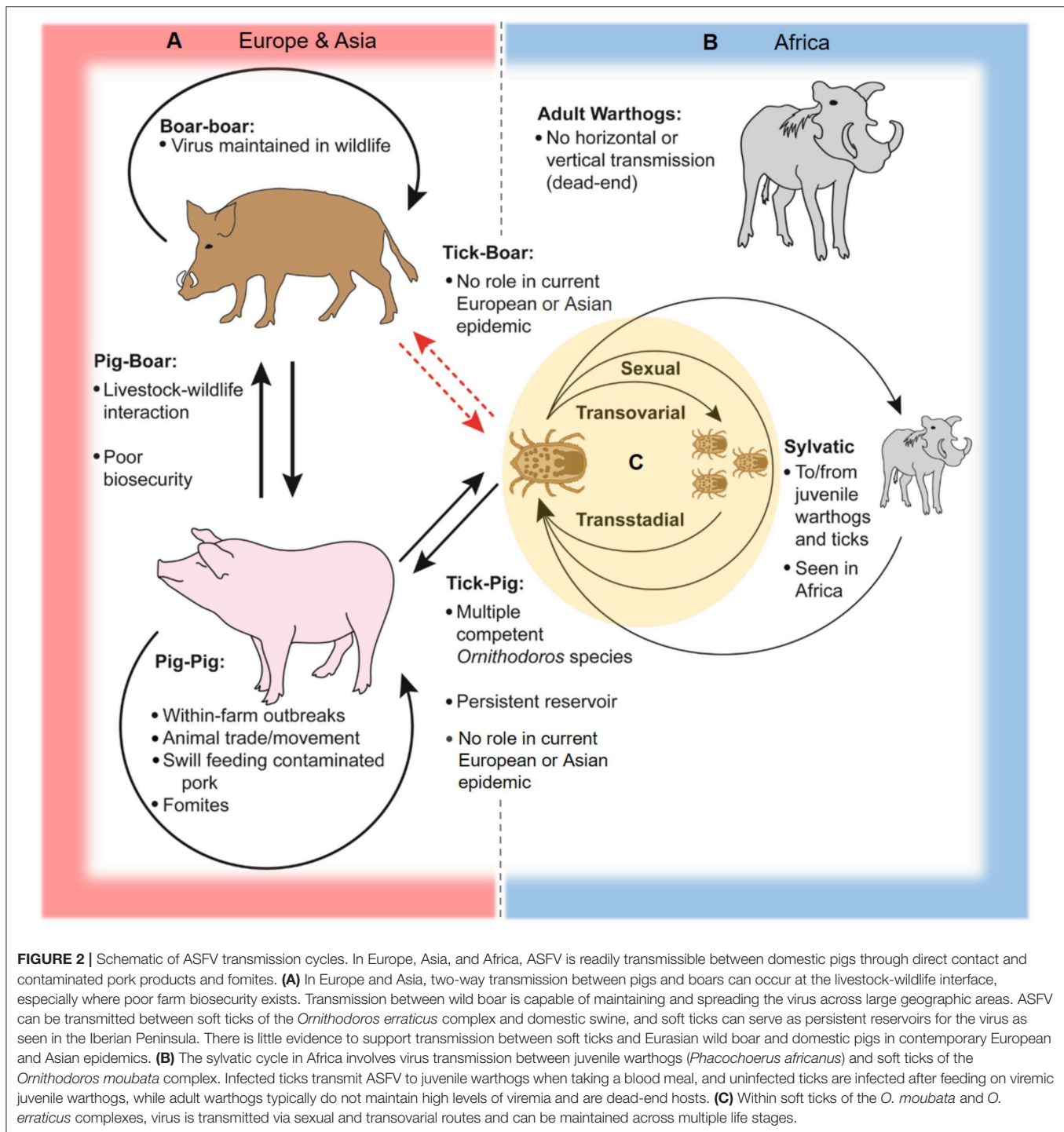
Tick-Pig (*Sus scrofa domesticus* and *Sus scrofa*) Transmission

Ornithodoros soft ticks including species of *O. moubata* complex in Africa and *O. erraticus* in Europe are capable of transmitting ASFV to domestic swine (*Sus scrofa domesticus*), and can become infected after feeding on viremic animals [(22, 25, 29, 44); Figure 2]. In Africa and Madagascar, infected ticks of the *O. moubata* complex have been isolated from pig sties and farms in locations affected by ASF outbreaks, including sites where little or no contact between wild and domestic swine occurs, suggesting an important role for soft ticks in disease maintenance in these areas (20, 45–47). A similar pattern was also observed in the Iberian Peninsula, where *O. erraticus* ticks were associated with the persistence of ASFV (7, 21–23, 40).

The genotype II Georgia 2007/1 strain responsible for the contemporary European epidemic has been experimentally demonstrated to replicate efficiently in live *O. erraticus* ticks (48). However, it is unlikely that a soft tick cycle plays a significant role in the ongoing outbreak in Europe and most likely also Asia, as soft ticks are largely absent in Central Europe and the Baltic nations, and most of the soft tick species in Eastern Europe and the Caucasus region do not infest domestic and wild swine (49). A study investigating potential contact between wild boar and soft ticks in Germany via serum screening for antibodies against *O. erraticus* in wild boar showed little evidence for feeding and infestation of wild boar by soft ticks, and limited interaction between these ASFV hosts is assumed (50).

Domestic (*Sus scrofa domesticus*) and Wild Boar (Eurasian Wild Pig; *Sus scrofa*) ASFV Infection in Domestic Swine

Infection with ASFV can produce a variety of clinical presentations ranging from chronic, subclinical, or low-level disease to hemorrhagic fever and peracute death, depending on viral strain, and host susceptibility (51). Studies of highly virulent Eurasian genotype II isolates have produced mortality rates of 100% in domestic pigs and wild boar, with disease rapidly progressing from non-specific clinical symptoms (fever, depression, anorexia, diarrhea) to death (52, 53). In contrast, the non-fatal genotype I isolates OUR T88/3 and OUR T88/4 obtained from *O. erraticus* ticks on a farm in Portugal produce no clinical disease after experimental infection of pigs (44). Genotype I ASFV strain NH/P68, isolated from a chronically-infected pig, is another example of a naturally occurring, non-fatal ASFV strain (54). However, attenuated ASFV strains including OUR T88/3 and NH/P68 can cause chronic infection in some pigs and have been associated with



chronic lesions affecting the skin and joints (54–58). Swine populations displaying increased resistance to clinical ASF have been previously described; however, offspring from these pigs reared in quarantine facilities showed no difference in survival rates compared to non-selected, susceptible animals after virulent ASFV challenge, suggesting resistance is not directly heritable (59). Clinical outcomes of ASFV infection

are therefore influenced by a variety of host, virus, and epidemiological factors.

Domestic Pig-Wild Boar Transmission

Domestic pigs readily transmit ASFV to other susceptible swine, and outbreaks of virulent strains display high levels of morbidity and mortality (22). Direct contact with infected pigs effectively

spreads disease to other wild and domestic pigs (22, 60); however, varying levels of transmission efficiency have been observed for high-, moderate-, and low-virulence strains, likely due to differences in levels of viremia and virus shedding (44, 60–63). Blood, body fluids, feces, and carcasses of infected pigs serve as indirect routes of infection (60). Animals which recover from infection with low or moderate virulent strains can become subclinical carriers potentially capable of spreading the virus to other pigs (60, 61, 64, 65). The illegal movement of infected pigs by producers or pork products has played a significant role in outbreaks of ASF in Africa, Europe, and Asia (9, 11, 66).

Eurasian wild boar are highly susceptible to the virulent ASFV genotype II isolates circulating in Europe (52, 53), and contact between infected wild boar and domestic pigs has been a significant contributing factor to the spread of ASFV in Eastern Europe, the Caucasus and the Russian Federation, where small-scale backyard pig farms with poor biosecurity are common (9). ASFV has been detected in wild boar throughout Eastern and Central Europe, and as far west as Belgium (1, 10). The existence of a geographically widespread wild pig population in which ASFV can circulate poses a significant challenge to disease control and eradication efforts.

Other Modes of Transmission

ASFV is stable under extreme environmental conditions, allowing it to be easily spread and transmitted. Modes of transmission other than direct contact with infected swine, tissues, carcasses or bites from infected soft ticks, include importation of infected pork products and contamination of fomites such as feed, equipment, vehicles, and clothing (22). ASFV can remain viable in a variety of animal feed ingredients under a range of environmental conditions, including those characteristic of trans-Atlantic shipping routes (67, 68), and efficient disease transmission through ASFV-contaminated liquids and plant-based animal feeds has been experimentally demonstrated (69). The movement of contaminated pork products and swill-feeding of domestic swine have been important epidemiological factors in ASFV outbreaks in the Caucasus and Russian Federation as well as the emergence of the disease in China (9, 11).

MOLECULAR PROPERTIES OF ASFV

ASFV has a large double-stranded DNA genome ranging from 170 to 190 kilobase pairs (kbp) that encodes more than 150 open reading frames (ORFs), depending on viral strain; it is the only known DNA arbovirus (70, 71). The observed significant differences in genome size are primarily due to gain or loss of gene copies belonging to the multigene families (MGFs) and variation within the number of tandem repeats in non-coding regions of the ASFV genome (70, 71). Mass spectrometry has identified 68 virion-associated structural proteins from purified virions of strain BA71V and up to 94 virion-associated polypeptides were detected in virions from 3 different mammalian cell lines infected with a recombinant OURT 88/3 strain; the precise function of a significant proportion of the structural and non-structural ASF viral proteins is

unknown (72, 73). The virion is ~200 nm in diameter and possesses a multi-layered structure consisting of the nucleoid, core shell, inner envelope, capsid, and a host-derived outer envelope (74). The p72 major capsid protein and four minor capsid proteins, M1249L, p17, p49, and H240R, make up the viral capsid (75).

Genotyping of ASFV has historically been based on the nucleotide sequence of a 478 bp variable region in the C-terminus of the viral p72 gene (76), though other viral genes have also been used to further characterize ASFV strains (77–79). Currently, there are 24 genotypes based on the major capsid protein p72, and 8 serotypes based on the viral hemagglutinin CD2-like protein (CD2v) and C-type lectin (80–83). All of the 24 ASFV genotypes have been identified in Africa (3). Strain virulence cannot be accurately predicted by p72 genotype (80). The first emergence of ASFV outside of Africa consisted of genotype I viruses, which are predominantly described in West Africa (22). Genotype II ASFV was introduced into the Caucasus in 2007, most likely from East Africa, and remains the current ASFV genotype circulating throughout Europe, the Russian Federation and Southeast Asia (22, 84).

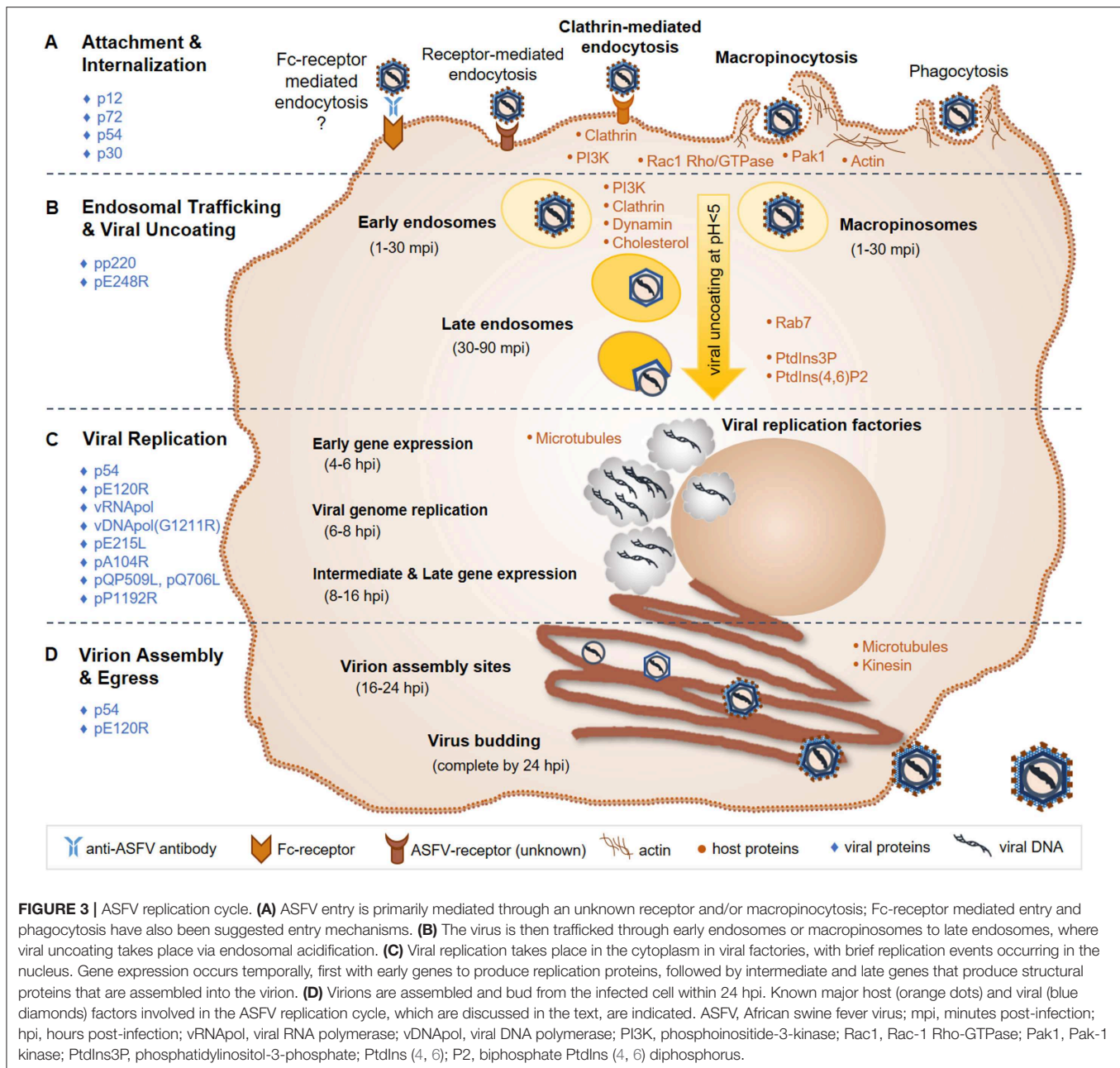
Details of virus-host interactions and events involved in the ASFV replication cycle have been reviewed previously (71, 85–88) and are summarized in the following sub-sections and **Figure 3**.

Target Cells

In swine, ASFV preferentially replicates in cells of the monocyte/macrophage lineage (89). It can also replicate in some established cell lines although with less efficiency (87, 90). The virulent ASFV BA71 strain was adapted to replicate in Vero cells (BA71V). Vero cells derived from African green monkey kidney, and have been widely used as a model for *in vitro* ASFV infection and replication studies (91). However, adaptation in cell culture can cause genetic modifications to the virus that result in attenuation of virulence and decreased fitness in primary swine macrophage cultures, which is the case for BA71V and other cell-adapted ASFV strains (92, 93). Therefore, only virus derived from infected animal tissues or propagated in primary swine macrophage cultures usually retains the natural characteristics and phenotype of the original virus (90).

ASFV Entry and Early Events in the Infectious Cycle

Several modes of cell entry for ASFV have been demonstrated (86, 94–96). Early studies on ASFV entry indicate receptor-dependent mechanisms including low pH and temperature-dependent events, and determined that ASFV binding to cell surfaces was saturable (97–100). Given the cell tropism of ASFV, several macrophage receptors have been implicated as playing a possible role including CD163, CD45, MHC II, and others, although no specific receptor for ASFV has yet been identified (101, 102). Earlier *in vitro* studies supported CD163 as being a significant receptor for ASFV, demonstrating that monoclonal antibodies could block infection (101). However, ASFV infection of gene-edited pigs lacking CD163 showed no difference in the course of infection or survival compared to wild type



CD163-expressing pigs, indicating that other receptors or entry mechanisms are critical (103).

Fc-receptor mediated entry of ASFV into cells has also been proposed, although the results from one study indicated Fc-receptors do not mediate ASFV infection of macrophages (104). Nonetheless, several studies suggest antibody-dependent enhancement of ASFV infection characterized by early and increased viremia and accelerated disease, supporting *in vivo* Fc-receptor involvement (105). Antibody-dependent enhancement of infection occurs through entry of macrophage-tropic microorganisms facilitated by IgG antibody-antigen complexes and Fc-receptor signaling, and has been demonstrated for several

viruses including porcine reproductive and respiratory virus, Dengue virus, and West Nile virus, among others (106–110). Accelerated disease has been observed in vaccinated swine compared to non-vaccinated controls following virulent ASFV challenge (111–113), and enhancement of ASFV infection was observed *in vitro* in the presence of sera from immunized animals, all of which coincide with the presence of non-neutralizing ASFV-antibodies (111, 114). Further investigations are warranted to elucidate the mechanisms involved in ASFV enhancement of infection and pathogenesis.

Other mechanisms including phagocytosis (115) and non-receptor mediated entry by macropinocytosis have also been

investigated (94, 116). Macropinocytosis is the non-selective, actin-dependent uptake of molecules, and is utilized by several large DNA viruses, including poxviruses and herpesviruses (117). ASFV apparently also utilizes macropinocytosis, demonstrated by the use of chemical inhibitors, purified labeled virions, and fluorescent and transmission electron microscopy to monitor early events of ASFV infection in swine macrophages (94, 116).

The current working model for ASFV entry includes both clathrin-mediated endocytosis and macropinocytosis (86, 95, 96). Actin modulation through phosphoinositide-3-kinase (PI3K), Rac-1 Rho-GTPase and Pak-1 kinase signaling is important for ASFV internalization via macropinocytosis (85, 94, 96, 118). PI3K is essential for ASFV infection, likely playing a critical role in endosomal trafficking of ASFV (85, 94, 118). Clathrin, dynamin, and cholesterol are required for ASFV transport through endosomes in both Vero cells and swine macrophages (118–120). Following internalization into early endosomes or macropinosomes, ASFV particles are transported to late endosomes where the cellular factor Rab7 was shown to play an important role (116, 118). ASF virions can be found in early endosomes between 1 and 30 min post-infection (mpi) and in late endosomes at 30–90 mpi. Increasing acidification through endosomal trafficking plays an essential role in the uncoating of the ASFV outer envelope and capsid. A pH below 5.0 was shown to be required for virion uncoating (116). Fusion then occurs between the ASFV inner envelope and late endosomal membranes, releasing the viral core into the cytoplasm where viral factories will subsequently form and ASFV replication takes place. Host cell phosphatidylinositol-3-phosphate (PtdIns3P) and biphosphate PtdIns (4, 6) diphosphorus are important for the progression of early infection events to the start of ASFV replication (85, 118).

ASFV Proteins Involved in the Initial Steps of Infection

ASFV structural proteins involved in virus attachment to permissive cells include p12, p72, and p54 (121–124). ASFV p30 is an early and abundantly expressed phosphoprotein and is necessary for virus internalization (124). The pp220 polyprotein is cleaved into 4 major protein components of the viral core, all of which are required for core detachment and release (125–127). The internal envelope protein pE248R is essential for viral fusion with endosomal membranes and core release (116, 128).

ASFV Gene Expression and Replication

Similar to other large DNA viruses such as poxviruses and herpesviruses, ASFV uses a temporal gene expression strategy (71, 85). The viral RNA polymerase recognizes and initiates the expression of early, intermediate and late genes throughout the respective stages of the ASFV replication cycle. Early gene expression occurs around 4–6 h post-infection (hpi), and produces proteins necessary for viral replication. At 6–8 hpi, ASFV replication is initiated via its own DNA polymerase encoded by gene G1211R. While ASFV replication primarily occurs in viral factories in the perinuclear region of the cytoplasm, an initial brief replication phase takes place in the cell nucleus (88). Intermediate and late gene expression then follows at 8–16 hpi producing structural proteins that are incorporated

into the virion. ASFV encoded E2 ubiquitin-conjugating enzyme [E215L; (129)], histone-like protein [pA104R; (130)], RNA helicases [QP509L and Q706L; (131)], and topoisomerase II [pP1192R; (132, 133)] have all been shown to localize to viral factories as well as diffusely within the cell cytoplasm and nucleus. Localization and expression studies along with siRNA knockdown experiments indicate that these viral factors play important roles during ASFV gene expression, genome replication and packaging (134).

Virion Assembly and Transport of Mature Virus Particles

Microtubules play an essential role in ASFV cellular transport and viral factory formation (85, 86, 135, 136). Microtubules and kinesin work together to support budding of ASF virions from the infected cell (136). ASFV p54 interacts with microtubules and is required for formation of viral factories and the recruitment of envelope precursors to virion assembly sites (137, 138). The viral capsid protein pE120R facilitates transport of mature virus particles from assembly sites to the plasma membrane, where the virus acquires its host-derived outer envelope (74, 139). Altogether, an entire ASFV infection cycle, from attachment and entry to budding of mature virus particles, is completed within 24 hpi (85).

ASFV Gene Functions and Virulence Factors

ASFV encodes for at least 150 proteins. So far, 38 ASFV proteins are associated with known or predicted functions in nucleotide metabolism, transcription, replication and repair; more than 24 ASFV proteins are involved in virion structure and morphogenesis, and at least 8 ASFV proteins are likely involved in host cell interactions (71). However, the functions of a large number of ASFV-encoded proteins still remain unknown. ASFV encodes several gene products involved in virulence and counteracting host antiviral responses. The ASFV protein DP96R has been shown to inhibit the cGAS-STING pathway, thereby blocking IFN- β production, a key mediator between innate and adaptive immune responses (140, 141); ASFV gene product I329L has been shown to inhibit toll-like receptor 3 signaling and type I interferon induction (142). In addition, ASFV proteins CD2v and I215L block the transcription of immunoregulatory genes, and ASFV proteins DP71L, A179L, A224L, and EP153R promote cell survival (71, 85). CD2v has also been shown to bind to host adaptor protein 1 (AP-1) and localizes around viral factories, which suggests a role in subversion of cell protein trafficking to promote viral replication and packaging (143). ASFV genes of the multigene families MGF360 and MGF505/530 are also associated with counteracting antiviral host responses involving interferon-associated mechanisms (144–146), and are host range determinants (147). MGF360 genes have the most copies and are the most variable among ASFV strains (71). Naturally attenuated ASFV strains typically lack multiple copies of MGF360 and MGF505/530 genes as well as the CD2v gene (70, 71). Furthermore, it has been demonstrated that targeted deletion of certain genes within MGF360 and MGF505, or of

CD2v, is capable of attenuating certain wild-type ASFV strains, but not all, indicating other ASFV virulence genes/factors are also important for the virulence of ASFV (148–151).

CONTROL OF ASFV

Successful prevention and mitigation of ASF outbreaks is hindered by multiple factors, including the lack of an effective vaccine, the broad geographic distribution of wild and feral swine and potential arthropod vectors capable of maintaining the virus, as well as the increasingly globalized nature of animal agriculture. As a result, ASF control strategies primarily focus on early detection, restriction on livestock movement, and culling of herds affected by or potentially exposed to the virus. The development of effective countermeasures for ASF will be essential in combatting current and future epidemics, and the associated trade restrictions.

Vaccines

Despite decades of research, a broadly protective, commercially available vaccine for ASFV remains elusive. Multiple vaccine development strategies have been employed, with varying levels of success. Inactivated whole viral antigen does not induce protective immunity (152). Subunit, vector-based, and DNA vaccines targeting specific viral proteins have produced inconsistent results, ranging from variable protection to enhancement of disease and accelerated mortality (105, 113, 153–155). Attenuated modified live virus (MLV) vaccines, derived from extensive viral passaging in cell lines or through targeted gene deletions, have been extensively investigated and can confer protection against homologous parental virus challenge (156), but generally provide little to no cross-protection against heterologous virulent strains (157). Additionally, MLV vaccines usually have a limited safety profile with modest to severe side effects causing arthritis, skin necrosis and chronic infections. Further research into the correlates of protection and basic ASFV immunology is needed to facilitate targeted, rationally-designed vaccine development (105, 157–159). A number of highly immunogenic ASFV antigens have been identified, yet the role of ASFV-specific cellular and humoral immune responses in protection from ASF is still not completely clear. Results regarding the role of ASFV-specific neutralizing antibodies in protection are conflicting, and high levels of non-neutralizing antibodies appear to have a detrimental effect (105, 160). For example, the presence of neutralizing antibodies does not always confer protection and in some cases immunization with ASF proteins is associated with enhanced ASFV infection and pathology, despite induction of antibodies which are neutralizing *in vitro* (105, 113, 153). Importantly, cell-mediated immunity, including induction of CD8⁺ T-cells and natural killer cells, appears to play an important role in protection against ASF (54, 161, 162), since pigs exposed to the low-virulence OUR/T88/3 strain and subsequently depleted of CD8⁺ lymphocytes were no longer protected from challenge with the virulent OUR/T88/1 isolate.

Basic research to elucidate ASFV gene functions and the mechanisms of ASFV replication, pathogenesis and immune

responses is critically needed to facilitate rational vaccine development (105, 157–159). This research will be important for identifying protective proteins as vaccine targets and feasible delivery systems that induce both humoral and cellular immune responses which correlate with protection. Other important elements needed for successful ASFV vaccine development are a permanent cell culture system for MLV vaccine production as well as the design of companion diagnostic assays that are capable of differentiating infected from vaccinated animals (DIVA) (157, 158).

Detection and Diagnosis

Since its discovery over 90 years ago, an array of diagnostic assays have been developed and employed for ASF. However, current methods for ASFV diagnosis often possess significant limitations such as (i) suboptimal analytical and clinical sensitivity/specificity, (ii) inadequate ability to detect early acute or chronically-infected animals, (iii) high cost, (iv) long time intervals for receiving results, and/or (v) the need for specialized equipment and high containment facilities (163). Consequently, the development of accurate, rapid, affordable, and field-deployable highly sensitive and specific diagnostic tests for ASFV remains a significant priority.

Detection of Virus and Viral Antigen

Methods for detection of virus and viral antigens include virus isolation and hemadsorption (HAD), fluorescent antibody testing, and antigen detection by ELISA or lateral flow tests (163, 164). A positive virus isolation or HAD test is considered definitive for ASFV; however, both assays are expensive, require primary cell cultures, and take >7 days to complete, and are therefore only performed by a few reference laboratories (163, 165, 166). Furthermore, not all ASFV isolates are hemadsorbing and some would therefore test negative in the HAD test. Direct fluorescence antibody testing and a commercial antigen-based ELISA and lateral flow tests are available, but their utility is hampered by lower sensitivity and specificity (163, 164, 167). Because of these limitations, PCR is often the best methodology for detecting virus in clinical samples.

Molecular Detection of ASFV DNA

Real-time quantitative PCR (qPCR) testing is the recommended method for screening and confirmatory testing during active infection, due to high sensitivity, specificity, and sample throughput (163, 164). Both, conventional and qPCR formats targeting conserved regions of the viral p72 gene and capable of detecting multiple genotypes have been developed and validated, though real-time qPCR assays are considered preferable (164, 168–171). The two OIE-recommended qPCR assays use TaqMan or Universal Probe Library (UPL) probes, the latter of which provides greater sensitivity for animals with low level viremia (164, 169, 171). The two qPCR assays recommended by the OIE possess significantly greater sensitivity than commercially-available antigen detection ELISAs, for both experimental and field isolates, and can be useful for detecting ASFV in poorly preserved or degraded samples where virus isolation and direct antigen detection may not be viable (164, 166, 167). In addition

to its use as a diagnostic method in swine, conventional and qPCR formats can be used to detect ASFV in *Ornithodoros* ticks, and are used in combination with sequencing to genotype viral isolates (40, 76, 172, 173).

Traditional real-time qPCR testing typically needs high-throughput thermocyclers and associated laboratory equipment such as automated RNA/DNA extraction instruments which are costly, difficult to transport, and require reliable access to electricity, as well as the use of reagents that must be kept cold (166, 174). Consequently, these assays are generally restricted to laboratory settings. Several challenges are associated with performing PCR in the field beyond the need for a portable, battery-operated thermocycler, which include performing nucleic acid extractions without a centrifuge/electricity, protecting samples against cross-contamination, and maintaining a cold chain for materials that may require refrigeration. Additionally, available portable thermocyclers for qPCR assays are low-throughput because they can only run a limited number of samples at one time. Selection of the proper thermostable PCR reagents and diluents can overcome some of the above mentioned issues (174).

Progress toward field-deployable molecular diagnostics for ASFV has involved research into novel DNA amplification and detection strategies, as well as the usage of portable equipment which can be run independent of electricity (e.g., GeneReach PockitTM or Biomeme FranklinTM), and lyophilized reagents which are stable at room temperature for several years (174). In addition, portable next generation sequencing (NGS) devices (e.g., Nanopore MinION) can be efficiently utilized to rapidly determine the genotype of PCR positive ASFV isolates and even sequence a significant part or the entire genome for downstream phylogenetic analyses.

The use of a commercial battery-powered portable thermocycler (T-COR 4TM) for the detection of ASFV via a real-time PCR assay has previously been evaluated (174, 175). In one study, reduced sensitivity of the portable thermocycler for clinical samples with very high Ct values on the gold standard qPCR was observed on a RT-PCR/PCR duplex assay for Classical and African swine fever viruses (175). In our hands, portable thermocyclers (e.g., GeneReach PockitTM or Biomeme FranklinTM) show comparable clinical and analytical sensitivity and specificity using ASF positive and negative experimental and field samples when compared to a laboratory thermocycler (Bio-Rad CFX 96). Rapid detection of ASFV is key to activate respective control measures. To address this need and provide near immediate detection of ASFV infected swine at the farm, fair, the sale barn, or the slaughter house, our group has developed a point of need (PON) molecular detection method using the USDA-approved qPCR ASFV assay for the detection of the ASFV p72 gene (170). The POCKITTM Nucleic Acid Analyzer (GeneReach USA) is a portable PCR device, which uses insulated isothermal polymerase chain reaction (iiPCR) technology and reports out plus/minus detection of the gene target for up to 8 samples within 1 h. DNA preparation is performed on the portable TacoTMMini (GeneReach, USA) automatic nucleic acid extraction system using the GeneReach total NA extraction kit using a magnetic bead extraction

protocol (113). EDTA whole blood was collected from swine experimentally infected with genotype II ASFV at various time points post-infection and the ASFV p72-specific qPCR was run side-by-side on the laboratory thermocycler BioRad CFX 96 and on the POCKITTM portable iiPCR device. The results from this side-by-side analysis demonstrated similar sensitivity and specificity of the laboratory and portable PCR devices for the detection of ASFV p72 in blood samples.

Isothermal amplification strategies are performed at a single temperature, thereby avoiding the need for thermal cycling of traditional laboratory thermocycler. Early research into a linear isothermal amplification assay for ASF by combining an oligonucleotide with an overlapping probe and the cleavase enzyme (Invader[®]) showed high specificity but poor sensitivity relative to other molecular diagnostic techniques (176). A study of loop-mediated isothermal amplification (LAMP) targeting the viral topoisomerase II gene (P1192R) showed good sensitivity near that of the OIE TaqMan real-time PCR assay and demonstrated the potential feasibility of a lateral flow device for detecting LAMP amplicons (177). Subsequent comparison of LAMP and TaqMan-based qPCR showed comparable sensitivity, depending on the cutoff value set for a positive LAMP reaction; the variability in reaction time to positivity for different samples on LAMP assays and its poor correlation with ASFV DNA levels as determined by qPCR Ct highlights the difficulty in establishing robust diagnostic parameters for LAMP assays (166). Two studies using the recombinase polymerase amplification (RPA) technique targeting the ASFV p72 gene have shown high sensitivity in this very rapid assay format that produces results in under 10 min; importantly, robust sensitivity could be maintained when the ASFV RPA assay was combined with a convenient lateral flow dipstick to detect ASFV amplicons (178, 179). Further validation of isothermal amplification assays for ASFV is needed to better understand the reliability and utility of these techniques as ASF diagnostic methods.

Detection of ASFV Antibodies

Due to the lack of an available vaccine, ASFV-specific antibodies are always the result of current or prior ASFV infection (or are maternally-derived). ASFV-specific antibodies in convalescent animals can persist for months or years (163, 164). Several immunogenic proteins of ASFV have been previously identified, including both structural and non-structural proteins (55, 180–185). A variety of serological tests for the detection of ASFV-specific antibodies have been developed using multiple formats including ELISAs, immunoblots, indirect fluorescent antibody tests (IFATs), indirect immunoperoxidase tests (IPTs), and lateral flow tests (LFTs); several of these tests are recommended by the OIE for disease surveillance and for determining freedom from ASFV infection prior to animal movement (163, 164, 182, 184–190). The above-mentioned serological assays are limited by a low sensitivity in detecting ASFV-infected animals with early-stage infections (<7 days post-infection) or swine which are infected with highly virulent strains that produce peracute ASF disease and death before the induction of ASFV-specific antibodies can occur (163, 164).

ELISA is the most commonly used method for high throughput ASF serological testing, with multiple commercial and in-house formats validated as fit-for-purpose by the OIE (164). Soluble antigens from the cytoplasmic fraction of ASFV-infected Vero cells are more sensitive than semi-purified viral p72 at detecting early antibody responses and can be used in an indirect ELISA format that is well-established but requires BSL-3 biocontainment facilities capable of handling live ASFV (164, 188). Utilization of recombinant ASFV proteins as ELISA antigens circumvents the need for virus propagation in BSL-3 containment and can provide comparable or even improved sensitivity and specificity, as well as better consistency, compared to the indirect ELISA using native ASFV antigens, especially with poorly preserved sera (184, 185, 191, 192). Variable sensitivity using sera from different geographic areas of East and West Africa have been demonstrated for recombinant ASFV protein ELISAs, depending on the ASFV protein used as target antigen (185). ELISAs have also been adapted to screen other clinical samples besides serum such as oral fluids or meat juice, which are easier or less invasive to collect than serum. A modified version of the OIE recommended indirect ELISA was able to detect ASFV-specific antibodies in oral fluids from pigs experimentally inoculated with the attenuated genotype I strain and challenged with virulent genotype II ASFV, albeit with reduced sensitivity relative to serum; this is likely due to the comparatively lower level of ASFV-specific antibodies in oral fluids (193). Indirect ELISAs using semi-purified p72 derived from genotype I ASFV isolate BA71V grown in Vero cell culture or using recombinant ASFV p30 have also shown positive reactivity with ASFV-specific antibodies present in feces from pigs infected with the attenuated Ken05/Tk1 isolate (194). Further evaluation of fecal, meat juice and oral fluid specimens collected from experimentally and field-infected pigs are needed to assess their viability and reliability as diagnostic samples for serological testing.

Confirmatory testing using an alternative serological or antigen/virus detection assay is recommended for ELISA-positive serum, especially for endemic areas, and/or poorly preserved samples (164). The IFAT is an established confirmatory test utilizing African green monkey kidney cells (Vero cells) infected with culture-adapted viral (e.g., BA71V) and a fluorophore-conjugated secondary antibody capable of detecting swine immunoglobulins (Ig). It provides a high level of specificity by allowing direct visualization of antibody reactivity with intracellular ASFV antigens in virus factories of infected cells, facilitating discernment from background noise (164, 189, 195). The ASFV IPT is conceptually similar to IFAT and has comparable sensitivity and specificity but instead uses a peroxidase-tagged secondary anti-swine Ig detection antibody, thereby avoiding the requirement for a fluorescent microscope and facilitating larger scale testing (164, 186). The IPT has been shown to possess greater sensitivity in detecting early ASFV-specific antibody responses than the OIE-recommended indirect ELISA and multiple commercial ELISAs (167). Both the IFAT and IPT are OIE-recommended confirmatory serological tests recommended for ELISA-positive samples from areas free of ASFV and for inconclusive ELISA samples from

endemic areas; the IPT is considered preferable over the IFAT (164). Immunoblots (IBs) or Western blots (WBs) use soluble cytoplasmic ASFV proteins as antigens, and can be used as an alternative to the IFAT and the IPT. They are highly specific and not too difficult to interpret since the immunoreactive proteins of ASFV detected by antibodies in the IB/WB test have been well-described (164, 187). Antibodies from positive animals maintain reactivity on IBs for longer than with the OIE-recommended indirect ELISA when the test serum is stored at room temperature or 37°C; the IB can be advantageous for poorly-preserved sera samples or where reliable refrigeration is not available (196). IBs using recombinant p54, a highly immunogenic ASFV protein expressed in *Escherichia coli* or baculovirus systems, have been described, and are easier to interpret than ASFV-infected cell-based IBs, and avoid the need for ASFV antigens produced in cells (182, 191). *E. coli*-expressed p30 has also been demonstrated to be a highly sensitive and specific antigen for IBs, capable of detecting serological responses as early as 6–8 days post-infection (197). IBs/WBs based on individual ASFV proteins do not offer the multiple ASFV antigen array present in ASFV-infected cell lysate. Therefore, future IB/WB approaches for ASF serological diagnosis should include multiple recombinant ASFV antigens in order to increase specificity and sensitivity.

CONCLUDING REMARKS

ASFV is a complex DNA arbovirus having a significant impact on the global swine industry. The lack of a safe and efficacious vaccine and the reliance on culling of herds to prevent disease spread has resulted in significant economic losses. Therefore, improved early detection, and on-farm biosecurity measures, as well as movement control continue to be of significant priority. Further studies on ASFV gene functions, virus and cellular factors involved in ASFV replication, pathogenesis, as well as host immune responses to determine the correlates of protection, will be critical for the development of a rationally-designed, safe, efficacious, and DIVA-compatible ASFV vaccine. In addition, given the vast distribution of susceptible soft tick vectors, wild boar, and feral pigs, methods to prevent and control ASFV establishment, and spread in populations of these species are also critically important.

ONLINE CITATIONS

ChinaDaily.com.cn, 9/11/2019, “Swine fever may affect pork for several years,” global.chinadaily.com.cn

FAO situation update, 02/19/2020, www.fao.org

FAO press release, 09/08/2019, “One year on, close to 5 million pigs lost to Asia’s swine fever outbreak”

Global Times, 09/18/2019, “A global battle against African swine fever,” www.globaltimes.cn

OIE WAHIS African Swine Fever (ASF) Report: September 13–26, 2019; www.oie.int

OIE WAHIS Interface, Disease information, Disease Timelines, 10/23/2019, www.oie.int

OIE WAHIS Interface, Disease information, Immediate notifications and Follow-ups, 09/21/2019, www.oie.int

Reuters Health News, 09/18/2019, “Thailand culls 200 pigs amid heightened fears over African swine fever,” www.reuters.com

Vet times, 11/19/2019, “African swine fever confirmed close to Germany,” www.vettimes.co.uk

AUTHOR CONTRIBUTIONS

All authors contributed to the conceptualization, writing and review of the manuscript, and are accountable for the content of this work.

REFERENCES

- Cwynar P, Stojkov J, Wlazlak K. African swine fever status in Europe. *Viruses*. (2019) 11. doi: 10.3390/v11040310
- Montgomery RE. On a form of swine fever occurring in British East Africa. *J Comp Pathol*. (1921) 34:59–191. doi: 10.1016/S0368-1742(21)80031-4
- Penrith ML, Bastos AD, Etter EM C, Beltran-Alcrudo D. Epidemiology of African swine fever in Africa today: sylvatic cycle versus socio-economic imperatives. *Transbound Emerg Dis*. (2019) 66:672–86. doi: 10.1111/tbed.13117
- Sanchez-Vizcaino JM, Mur L, Martinez-Lopez B. African swine fever (ASF): five years around Europe. *Vet Microbiol*. (2013) 165:45–50. doi: 10.1016/j.vetmic.2012.11.030
- Cisek AA, Dabrowska I, Gregorczyk KP, Wyzewski Z. African swine fever virus: a new old enemy of Europe. *Ann Parasitol*. (2016) 62:161–7. doi: 10.1016/ap6203.49
- Costard S, Wieland B, de Glanville W, Jori F, Rowlands R, Vosloo W, et al. African swine fever: how can global spread be prevented? *Philos Trans R Soc Lond B Biol Sci*. (2009) 364:2683–96. doi: 10.1098/rstb.2009.0098
- Boinas FS, Wilson AJ, Hutchings GH, Martins C, Dixon LJ. The persistence of African swine fever virus in field-infected *Ornithodoros erraticus* during the ASF endemic period in Portugal. *PLoS ONE*. (2011) 6:e20383. doi: 10.1371/journal.pone.0020383
- Laddomada A, Rolesu S, Loi F, Cappai S, Oggiano A, Madrau MP, et al. Surveillance and control of African swine fever in free-ranging pigs in Sardinia. *Transbound Emerg Dis*. (2019) 66:1114–9. doi: 10.1111/tbed.13138
- Gogin A, Gerasimov V, Malogolovkin A, Kolbasov D. African swine fever in the North Caucasus region and the Russian Federation in years 2007–2012. *Virus Res*. (2013) 173:198–203. doi: 10.1016/j.virusres.2012.12.007
- Linden A, Licoppe A, Volpe R, Paternostre J, Lesenfans C, Cassart D, et al. Summer 2018: African swine fever virus hits north-western Europe. *Transbound Emerg Dis*. (2019) 66:54–5. doi: 10.1111/tbed.13047
- Zhou X, Li N, Luo Y, Liu Y, Miao F, Chen T, et al. Emergence of African swine fever in China, 2018. *Transbound Emerg Dis*. (2018) 65:1482–4. doi: 10.1111/tbed.12989
- Lu G, Pan J, Zhang G. African swine fever virus in Asia: its rapid spread and potential threat to unaffected countries. *J Infection*. (2020) 80:350–71. doi: 10.1016/j.jinf.2019.11.011
- Golnar AJ, Martin E, Wormington JD, Kading RC, Teel PD, Hamer SA, et al. Reviewing the potential vectors and hosts of african swine fever virus transmission in the United States. *Vector Borne Zoonotic Dis*. (2019) 19:512–24. doi: 10.1089/vbz.2018.2387
- Plowright W, Parker J, Pierce MA. African swine fever virus in ticks (*Ornithodoros moubata*, Murray) collected from animal burrows in Tanzania. *Nature*. (1969) 221:1070–3. doi: 10.1038/2211071a0
- Plowright W, Perry CT, Peirce MA. Transovarial infection with African swine fever virus in the argasid tick, *Ornithodoros moubata* porcinus, Walton. *Res Vet Sci*. (1970) 11:582–4. doi: 10.1016/S0034-5288(18)34259-0

FUNDING

This research was funded by grants from the U.S. Department of Homeland Security under Grant Award No. DHS-2010-ST-061-AG0001 for the Center of Excellence for Emerging and Zoonotic Animal Diseases, National Pork Board grant #16-021, #16-022 and #16-184, and the State of Kansas National Bio- and Agro-Defense Facility transition funds. WCW is supported by US Department of Agriculture.

ACKNOWLEDGMENTS

The authors thank Mal Rooks Hoover for her assistance with the figure illustrations.

- Plowright W, Perry CT, Peirce MA, Parker J. Experimental infection of the argasid tick, *Ornithodoros moubata* porcinus, with African swine fever virus. *Arch Gesamte Virusforsch*. (1970) 31:33–50. doi: 10.1007/BF01241664
- Plowright W, Perry CT, Greig A. Sexual transmission of African swine fever virus in the tick, *Ornithodoros moubata* porcinus. *Walton Res Vet Sci*. (1974) 17:106–13. doi: 10.1016/S0034-5288(18)33716-0
- Kleiboeker SB, Scoles GA. Pathogenesis of African swine fever virus in *Ornithodoros* ticks. *Anim Health Res Rev*. (2001) 2:121–8. doi: 10.1079/AHRR200133
- Jori F, Bastos AD. Role of wild suids in the epidemiology of African swine fever. *Ecohealth*. (2009) 6:296–310. doi: 10.1007/s10393-009-0248-7
- Ravaomanana J, Michaud V, Jori F, Andriatsimahavandy A, Roger F, Albina E, et al. First detection of African swine fever virus in *Ornithodoros porcinus* in Madagascar and new insights into tick distribution and taxonomy. *Parasit Vectors*. (2010) 3:115. doi: 10.1186/1756-3305-3-115
- Perez-Sanchez R, Astigarraga A, Oleaga-Perez A, Encinas-Grandes A. Relationship between the persistence of African swine fever and the distribution of *Ornithodoros erraticus* in the province of Salamanca, Spain. *Vet Rec*. (1994) 135:207–209. doi: 10.1136/vr.135.9.207
- Costard S, Mur L, Lubroth J, Sanchez-Vizcaino JM, Pfeiffer DU. Epidemiology of African swine fever virus. *Virus Res*. (2013) 173:191–7. doi: 10.1016/j.virusres.2012.10.030
- Boinas F, Ribeiro R, Madeira S, Palma M, de Carvalho IL, Nuncio S, et al. The medical and veterinary role of *Ornithodoros erraticus* complex ticks (Acari: Ixodidae) on the Iberian Peninsula. *J Vector Ecol*. (2014) 39:238–48. doi: 10.1111/jvec.12098
- Grieg A. The localization of African swine fever virus in the tick *Ornithodoros moubata* porcinus. *Arch Gesamte Virusforsch*. (1972) 36:240–7. doi: 10.1007/BF01241546
- Kleiboeker SB, Burrage TG, Scoles GA, Fish D, Rock DL. African swine fever virus infection in the argasid host, *Ornithodoros porcinus* porcinus. *J Virol*. (1998) 72:1711–24. doi: 10.1128/JVI.72.3.1711-1724.1998
- Groocock CM, Hess WR, Gladney WJ. Experimental transmission of African swine fever virus by *Ornithodoros coriaceus*, an argasid tick indigenous to the United States. *Am J Vet Res*. (1980) 41:591–4.
- Hess WR, Endris RG, Haslett TM, Monahan MJ, McCoy JP. Potential arthropod vectors of African swine fever virus in North America and the Caribbean basin. *Vet Parasitol*. (1987) 26:145–55. doi: 10.1016/0304-4017(87)90084-7
- Hess WR, Endris RG, Lousa A, Caidado JM. Clearance of African swine fever virus from infected tick (Acari) colonies. *J Med Entomol*. (1989) 26:314–7. doi: 10.1093/jmedent/26.4.314
- Endris RG, Hess WR. Experimental transmission of African swine fever virus by the soft tick *Ornithodoros (Pavlovskyella) maroccanus* (Acari: Ixodoidea: Argasidae). *J Med Entomol*. (1992) 29:652–6. doi: 10.1093/jmedent/29.4.652
- Endris RG, Hess WR, Caiado JM. African swine fever virus infection in the Iberian soft tick, *Ornithodoros (Pavlovskyella) maroccanus* (Acari: Argasidae). *J Med Entomol*. (1992) 29:874–8. doi: 10.1093/jmedent/29.5.874

31. Rennie L, Wilkinson PJ, Mellor PS. Effects of infection of the tick *Ornithodoros moubata* with African swine fever virus. *Med Vet Entomol.* (2000) 14:355–60. doi: 10.1046/j.1365-2915.2000.00251.x
32. Endris RG, Hess WR. Attempted transovarial and venereal transmission of African swine fever virus by the Iberian soft tick *Ornithodoros* (Pavlovskyella) maroccanus (Acari: Ixodoidea: Argasidae). *J Med Entomol.* (1994) 31:373–81. doi: 10.1093/jmedent/31.3.373
33. Burrage TG. African swine fever virus infection in *Ornithodoros* ticks. *Virus Res.* (2013) 173:131–9. doi: 10.1016/j.virusres.2012.10.010
34. Burrage TG, Lu Z, Neilan JG, Rock DL, Zsak L. African swine fever virus multigene family 360 genes affect virus replication and generalization of infection in *Ornithodoros porcinus* ticks. *J Virol.* (2004) 78:2445–53. doi: 10.1128/JVI.78.5.2445-2453.2004
35. Rowlands RJ, Duarte MM, Boinas F, Hutchings G, Dixon LK. The CD2v protein enhances African swine fever virus replication in the tick vector, *Ornithodoros erraticus*. *Virology.* (2009) 393:319–28. doi: 10.1016/j.virol.2009.07.040
36. Kleiboeker SB, Scoles GA, Burrage TG, Sur J. African swine fever virus replication in the midgut epithelium is required for infection of *Ornithodoros* ticks. *J Virol.* (1999) 73:8587–98. doi: 10.1128/JVI.73.10.8587-8598.1999
37. Rennie L, Wilkinson PJ, Mellor PS. Transovarial transmission of African swine fever virus in the argasid tick *Ornithodoros moubata*. *Med Vet Entomol.* (2001) 15:140–6. doi: 10.1046/j.1365-2915.2001.00282.x
38. Mellor PS, Wilkinson PJ. Experimental transmission of African swine fever virus by *Ornithodoros savignyi* (Audoin). *Res Vet Sci.* (1985) 39:353–6. doi: 10.1016/S0034-5288(18)31726-0
39. Endris RG, Haslett TM, Hess WR. Experimental transmission of African swine fever virus by the tick *Ornithodoros* (Alectorobius) *puertoricensis* (Acari: Argasidae). *J Med Entomol.* (1991) 28:854–8. doi: 10.1093/jmedent/28.6.854
40. Basto AP, Nix RJ, Boinas F, Mendes S, Silva MJ, Cartaxeiro C, et al. Kinetics of African swine fever virus infection in *Ornithodoros erraticus* ticks. *J Gen Virol.* (2006) 87(Pt 7): 1863–71. doi: 10.1099/vir.0.81765-0
41. Thomson GR, Gainaru MD, Van Dellen AF. Experimental infection of warthogs (*Phacochoerus aethiopicus*) with African swine fever virus. *Onderstepoort J Vet Res.* (1980) 47:19–22.
42. Anderson EC, Hutchings GH, Mukarati N, Wilkinson PJ. African swine fever virus infection of the bushpig (*Potamochoerus porcus*) and its significance in the epidemiology of the disease. *Vet Microbiol.* (1998) 62:1–15. doi: 10.1016/S0378-1135(98)00187-4
43. Oura CAL, Powell PP, Anderson E, Parkhouse RM E. The pathogenesis of African swine fever in the resistant bupig. *J Gen Virol.* (1998) 79:1439–43. doi: 10.1099/0022-1317-79-6-1439
44. Boinas FS, Hutchings GH, Dixon LK, Wilkinson PJ. Characterization of pathogenic and non-pathogenic African swine fever virus isolates from *Ornithodoros erraticus* inhabiting pig premises in Portugal. *J Gen Virol.* (2004) 85(Pt 8):2177–87. doi: 10.1099/vir.0.80058-0
45. Wilkinson PJ, Pegram RG, Perry BD, Lemche J, Schels HF. The distribution of African swine fever virus isolated from *Ornithodoros moubata* in Zambia. *Epidemiol Infect.* (1988) 101:547–64. doi: 10.1017/S0950268800029423
46. Haresnape JM, Wilkinson PJ, Mellor PS. Isolation of African swine fever virus from ticks of the *Ornithodoros moubata* complex (Ixodoidea: Argasidae) collected within the African swine fever enzootic area of Malawi. *Epidemiol Infect.* (1988) 101:173–85. doi: 10.1017/S0950268800029332
47. Haresnape JM, Wilkinson PJ. A study of African swine fever virus infected ticks (*Ornithodoros moubata*) collected from three villages in the ASF enzootic area of Malawi following an outbreak of the disease in domestic pigs. *Epidemiol Infect.* (1989) 102:507–22. doi: 10.1017/S0950268800302223
48. Diaz AV, Netherton CL, Dixon LK, Wilson AJ. African Swine Fever Virus Strain Georgia 2007/1 in *Ornithodoros erraticus* Ticks. *Emerg Infect Dis.* (2012) 18:1026–8. doi: 10.3201/eid1806.111728
49. Frant M, Wozniakowski G, Pejsak Z. African Swine Fever (ASF) and Ticks. No risk of tick-mediated ASF spread in Poland and Baltic states. *J Vet Res.* (2017) 61:375–80. doi: 10.1515/jvetres-2017-0055
50. Pietschmann J, Mur L, Blome S, Beer M, Perez-Sanchez R, Oleaga A, et al. African swine fever virus transmission cycles in Central Europe: evaluation of wild boar-soft tick contacts through detection of antibodies against *Ornithodoros erraticus* saliva antigen. *BMC Vet Res.* (2016) 12:1. doi: 10.1186/s12917-015-0629-9
51. Blome S, Gabriel C, Beer M. Pathogenesis of African swine fever in domestic pigs and European wild boar. *Virus Res.* (2013) 173:122–30. doi: 10.1016/j.virusres.2012.10.026
52. Gabriel C, Blome S, Malogolovkin A, Parilov S, Kolbasov D, Teifke JP, et al. Characterization of African swine fever virus Caucasus isolate in European wild boars. *Emerg Infect Dis.* (2011) 17:2342–5. doi: 10.3201/eid1712.110430
53. Blome S, Gabriel C, Dietze K, Breithaupt A, Beer M. High virulence of African swine fever virus caucasus isolate in European wild boars of all ages. *Emerg Infect Dis.* (2012) 18:708. doi: 10.3201/eid1804.111813
54. Leitao A, Cartaxeiro C, Coelho R, Cruz B, Parkhouse RM, Portugal F, et al. The non-haemadsorbing African swine fever virus isolate ASFV/NH/P68 provides a model for defining the protective anti-virus immune response. *J Gen Virol.* (2001) 82(Pt 3):513–23. doi: 10.1099/0022-1317-82-3-513
55. Reis AL, Parkhouse RM, Penados AR, Martins C, Leitao A. Systematic analysis of longitudinal serological responses of pigs infected experimentally with African swine fever virus. *J Gen Virol.* (2007) 88(Pt 9):2426–34. doi: 10.1099/vir.0.82857-0
56. King K, Chapman D, Argilaguet JM, Fishbourne E, Hutet E, Cariolet R, et al. Protection of European domestic pigs from virulent African isolates of African swine fever virus by experimental immunisation. *Vaccine.* (2011) 29:4593–600. doi: 10.1016/j.vaccine.2011.04.052
57. Abrams CC, Goatley L, Fishbourne E, Chapman D, Cooke L, Oura CA, et al. Deletion of virulence associated genes from attenuated African swine fever virus isolate OUR T88/3 decreases its ability to protect against challenge with virulent virus. *Virology.* (2013) 443:99–105. doi: 10.1016/j.virol.2013.04.028
58. Mulumba-Mfumu LK, Goatley LC, Saegerman C, Takamatsu HH, Dixon LK. Immunization of African indigenous pigs with attenuated genotype I African swine fever virus OURT88/3 induces protection against challenge with virulent strains of genotype I. *Transbound Emerg Dis.* (2015) 63:e323–7. doi: 10.1111/tbed.12303
59. Penrith ML, Thomson GR, Bastos AD, Phiri OC, Lubisi BA, Du Plessis EC, et al. An investigation into natural resistance to African swine fever in domestic pigs from an endemic area in southern Africa. *Rev Sci Tech.* (2004) 23:965–77. doi: 10.20506/rst.23.3.1533
60. Guinat C, Gogin A, Blome S, Keil G, Pollin R, Pfeiffer DU, et al. Transmission routes of African swine fever virus to domestic pigs: current knowledge and future research directions. *Vet Rec.* (2016) 178:262–7. doi: 10.1136/vr.103593
61. Gallardo C, Soler A, Nieto R, Sanchez MA, Martins C, Pelayo V, et al. Experimental transmission of african swine fever (ASF) low virulent isolate NH/P68 by surviving pigs. *Transbound Emerg Dis.* (2015) 62:612–22. doi: 10.1111/tbed.12431
62. Guinat C, Gubbins S, Vergne T, Gonzales JL, Dixon L, Pfeiffer DU. Experimental pig-to-pig transmission dynamics for African swine fever virus, Georgia 2007/1 strain. *Epidemiol Infect.* (2016) 144:25–34. doi: 10.1017/S0950268815000862
63. Olesen AS, Lohse L, Boklund A, Halasa T, Gallardo C, Pejsak Z, et al. Transmission of African swine fever virus from infected pigs by direct contact and aerosol routes. *Vet Microbiol.* (2017) 211:92–102. doi: 10.1016/j.vetmic.2017.10.004
64. Petrov A, Forth JH, Zani L, Beer M, Blome S. No evidence for long-term carrier status of pigs after African swine fever virus infection. *Transbound Emerg Dis.* (2018) 65:1318–28. doi: 10.1111/tbed.12881
65. Eble PL, Hagenshaars TJ, Weesendorp E, Quak S, Moonen-Leusen HW, Loeffen WL, et al. Transmission of African swine fever virus via carrier (survivor) pigs does occur. *Vet Microbiol.* (2019) 237:108345. doi: 10.1016/j.vetmic.2019.06.018
66. Penrith ML, Vosloo W, Jori F, Bastos AD. African swine fever virus eradication in Africa. *Virus Res.* (2013) 173:228–46. doi: 10.1016/j.virusres.2012.10.011
67. Dee SA, Bauermann FV, Niederwerder MC, Singrey A, Clement T, de Lima M, et al. Correction: survival of viral pathogens in animal feed ingredients under transboundary shipping models. *PLoS ONE.* (2018) 13:e0208130. doi: 10.1371/journal.pone.0208130
68. Stoian AMM, Zimmerman J, Ji J, Hefley TJ, Dee S, Diel DG, et al. Half-life of African swine fever virus in shipped feed. *Emerg Infect Dis.* (2019) 25:2261–3. doi: 10.3201/eid2512.191002

69. Niederwerder MC, Stoian AMM, Rowland RRR, Dritz SS, Petrovan V, Constance LA, et al. Infectious dose of African swine fever virus when consumed naturally in liquid or feed. *Emerg Infect Dis.* (2019) 25:891–7. doi: 10.3201/eid2505.181495
70. Chapman DA, Tcherepanov V, Upton C, Dixon LK. Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever virus isolates. *J Gen Virol.* (2008) 89(Pt 2):397–408. doi: 10.1099/vir.0.83343-0
71. Dixon LK, Chapman DA, Netherton CL, Upton C. African swine fever virus replication and genomics. *Virus Res.* (2013) 173:3–14. doi: 10.1016/j.virusres.2012.10.020
72. Alejo A, Matamoros T, Guerra M, Andres G. A proteomic atlas of the African swine fever virus particle. *J Virol.* (2018) 14714. doi: 10.1128/JVI.01293-18
73. Kessler C, Forth JH, Keil GM, Mettenleiter TC, Blome S, Karger A. The intracellular proteome of African swine fever virus. *Sci Rep.* (2018) 8:14714. doi: 10.1038/s41598-018-32985-z
74. Salas ML, Andres G. African swine fever virus morphogenesis. *Virus Res.* (2013) 173:29–41. doi: 10.1016/j.virusres.2012.09.016
75. Wang N, Zhao D, Wang J, Zhang Y, Wang M, Gao Y, et al. Architecture of African swine fever virus and implications for viral assembly. *Science.* (2019) 366:640–4. doi: 10.1126/science.aaz1439
76. Bastos AD, Penrith ML, Cruciere C, Edrich JL, Hutchings G, Roger F, Couacy-Hymann E, Thomson RG. Genotyping field strains of African swine fever virus by partial p72 gene characterisation. *Arch Virol.* (2003) 148:693–706. doi: 10.1007/s00705-002-0946-8
77. Nix RJ, Gallardo C, Hutchings G, Blanco E, Dixon LK. Molecular epidemiology of African swine fever virus studied by analysis of four variable genome regions. *Arch Virol.* (2006) 151:2475–94. doi: 10.1007/s00705-006-0794-z
78. Gallardo C, Mwaengo DM, Macharia JM, Arias M, Taracha EA, Soler A, et al. Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes. *Virus Genes.* (2009) 38:85–95. doi: 10.1007/s11262-008-0293-2
79. Gallardo C, Anchuelo R, Pelayo V, Poudevigne F, Leon T, Nzoussi J, et al. African swine fever virus p72 genotype IX in domestic pigs, Congo, 2009. *Emerg Infect Dis.* (2011) 17:1556–8. doi: 10.3201/eid1708.101877
80. Malogolovkin A, Burmakina G, Titov I, Sereda A, Gogin A, Baryshnikova E, et al. Comparative analysis of African swine fever virus genotypes and serogroups. *Emerg Infect Dis.* (2015) 21:312–5. doi: 10.3201/eid2102.140649
81. Malogolovkin A, Burmakina G, Tulman ER, Delhon G, Diel DG, Salnikov N, et al. African swine fever virus CD2v and C-type lectin gene loci mediate serological specificity. *J Gen Virol.* (2015) 96(Pt 4):866–73. doi: 10.1099/jgv.0.000024
82. Achenbach JE, Gallardo C, Nieto-Pelegrin E, Rivera-Arroyo B, Degefa-Negi T, Arias M, et al. Identification of a new genotype of African swine fever virus in domestic pigs from Ethiopia. *Transbound Emerg Dis.* (2016) 64:1393–404. doi: 10.1111/tbed.12511
83. Quembo CJ, Jori F, Vosloo W, Heath L. Genetic characterization of African swine fever virus isolates from soft ticks at the wildlife/domestic interface in Mozambique and identification of a novel genotype. *Transbound Emerg Dis.* (2018) 65:420–31. doi: 10.1111/tbed.12700
84. Rowlands RJ, Michaud V, Heath L, Hutchings G, Oura C, Vosloo W, et al. African swine fever virus isolate, Georgia, 2007. *Emerg Infect Dis.* (2008) 14:1870–4. doi: 10.3201/eid1412.080591
85. Munoz-Moreno R, Galindo I, Cuesta-Geijo MA, Barrado-Gil L, Alonso C. Host cell targets for African swine fever virus. *Virus Res.* (2015) 209:118–27. doi: 10.1016/j.virusres.2015.05.026
86. Galindo I, Alonso C. African swine fever virus: a review. *Viruses.* (2017) 9:E103. doi: 10.3390/v9050103
87. Sanchez EG, Riera E, Nogal M, Gallardo C, Fernandez P, Bello-Morales R, et al. Phenotyping and susceptibility of established porcine cells lines to African swine fever virus infection and viral production. *Sci Rep.* (2017) 7:10369. doi: 10.1038/s41598-017-09948-x
88. Simoes M, Freitas FB, Leitao A, Martins C, Ferreira F. African swine fever virus replication events and cell nucleus: new insights and perspectives. *Virus Res.* (2019) 270:197667. doi: 10.1016/j.virusres.2019.197667
89. Gomez-Villamandos JC, Bautista MJ, Sanchez-Cordon PJ, Carrasco L. Pathology of African swine fever: the role of monocyte-macrophage. *Virus Res.* (2013) 173:140–9. doi: 10.1016/j.virusres.2013.01.017
90. Carrascosa AL, Bustos MJ, de Leon P. Methods for growing and titrating African swine fever virus: field and laboratory samples. *Curr Protoc Cell Biol Chapter.* (2011) 26:Unit 26.14. doi: 10.1002/0471143030.cb2614s53
91. Enjuanes L, Carrascosa AL, Moreno MA, Vinuela E. Titration of African swine fever (ASF) virus. *J Gen Virol.* (1976) 32:471–7. doi: 10.1099/0022-1317-32-3-471
92. Krug PW, Holinka LG, O'Donnell V, Reese B, Sanford B, Fernandez-Sainz I, et al. The progressive adaptation of a georgian isolate of African swine fever virus to vero cells leads to a gradual attenuation of virulence in swine corresponding to major modifications of the viral genome. *J Virol.* (2015) 89:2324–32. doi: 10.1128/JVI.03250-14
93. Rodriguez JM, Moreno LT, Alejo A, Lacasta A, Rodriguez F, Salas ML. Genome sequence of African swine fever virus BA71, the virulent parental strain of the nonpathogenic and tissue-culture adapted BA71V. *PLoS ONE.* (2015) 10:e0142889. doi: 10.1371/journal.pone.0142889
94. Sanchez EG, Quintas A, Perez-Nunez D, Nogal M, Barroso S, Carrascosa AL, et al. African swine fever virus uses macropinocytosis to enter host cells. *PLoS Pathog.* (2012) 8:e1002754. doi: 10.1371/journal.ppat.1002754
95. Andres G. African swine fever virus gets undressed: new insights on the entry pathway. *J Virol.* (2017) 91. doi: 10.1128/JVI.01906-16
96. Sanchez EG, Perez-Nunez D, Revilla Y. Mechanisms of entry and endosomal pathway of African swine fever virus. *Vaccines.* (2017) 5:42. doi: 10.3390/vaccines5040042
97. Valdeira ML, Geraldine A. Morphological study on the entry of African swine fever virus into cells. *Biol Cell.* (1985) 55:35–40. doi: 10.1111/j.1768-322X.1985.tb00407.x
98. Alcamí A, Carrascosa AL, Vinuela E. The entry of African swine fever virus into Vero cells. *Virology.* (1989) 171:68–75. doi: 10.1016/0042-6822(89)90511-4
99. Alcamí A, Carrascosa AL, Vinuela E. Saturable binding sites mediate the entry of African swine fever virus into Vero cells. *Virology.* (1989) 168:393–8. doi: 10.1016/0042-6822(89)90281-X
100. Alcamí A, Carrascosa AL, Vinuela E. Interaction of African swine fever virus with macrophages. *Virus Res.* (1990) 17:93–104. doi: 10.1016/0168-1702(90)90071-1
101. Sanchez-Torres C, Gomez-Puertas PM, Gomez-del-Moral AF, Escribano JM, Ezquerro A, Dominguez J. Expression of porcine CD163 on monocytes/macrophages correlates with permissiveness to African swine fever infection. *Archiv Virol.* (2003) 148:2307–23. doi: 10.1007/s00705-003-0188-4
102. Lithgow P, Takamatsu H, Werling D, Dixon L, Chapman D. Correlation of cell surface marker expression with African swine fever virus infection. *Vet Microbiol.* (2014) 168:413–9. doi: 10.1016/j.vetmic.2013.12.001
103. Popescu L, Gaudreault NN, Whitworth KM, Murgia MV, Nietfeld JC, Mileham A, et al. Genetically edited pigs lacking CD163 show no resistance following infection with the African swine fever virus isolate, Georgia 2007/1. *Virology.* (2017) 501:102–6. doi: 10.1016/j.virol.2016.11.012
104. Alcamí A, Vinuela E. Fc receptors do not mediate African swine fever virus replication in macrophages. *Virology.* (1991) 181:756–9. doi: 10.1016/0042-6822(91)90912-U
105. Gaudreault NN, Richt JA. Subunit vaccine approaches for African swine fever virus. *Vaccines.* (2019) 7:E56. doi: 10.3390/vaccines7020056
106. Halstead SB, Chow JS, Marchette NJ. Immunological enhancement of dengue virus replication. *Nat New Biol.* (1973) 243:24–6.
107. Yoon KJ, Wu LL, Zimmerman JJ, Hill HT, Platt KB. Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. *Viral Immunol.* (1996) 9:51–63. doi: 10.1089/vim.1996.9.51
108. Oliphant T, Nybakken GE, Engle M, Xu Q, Nelson CA, Sukupolvi-Petty S, et al. Antibody recognition and neutralization determinants on domains I and II of West Nile virus envelope protein. *J Virol.* (2006) 80:12149–59. doi: 10.1128/JVI.01732-06
109. Halstead SB, Mahalingam S, Marovich MA, Ubol S, Mosser DM. Intrinsic antibody-dependent enhancement of microbial infection in macrophages:

- disease regulation by immune complexes. *Lancet Infect Dis.* (2010) 10:712–22. doi: 10.1016/S1473-3099(10)70166-3
110. Taylor A, Foo SS, Bruzzone R, Dinh LV, King NJ, Mahalingam S. Fc receptors in antibody-dependent enhancement of viral infections. *Immunol Rev.* (2015) 268:340–64. doi: 10.1111/immr.12367
 111. Argilaguet JM, Perez-Martin E, Gallardo C, Salguero FJ, Borrego B, Lacasta A, et al. Enhancing DNA immunization by targeting ASFV antigens to SLA-II bearing cells. *Vaccine.* (2011) 29:5379–85. doi: 10.1016/j.vaccine.2011.05.084
 112. Blome S, Gabriel C, Beer M. Modern adjuvants do not enhance the efficacy of an inactivated African swine fever virus vaccine preparation. *Vaccine.* (2014) 32:3879–82. doi: 10.1016/j.vaccine.2014.05.051
 113. Sunwoo SY, Perez-Nunez D, Morozov I, Sanchez EG, Gaudreault NN, Trujillo JD, et al. DNA-protein vaccination strategy does not protect from challenge with African swine fever virus Armenia 2007 strain. *Vaccines.* (2019) 7:12. doi: 10.3390/vaccines7010012
 114. Perez-Nunez D, Sunwoo SY, Sanchez EG, Haley N, Garcia-Belmonte R, Nogal M, et al. Evaluation of a viral DNA-protein immunization strategy against African swine fever in domestic pigs. *Vet Immunol Immunopathol.* (2019) 208:34–43. doi: 10.1016/j.vetimm.2018.11.018
 115. Basta S, Gerber H, Schaub A, Summerfield A, McCullough KC. Cellular processes essential for African swine fever virus to infect and replicate in primary macrophages. *Vet Microbiol.* (2010) 140:9–17. doi: 10.1016/j.vetmic.2009.07.015
 116. Hernaez B, Guerra M, Salas ML, Andres G. African swine fever virus undergoes outer envelope disruption, capsid disassembly and inner envelope fusion before core release from multivesicular endosomes. *PLoS Pathog.* (2016) 12:e1005595. doi: 10.1371/journal.ppat.1005595
 117. Mercer J, Helenius A. Virus entry by macropinocytosis. *Nat Cell Biol.* (2009) 11:510–520. doi: 10.1038/ncb0509-510
 118. Cuesta-Geijo MA, Galindo I, Hernaez B, Quetglas JI, Dalmau-Mena I, Alonso C. Endosomal maturation, Rab7 GTPase and phosphoinositides in African swine fever virus entry. *PLoS ONE.* (2012) 7:e48853. doi: 10.1371/journal.pone.0048853
 119. Hernaez B, Alonso C. Dynamin- and clathrin-dependent endocytosis in African swine fever virus entry. *J Virol.* (2010) 84:2100–9. doi: 10.1128/JVI.01557-09
 120. Galindo I, Cuesta-Geijo MA, Hlavova K, Munoz-Moreno R, Barrado-Gil L, Dominguez J, et al. African swine fever virus infects macrophages, the natural host cells, via clathrin- and cholesterol-dependent endocytosis. *Virus Res.* (2015) 200:45–55. doi: 10.1016/j.virusres.2015.01.022
 121. Carrascosa AL, Sastre I, Vinuela E. African swine fever virus attachment protein. *J Virol.* (1991) 65:2283–9. doi: 10.1128/JVI.65.5.2283-2289.1991
 122. Angulo A, Vinuela E, Alcamí A. Inhibition of African swine fever virus binding and infectivity by purified recombinant virus attachment protein p12. *J Virol.* (1993) 67:5463–71. doi: 10.1128/JVI.67.9.5463-5471.1993
 123. Gomez-Puertas P, Rodriguez F, Oviedo JM, Ramiro-Ibanez F, Ruiz-Gonzalvo F, Alonso C, et al. Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. *J Virol.* (1996) 70:5689–94. doi: 10.1128/JVI.70.8.5689-5694.1996
 124. Gomez-Puertas P, Rodriguez F, Oviedo JM, Brun A, Alonso C, Escribano JM. The African swine fever virus proteins p54 and p30 are involved in two distinct steps of virus attachment and both contribute to the antibody-mediated protective immune response. *Virology.* (1998) 243:461–71. doi: 10.1006/viro.1998.9068
 125. Andres G, Alejo A, Salas J, Salas ML. African swine fever virus polyproteins pp220 and pp62 assemble into the core shell. *J Virol.* (2002) 76:12473–82. doi: 10.1128/JVI.76.24.12473-12482.2002
 126. Andres G, Garcia-Escudero R, Salas ML, Rodriguez JM. Repression of African swine fever virus polyprotein pp220-encoding gene leads to the assembly of icosahedral core-less particles. *J Virol.* (2002) 76:2654–66. doi: 10.1128/JVI.76.6.2654-2666.2002
 127. Alejo A, Andres G, Salas ML. African swine fever virus proteinase is essential for core maturation and infectivity. *J Virol.* (2003) 77:5571–7. doi: 10.1128/JVI.77.10.5571-5577.2003
 128. Rodriguez I, Nogal ML, Redrejo-Rodriguez M, Bustos MJ, Salas ML. The African swine fever virus virion membrane protein pE248R is required for virus infectivity and an early postentry event. *J Virol.* (2009) 83:12290–300. doi: 10.1128/JVI.01333-09
 129. Freitas FB, Frouco G, Martins C, Ferreira F. African swine fever virus encodes for an E2-ubiquitin conjugating enzyme that is mono- and di-ubiquitinated and required for viral replication cycle. *Sci Rep.* (2018) 8:3471. doi: 10.1038/s41598-018-21872-2
 130. Frouco G, Freitas FB, Coelho J, Leitão A, Martins C, Ferreira F. DNA-binding Properties of African swine fever virus pA104R, a histone-like protein involved in viral replication and transcription. *J Virol.* (2017) 91:e02498-16. doi: 10.1128/JVI.02498-16
 131. Freitas FB, Frouco G, Martins C, Ferreira F. The QP509L and Q706L superfamily II RNA helicases of African swine fever virus are required for viral replication, having non-redundant activities. *Emerging Microbes & Infections.* (2019) 8:291–302. doi: 10.1080/22221751.2019.1578624
 132. Coelho J, Martins C, Ferreira F, Leitao A. African swine fever virus ORF P1192R codes for a functional type II DNA topoisomerase. *Virology.* (2015) 474:82–93. doi: 10.1016/j.virol.2014.10.034
 133. Freitas FB, Frouco G, Martins C, Leitao A, Ferreira F. *In vitro* inhibition of African swine fever virus-topoisomerase II disrupts viral replication. *Antiviral Res.* (2016) 134:34–41. doi: 10.1016/j.antiviral.2016.08.021
 134. Karger A, Pérez-Núñez D, Urquiza J, Hinojar P, Alonso C, Freitas FB, et al. An Update on African swine fever virology. *Viruses.* (2019) 11:864. doi: 10.3390/v11090864
 135. de Matos AP, Carvalho ZG. African swine fever virus interaction with microtubules. *Biol Cell.* (1993) 78:229–34. doi: 10.1016/0248-4900(93)90134-Z
 136. Jouvenet N, Monaghan P, Way M, Wileman T. Transport of African swine fever virus from assembly sites to the plasma membrane is dependent on microtubules and conventional kinesin. *J Virol.* (2004) 78:7990–8001. doi: 10.1128/JVI.78.15.7990-8001.2004
 137. Alonso C, Miskin J, Hernaez B, Fernandez-Zapatero P, Soto L, Canto C, et al. African swine fever virus protein p54 interacts with the microtubular motor complex through direct binding to light-chain dynein. *J Virol.* (2001) 75:9819–27. doi: 10.1128/JVI.75.20.9819-9827.2001
 138. Rodriguez JM, Garcia-Escudero R, Salas ML, Andres G. African swine fever virus structural protein p54 is essential for the recruitment of envelope precursors to assembly sites. *J Virol.* (2004) 78:4299–1313. doi: 10.1128/JVI.78.8.4299-4313.2004
 139. Andres G, Garcia-Escudero R, Vinuela E, Salas ML, Rodriguez JM. African swine fever virus structural protein pE120R is essential for virus transport from assembly sites to plasma membrane but not for infectivity. *J Virol.* (2001) 75:6758–68. doi: 10.1128/JVI.75.15.6758-6768.2001
 140. Wang X, Wu J, Wu Y, Chen H, Zhang S, Li J, et al. Inhibition of cGAS-STING-TBK1 signaling pathway by DP96R of ASFV China 2018/1. *Biochem Biophys Res Commun.* (2018) 506:437–43. doi: 10.1016/j.bbrc.2018.10.103
 141. Garcia-Belmonte R, Perez-Nunez D, Pittau M, Richt JA, Revilla Y. African swine fever virus Armenia/07 virulent strain controls IFN-beta production through cGAS-STING pathway. *J Virol.* (2019) 93. doi: 10.1128/JVI.02298-18
 142. de Oliveira VL, Almeida SC, Soares HR, Crespo A, Marshall-Clarke S, Parkhouse RM. A novel TLR3 inhibitor encoded by African swine fever virus (ASFV). *Arch Virol.* (2011) 156:597–609. doi: 10.1007/s00705-010-0894-7
 143. Perez-Nunez D, Garcia-Urdiales E, Martinez-Bonet M, Nogal ML, Barroso S, Revilla Y, et al. CD2v Interacts with adaptor protein AP-1 during African swine fever infection. *PLoS ONE.* (2015) 10:e0123714. doi: 10.1371/journal.pone.0123714
 144. Alfonso P, Rivera J, Hernaez B, Alonso C, Escribano JM. Identification of cellular proteins modified in response to African swine fever virus infection by proteomics. *Proteomics.* (2004) 4:2037–46. doi: 10.1002/pmic.2003.00742
 145. Correia S, Ventura S, Goodbourn S, Parkhouse RM. African swine fever virus include multiple mechanisms for the manipulation of interferon responses. *Cytokine.* (2013) 63:256. doi: 10.1016/j.cyto.2013.06.058
 146. Golding JP, Goatley L, Goodbourn S, Dixon LK, Taylor G, Netherton CL. Sensitivity of African swine fever virus to type I interferon is linked to genes within multigene families 360 and 505. *Virology.* (2016) 493:154–61. doi: 10.1016/j.virol.2016.03.019
 147. Zsak L, Lu Z, Burrage TG, Kutish GF, Moore DM, et al. African swine fever virus multigene family 360 and 530 genes are

- novel macrophage host range determinants. *J Virol.* (2001) 75:3066–76. doi: 10.1128/JVI.75.7.3066-3076.2001
148. Borca MV, Carrillo C, Zsak L, Laegreid WW, Kutish GF, Neilan JG, et al. Deletion of a CD2-like gene, 8-DR, from African swine fever virus affects viral infection in domestic swine. *J Virol.* (1998) 72:2881–9. doi: 10.1128/JVI.72.4.2881-2889.1998
 149. O'Donnell V, Holinka LG, Gladue DP, Sanford B, Krug PW, Lu X, et al. African swine fever virus georgia isolate harboring deletions of MGF360 and MGF505 genes is attenuated in swine and confers protection against challenge with virulent parental virus. *J Virol.* (2015) 89:6048–56. doi: 10.1128/JVI.00554-15
 150. Reis AL, Abrams CC, Goatley LC, Netherton C, Chapman DG, Sanchez-Cordon P, et al. Deletion of African swine fever virus interferon inhibitors from the genome of a virulent isolate reduces virulence in domestic pigs and induces a protective response. *Vaccine.* (2016) 34:4698–705. doi: 10.1016/j.vaccine.2016.08.011
 151. Monteagudo PL, Lacasta A, Lopez E, Bosch L, Collado J, Pina-Pedrero S, et al. BA71DeltaCD2: a new recombinant live attenuated African swine fever virus with cross-protective capabilities. *J Virol.* (2017) 91:e01058–17. doi: 10.1128/JVI.01058-17
 152. Forman AJ, Wardley RC, Wilkinson PJ. The immunological response of pigs and guinea pigs to antigens of African swine fever virus. *Arch Virol.* (1982) 74:91–100. doi: 10.1007/BF01314703
 153. Neilan JG, Zsak L, Lu Z, Burrage TG, Kutish GF, Rock DL. Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. *Virology.* (2004) 319:337–42. doi: 10.1016/j.virol.2003.11.011
 154. Argilaguet JM, Perez-Martin E, Nofrarias M, Gallardo C, Accensi F, Lacasta A, et al. DNA vaccination partially protects against African swine fever virus lethal challenge in the absence of antibodies. *PLoS ONE.* (2012) 7:e40942. doi: 10.1371/journal.pone.0040942
 155. Argilaguet JM, Perez-Martin E, Lopez S, Goethe M, Escibano JM, Giesow K, et al. BacMam immunization partially protects pigs against sublethal challenge with African swine fever virus. *Antiviral Res.* (2013) 98:61–5. doi: 10.1016/j.antiviral.2013.02.005
 156. Borca MV, Ramirez-Medina E, Silva E, Vuono E, Rai A, Pruitt S, et al. Development of a highly effective African swine fever virus vaccine by deletion of the I177L gene results in sterile immunity against the current epidemic Eurasia strain. *J Virol.* (2020). doi: 10.1128/JVI.02017-19
 157. Sanchez EG, Perez-Nunez D, Revilla Y. Development of vaccines against African swine fever virus. *Virus Res.* (2019) 265:150–5. doi: 10.1016/j.virusres.2019.03.022
 158. Arias MA, de la Torre, Dixon L, Gallardo C, Jori F, Laddomada A, et al. Approaches and perspectives for development of African swine fever virus vaccines. *Vaccines.* (2017) 5:35. doi: 10.3390/vaccines5040035
 159. Rock DL. Challenges for African swine fever vaccine development—“... perhaps the end of the beginning.” *Vet Microbiol.* (2017) 206:52–58. doi: 10.1016/j.vetmic.2016.10.003
 160. Escibano JM, Galindo I, Alonso C. Antibody-mediated neutralization of African swine fever virus: myths and facts. *Virus Res.* (2013) 173:101–9. doi: 10.1016/j.virusres.2012.10.012
 161. Oura CA, Denyer MS, Takamatsu H, Parkhouse RM. *In vivo* depletion of CD8+ T lymphocytes abrogates protective immunity to African swine fever virus. *J Gen Virol.* (2005) 86(Pt 9):2445–50. doi: 10.1099/vir.0.81038-0
 162. Takamatsu HH, Denyer MS, Lacasta A, Stirling CM, Argilaguet JM, Netherton CL, et al. Cellular immunity in ASFV responses. *Virus Res.* (2013) 173:110–21. doi: 10.1016/j.virusres.2012.11.009
 163. Beltrán-Alcrudo, D AM, Gallardo, C, Kramer, S, et al. *African Swine Fever: Detection and Diagnosis – A Manual for Veterinarians*. 19th ed. Rome: Food and Agriculture Organization of the United Nations (2017).
 164. Sánchez-Vizcaino JM, Heath DL. African swine fever (infection with African swine fever virus). In: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. 8th ed. Paris: Office International des Epizooties (2019). Available online at: https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.08.01_ASF.pdf
 165. de Leon P, Bustos MJ, Carrascosa AL. Laboratory methods to study African swine fever virus. *Virus Res.* (2013) 173:168–79. doi: 10.1016/j.virusres.2012.09.013
 166. Oura CA, Edwards L, Batten CA. Virological diagnosis of African swine fever—comparative study of available tests. *Virus Res.* (2013) 173:150–8. doi: 10.1016/j.virusres.2012.10.022
 167. Gallardo C, Nieto R, Soler A, Pelayo V, Fernandez-Pinero J, Markowska-Daniel I, et al. Assessment of African swine fever diagnostic techniques as a response to the epidemic outbreaks in eastern European union countries: how to improve surveillance and control programs. *J Clin Microbiol.* (2015) 53:2555–65. doi: 10.1128/JCM.00857-15
 168. Agüero M, Fernandez J, Romero L, Sanchez Mascaraque C, Arias M, Sanchez-Vizcaino JM. Highly sensitive PCR assay for routine diagnosis of African swine fever virus in clinical samples. *J Clin Microbiol.* (2003) 41:4431–4. doi: 10.1128/JCM.41.9.4431-4434.2003
 169. King DP, Reid SM, Hutchings GH, Grierson SS, Wilkinson PJ, Dixon LK, et al. Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus. *J Virol Methods.* (2003) 107:53–61. doi: 10.1016/S0166-0934(02)00189-1
 170. Zsak L, Borca MV, Risatti GR, Zsak A, French RA, Lu Z, et al. Preclinical diagnosis of African swine fever in contact-exposed swine by a real-time PCR assay. *J Clin Microbiol.* (2005) 43:112–9. doi: 10.1128/JCM.43.1.112-119.2005
 171. Fernandez-Pinero J, Gallardo C, Elizalde M, Robles A, Gomez C, Bishop R, et al. Molecular diagnosis of African swine fever by a new real-time PCR using universal probe library. *Transbound Emerg Dis.* (2013) 60:48–58. doi: 10.1111/j.1865-1682.2012.01317.x
 172. Basto AP, Portugal RS, Nix RJ, Cartaxeiro C, Boinas F, Dixon LK, et al. Development of a nested PCR and its internal control for the detection of African swine fever virus (ASFV) in *Ornithodoros erraticus*. *Arch Virol.* (2006) 151:819–26. doi: 10.1007/s00705-005-0654-2
 173. Bastos AD, Arnot LF, Jacquier MD, Maree S. A host species-informative internal control for molecular assessment of African swine fever virus infection rates in the African sylvatic cycle *Ornithodoros* vector. *Med Vet Entomol.* (2009) 23:399–409. doi: 10.1111/j.1365-2915.2009.00828.x
 174. Liu L, Atim S, LeBlanc N, Rauh R, Esau M, Chenais E, et al. Overcoming the challenges of pen-side molecular diagnosis of African swine fever to support outbreak investigations under field conditions. *Transbound Emerg Dis.* (2019) 66:908–14. doi: 10.1111/tbed.13103
 175. Liu L, Luo Y, Accensi F, Ganges L, Rodriguez F, Shan H, et al. Pre-clinical evaluation of a real-time PCR assay on a portable instrument as a possible field diagnostic tool: experiences from the testing of clinical samples for African and classical swine fever viruses. *Transbound Emerg Dis.* (2017) 64:e31–5. doi: 10.1111/tbed.12538
 176. Hjertner B, Meehan B, McKillen J, McNeilly F, Belak S. Adaptation of an Invader assay for the detection of African swine fever virus DNA. *J Virol Methods.* (2005) 124:1–10. doi: 10.1016/j.jviromet.2004.10.009
 177. James HE, Ebert K, McGonigle R, Reid SM, Boonham N, Tomlinson JA, et al. Detection of African swine fever virus by loop-mediated isothermal amplification. *J Virol Methods.* (2010) 164:68–74. doi: 10.1016/j.jviromet.2009.11.034
 178. Wang J, Wang J, Geng Y, Yuan W. A recombinase polymerase amplification-based assay for rapid detection of African swine fever virus. *Can J Vet Res.* (2017) 81:308–12.
 179. Miao F, Zhang J, Li N, Chen T, Wang L, Zhang F, et al. Rapid and sensitive recombinase polymerase amplification combined with lateral flow strip for detecting African swine fever virus. *Front Microbiol.* (2019) 10:1004. doi: 10.3389/fmicb.2019.01004
 180. Tabares E, Marcotegui MA, Fernandez M, Sanchez-Botija C. Proteins specified by African swine fever virus. I. Analysis of viral structural proteins and antigenic properties. *Arch Virol.* (1980) 66:107–17. doi: 10.1007/BF01314979
 181. Tabares E, Martinez J, Ruiz Gonzalvo F, Sanchez-Botija C. Proteins specified by African swine fever virus. II. Analysis of proteins in infected cells and antigenic properties. *Arch Virol.* (1980) 66:119–32. doi: 10.1007/BF01314980
 182. Alcaraz C, Rodriguez F, Oviedo JM, Eiras A, De Diego M, Alonso C, et al. Highly specific confirmatory western blot test for African swine fever virus antibody detection using the recombinant virus protein p54. *J Virol Methods.* (1995) 52:111–9. doi: 10.1016/0166-0934(94)00150-F
 183. Kollnberger SD, Gutierrez-Castaneda B, Foster-Cuevas M, Corteyn A, Parkhouse RM E. Identification of the principal serological immunodeterminants of African swine fever virus by screening

- a virus cDNA library with antibody. *J General Virol.* (2002) 83:1331–42. doi: 10.1099/0022-1317-83-6-1331
184. Gallardo C, Blanco E, Rodriguez JM, Carrascosa AL, Sanchez-Vizcaino JM. Antigenic properties and diagnostic potential of African swine fever virus protein pp62 expressed in insect cells. *J Clin Microbiol.* (2006) 44:950–6. doi: 10.1128/JCM.44.3.950-956.2006
 185. Gallardo C, Reis AL, Kalema-Zikusoka G, Malta J, Soler A, Blanco E, et al. Recombinant antigen targets for serodiagnosis of African swine fever. *Clin Vaccine Immunol.* (2009) 16:1012–20. doi: 10.1128/CVI.00408-08
 186. Pan IC, Huang TS, Hess WR. New method of antibody detection by indirect immunoperoxidase plaque staining for serodiagnosis of African swine fever. *J Clin Microbiol.* (1982) 16:650–5. doi: 10.1128/JCM.16.4.650-655.1982
 187. Pastor MJ, Laviada MD, Sanchez-Vizcaino JM, Escribano JM. Detection of African swine fever virus antibodies by immunoblotting assay. *Can J Vet Res.* (1989) 53:105–107.
 188. Pastor MJ, Arias M, Escribano JM. Comparison of two antigens for use in an enzyme-linked immunosorbent assay to detect African swine fever antibody. *Am J Vet Res.* (1990) 51:1540–3.
 189. Cubillos C, Gomez-Sebastian S, Moreno N, Nunez MC, Mulumba-Mfumu LK, Quembo CJ, et al. African swine fever virus serodiagnosis: a general review with a focus on the analyses of African serum samples. *Virus Res.* (2013) 173:159–67. doi: 10.1016/j.virusres.2012.10.021
 190. Sastre P, Perez T, Costa S, Yang X, Raber A, Blome S, et al. Development of a duplex lateral flow assay for simultaneous detection of antibodies against African and Classical swine fever viruses. *J Vet Diagn Invest.* (2016) 28:543–9. doi: 10.1177/1040638716654942
 191. Oviedo JM, Rodriguez F, Gomez-Puertas P, Brun A, Gomez N, Alonso C, et al. High level expression of the major antigenic African swine fever virus proteins p54 and p30 in baculovirus and their potential use as diagnostic reagents. *J Virol Methods.* (1997) 64:27–35. doi: 10.1016/S0166-0934(96)02140-4
 192. Perez-Filgueira DM, Gonzalez-Camacho F, Gallardo C, Resino-Talavan P, Blanco E, Gomez-Casado E, et al. Optimization and validation of recombinant serological tests for African Swine Fever diagnosis based on detection of the p30 protein produced in *Trichoplusia ni* larvae. *J Clin Microbiol.* (2006) 44:3114–21. doi: 10.1128/JCM.00406-06
 193. Mur L, Gallardo C, Soler A, Zimmermann J, Pelayo V, Nieto R, et al. Potential use of oral fluid samples for serological diagnosis of African swine fever. *Vet Microbiol.* (2013) 165:135–9. doi: 10.1016/j.vetmic.2012.12.034
 194. Nieto-Pelegrin E, Rivera-Arroyo B, Sanchez-Vizcaino JM. First detection of antibodies against African swine fever virus in faeces samples. *Transbound Emerg Dis.* (2015) 62:594–602. doi: 10.1111/tbed.12429
 195. Botija C. Diagnosis of African swine fever by immunofluorescence. *Bull Off Int Epizoot.* (1971) 73:1025–36.
 196. Arias M, Escribano JM, Sanchez-Vizcaino JM. Persistence of African swine fever antibody reactivity on ELISA and immunoblotting assays. *Vet Rec.* (1993) 133:189–90. doi: 10.1136/vr.133.8.189
 197. Kazakova AS, Imatdinov IR, Dubrovskaya OA, Imatdinov AR, Sidlik MV, Balyshv VM, et al. Recombinant Protein p30 for serological diagnosis of African swine fever by immunoblotting assay. *Transbound Emerg Dis.* (2017) 64:1479–92. doi: 10.1111/tbed.12539

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Gaudreault, Madden, Wilson, Trujillo and Richt. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Protection of Cattle Elicited Using a Bivalent Lumpy Skin Disease Virus-Vectored Recombinant Rift Valley Fever Vaccine

David B. Wallace^{1,2*}, A. Mather¹, P. D. Kara¹, Leeann Naicker³, Nobalanda B. Mokoena³, A. Pretorius¹, T. Nefefe¹, N. Thema¹ and Shawn Babiuk^{4,5}

¹ Vaccines and Diagnostics Development Programme, ARC-Onderstepoort Veterinary Institute, Pretoria, South Africa,

² Department Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa,

³ Onderstepoort Biological Products SOC Ltd., Pretoria, South Africa, ⁴ National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, MB, Canada, ⁵ Department of Immunology, University of Manitoba, Winnipeg, MB, Canada

OPEN ACCESS

Edited by:

Michael Kogut,
United States Department of
Agriculture, United States

Reviewed by:

Paulo Henrique Verardi,
University of Connecticut,
United States
Benjamin Lamp,
University of Veterinary Medicine
Vienna, Austria

*Correspondence:

David B. Wallace
WallaceD@arc.agric.za

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 08 January 2020

Accepted: 17 April 2020

Published: 19 May 2020

Citation:

Wallace DB, Mather A, Kara PD, Naicker L, Mokoena NB, Pretorius A, Nefefe T, Thema N and Babiuk S (2020) Protection of Cattle Elicited Using a Bivalent Lumpy Skin Disease Virus-Vectored Recombinant Rift Valley Fever Vaccine. *Front. Vet. Sci.* 7:256. doi: 10.3389/fvets.2020.00256

Lumpy skin disease and Rift Valley fever are two high-priority livestock diseases which have the potential to spread into previously free regions through animal movement and/or vectors, as well as intentional release by bioterrorists. Since the distribution range of both diseases is similar in Africa, it makes sense to use a bivalent vaccine to control them. This may lead to the more consistent and sustainable use of vaccination against Rift Valley fever through a more cost-effective vaccine. In this study, a recombinant lumpy skin disease virus was constructed in which the thymidine kinase gene was used as the insertion site for the Gn and Gc protective glycoprotein genes of Rift Valley fever virus using homologous recombination. Selection markers, the enhanced green fluorescent protein and *Escherichia coli* guanidine phosphoribosyl transferase (gpt), were used for selection of recombinant virus and in a manner enabling a second recombination event to occur upon removal of the gpt selection-pressure allowing the removal of both marker genes in the final product. This recombinant virus, LSD-RVF.mf, was selected to homogeneity, characterized and evaluated in cattle as a vaccine to show protection against both lumpy skin disease and Rift Valley fever in cattle. The results demonstrate that the LSD-RVF.mf is safe, immunogenic and can protect cattle against both diseases.

Keywords: lumpy skin disease, recombination, vaccine, Rift Valley fever, cattle

INTRODUCTION

Capripoxviruses are large double-stranded DNA viruses in the family Poxviridae comprised of sheepox, goatpox and lumpy skin disease viruses. Lumpy skin disease (LSD) causes fever and skin lesions in cattle leading to major economic losses as a result of high morbidity, and occasionally mortality (1). No country in sub-Saharan Africa claimed freedom from this disease since its discovery (OIE, HandiSTATUS, 02/02/05). LSD has rapidly spread from its historical range in most of Africa, through the Middle East into Eastern Europe (2) and many countries in Asia (3), currently as far as China, Bangladesh and India. The disease is most likely spread by insect and other arthropod vectors via mechanical transmission and is not highly contagious in the absence of potential vectors. This makes control of LSD difficult and impractical, even in non-endemic

countries experiencing occasional incurrences, which have often used stamping-out through slaughter of infected cattle. Effective control can, however, be achieved through vaccination using live attenuated vaccines, as was more recently demonstrated in Europe using mass vaccination (4). Lumpy skin disease and sheep and goat pox are ranked in the top 20 global diseases for impact on the poor (5).

Rift Valley fever virus (RVFV) is an RNA virus and member of the Phenuiviridae family (6) originally described in the 1930's in Kenya in the Rift Valley (7). The geographic distribution of Rift Valley fever (RVF) was limited to sub-Saharan Africa until outbreaks in Egypt in the 1970's (8). In 2000, RVFV spread into Yemen and Saudi Arabia (9). Fortunately, the outbreak was contained and it appears that RVFV is not currently present in the Middle East. However, there is always the risk of new outbreaks, which could become endemic. Rift Valley fever virus is a vector-borne pathogen spread by mosquitos and can infect a wide range of ruminants including cattle, sheep, goats and camels. In livestock, disease is characterized by fever, diarrhea, and abortions. Unlike LSDV, RVFV is a zoonotic virus that regularly causes epidemics among people (10, 11). As a zoonotic disease, RVF is ranked in the top 10 globally. The virus is also of major economic importance in livestock across sub-Saharan Africa and more recently in the Middle East, primarily as a result of trade embargoes on exports of mainly sheep from Africa to the Arabian Peninsula. There are vaccines available for controlling RVF and numerous experimental vaccines in development (12, 13). Since both LSDV and RVFV infect cattle and share the same geographic distribution in Africa, a bivalent vaccine able to protect against both diseases would be advantageous. This can be achieved by either formulating a vaccine combining a live attenuated LSD vaccine strain together with a live attenuated RVF vaccine or by generating a single, recombinant vaccine with protective components of both viruses.

Previously, an LSD-vectored candidate vaccine expressing the RVFV Gn (G2) and Gc (G1) glycoproteins was developed by the Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) in collaboration with Onderstepoort Biological Products (OBP Ltd). This vaccine utilizes the South African live attenuated Onderstepoort LSD vaccine as vector, which co-expresses the RVFV structural glycoprotein (GP) genes under control of the vaccinia virus P7.5K early/late promoter inserted into the LSDV viral thymidine kinase gene (14). Two selectable marker genes are also present (the *E. coli* guanine phosphoribosyl transferase (*gpt*) gene and the enhanced green fluorescent protein (EGFP) gene), which assist with the initial stages of recombinant virus selection. This bivalent LSD-RVF vaccine has been shown to fully protect mice and 1-year old Merino sheep against virulent RVFV challenge, in preliminary safety and efficacy tests (15).

However, not only is this bivalent vaccine classified as a genetically modified organism (GMO), but it also retains the dominant selection marker gene (*gpt*) conferring drug resistance, in addition to the EGFP marker gene. In order to satisfy environmental concerns over the use of products developed from micro-organisms expressing resistance genes and the sharp rise in pathogens in the field acquiring drug

resistance (e.g., XDR tuberculosis) (16) it is required that both marker genes are removed in order to satisfy regulatory agencies requirements for licensing the vaccine. This is routinely achieved for other poxvirus-vectored vaccine constructs using transient dominant selection (17, 18). As described in this paper, this method was employed to produce a selection marker-free LSD-RVF candidate vaccine, LSD-RVF.mf. Furthermore, this vaccine requires evaluation in the target species (cattle) to demonstrate its safety and efficacy, as there are numerous examples of vaccines which demonstrated protection in animal models, but failed when used in the target species.

The advantages of using a bivalent LSD-RVF.mf vaccine over conventional vaccination approaches include: (1) a vaccine which is cost effective; (2) since RVF outbreaks are cyclical, producers are reluctant to vaccinate as it is expensive, and thus having a bivalent capripoxvirus vaccine removes these economic reasons for not vaccinating; and (3) the bivalent vaccine is able to differentiate infected from vaccinated animals (DIVA) for RVF, allowing for serological surveillance testing to still be performed when it is in use. The impact of using the bivalent LSD-RVF.mf vaccine includes: (1) preventing mortality and debilitating disease in cattle caused by LSD and RVF in all regions of sub-Saharan Africa where the diseases both occur, leading to improved production and economic development; (2) indirectly protecting people from RVF virus by decreasing the viral loads in livestock; and (3) helping provide an effective barrier to further spread of RVFV into non-endemic countries.

Determining the precise impact of these diseases on incomes of the poor would require a dedicated study. However, bearing in mind the fact that LSD and RVF viruses are OIE listed diseases and the current vaccine uptake of individual vaccines against these diseases is relatively high, this vaccine could become more widely used and have greater benefit in developing countries than most other livestock vaccines currently available. A recent study determined that vaccination against LSD is cost effective and yields a significant investment return to both smallholder and large commercial producers (19).

Since currently used live attenuated LSD and RVF vaccines are effective following a single dose, any new vaccine must also be effective following a single administration in order to be used in the field. Therefore, the LSD-RVF.mf vaccine was evaluated in cattle for its ability to provide protection using a single administration against both virulent LSD and RVF challenge and the results are presented here.

MATERIALS AND METHODS

Cells and Viruses

Cells

Primary fetal bovine testes (FBT) cells were prepared according to standard techniques (20). These were used for construction and selection of the recombinants and were propagated in a 1:1 combination of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (Gibco, USA) containing 8% FCS and antibiotics (100 µg/ml penicillin, 100 µg/ml streptomycin and 250 µg/ml amphotericin B) (Lonza, USA).

Focus selection and titrations were performed using Madin-Darby bovine kidney (MDBK) cells (obtained from the American Type Tissue Culture Collection, no. CCL-22, USA) and baby hamster kidney (BHK) cells (obtained from the American Type Tissue Culture Collection, no. CCL-10, USA) for preparation of the RVFV challenge strain, as below.

Viruses

The South African Neethling vaccine strain of LSDV, produced by Onderstepoort Biological Products (OBP LTD), was used as parental virus (21) for the generation of recombinants.

The virulent LSDV Warmbaths field isolate (LSDV_WB) (GenBank Accession no. AF409137) was used as the inoculum strain for the LSDV challenge (22).

The M35/74 field strain of RVFV was used as the inoculum for the RVFV challenge (23). A freeze-dried ampule containing the virus was reconstituted in 1 ml Eagle's Minimum Essential Medium (EMEM) (Gibco, USA) and seeded onto a 90% confluent monolayer of BHK cells in EMEM (with 5% FCS and antibiotics). The infected cells were incubated at 37°C and were freeze-thawed between −20°C and room temperature (RT) three times 2 days after cytopathic effects (CPE) were observed. The lysed cell/virus-containing medium was then aliquoted into 15 ml sterile conical tubes and stored at −20°C, and aliquots removed on two separate occasions for titration on BHK cells. On Day 0 of the trial, the required volume of inoculum was removed from a tube after thawing at RT, followed by mixing, and dilution to the required virus concentration in EMEM.

High-titer virus stocks of the commercial LSD vaccine and the recombinant LSD-RVFV vaccine were prepared and obtained from OBP LTD, following their routine manufacturing protocols (details of this procedure is protected intellectual property of the manufacturer).

Plasmid Design

The insertion vector, pLS(EG)-RV, was constructed along similar lines to pLSEG-RVFV (14), except the transfer vector, pLS(EG), was developed with insertion of the positive and visual selection marker genes, the *E. coli* gpt and EGFP genes respectively, exterior to the TK-L and TK-R LSDV thymidine kinase (TK) gene flanking regions (**Supplementary Material 1**).

Recombinant Virus Construction, Selection and Characterization

The recombinant marker-free virus was generated, selected and characterized according to the improved method described by Wallace et al. (24), with the addition of a second round of selection in the absence of mycophenolic acid (MPA) selection pressure, resulting in a product free from both marker genes [known as transient dominant selection (17, 18)]. However, as a potential by-product of this selection method is a reconstituted parental virus (with an intact TK gene and no RVFV GP genes), a method was devised to enable selection of homogeneous marker-free recombinant virus expressing the RVFV GPs—in effect, an “intracellular” ELISA, as described below.

“Intracellular” ELISA

Ninety-six well cell culture plates (Nunc, Denmark) were seeded with FBT cells and once 80–90% confluent, growth medium was removed, replaced with maintenance medium and infected with half aliquots of pre-selected foci (the remainder of each focus being frozen at −20°C) in duplicate (uninfected cells and parental LSDV-infected cells were included as controls).

Once the cells were heavily infected (displaying >80% cytopathogenic effect [cpe]), they were washed gently in PBS, fixed with ice-cold 70% acetone for 10 min (min) and allowed to dry overnight at 4°C. The fixed cells were then incubated at 37°C for 1 h in 10% fat-free milk powder (Elite, Clover, South Africa) (diluted in PBS) (300 µl/well), washed three times in Tris-buffered saline (with Tween 20, pH 8.0) (TST) wash buffer (Sigma, USA) (300 µl/well) and then incubated at 37°C for 1 h with 100 µl/well positive RVFV polyclonal sheep serum diluted 1:50 in 10% fat-free milk powder. The washing was repeated (as above) and the cells incubated for 1 h at 37°C with 100 µl/well Protein G-conjugated horseradish immunoperoxidase (Invitrogen, USA) diluted 1:10 000 in 10% fat-free milk powder. After another round of washing, 100 µl/well of TMB Ready-to-use substrate (Life Technologies [ThermoFisher Scientific, USA]) was added for 10 min and thereafter the color reaction development was stopped using sulphuric acid (Merck, USA) and the OD values were determined using a BioTek ELISA reader (Model: ELX808) (BioTek, USA) at 450 nm.

OD values for each duplicate set of wells were averaged and using the negative control well values as a guide, wells with the highest average OD values were selected as those containing recombinant virus at or close to homogeneity and expressing high levels of the RVFV GPs.

Frozen half-aliquots of foci, selected due to their high OD values, as above, were removed from −20°C storage, thawed and frozen three times, and added to fresh uninfected FBT cells. Released virus was propagated and once cells displayed 100% cpe, viral and cellular DNA was purified using the Roche Magna Pure Total nucleic acid extraction kit (Roche, Switzerland) and a Roche MagNA Pure LC 2.0 extractor according to the manufacturer's instructions. Viral foci were then characterized for homogeneity (free from parental LSDV virus and EGFP and gpt marker genes) using PCR-amplification of the target insertion region, as described next.

Polymerase Chain Reaction (PCR) Assay

Detection of recombinant viruses containing the RVFV GP genes and selection to homogeneity was performed using conventional PCR. In brief, a primer pair (MP1-F: 5′ – CTC CTG TAT TTA TAG AAC CTA – 3′; MP1-R: 5′ – GCA TTA TCA TTA TCG TCA TCA TC – 3′) was designed to amplify flanking regions of the LSDV TK gene (product size ~ 1.9 kilo-base pairs [kbp]), into which the RVFV GP genes were inserted (**Supplementary Material 1**). Amplification was carried out using a GeneAmp 2400 thermocycler (Perkin-Elmer, USA) in a 25 µl reaction volume consisting of 2.5 µl 10X PCR buffer (containing 20 mM MgCl₂), 2 µl 2.5 mM dNTPs, 0.25 U Taq DNA polymerase (TaKaRa Ex Taq™) (Takara Biomedicals, Japan), 0.5 µl of each primer [20 pmoles

each] [Gibco-BRL, UK], 1 µl template DNA (~ 0.1 ng) and 18.4 µl sterile distilled water. Template DNA was denatured for 45 s (sec) at 95°C, primer annealing was carried out at 62°C for 45 sec, and strand extension was at 72°C for 7 min (repeated through 35 amplification cycles) (preceded by an initial denaturation step at 95°C for 60 s and a final elongation step at 72°C for 7 min and then holding at 4°C). PCR products were separated on a 2% agarose gel containing Ethidium bromide (EtBr).

Indirect Immunofluorescence Assay (IFA)

The IFA was used to detect the expression of the RVFV glycoprotein genes from the recombinant virus using the method described by Wallace (25). The slides were viewed and images captured using an Olympus BX41 phase contrast microscope (Olympus, USA) and an Olympus DX10 digital camera (Olympus, Japan).

Animal Selection, Care, and Housing

The cattle trial was conducted with the approval of the Agricultural Research Council-Onderstepoort Veterinary Institute Animal Ethics Committee (approval no. AEC 4.17) and the South African Department of Agriculture, Forestry and Fisheries (DAFF) (Section 20 of the South African Animal Diseases Act [Act No 35 of 1984] permit no. 12/11/1/15).

Fifty-six male Holstein-Friesian cross calves, ~8 months old, were pre-screened for exposure/presence of LSDV and RVFV using a conventional PCR for LSDV (26) and a real-time RT-PCR for RVFV (27), respectively. Serology was also performed for detecting antibodies to either pathogen. Forty calves were then selected, purchased and delivered to the ARC-Transboundary Animal Diseases (TADs) biosafety containment level-3 facilities, of which twenty were used in this trial.

They were housed in four individual stalls with five animals per stall. During a 2-weeks acclimatization period, the calves were monitored for overall health and well-being. Rectal temperatures were recorded throughout this period and throughout the trial. The calves were fed daily on a balanced maintenance diet of bovine pellets and provided with fresh, clean water *ad-libitum*. Within the 1st week of arrival, serum samples were obtained from each calf and retested for the presence of LSDV and RVFV antibodies, as described. For blood collection, animals were first mildly sedated with Xylazine-hydrochloride (RompunTM, Bayer AH, USA) and once calm they were bled via the jugular vein into EDTA or serum tubes. At the conclusion of the trial, all calves were euthanised using an overdose of sodium pentobarbitone (Sigma-Aldrich, USA).

Vaccination and LSD Challenge

The calves were inoculated as described in Table 1. The 10 calves in Group 1 were mock-vaccinated with 2.0 ml vaccine diluent subcutaneously (SC) (obtained from OBP Ltd, South Africa) as negative controls, while calves in Group 2 were inoculated SC with 2.0 ml of the LSD-RVF.mf construct at a titer of 1.0×10^3 TCID₅₀/ml.

At 21 day's post-inoculation (dpi), five of the mock-vaccinated calves in Group 1 (D1-1 to D1-5) and five of the calves vaccinated

with the LSD-RVF.mf construct in Group 2 (A1-1 to A1-5) were challenged with 2.0 ml of LSDV_WB at a titer of 5.0×10^4 TCID₅₀/ml, clustered at two sites SC, and 2.0 ml intravenously (IV) at a titer of 5.0×10^5 TCID₅₀/ml.

Detection of LSDV Viremia

Whole blood was collected from calves on the day of inoculation and 7 and 14 dpi, and similarly, equivalent days' post-challenge (dpc). The blood was aliquoted into sterile 1.5 ml tubes (Eppendorf, Germany). An equal volume of Lysis/binding buffer from the MagNA Pure LC total nucleic acid isolation kit (Roche, Switzerland) was added to the blood, which was then frozen at -20°C until further use. Once the samples were thawed, total nucleic acid extraction was performed using the kit (following its "Total NA HP" protocol) and a Roche MagNA Pure LC 2.0 extractor, both according to the manufacturer's instructions.

Detection of the extracted LSDV DNA was achieved using a conventional PCR and a GeneAmp 2400 thermocycler (Perkin Elmer, USA). The method was performed as described by Viljoen et al. (26). The primer pair (OP3: 5'-CAC CAG AGC CGA TAA C-3'; OP49: 5'-GTG CTA TCT AGT GCA GCT AT-3') was used. This primer pair amplifies a 450 base pair (bp) region within the wild type LSDV TK gene. Amplification was carried out using a GeneAmp 2400 thermocycler (Perkin-Elmer, USA) in a 25 µl reaction volume consisting of 2.0 µl 10X HotStarTaq PCR buffer, 1 µl 2.5 mM dNTPs, 2 units HotStarTaq polymerase (Qiagen, Germany), 1.0 µl of each primer [20 pmoles each] [Gibco-BRL, UK], 5.0 µl template DNA (1 ng) and 14.8 µl sterile DNase/RNase free water. Template DNA was denatured for 45 s (sec) at 94°C, primer annealing was carried out at 53°C for 45 s, and strand extension was at 75°C for 60 s (repeated through 35 amplification cycles) (preceded by an initial denaturation step at 95°C for 7 min). PCR products were separated on a 2% agarose gel containing Ethidium bromide (EtBr).

Detection of LSDV in Skin Lesions

Biopsies of skin lesions were collected from the calves as described by Tuppurainen et al. (28), with some modifications: instead of suturing the wound, the site was sprayed with antiseptic spray (Zerpar, Bayer AH, USA) daily for 3 days following the procedure. Prior to the biopsy, the area was first sterilized using a cotton swab soaked in 70% ethanol. Skin lesion samples were sent for immunohistopathology evaluation specific for detection of LSDV antigen and gross cellular pathology related to virus infection (Idexx Laboratories, South Africa).

Humoral Immunity

Sera was recovered from blood collected from all calves on the day of inoculation, and 7, 11, 17, and 21 dpi, the day of challenge and 8, 11, 14, and 21 dpc. Detection of antibodies in sera to LSDV was achieved using the virus neutralization test (VNT) and the constant virus-varying serum method (29). For RVFV antibody

TABLE 1 | Calf identity numbers, group numbers, the inoculum used for both inoculation and challenge and their relevant titres and routes and day of challenge.

Animal number	Group	Inoculum (titer)	Day of challenge and challenge virus	Titer
D1-1	1	diluent* (2.0 ml, SC)	Day 21 post-inoculation. LSDV Warmbaths isolate	5 × 10 ⁴ TCID ₅₀ /ml (2.0 ml, SC) & 5 × 10 ⁵ TCID ₅₀ /ml (2.0 ml, IV)
D1-2				
D1-3				
D1-4				
D1-5				
D2-1		diluent* (2.0 ml, SC)	Day 28 post-inoculation. RVFV strain M35/74	3.0 × 10 ⁶ TCID ₅₀ , 1.0 ml IV
D2-2				
D2-3				
D2-4				
D2-5				
A1-1	2	LSD-RVF.mf (1.0 × 10 ³ TCID ₅₀)	Day 21 post-inoculation. LSDV Warmbaths isolate	5 × 10 ⁴ TCID ₅₀ /ml (2.0 ml, SC) & 5 × 10 ⁵ TCID ₅₀ /ml (2.0 ml, IV)
A1-2				
A1-3				
A1-4				
A1-5				
C2-1		LSD-RVF.mf (1.0 × 10 ³ TCID ₅₀)	Day 28 post-inoculation. RVFV strain M35/74	3.0 × 10 ⁶ TCID ₅₀ , 1.0 ml, IV
C2-2				
C2-3				
C2-4				
C2-5				

*2.0 ml of vaccine diluent, obtained from Onderstepoort Biological products.

detection, the standard VNT (30) was used and two ELISAs—a RVFV immunoglobulin M (IgM) capture ELISA (31) and an immunoglobulin G (IgG) indirect ELISA (32).

Cellular Immunity

Whole blood was collected on the day of inoculation, 7 and 14 dpi, the day of challenge and 7 and 14 dpc. Lymphocytes were isolated and used for bovine IFN- γ ELISPOT and phenotype analysis as described previously by Kara et al. (22). In both assays PMBCs (2 × 10⁵ cells/well) were stimulated in triplicate with the LSDV_WB isolate (1 × 10⁵ pfu/well), inactivated whole RVFV antigen (0.5% [V/V] formaldehyde solution) (10 μ g/ml, from OBP LTD, Smithburn vaccine strain) and positive ConA antigen (5 μ g/ml, Sigma Aldrich, USA). Responses were measured using the bovine IFN- γ ELISPOT kit (Mabtech AB, Sweden) as per the manufacturer's instructions and spot-forming cells (SFC) were enumerated using an automated ELISPOT reader (Zeiss KS ELISPOT Compact 4.5, Germany). Antibodies used for phenotype determination (at a 1:100 dilution) were mouse anti-bovine CD45Ro-PE (cell line IL-A116, IgG3, BioRad), mouse anti-bovine CD8 α -FITC (Clone 38.65, IgG1, BioRad), mouse anti-bovine CD4 primary antibody (cell line GC50A1, IgM, VMRD Inc, USA) and goat anti-mouse IgM-APC secondary antibody (1:8 dilution; Invitrogen, UK). Flow cytometry data acquisition was performed using a MACSQuant[®] Analyzer 10 (Miltenyi Biotec) and data were analyzed using Kaluza 2.1 software (Beckman Coulter, USA). Isotypic controls were included for all isotypes used. The significance of differences

between the average values per group was determined by means of the Student's *t*-test. Differences with $P \leq 0.05$ were considered significant.

RVF Vaccination and Challenge

Twenty-eight dpi the remaining five mock-vaccinated calves in Group 1 (D2-1 to D2-5) and the five calves inoculated with the LSD-RVF.mf construct in Group 2 (C2-1 to C2-5) were challenged intravenously with the virulent RVFV M35/74 strain at a titer of 3.0 × 10⁶ TCID₅₀/ml in 1.0 ml (Table 1).

Sera were recovered from blood collected from all calves on the day of inoculation, and 7, 11, 17, and 21 dpi, the day of challenge and 3, 6, 9, and 14 dpc, and were evaluated for RVFV-specific antibodies. Whole blood was collected pre-challenge for CMI testing, as described.

Detection of RVF Viremia

A RVFV real-time RT-PCR (27) was used for the detection of RVFV in serum samples that were recovered on the day of challenge and 3, 6, 9, 14, and 21 dpc.

Total nucleic acid was extracted from serum samples using the MagNA Pure Total Nucleic Acid Kit (High Performance) (Roche, Switzerland). The real-time RT-PCR assay was performed using the LC 480 One-step RNA Master kit (Roche, Switzerland), as per the manufacturer's instructions. The following primers and probe were used: RVS (Forward): 5'- AAA GGA ACA ATG GAC TCT GGT CA-3'; RVA (Reverse): 5'- CAC TTC TTA CTA CCA TGT

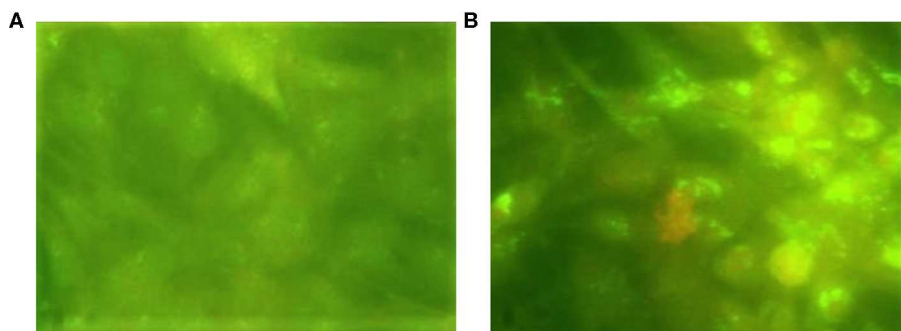


FIGURE 1 | LSD-RVF.mf recombinant foci immunostaining of **(A)** Negative control—an unrelated LSDV-vectored recombinant construct (expressing an antigen of *Mycoplasma mycoides* subspecies *mycoides* small colony) and **(B)** Sub-culture foci 2a.2 (x100).

CCT CCA AT–3′; RVP (Probe): 5′ –FAM- AAA GCT TTG ATA TCT CTC AGT GCC CCA A –BHQ–3′. The real-time RT-PCR reaction mix and the cycling conditions are listed in **Supplementary Material 2**.

RESULTS

Recombinant Virus Construction, Selection and Characterization

The OBP vaccine strain of LSDV was used as the parental virus with the pLS(EG)-RV insertion plasmid containing the RVFV GP genes and EGFP and *E. coli* gpt selection marker genes to generate recombinant virus through the use of homologous recombination (**Supplementary Material 1**) (24). Selection of foci with EGFP expression under mycophenolic acid (MPA) selection pressure was achieved through multiple rounds of plaque picking, followed by the progeny virions being grown in the absence of MPA to allow for removal of the selection markers. EGFP fluorescence was rapidly lost from these foci, indicating occurrence of a second recombination event resulting in either reversion to parental virus, or recombinant viruses containing the RVFV GP genes, but with the loss of the EGFP and gpt selection marker genes - LSD-RVF.mf.

Multiple foci lacking EGFP expression were selected, half portions of each were frozen at –20°C and the remainder subjected to the “Intracellular ELISA” technique developed and described under the Materials and Methods. Wells containing foci showing signals (OD values) for the positive RVFV antiserum which were significantly higher than in duplicate wells for the negative serum were deemed to be positive for expression of the RVFV GPs and were further selected and sub-cultured after filtration and sonication (**Supplementary Material 3**). DNA was extracted from a number of sub-cultured foci resulting from focus 2a.2 and PCR was performed to confirm homogeneity (**Supplementary Material 4**). The presence of amplification products of the correct size (~5.5 kbp) for the recombinant virus and absence of any amplification products of 1.9 kbp, indicative of parental virus, provided clear evidence that the sub-cultures were pure and thus free of parental virus.

Recombinant virus foci were also confirmed for expression of the RVFV GPs using the IFA, as shown in **Figure 1**. Only the LSD-RVF.mf foci were positive for RVFV protein expression.

Sub-culture focus 2a.2_6 was finally selected for a stability study, in which it was passaged 10x in cell culture and retested for RVFV GP expression and homogeneity (data not shown), and it was shown to be stable, homogeneous and expressing the RVFV GPs. This recombinant virus was then grown to high titres, aliquots made and stored at –20°C, until further use.

Vaccine Safety in Cattle

All animals tested negative for the presence of either LSDV or RVFV by the methods used for pre-screening, and were deemed to be in good health at the outset of the trial.

No erythema or abscesses were observed in any of the negative control animals, post-inoculation. Two of the 10 animals in the vaccinated group (with LSD-RVF.mf) (animals A1-3 and C2-1) developed a mild local reaction at the site of inoculation at 7 dpi. In animal A1-3, this presented as a swelling (2 × 3 cm in size), which persisted to 15 dpi, after which it began to resolve and by 26 dpi it was completely resolved. Animal C2-1 presented with a single injection-site swelling, 1.5 × 1.5 cm in size. A biopsy sample from this region was not positive for LSDV antigen via immunohistochemistry (IHC) staining. No pyrexia (temperature > 39.5°C) was observed in any of the vaccinated animals (**Figure 2**), nor was it possible to detect LSDV nucleic acid in blood via conventional PCR from the vaccinated group of animals at 7 or 11 dpi.

Protection Against LSDV Challenge LSD Clinical Evaluation

Following virus challenge with the virulent LSDV_WB isolate, an increase in rectal temperatures indicative of pyrexia (>39.5°C) was observed in 4/5 of the mock-vaccinated negative control calves (**Figure 3A**). Animal D1-4 and D1-5, at 8 dpc and 4 dpc, respectively, had temperatures above 40°C. The febrile response lasted for 4 days in animal D1-5, while the temperature normalized the following day in animal D1-4. In the calves vaccinated with LSD-RVF.mf (**Figure 3B**) an increase in rectal temperatures indicative of pyrexia was observed in all calves, except animal A1-5, at 24 h post-challenge. The febrile response

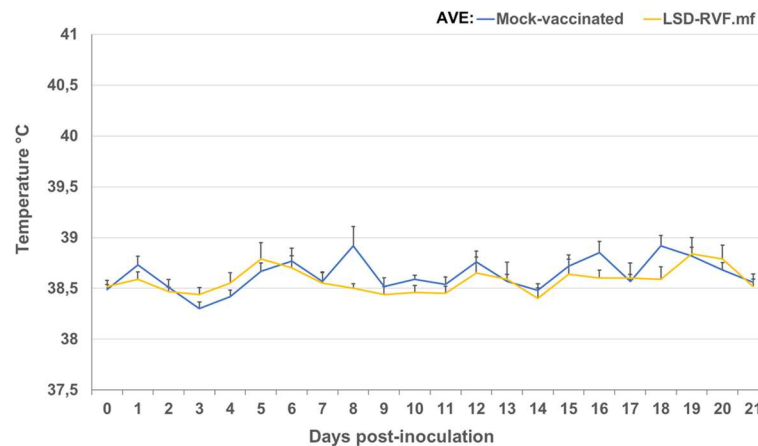


FIGURE 2 | Average temperatures of calves following vaccination with LSD-RVF.mf vaccine and mock vaccination with PBS. No increased rectal temperatures indicative of pyrexia ($>39.5^{\circ}\text{C}$) were observed for any of the calves.

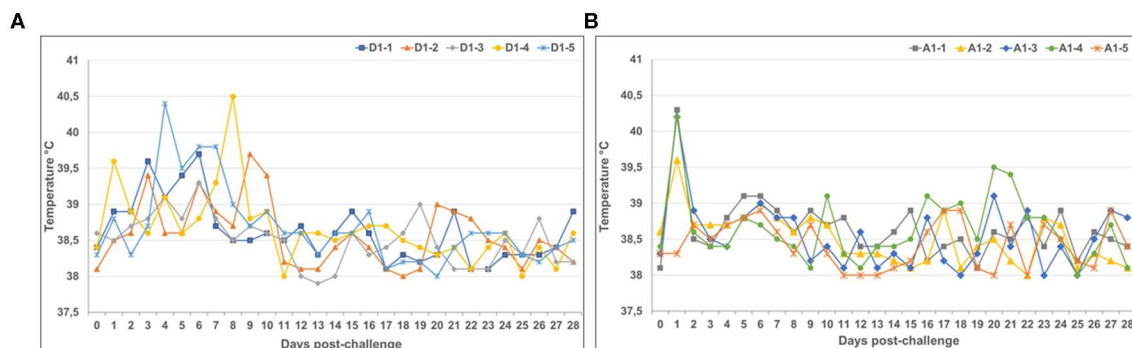


FIGURE 3 | (A) Temperatures of mock-vaccinated calves following challenge with the virulent LSDV_{WB} isolate. Increases in rectal temperatures indicative of pyrexia ($>39.5^{\circ}\text{C}$) were observed in calf D1-4 and D1-5 at 8 and 4 dpc, respectively. Febrile responses lasted for 4 days in animal D1-5, while the temperature normalized the following day in animal D1-4. **(B)** Temperatures of calves vaccinated with LSD-RVF.mf following challenge with the virulent LSDV_{WB} isolate. Increases in rectal temperatures indicative of pyrexia were observed in all calves, except animal A1-5, at 1 dpc. Febrile responses (in the range of $39.6\text{--}40.3^{\circ}\text{C}$) lasted for a single day, with temperatures normalizing by the following day.

(in the range of $39.6\text{--}40.3^{\circ}\text{C}$) lasted for a single day with temperatures normalizing the following day.

LSD Viremia

Viremia was assessed using detection of LSDV DNA in blood using conventional PCR. Viral nucleic acid was not detected in the control or the LSD-RVF.mf-vaccinated group via conventional PCR at any time points tested, post-challenge.

LSDV-Specific Antibody Responses Following Vaccination and Challenge

Sera were evaluated at multiple time points following vaccination and challenge. No measurable neutralizing antibodies were detected in the mock-vaccinated group after inoculation, however, neutralizing antibody titres were observed in all five animals by 11 dpc, with the highest levels observed at 21 dpc

(**Figure 4A**). Following vaccination with LSD-RVF.mf, LSDV-specific virus neutralizing antibodies were detected as early as 11 dpi in animals A1-2 and A1-5 (**Figure 4B**). All five animals sero-converted by 17 dpi. A significant increase in titres were observed following challenge in three animals by 14 dpc.

LSDV- and RVFV-Specific Cellular Immune Responses

PBMCs were collected from calves on the day of inoculation (day 0), 7 and 14 dpi, and 7 and 14 dpc (**Figure 5**). ELISPOT analyses showed increased levels of IFN- γ -producing cells at 14 dpi in two animals (A1-3 and A1-4) from Group 2 (the vaccinated and LSDV-challenged group: animals A1-1 to A1-5) when stimulated with LSDV antigen (**Figure 5A**). Increased numbers of IFN- γ -producing cells were detected in Group 2 calves post-challenge, with higher expression levels in the vaccinated group being

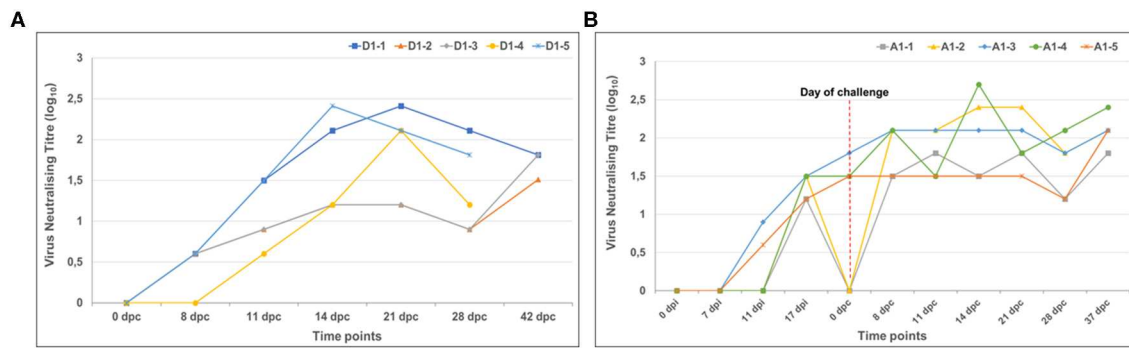


FIGURE 4 | LSDV-neutralizing antibody titres in calves. **(A)** Mock-vaccinated animals had no detectable virus neutralizing antibody levels at any days' post-inoculation (dpi) and are therefore not represented on the graph. However, neutralizing antibody titres were observed in all animals by 11 days' post challenge (dpc) with the highest levels observed at 14 and 21 dpc. **(B)** All calves inoculated with the LSD-RVF.mf vaccine sero-converted by 17 dpi with two animals sero-converting as early as 11 dpi. A significant increase in titres were observed following challenge in three animals by 14 dpc.

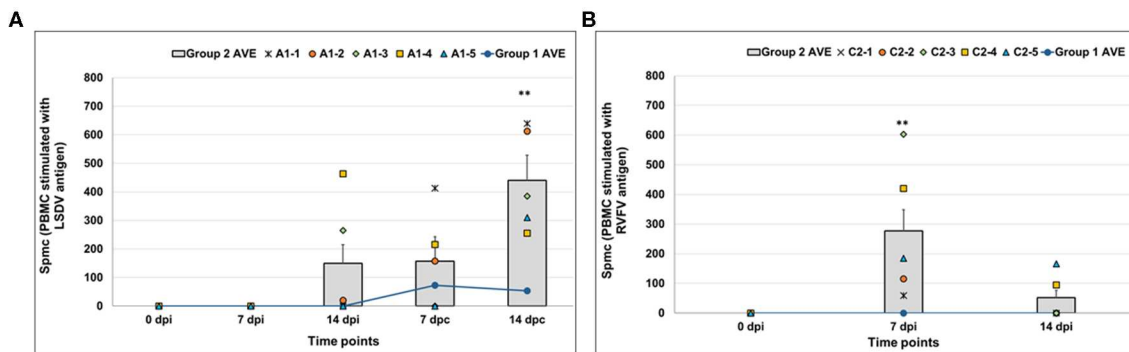


FIGURE 5 | Interferon- γ (IFN- γ) responses in PBMCs collected from calves. **(A)** Group 2 (vaccinated and LSDV challenged) animals' PBMCs stimulated with LSDV. **(B)** Group 2 (vaccinated and RVFV challenged) animals stimulated with Rift Valley fever virus (RVFV) antigen. Averages for the Group 1 (mock-vaccinated control animals: D1-1 to D1-5) are depicted in the line graph in both panels. PBMCs were isolated for IFN- γ responses on the day of inoculation (Day 0), 7 and 14 dpi, and 7 and 14 dpc. The average spots per million cells (sPMC) for 5 calves are represented for ELISPOT as a bar graph while individual animals' data are also indicated at each time point. Significant differences, as compared to the average 0 dpi data with $P \leq 0.01$, are indicated with**.

recorded (Figure 5A). Numbers of IFN- γ -producing cells were significantly increased at 7 dpi when stimulated with RVFV antigen (Figure 5B) in Group 2 animals (vaccinated and RVFV challenged animals: C2-1 to C2-5). Animals in Group 1 (mock-vaccinated: D1-1 to D1-5) challenged with LSDV also showed an increase in IFN- γ -producing cells post-challenge when stimulated with LSDV antigen (Figure 5A, line graph), while IFN- γ secretion was absent in the PBMCs of the same animals after stimulation with RVFV antigen. The vaccinated group challenged with virulent RVFV (Group 2: C2-1 to C2-5) was not immunologically monitored after challenge due to safety risks. Phenotypic analysis to determine memory T cells indicated that animals in Group 2 (vaccinated and LSDV-challenged animals: A1-1 to A1-5) had a significant increase in both CD4⁺ CD45Ro⁺ and CD8⁺ CD45Ro⁺ memory T cells at 7 dpi and 14 dpc after stimulation with LSDV antigen (Figures 6A,B). PBMCs stimulated with RVFV antigen only showed an increase in CD8⁺ CD45Ro⁺ memory T cells at 14 dpi, while no CD4⁺ memory

T cells were detected (Supplementary Material 5). No memory cells specific to LSDV or RVFV antigen were detected in Group 1 (mock-vaccinated animals: D1-1 to D1-5), as indicated in Figures 6A,B, line graph.

Protection Against RVF Challenge

RVF Clinical Evaluation

Animals in the control (mock-vaccinated) group and the vaccinated group responded with pyrexia following challenge, although the mean rectal temperatures were higher in the control group, with peak temperatures occurring in all animals 3 days' post-challenge (dpc) followed by a decline back to baseline (Figure 7A). Animals C2-2 and C2-3 in the vaccinated group developed fevers above 41°C at 3 dpc (Figure 7B).

All animals in the control group displayed typical clinical signs of RVFV infection, such as inappetence and occasional sternal recumbency. Control animal D2-2's temperature peaked

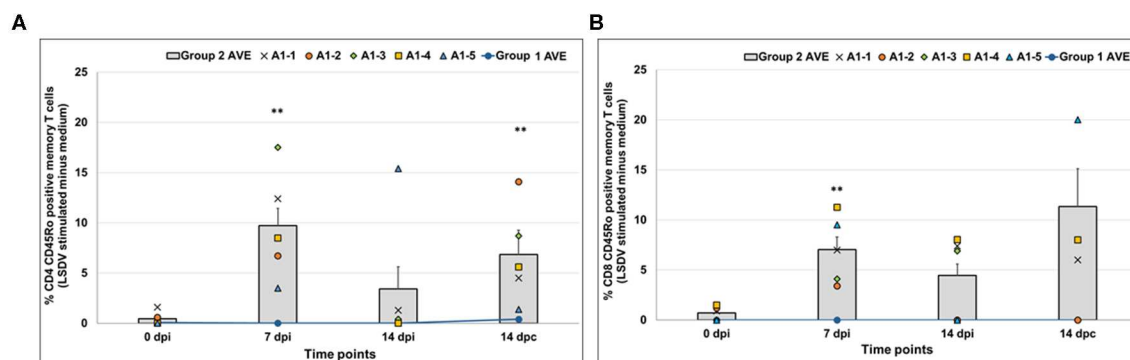


FIGURE 6 | Phenotypic analysis of memory T cells in PBMCs collected from calves. **(A)** CD4+ CD45RO+ cells and **(B)** CD8+ CD45RO+ cells in Group 2 (vaccinated and LSDV challenged animals) when PBMCs were stimulated with LSDV. Averages for the Group 1 (mock-vaccinated control animals: D1-1 to D1-5) are depicted in the line graph in both panels. PBMCs stimulated with antigen were normalized against unstimulated PBMCs. Significant differences, as compared to the negative control group with $P \leq 0.01$, are indicated with**.

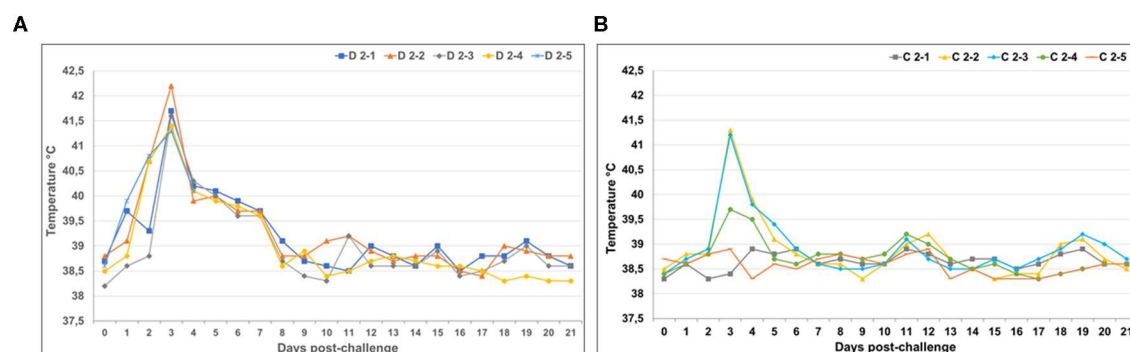


FIGURE 7 | Temperature responses in vaccinated and mock-vaccinated animals following virulent RVFV challenge. **(A)** Rectal temperatures of individual animals in the control mock-vaccinated group. **(B)** Rectal temperatures of individual animals in the vaccinated group.

at 42.2°C and it developed diarrhea 6 dpc. One control animal (D2-5) died at 5 dpc and was immediately necropsied. The carcass was bloated and partially autolyzed. The liver had multiple coalescing pale areas of necrosis surrounded by hemorrhagic hepatic parenchyma. Petechiae of the mucosa of the gall bladder and diffuse hemorrhaging of lymph nodes were observed – thus, the cause of death was linked to acute RVFV infection. Liver, kidney and spleen samples were sent for histopathology and IHC staining and evaluation related to RVFV infection (Idexx Laboratories, South Africa). Positive staining for RVFV antigen was detected in pooled organ samples from this animal, further supporting cause of death linked to RVFV infection.

None of the animals in the vaccinated group showed clinical symptoms, besides pyrexia, typical of RVFV infection. No gross pathology consistent with RVFV infection was observed in organs of the remainder of the animals in the control group or in any of the animals in the vaccinated group, upon necropsy following euthanasia.

RVF Viremia

Viremia was assessed by detecting RVFV genomic sequences in sera using qRT-PCR. Viral genomes were detected in the mock-vaccinated calves 3 dpc in four of the five animals (not detected in animal D2-1) (**Figure 8A**). Peak levels of RVFV occurred at this time point for most animals as indicated by low Ct values. Animal D2-5 displayed the lowest Ct value at 3 dpc, and succumbed to RVFV infection by 5 dpc. In contrast, RVFV was detected in two of the five animals (C2-2 and C2-3) in the vaccinated group and displayed higher Ct values than the mock-vaccinated group at 3 dpc (**Figure 8B**). By 6 dpc, RVFV was detected in only one animal (C2-1) at a low level, as indicated by the high Ct value.

RVFV-Specific Antibody Responses Following Vaccination and Challenge

The mock-vaccinated animals had no detectable virus neutralizing antibody levels post-inoculation. Virus neutralizing titres post-challenge were higher in the vaccinated group than in the mock-vaccinated group by 6 dpc (**Figure 9A**). Rift Valley

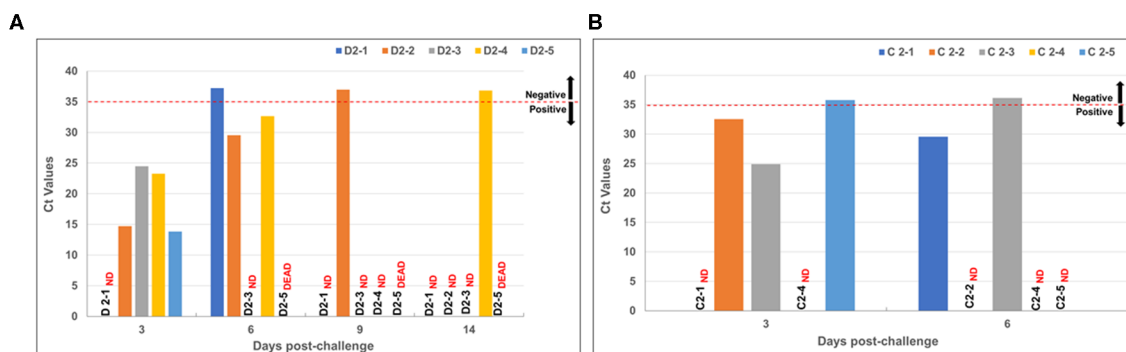


FIGURE 8 | Viremia in vaccinated and mock-vaccinated calves following virulent RVFV challenge. *ND*, not detected and indicates the animal number. Dashed line (---) denotes cut-off point between positive (below line) and negative (above line). **(A)** - qRT-PCR Ct values for mock-vaccinated animals and **(B)** vaccinated animals after challenge with RVFV.

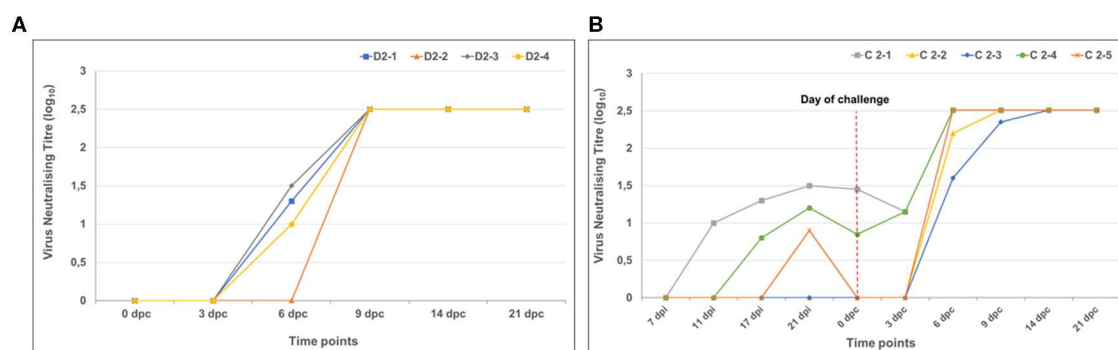


FIGURE 9 | RVFV neutralizing antibody titres in calves. **(A)** Mock-vaccinated animals had no detectable virus neutralizing antibody levels at any days' post-inoculation (dpi) and are therefore not represented on the graph. Highest levels of neutralizing antibody titres were observed in all animals by 9 dpc. Animal D2-5 is not shown on the graph as neutralizing antibodies were not detected at 3 day's post-challenge (dpc), however, the animal died of acute RVF at 5 dpc. **(B)** Three calves inoculated with the LSD-RVF.mf vaccine sero-converted post-vaccination. A significant increase in titres were observed following challenge in all animals by 9 dpc (testing was performed up to a limit of 1:320 serum dilution, thus all final titres are higher).

fever virus neutralizing antibodies were present in three of the five calves (**Figure 9B**) after vaccination, which increased significantly after challenge by 6 dpc.

The sera were also evaluated for antibodies to RVFV using two ELISAs (an IgG and IgM ELISA) utilizing recombinant RVFV nucleoprotein (rNP) as antigen (**Figures 10, 11**). As expected, first IgM antibodies were detected, from 3 dpc, and then IgG antibodies, from 6 dpc, since the recombinant LSDV-vectored RVF vaccine expresses the viral GPs, and not the NP. IgM antibody levels rapidly decreased from 9 to 14 dpc, then rose around 21 dpc. The IgG antibody levels (**Figure 11**) rapidly rose between 14 and 21 dpc for both mock-vaccinated and vaccinated groups, although not for all animals.

DISCUSSION

Lumpy skin disease is no longer an “African” disease, as it has spread into the Middle East, Eastern Europe, the Balkans, Russia and Kazakhstan, with rapid recent spread into China, Bangladesh and India, which significantly increases the risk

of LSD spreading into the remaining free countries in Asia. Currently, only the Americas, Western Europe, Australia, New Zealand, Indo-Pacific islands, amongst others, can claim to be LSD-free. However, these regions face a constant threat of inadvertent or intentional introduction.

In contrast, so far Rift Valley fever has remained within Africa, except for infrequent spread to Saudi Arabia and Yemen in the Middle East. It is possible that Rift Valley fever virus could spread into new regions, including the Americas, as was the case with West Nile virus. Increasing socio-political unrest and bioterrorism have drastically increased the risk for further spread of the disease.

Vaccination is the proven method for controlling both human and animal diseases, and its use has been demonstrated to successfully control LSD and RVF. Although efficacious vaccines exist for both, due to the sporadic nature of RVF outbreaks requiring factors such as weather and mosquito vectors acting together to produce a “perfect storm,” vaccination against the disease is not practiced consistently. This results in increasing the susceptible ruminant population over time. Outbreaks of LSD,

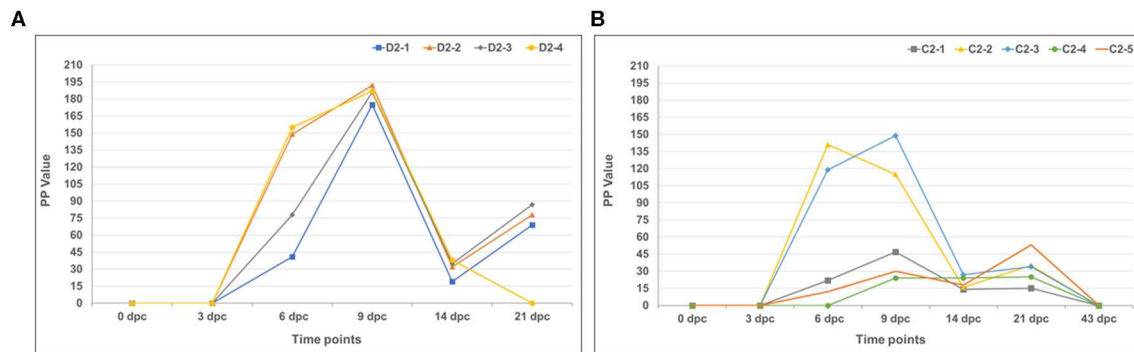


FIGURE 10 | Immunoglobulin M (IgM) host antibody responses demonstrated using a RVFV-specific recombinant nucleoprotein (rNP) capture enzyme-linked immunosorbent assay (ELISA). **(A)** Mock-vaccinated calves and **(B)** LSD-RVF.mf-vaccinated calves.

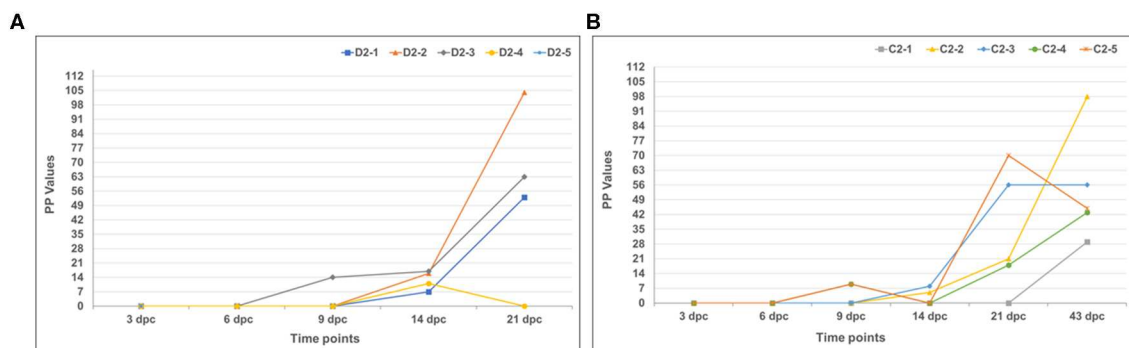


FIGURE 11 | Immunoglobulin G (IgG) host antibody responses demonstrated using a RVFV-specific recombinant nucleoprotein (rNP) indirect enzyme-linked immunosorbent assay (ELISA). **(A)** Mock-vaccinated calves and **(B)** LSD-RVF.mf-vaccinated calves.

by contrast, generally occur annually, therefore vaccination is practiced more regularly. For many smallholder farmers across Africa, the high cost of vaccines, lack of education on their many benefits, and limited access and available infrastructure to enable their proper storage at low temperatures, are some factors which impede their regular use. In addition, not all governments provide aid to support vaccination programs. The development and use of multivalent and combination vaccines in the medical and veterinary field have offered a partial solution to these problems. Lumpy skin disease virus has been investigated for its ability to perform as a stable and versatile vaccine delivery platform, as has been the case for other poxviruses such as vaccinia virus and canarypox virus, but with the added advantage of providing additional protection against LSD – and, sheep- and goat-pox (12). Since vaccination for LSD is routinely practiced, having a bivalent- or multivalent-vectored vaccine based on LSDV has advantages compared to conventional vaccination using multiple vaccines.

In the past, we developed and evaluated recombinant LSDV-vectored vaccines, including one expressing the protective glycoproteins (GPs) of RVFV, with retention of the selectable markers used for their generation and selection to homogeneity (15). However, due to environmental concerns regarding release

of products containing genes that confer resistance to drugs (such as the *E.coli* gpt gene), we have since redesigned our transfer vector plasmid to enable a second round of recombination after removal of selection pressure (called transient dominant selection) to remove the markers, resulting in a marker-free recombinant. This was performed in our laboratory for a new recombinant vaccine construct expressing the protective GPs of RVFV, called LSD-RVF.mf, as described in this paper.

This study demonstrates that the LSD-RVF.mf vaccine was safe in cattle. It was not detected in the blood following vaccination, indicating that the vaccine is attenuated, unlike virulent LSDV, which can be detected in blood following infection. The LSD-RVF.mf vaccine elicited protective immunity against both LSDV and RVFV challenge. It is likely that both antibody (humoral) and cell-mediated immunity elicited by the LSD-RVF.mf vaccine are important for protection against both diseases. Low levels of neutralizing antibodies specific for LSDV and RVFV were elicited by the LSD-RVF.mf vaccine prior to challenge. Protection against RVFV challenge in the vaccinated group of animals appears to be associated with neutralizing antibody development to the virus.

Cellular immunity was measured using the same parameters as described in a related study (22). ELISPOT assays were used

to determine the number of IFN- γ -producing cells in calves and the results demonstrated that LSD-RVFV.mf-vaccinated animals had higher numbers of IFN- γ -producing cells compared to calves in the control groups following challenge with LSDV and stimulation with LSDV antigen. The increased number of IFN- γ -producing cells correlated with increased CD4+ and CD8+ memory T cells in PBMCs of the vaccinated animals post-challenge. Production of IFN- γ by memory T cells is a key element required for the control of virus infections (33). It was also demonstrated that the PBMCs of vaccinated calves produced IFN- γ 7 dpi when stimulated with RVFV antigen and a RVFV-specific memory CD8+ T cell response was detected at 14 dpi. The production of IFN- γ may be considered as a key factor for survival in RVFV infection. It has been shown that IFN- γ treatment can reduce RVF infection in rhesus monkeys (34) and in other monkey models early production of IFN- γ and CD8+ T cells were detected in survivors (35). The source of IFN- γ at 7 dpi is most likely NK cells (36), but CD8+ T cells cannot be ruled out, since elevated levels of both were detected in the PBMCs of calf C2-4 at 7 dpi.

This study demonstrates that the currently used commercial OBP LSD vaccine can be used as a vaccine vector in cattle for RVFV protective antigens. This vaccine has DIVA capability for RVF enabling improved surveillance of RVF. This vaccine also has the potential to improve vaccination coverage for RVF, as the current vaccines are not used consistently, due mainly to cost factors. Further work is required for registration of this vaccine to allow it to be used in the field, although it has undergone small-scale batch formulation and the process parameters at a small scale have been determined by Onderstepoort Biological Products (OBP Ltd) in South Africa. The vaccine is safe to use and protects cattle against LSD and RVF, as demonstrated in this pilot phase cattle trial. However, recent updates in regulatory requirements for the biological license application for new veterinary vaccines necessitates additional regulatory process undertakings toward product registration for a marketing license in terms of the Medicines and Related Substances Act, 1965 (Act No. 101 of 1965), as gazetted by the government of South Africa. This will require vaccine pilot batch production, including master seed characterization and clinical validation of this vaccine batch in cattle for safety and efficacy. Plans are in progress to enable these additional studies.

REFERENCES

1. Babiuk S, Bowden TR, Boyle DB, Wallace DB, Kitching RP. Capripoxviruses: an emerging worldwide threat to sheep, goats and cattle. *Transbound Emerg Dis.* (2008) 55:263–72. doi: 10.1111/j.1865-1682.2008.01043.x
2. Tuppurainen ESM, Venter EH, Shisler JL, Gari G, Mekonnen GA, Juleff N, et al. Review: capripoxvirus diseases: current status and opportunities for control. *Transbound Emerg Dis.* (2017) 64:729–45. doi: 10.1111/tbed.12444
3. Machado G, Korennoy F, Alvarez J, Picasso-Risso C, Perez A, Van der Waal K. Mapping changes in the spatiotemporal distribution of lumpy skin disease virus. *Transbound Emerg Dis.* (2019) 66:2045–57. doi: 10.1111/tbed.13253
4. Tuppurainen ESM, Antoniou SE, Tsiamadis E, Topkaridou M, Labus T, Debeljak Z, et al. Field observations and experiences gained from the

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by ARC-OVI Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

DW constructed the insertion vector, generated and selected the recombinant virus construct to homogeneity (with assistance of Mr. Maanda Phosiwa), was directly involved in most of the cattle trial (and, data analysis) and co-wrote this manuscript. AM and PK co-conceptualized, planned and executed the trial, and analyzed data and writing, review and editing of the original draft manuscript. AP planned and helped analyse the CMI work, as did NT and TN, both of whom also performed the work. AP, NT, and TN contributed to writing the manuscript. SB assisted with the cattle trial planning and helped analyse the resulting data and co-wrote the manuscript. LN and NM designed and executed vaccine formulation experiments, manufactured vaccine, and challenge strains used in the cattle trial. NM contributed to the design of the cattle trial.

FUNDING

This work was generously supported by the GALVmed organization (recombinant virus construction, selection, and characterization) (under grant no. 710/CAP/07/003) and the Canadian International Development Research Centre in collaboration with Global Affairs Canada, within the Canadian International Food Security Research Fund (CIFSRF) Grant no. 107848-002 (cattle trial). IDRC have also kindly agreed to pay the APF.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2020.00256/full#supplementary-material>

implementation of control measures against lumpy skin disease in South-East Europe between 2015 and 2017. *Prev Vet Med.* (2018) 18:30548–30548. doi: 10.1016/j.prevetmed.2018.12.006

5. Perry BD, Randolph TF, McDermott JJ, Sones KR, Thornton PK. *Investing in Animal Health Research to Alleviate Poverty*. Nairobi: ILRI (2002). p. 68–75.
6. Abudurexiti A, Adkins S, Alioto D, Alkhovsky SV, Avšič Županc T, Ballinger MJ, et al. Taxonomy of the order Bunyavirales. *Arch Virol.* (2019) 164:1949–65. doi: 10.1007/s00705-019-04253-6
7. Daubney R, Hudson JR. Enzootic hepatitis or rift valley fever. *J Path and Bact.* (1931) 34:545. doi: 10.1002/path.1700340418
8. Davies FG. Risk of a Rift Valley fever epidemic at the haj in Mecca, Saudi Arabia. *Rev Sci Tech.* (2006) 25:137–47. doi: 10.20506/rst.25.1.1648

9. Centers for Disease Control and Prevention (CDC). Update: outbreak of rift valley fever—Saudi Arabia, August–November 2000. *Morb Mortal Wkly Rep.* (2000) 49:982–5.
10. Swanepoel R, Coetzee JAW. Rift Valley Fever. In: Coetzee JAW, Thomson GR, Tustin RC, editors. *Infectious Diseases of Livestock with Special Reference to Southern Africa. Volume 1.*, Cape Town: Oxford University Press (1994). p. 688–717.
11. Shoemaker T, Boulianne C, Vincent MJ, Pezzanite L, Al-Qahtani MM, Al-Mazrou Y, et al. Genetic analysis of viruses associated with emergence of Rift Valley Fever virus in Saudi Arabia and Yemen, 2000–1. *Emerg Infect Dis.* (2002) 8:1415–20. doi: 10.3201/eid0812.020195
12. Boshra H, Truong T, Nfon C, Gerdtz V, Tikoo S, Babiuk LA, et al. Capripoxvirus-vectored vaccines against livestock diseases in Africa. *Antiviral Res.* (2013) 98:217–27. doi: 10.1016/j.antiviral.2013.02.016
13. Faburay B, Wilson WC, Gaudreault NN, Davis AS, Shivanna V, Bawa B, et al. A recombinant Rift Valley Fever virus glycoprotein subunit vaccine confers full protection against Rift Valley Fever challenge in sheep. *Sci Rep.* (2016) 6:27719. doi: 10.1038/srep27719
14. Wallace DB, Viljoen GJ. Immune responses to recombinants of the South African vaccine strain of lumpy skin disease virus generated by using thymidine kinase gene insertion. *Vaccine.* (2005) 23:3061–7. doi: 10.1016/j.vaccine.2004.10.006
15. Wallace DB, Ellis CE, Espach A, Smith SJ, Greyling RR, Viljoen GJ. Protective immune responses induced by different recombinant vaccines to Rift Valley fever. *Vaccine.* (2006) 24:7181–9. doi: 10.1016/j.vaccine.2006.06.041
16. Singh JA, Upshur R, Padayatchi N. XDR-TB in South Africa: no time for denial or complacency. *PLoS Med.* (2007) 4:e50. doi: 10.1371/journal.pmed.0040050
17. Falkner FG, Moss B. Transient dominant selection of recombinant vaccinia viruses. *J Virol.* (1990) 64:3108–11. doi: 10.1128/JVI.64.6.3108-3111.1990
18. Scheiflinger F, Dorner F, Falkner FG. Transient marker stabilisation: a general procedure to construct marker-free recombinant vaccinia virus. *Arch Virol.* (1998) 143:467–74. doi: 10.1007/s007050050303
19. Molla W, de Jong CM, Gari G, Frankena K. Economic impact of LSD and cost effectiveness of vaccination for the control of outbreaks in Ethiopia. *Prev Vet Med.* (2017) 147:100–7. doi: 10.1016/j.prevetmed.2017.09.003
20. Freshney RI. Chapter 9, Disaggregation of the tissue and Primary Culture. In Alan R. Liss, Inc., editor. *Culture of Animal Cells: A Manual of Basic Technique.* New York, NY: Alan R. Liss, Inc., (1987). p. 107–126.
21. Weiss KE. Lumpy Skin Disease virus. *Virology Monographs.* (1968) 3:111–31. doi: 10.1007/978-3-662-39771-8_3
22. Kara PD, Mather AS, Pretorius A, Chetty T, Babiuk S, Wallace DB. Characterisation of putative immunomodulatory gene knockouts of lumpy skin disease virus in cattle towards an improved vaccine. *Vaccine.* (2018) 36:4708–15. doi: 10.1016/j.vaccine.2018.06.017
23. Barnard BJH. Rift Valley fever vaccine - antibody and immune response in cattle to a live and an inactivated vaccine. *J S Afr Vet Assoc.* (1979) 50:155–7.
24. Wallace DB, Weyer J, Nel LH, Viljoen GJ. Improved method for the generation and selection of homogeneous lumpy skin disease virus (SA-Neethling) recombinants. *J Virol Methods.* (2007) 146:52–60. doi: 10.1016/j.jviromet.2007.06.004
25. Wallace DB. An evaluation of the vaccine-vector potential of thymidine kinase-disrupted recombinants of lumpy skin disease virus (South African vaccine). (Philosophiae Doctor thesis), University of Pretoria, Pretoria, Gauteng (2005).
26. Viljoen GJ, Nel LH, Crowther JR (eds.). Disease diagnosis using PCR specific procedures for important veterinary pathogens. In: *Molecular Diagnostic PCR Handbook.* Dordrecht: Springer (2005). p. 120–201.
27. Drosten C, Götting S, Schilling S, Asper M, Panning M, Schmitz H, et al. Rapid detection and quantification of RNA of Ebola and Marburg Viruses, Lassa virus, Crimean-Congo haemorrhagic fever virus, Rift valley fever virus, Dengue virus, and Yellow Fever Virus by Real-time Reverse transcription-PCR. *J Clin Microbiol.* (2002) 40:2323–30. doi: 10.1128/JCM.40.7.2323-2330.2002
28. Tuppurainen ES, Venter EH, Coetzee JA. The detection of lumpy skin disease virus in samples of experimentally infected cattle using different diagnostic techniques. *Onderstepoort J Vet Res.* (2005) 72:153–64. doi: 10.4102/ojvr.v72i2.213
29. Kitching RP. Chapter 2.4.14 on “Lumpy Skin Disease”. In: OIE Biological Standards Commission, International Committee of the OIE, editors. *World Organisation for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th edition vol. 2.* Paris: OIE (2008).
30. Gerdes TGH. Chapter 2.1.14 on “Rift Valley fever”. In: OIE Biological Standards Commission, International Committee of the OIE, editors. *World Organisation for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th edition vol. 1.* Paris: OIE (2008).
31. Williams R, Ellis CE, Smith SJ, Potgieter CA, Wallace D, Mareledwane VE, et al. Validation of an IgM antibody capture ELISA based on a recombinant nucleoprotein for identification of domestic ruminants infected with Rift Valley fever virus. *J Virol Methods.* (2011) 177:140–6. doi: 10.1016/j.jviromet.2011.07.011
32. van Vuren PJ, Potgieter AC, Paweska JT, van Dijk AA. Preparation and evaluation of a recombinant Rift Valley fever virus N protein for the detection of IgG and IgM antibodies in humans and animals by indirect ELISA. *J Virol Methods.* (2007) 140:106–14. doi: 10.1016/j.jviromet.2006.11.005
33. Gray JJ, Westerhof LM, MacLeod MKL. The roles of resident, central and effector memory CD4 T-cells in protective immunity following infection or vaccination. *Immunol.* (2018) 154:574–81. doi: 10.1111/imm.12929
34. Morrill JC, Czarniecki CW, Peters CJ. Recombinant human interferon-gamma modulates Rift Valley fever virus infection in the rhesus monkey. *J Interferon Res.* (1991) 11:297–304. doi: 10.1089/jir.1991.11.297
35. Wonderlich ER, Caroline AL, McMillen CM, Walters AW, Reed DS, Barratt-Boyes SM, et al. Peripheral blood biomarkers of disease outcome in a monkey model of Rift Valley Fever encephalitis. *J Virol.* (2018) 92:e01662–e01617. doi: 10.1128/JVI.01662-17
36. Lathan R, Simon-Chazottes D, Jouvion G, Godon O, Malissen M, Flamand M, et al. Innate Immune Basis for Rift Valley Fever Susceptibility in Mouse Models. *Sci Rep.* (2017) 7:7096. doi: 10.1038/s41598-017-07543-8

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Wallace, Mather, Kara, Naicker, Mokoena, Pretorius, Nefefe, Thema and Babiuk. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Livestock Challenge Models of Rift Valley Fever for Agricultural Vaccine Testing

Andrea Louise Kroeker^{1†}, Shawn Babiuk^{1,2}, Bradley S. Pickering^{1,3}, Juergen A. Richt⁴ and William C. Wilson⁵

¹ Canadian Food Inspection Agency, National Centre for Foreign Animal Disease, Winnipeg, MB, Canada, ² Department of Immunology, University of Manitoba, Winnipeg, MB, Canada, ³ Department of Medical Microbiology, University of Manitoba, Winnipeg, MB, Canada, ⁴ Center of Excellence for Emerging and Zoonotic Animal Diseases (CEEZAD), Manhattan, KS, United States, ⁵ USDA, Arthropod-Borne Animal Diseases Research Unit (ABADRU), Manhattan, KS, United States

OPEN ACCESS

Edited by:

Armanda Bastos,
University of Pretoria, South Africa

Reviewed by:

Jeroen Kortekaas,
Central Veterinary Institute,
Netherlands
Gema Lorenzo,
Instituto Nacional de Investigación y
Tecnología Agraria y Alimentaria
(INIA), Spain

*Correspondence:

Andrea Louise Kroeker
Andrea.Kroeker@usask.ca

†Present address:

Andrea Louise Kroeker,
VIDO-InterVac, University of
Saskatchewan, Saskatoon,
SK, Canada

Specialty section:

This article was submitted to
Veterinary Infectious Diseases,
a section of the journal
Frontiers in Veterinary Science

Received: 25 September 2019

Accepted: 07 April 2020

Published: 27 May 2020

Citation:

Kroeker AL, Babiuk S, Pickering BS,
Richt JA and Wilson WC (2020)
Livestock Challenge Models of Rift
Valley Fever for Agricultural Vaccine
Testing. *Front. Vet. Sci.* 7:238.
doi: 10.3389/fvets.2020.00238

Since the discovery of Rift Valley Fever virus (RVFV) in Kenya in 1930, the virus has become widespread throughout most of Africa and is characterized by sporadic outbreaks. A mosquito-borne pathogen, RVFV is poised to move beyond the African continent and the Middle East and emerge in Europe and Asia. There is a risk that RVFV could also appear in the Americas, similar to the West Nile virus. In light of this potential threat, multiple studies have been undertaken to establish international surveillance programs and diagnostic tools, develop models of transmission dynamics and risk factors for infection, and to develop a variety of vaccines as countermeasures. Furthermore, considerable efforts to establish reliable challenge models of Rift Valley fever virus have been made and platforms for testing potential vaccines and therapeutics in target species have been established. This review emphasizes the progress and insights from a North American perspective to establish challenge models in target livestock such as cattle, sheep, and goats in comparisons to other researchers' reports. A brief summary of the potential role of wildlife, such as buffalo and white-tailed deer as reservoir species will also be discussed.

Keywords: Rift Valley Fever virus, RVFV, vaccine, cattle, sheep, goat, deer, ruminants

BACKGROUND AND INTRODUCTION

In the last 20 years there has been a re-emergence of various well-known arboviral diseases, many of which are zoonotic in nature, such as West Nile, Japanese encephalitis, Rift Valley fever, Venezuelan equine encephalitis, and Eastern equine encephalitis (1). Among these, Rift Valley fever (RVF) is considered a significant threat to animal and public health, economy, and food (2–4). Rift Valley fever was first reported in Kenya in 1930 (5), and has since created sporadic outbreaks in cattle and small ruminants with associated zoonotic spread to humans in sub-Saharan Africa. The disease first came to global attention during an outbreak in Egypt in 1977–78, involving at least 200,000 human infections (6). It was during the Egyptian outbreak when ocular degeneration was first associated with RVFV infections in humans (7). Global concerns were raised when RVF virus (RVFV) spread to Saudi Arabia and Yemen in 2000 (8) as well as during an outbreak in East Africa (Kenya, Tanzania, Somalia, and the Sudan) in 2006/7 (9–13). Since then there have been outbreaks in Madagascar (3), Mauritania 2010 (14), Namibia 2010 (15), South Africa 2008–2011 (16), Mozambique 2014 (17), Republic of Niger 2016 (18), and Kenya 2018 (WHO). The presence of

the disease, especially during outbreaks, has significant socio-economic impact in endemic regions (19, 20). This along with the potential risk of RVFV importation into Europe and the U.S. (4) as well as its potential use as a biological weapon (21, 22), has led to intensified research on developing mitigation strategies. Developing such strategies requires a detailed understanding of the mosquito-transmitted virus that causes RVF disease.

RVFV is endemic in Sub-Saharan Africa and continues to cause sporadic outbreaks that are of veterinary and public health concern. Although RVFV is primarily a disease of domestic and wild animals, there can be spillover to humans during outbreaks than can lead in rare cases to lethal hemorrhagic disease in humans. RVFV outbreaks occur in 7–10 year cycles presumably as the number of seropositive animals in the population decrease, and/or ideal weather conditions for the mosquito vectors are present. It has also been suggested that recently emerged strains of RVFV might be more virulent to humans (23). Thus, RVF is clearly a disease that requires a “One Health” approach to mitigation strategies (24, 25) as it is a potential threat to animal and public health, economy, and food security (4, 25–27).

Outbreaks of RVF occur when conditions are ideal for mosquito expansion and virus transmission. *Aedes mcintoshi* mosquito species are thought to initiate epizootic outbreaks because of their transovarial transmission capability (28). Once the outbreak has been established, it can then be maintained by *Aedes* and other species (e.g., *Culex* and *Mansonia*) which can both replicate and transmit the virus (29). Although this is a well-accepted hypothesis for RVFV maintenance, transovarial transmission has only been demonstrated in one study. Alternatively, the mosquito to animal transmission cycle could be continuous at low levels and only become observed when ideal environmental conditions occur. The importance of understanding the potential role of transovarial transmission in mosquito-borne viruses has been reviewed (30). An increasing number of studies have also identified other species of mosquitoes that are either susceptible to RVFV and/or are capable of transmitting RVFV in the *Anopheles*, *Mansonia*, and other mosquito genera (13, 31). North American species such as *Aedes canadensis*, *Aedes taeniorhynchus*, and *Culex tarsalis* (32–34) and the stable fly species *Stomoxys calcitrans* (33) have also been shown to be capable of transmitting RVFV. The control of mosquitoes involved in RVFV transmission is complex because there are numerous mosquito species present in endemic and non-endemic areas that are capable of virus infection and transmission [reviewed in Linthicum et al. (29)], and continuous low-level transmission of RVFV to domestic and wild animals in endemic areas may also help maintain the virus. Other species that may play a role in RVFV ecology and have been reported to be susceptible to RVFV are mice, rats, shrews, dormice, and bats (35–40). Additional wild animal species that have been investigated include the African buffalo, primates, elephants, rhinoceros, deer, and coyotes (41–45). However, it is difficult to determine the role of susceptible wild animals in maintenance and transmission of RVFV. Based on a risk model, transmission and seroprevalence rates in both domestic and wild animals correlate positively with the risk of zoonotic infection of people (46).

RVFV is in the order *Bunyavirales* (*Phenuiviridae*; genus *Phlebovirus*), with a genome consisting of three negative-sense, single stranded RNA segments; L (large), M (medium), and S (small). The L segment encodes the RNA-dependent RNA polymerase (47). The M segment encodes the precursor protein of two structural glycoproteins, Gn and Gc, which are present on the virus surface. Cleavage of the precursor protein leads to two additional non-structural proteins of 78 kDa (P78 or LGp) and 14 kDa (NSm) in molecular mass (48–51). The Gn and Gc form heterodimers on the virus surface (52) and are involved in attachment of the virus to the host cell (53, 54). The NSm has been shown to inhibit apoptosis but is not essential for virus replication (55). Although also not critical for RVFV virulence, lack of NSm did reduce mortality and increase the number of animals demonstrating neurological disease in subcutaneously infected rats (56) and NSm mutated viruses were attenuated in intraperitoneally infected mice (57). The LGp/P78 protein, which is not associated with RVFV virulence in mice, is packaged into viruses grown in C6/36 (*Aedes albopictus*) insect cells, but not in mammalian cells, and is a major determinant of virus dissemination in mosquitoes (57, 58). Interestingly, additional studies showed that NSm is involved in virus replication and dissemination in *Aedes aegypti* mosquitoes (59, 60). The S segment utilizes an ambisense strategy encoding the nucleocapsid (N) protein in the anti-sense direction and the NSs protein in the sense direction (61). The N protein is the most abundant protein in the virion and plays a key role in transcription and replication and reconstitutes the ribonucleoprotein (RNP) complex together with the vRNA and the L protein (62). The N protein is immuno-dominant and is used as an antigen for diagnostic assays (63). The NSs protein has immunomodulatory functions and acts as interferon-antagonist via the inhibition of host gene transcription (64–66). The NSs protein is produced early during RVFV infection and has also a positive effect on viral replication and RNA transcription (67). The above described studies indicate that both, LGp/P78 and NSm seem important for virus maintenance in mammalian and insect hosts, and that NSs is an important virulence factor. This information led to the development of a NSm and NSs double deleted virus that was shown to be attenuated in rats (68). When used as a vaccine, RVF virus containing NSm and NSs deletions were shown to be safe and non-teratogenic in pregnant sheep as well as protective against the development of viremia and RVF disease (69). These findings are supportive of the mechanistic studies in but since just rodent model systems; however, there have been only a few studies directed at understanding of the molecular basis of RVFV virulence and molecular pathogenesis in target livestock species.

RVFV research conducted in target livestock species has been primarily focused on the development of diagnostics and vaccines. As a result of the 2006/2007 RVF outbreak in Kenya (12, 70), this disease garnered increased attention from global scientific communities. This has led to an increased focus on identifying North American mosquito species that are capable of being infected and able to transmit RVFV, as well as improving RVFV diagnostic tools (71–75), developing better risk models for RVF (76–79) and evaluating these models using seroprevalence data (46). Several studies have also published data demonstrating

TABLE 1 | Rates of mortality in animals during recent RVFV outbreaks.

	Cattle	Sheep	Goats	Camels	Buffaloes	Wild species
Niger 2016 (18)				X		
Mozambique 2014 (17)	x	8 abortions; 7 neonatal deaths	42 abortions; 5 neonatal deaths	x	x	X
Senegal 2013 (93)	0% mortality	x	19–33% mortality	x	x	37.5% mortality
South Africa 2010–11* (94)	61% mortality	62% mortality	55% mortality	100% mortality	100% mortality	100% mortality
Kenya 2006–07 (12)		14% mortality		X	x	X
Mauritania 2003 (9)	x	70% abortions		X	x	X
Mauritania 1998 (95)	4.6% abortions	9.7% abortions	47.4% abortions	20.6% abortions	x	X

*The field-study sites were selected for farms that had high numbers of deaths (96).

%mortality, deaths/confirmed cases; x, no data available.

that RVFV circulates in endemic countries during inter-epidemic periods (43, 80, 81).

Currently, there are modified live virus (MLV) or attenuated (82, 83) and inactivated/killed vaccines licensed for veterinary use in RVFV endemic countries and one attenuated MLV vaccine with a conditional license in the United States (USDA-APHIS, CVB Notice 13-12). Since various RVFV vaccine approaches were recently reviewed in several publications (25, 84–86), they will be only discussed briefly here. Other recent reviews have focused on the molecular biology, reassortment capacity, diagnostics, and vaccines (86, 87). Thus, this review will focus on the development of target livestock infection models.

DEVELOPING RVFV INFECTIONS IN TARGET SPECIES

The animals most susceptible to RVFV consist of ruminants such as sheep, goats and cattle, as well as camels, buffaloes, and humans (88). These animals all produce viremia upon infection with clinical signs that typically range from asymptomatic to moderate and high severity to death; in addition, pregnant animals suffer from high rates of abortion (17, 89–91). Although the documentation of confirmed RVFV cases, deaths and abortions in animals has been sparse, recent studies with confirmed RVFV antibody status have provided estimates of animal mortality rates during different outbreaks [(92); see Table 1].

Early work on RVFV livestock infections was done in South Africa (89, 90, 97), and a few RVFV experimental infections of livestock were conducted in the 1970/80s at the Plum Island Animal Disease Center (USDA) (98). These early studies included safety and efficacy trials for both inactivated (98) and a mutagen-attenuated MLV vaccine (99, 100). No RVFV infection studies using livestock had been conducted on the mainland of North America since these earlier studies. In 2006, the Canadian Food Inspection Agency (CFIA) and the United States Department of Agriculture (USDA) were tasked with developing target animal infection models to develop and evaluate diagnostic and control strategies. The procedures and models developed through this cooperative research were then transferred to the

Biosecurity Research Institute (BRI) at Kansas State University (KSU) through collaboration with the Center of Excellence for Emerging and Zoonotic Animal Diseases (CEEZAD) at KSU. The results of this ongoing three-way collaboration are reviewed here and discussed within context of the literature.

There are several traits desirable in a veterinary RVFV vaccine, but at a minimum the vaccine should protect against abortions in pregnant animals and should prevent viremia to avoid transmission. In addition, the vaccine should be safe and efficacious in the most vulnerable target species and groups, that is, in fetal and newborn animals. For example, RVFV can be transmitted vertically from pregnant ewes to their fetus (101). Newborn lambs also remain highly susceptible to RVFV after they have been weaned as they lose any protection from maternally-derived antibodies. As weaning can occur any time between 3 weeks and 4 months, the earlier a vaccine can be given, the better. Therefore, in developing challenge models, these are important aspects of the disease that should be considered. For example, previous studies have utilized pregnant ewe models to evaluate whether experimental attenuated or MLV vaccines cross the placenta (102) and are safe to administer during pregnancy. Although this model is highly susceptible to RVFV and is useful for evaluating vaccine safety, using pregnant animals in high containment animal rooms (i.e., BSL-3Ag) is logistically challenging. Therefore, alternative models have also been developed and will be discussed.

SHEEP MODEL DEVELOPMENT

Several challenge models have been developed in 2–3 month old lambs that display significant pathology that is typical of RVFV. For example, Kortekaas et al. has utilized intravenous (103–105) and intraperitoneal inoculation (106) of the recombinant 35/74 RVFV isolate grown in mammalian cells in Texel, Romane, and other European breeds at 10^5 TCID₅₀. The challenge controls in these studies developed peracute clinical signs and fever, viremia for 4–6 days with peak titers of 10^5 – 10^6 TCID₅₀, and virus was detected in the liver. Clinical markers also indicated elevated levels of plasma alkaline phosphatase and alanine transferase (hepatic dysfunction) as well as blood urea nitrogen and

TABLE 2 | Summary of included sheep studies involving RVFV challenge.

Breed	Age	Virus isolate	Route	Infection dose	Cells*	Viremia length	Viremia peak	Organs infected**	Clinical chemistry***	Shedding	Deaths	References
Dorper-Katahdin X	4–5m	SA01	SC	10 ⁶ pfu	I	2d	10 ⁴ pfu/ml	B, L, S, H	BUN	No	N	(113)
Dorper-Katahdin X	4–5m	Ken06	SC	10 ⁶ pfu	I	5d	10 ⁷ pfu/ml	B, L, S, H	AST, BUN	Nasal VI	No	
Polypay	4–5m	Ken06	SC	2 × 10 ⁶ pfu	I	4d	10 ⁷ pfu/ml	L, S	AST, BUN	n.a.	3/5	
Rideau-Arcott	4–6m	ZH501	SC	10 ⁵ pfu	M	3d	10 ⁵ pfu/ml	n.a.	n.a.	n.a.	No	(112)
Rideau-Arcott	4–6m	ZH501	SC	10 ⁵ pfu	I	2d	10 ⁵ pfu/ml	n.a.	n.a.	n.a.	No	
Rideau-Arcott	4–6m	ZH501	SC	10 ⁷ pfu	M	1d	10 ³ pfu/ml	n.a.	n.a.	n.a.	No	
Rideau-Arcott	4–6m	ZH501	SC	10 ⁷ pfu	I	4d	10 ⁵ pfu/ml	n.a.	n.a.	n.a.	No	
Dorper	4–6m	56/74	SC	10 ⁶ pfu	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	No	(115)
Texel-X	2–3m	rec35/74	IV	10 ⁵ TCID ₅₀	M	n.a.	n.a.	L, S	n.a.	n.a.	3/7	(105)
Romane	2–3m	rec35/74	IV	10 ⁵ TCID ₅₀	M	n.a.	10 ⁶ TCID ₅₀	L, S, K, A, Lu, LN	n.a.	n.a.	3/8	(103)
Texel	2–3m	rec35/74	IV	10 ⁵ TCID ₅₀	M	n.a.	n.a.	L, B	n.a.	n.a.	1/8	(104)
European	6 wks	rec35/74	IP	10 ⁵ TCID ₅₀	M	9d	10 ⁵ TCID ₅₀	L	ALP, CK, BUN	n.a.	1/6	(106)
n.a.	2m	56/74	SC	10 ⁶ TCID ₅₀	I	5d	10 ⁶ TCID ₅₀	n.a.	n.a.	n.a.	2/8	(111)
Colmenarena	3m	56/74	SC	10 ⁵ TCID ₅₀	I	4d	10 ⁶ TCID ₅₀	n.a.	BUN, ALB	n.a.	1/5	(110)
Ripollesa	2–3m	56/74	SC	10 ⁵ TCID ₅₀	M	4d	10 ⁶ TCID ₅₀	n.d.	n.a.	Nasal and oral VI	No	(109)
Ripollesa	2–3m	56/74	SC	10 ⁵ TCID ₅₀	M	n.a.	n.a.	K	n.a.	Nasal and oral RNA	No	(107)
Ripollesa	2–3m	252/75	SC	10 ⁵ TCID ₅₀	M	n.a.	n.a.	K	n.a.	Nasal and oral RNA	No	
Ripollesa	2–3m	AN1830	SC	10 ⁵ TCID ₅₀	M	n.a.	n.a.	K	n.a.	Nasal and oral RNA	No	
Ripollesa	2–3m	AR20368	SC	10 ⁵ TCID ₅₀	M	n.a.	n.a.	K	n.a.	Nasal and oral RNA	No	

*M, mammalian cell culture; I, insect cells culture.

**B, brain; L, liver; S, spleen; H, heart; Lu, lung; LN, lymph node; K, kidney; A, adrenal gland.

***ALP, alkaline phosphatase; CK, creatinine kinase; BUN, blood urea nitrogen.

creatinine (renal dysfunction) (106). Pathology varied between different individual animals as is typically seen in studies with ruminants, but heavily affected the liver, and could also include abdominal hemorrhage, pulmonary edema and petechiae in the spleen, heart and lungs (104). Notably, these studies indicated an overall rate of 20% mortality with up to 70% mortality in some studies. Other pathological findings have been found in a study where 2–3 month-old lambs were infected with various field isolates (56/74, 252/75, AN1830, AR20368) and included the development of corneal opacity (107). A study with the Zinga isolate also induced severe clinical signs characterized by hyperactivity, watery and mucoid nasal discharges, projectiles and bloody diarrhea, external hemorrhage and neurological signs (108).

Other groups have developed 2–3 month old challenge models with the 56/74 RVFV isolate in Ripolessa (107, 109), Colmenarena (110) and other (111) sheep breeds using subcutaneous inoculation at 10^5 – 10^6 TCID₅₀. Similarly, these animals also developed clinical signs and fever and viremia for 4–5 days with peak titers of 10^5 – 10^6 TCID₅₀. Interestingly, two of these studies detected oral and nasal shedding of viral RNA between one and 7 days post infection (107, 109) with a few samples also leading to viral isolation (109); one of the uninfected sentinel animals even became seropositive suggesting that horizontal transmission may have occurred. The only other study to report horizontal transmission in sheep is after challenge with the Zagazig strain (98). While the studies by Busquets utilized passage 12 virus stocks grown in mammalian cells and had no deaths, the two studies by Chrun and Lorenzo had ~20% mortality and both used passage 5 mosquito-cell derived viral stocks. Since these three studies were performed independently in different breeds of sheep, it is not possible to directly compare these results; however, it is interesting to note that the age, viral strain, route, and dose were all similar in these studies, leaving breed, source of virus, and virus passage history as the main differences.

Challenge models have also been developed in older lambs at 4–6 months of age. For example, Suffolk and Arcott-Rideau breeds were challenged subcutaneously with 10^5 or 10^7 pfu of the ZH-501 RVFV isolate and compared virus stocks that had been grown in either mammalian Vero or mosquito C6/36 cells (112). While the mosquito-cell grown virus produced a robust and consistent infection, the mammalian-cell produced virus had reduced efficacy with viremia only present on day 1 and lower titers (10^2 pfu/ml serum) (112). In contrast to the acute illness seen in the 2–3 month old lambs, 4–6 month old sheep only produced a mild, self-resolving disease with transient pyrexia during the first week after infection and had no obvious gross pathology at 7 days post infection.

At KSU, 4–6 month-old Dorper-Katahdin and Polypay sheep were inoculated subcutaneously with 10^6 pfu passage-2 mosquito-derived virus stock using either RVFV isolate Kenya-128B-15 (Ken06) or SA01-1322 (SA01) (113). SA01 originated from the Saudi Arabian outbreak in 2001 (8) and Ken06 from the Kenyan outbreak in 2006 (114) which had affected an unusually large number of people and was speculated to possibly be more pathogenic. Both RVFV strains produced detectable viremia

between days 1 and 5, and both strains produced gross pathology and histopathology consistent with RVFV virus infection at between days 3–5 (113). However, the Ken06 group tended to have higher viremia and serum aspartate aminotransferase (AST) levels indicating that liver damage was significantly higher in Ken06 infected lambs as compared to SA1 infected animals. Virus isolation from nasal swabs detected infectious virus in a three out of six animals infected with the Ken06 strain while no shedding was seen in SA01-infected lambs. Histopathology and viral antigen was detectable in a wide variety of organs including the spleen, liver, adrenal gland, and kidney during the first week after infection, although no specific differences were attributed to one isolate compared to the other. In addition, histopathology without antigen staining was detected in the brain, intestine, and the eye at later time points, and infectious virus could also be isolated from several tissues including the spleen and the liver between 3–5 days post challenge (25, 113). Three of five animals inoculated with Ken06 had large necrotic foci in the liver, hemorrhage in the liver and spleen, and pulmonary edema.

A summary of the discussed sheep models can be found in Table 2.

GOAT MODEL DEVELOPMENT

Initial experimental goat infection studies were performed with RVFV strain ZH501 (112) and were intended to establish the dose required for the induction of viremia, the timing of viremia and a comparison of inoculation virus grown in mammalian and insect cells. In this study, viremia occurred very quickly in goats, appearing on the first day post infection after SQ inoculation and lasted 2–5 days. By comparing an inoculation dose of 10^5 pfu and 10^7 pfu of the ZH-501 strain (112) we determined that the higher dose achieved more robust and reliable titers. A second study in Boer goats focused on the characterization of innate and adaptive immune responses in the blood after RVFV infection (116). Flow cytometry indicated that after RVFV infection there was a decline in CD5+ (T cells), CD172+ cells (monocytes and dendritic cells), and CD8+ T cells (cytotoxic T lymphocytes) and an increase in CD21+ cells (B cells). Interestingly, these effects were more pronounced in goats infected with mosquito cell-grown virus compared to goats infected with mammalian cell-grown virus (116). In addition, cytokine profiling in the blood demonstrated an increase of interleukin-12 at 1 dpi, an increase of IFN- γ at 2 dpi, and a steady increase of TNF- α , IL-6 and IL-1 β up until the end of the observation period at 21 dpi (116). A Kenyan RVFV isolate (“Ken-UAP,” Genbank #MH175203.1, MH175204.1, MH175205.1) that had been proposed to be more pathogenic than ZH501 has also been tested in goats (117). Although the Ken-UAP and ZH501 RVFV isolates were not compared directly in the same goat breed, viremia titers after subcutaneous inoculations of each were comparable (112, 117).

Several novel alternative routes of infection have also been explored. For example, numerous manuscripts describing arbovirus infections have demonstrated that mosquito saliva can modulate the pathogenicity of the virus upon infection (118–123). To test the effect of saliva on RVFV infection in

TABLE 3 | Summary of included goat studies involving RVFV challenge.

Breed	Age	Virus isolate	Route	Infection dose	Cells*	Viremia length	Viremia peak	Organs infected**	Clinical chemistry	Shedding	Deaths	References
BoerX		ZH501	SC	10 ⁷ pfu	M	n.a.	n.a.	n.a.	n.a.	n.a.	No	(116)
BoerX		ZH501	SC	10 ⁷ pfu	M	2d	10 ³ pfu/ml	n.a.	n.a.	n.a.	No	(112)
Galla	4–6m	56/74	SC	10 ⁷ pfu	I	n.a.	10 ⁴ pfu/ml	n.a.	n.a.	n.a.	No	(115)
Nubian	4–6m	KenUAP	SC	10 ⁷ pfu	I	3d	10 ³ pfu/ml	M, P, R, Olf, Tri, CB, MB	n.a.	Nasal RNA	No	(117)
Nubian	4–6m	KenUAP	mosSC	10 ⁷ pfu	I	3d	10 ³ pfu/ml	M, P, R, S, L, Olf, Tri, BS, CB, MB, Cer	n.a.	Nasal RNA	No	
Nubian	4–6m	KenUAP	IN	10 ⁷ pfu	I	3d	10 ⁵ pfu/ml	M, P, R, S, Olf, Tri, MB, CB, MB	n.a.	Nasal RNA	No	
LaMancha	4–6m	KenUAP	SC	10 ⁷ pfu	I	2d	10 ³ pfu/ml	No	n.a.	No	No	
LaMancha	4–6m	KenUAP	IN	10 ⁷ pfu	I	2d	10 ³ pfu/ml	No	n.a.	No	No	
LaMancha	4–6m	KenUAP	IN	10 ⁷ pfu	M	2d	10 ³ pfu/ml	No	n.a.	No	No	

*M, mammalian cell culture; I, insect cells culture.

**L, liver; S, spleen; H, heart; Lu, lung; M/P/R, mesenteric/prescapular/retropharyngeal lymph node; Olf, olfactory bulb; Tri, trigeminal ganglion; CB, cerebellum; Cer, cerebrum; MB, midbrain; BS, brainstem.

TABLE 4 | Summary of included cattle studies involving RVFV challenge.

Breed	Age	Virus isolate	Route	Infection dose	Cells*	Viremia length	Viremia peak	Organs infected**	Clinical chemistry***	Shedding	Deaths	References
Holstein	4–6m	56/74	SC	10 ⁷ pfu	I	n.a.	10 ⁵ pfu/ml	n.a.	n.a.	n.a.	No	(115)
Hereford-Angus	4–5m	SA01	SC	2 × 10 ⁶ pfu	I	2d	10 ³ pfu/ml	B, K, S, L	ALP		No	(125)
Hereford-Angus	4–5m	Ken06	SC	2 × 10 ⁶ pfu	I	4d	10 ³ pfu/ml	B, K, S, L	ALP		No	
Holstein	3–6m	KenUAP	ID	10 ⁷ pfu	I	2d	10 ³ pfu/ml	S, P, R, Tu	ALP, ALB	No	No	(126)
Holstein	3–6m	KenUAP	IN	10 ⁷ pfu	I	4d	10 ⁶ pfu/ml	M, R, S, L, K, Lu, Tu, Tr, I, H, BS, MB, CB, CSF	ALP, ALB, BUN	No	No	
Holstein	3–6m	KenUAP	SC-ID-IN	v	I	1d	10 ² pfu/ml	L, Tu, Olf, Tri	ALP, ALB	Nasal RNA	No	

*M, mammalian cell culture; I, insect cells culture.

**B, brain; L, liver; S, spleen; H, heart; Lu, lung; R/P/M, retropharyngeal/prescapular/mesenteric lymph node; K, kidney; Tu, turbinate; Tr, trachea; BS, brainstem; MB, midbrain; CB, cerebellum; CSF, cerebral spinal fluid; Olf, olfactory bulb; Tri, trigeminal ganglion; I, ileum.

***ALP, alkaline phosphatase; ALB, albumin; BUN, blood urea nitrogen.

goats, we mimicked a methodology developed by Le Coupanec et al. (119) in mice; first, we allowed naïve mosquitoes to feed at a shaved site on the goats' skin and second, we then injected a known amount of virus subcutaneously (SQ) into the same area. Although the "mosquito-SQ" infection did not result in significant differences in viremia or antibody titers when compared to the "only SQ" infection, we noted that the "mosquito SQ" group retained higher levels of viral RNA in tissues at 28 days post challenge (117). Interestingly, viremia is delayed by 1 day when the virus is inoculated intranasally, suggesting that it has a longer transition (i.e., 48 h) route to travel to the bloodstream (117). Seroconversion kinetics are similar to that in sheep and cattle, occurring at 4–5 days post infection, and producing robust antibody titers at 21–28 days post infection. The tissues that are infected by RVFV may differ with breed, route of inoculation, and RVFV strain. However, spleen, liver, and lymph nodes are consistently positive and reliable targets for RVF diagnosis (112, 117). Other tissues that may be infected by RVFV include a variety of CNS regions such as the olfactory bulb, the trigeminal nerve, the cerebellum, the midbrain and the brainstem. The development of clinical signs varied from asymptomatic to mild and most commonly consisted of fever and diarrhea. To assist in identifying and quantifying other more subtle signs of disease, a RVFV clinical scoring sheet was developed for ruminants (see **Supplementary Table 1**). Using the clinical scoring sheet, subcutaneous infection of Nubian goats demonstrated slightly higher clinical scores than intranasal infection (**Supplementary Figures 2A,C**). In contrast, LaMancha goats had a higher clinical score after intranasal infection when compared to a subcutaneous infection, and a higher clinical score when infected with mosquito cell grown virus compared to mammalian cell grown virus (**Supplementary Figures 2E,F**). Different clinical outcomes after RVFV infection are also seen amongst different experimental groups with Boer goats remaining almost asymptomatic with mild clinical signs with ZH501, whereas Nubian and LaMancha goats exhibited clear clinical signs with Ken06 (**Supplementary Figures 2A–I**).

A few other goat breeds have been successfully used for RVFV model development or RVFV vaccine testing including the Galla (115) and Saanen (124) goat breed. Both experiments produced viremia but no clinical signs.

A summary of the discussed goat models can be found in **Table 3**.

CATTLE MODEL DEVELOPMENT

At KSU, an initial cattle model was established using an Angus or Hereford cross (125), which are commonly bred in North America or Europe for beef production and could be sourced from local farms. Similar in design to our study with sheep, a subcutaneous injection of mosquito-cell grown Kenya-128B-15 (Ken06) or SA01-1322 (SA01) at a titer of 10^6 pfu was inoculated (125). There was variation in the responses to RVFV infection. Most of the infected animals had detectable viremia at least 1 day post infection (4 of 5), but some were asymptomatic, some were febrile and one animal died of infection. There was detectable

virus in nasal swabs during the peak of viremia but no evidence of contact transmission to the contact control animals (125).

In an effort to increase the reliability of the infection in cattle, a second study was undertaken in Holstein calves in which we used three different routes of infections, including intradermal, intranasal and a combination of subcutaneous, intradermal, and intranasal. Despite an adherence to subcutaneous RVFV infections in a majority of manuscripts, we tested whether an intradermal challenge model could result in an enhanced clinical course of RVFV infection. Our results indicated that the degree of viremia was similar to that of a subcutaneous infection, although far fewer tissues tested positive for virus presence in the intradermal model. After intradermal inoculation, infectious virus was only found in turbinates, prescapular lymph nodes, and retropharyngeal lymph nodes (126). Although ruminants are not known to become infected intranasally in the wild, they are quite susceptible to intranasal infection. Intranasal inoculation of cattle led to high titers of viremia with peak titers of 6×10^5 pfu/ml blood and produced infectious virus in a variety of tissues including spleen, liver, kidney, lymph nodes, heart, thyroid, turbinates, and cerebellum (126). We speculated that during intranasal and subcutaneous/intradermal RVFV inoculations, the virus may follow different pathways to reach the bloodstream (117). This led us to hypothesize that combining these three routes could produce an additive effect and increase viremia. However, when cattle were infected using the intranasal, intradermal, and subcutaneous inoculation routes at the same time, this method produced less viremia than using each route individually. All three routes of infection all produced viremia in all animals as well as mild but observable clinical signs such as fever, a depressed disposition, and a lessened appetite in some animals (**Supplementary Figure 1**). Viral RNA was detected in nasal swabs but no infectious virus was present (126).

In a third study, Warimwe et al. challenged 4–6 month-old Holstein-Friesian cattle with 10^7 pfu RVFV 56/74IN subcutaneously. Similar to the experiments performed at NCFAD and KSU, control cattle developed fever and viremia, with peak viremia levels of 10^5 relative pfu (115).

A summary of the discussed cattle models can be found in **Table 4**.

DEER

The role of wildlife as maintenance hosts has been and continues to be a concern in endemic regions in Africa and the Arabian Peninsula (43, 46), and is also of critical significance if the RVFV emerges in other, previously non-endemic regions. To predict the potential of North American wildlife to act as RVFV maintenance hosts, a panel of available wildlife-based cell lines were assessed for RVFV susceptibility (42). Cells derived from white-tailed deer (WTD, *Odocoileus virginianus*), an important wildlife species in North America, were found to be susceptible to RVFV infection. The abundance and wide distribution of WTD in North America and their known susceptibility to other vector-borne diseases is a serious concern (127–129). Risk models for RVFV have also addressed the issue of the potential role that

WTD could play if RVFV were introduced to North America (130–133). To address this concern, a group of young, farm-reared WTD were experimentally infected with 10^6 pfu of the Ken06 RVFV strain at the KSU BSL-3Ag facility using specially designed and constructed pens. Surprisingly, WTD were found to be highly susceptible to RVFV infection with lethality in two of the four animals after subcutaneous inoculation. A sentinel contact control animal, which was co-housed with the principally infected deer also became RVFV infected and had to be humanely euthanized due to severe clinical signs. All dead/euthanized animals had severe clinical signs including bloody diarrhea, which most likely caused the transmission of the virus via the oronasal route to the uninfected contact control animal (134).

OVERVIEW ON FACTORS INFLUENCING RVFV INFECTION IN RUMINANTS

- (a) Age: The age of the animal is arguably one of the most important parameters to consider in a RVFV challenge model. All young ruminants (<3 months) are highly susceptible to RVFV infection and typically succumb to acute liver failure (89, 135–137). Recent studies at KSU and NCFAD have opted to develop challenge models in animals old enough to be weaned (4–6 months) due to the logistics of working in high containment (112, 113, 117, 125, 126). However, numerous vaccine studies have also successfully used younger animals at 2–3 months of age (104) as well as pregnant ewes to induce protective immunity (138).
- (b) Isolate and passage history: Experiments at CFIA used a low passage ZH-501 strain kindly provided by Stuart Nichol, Centers for Disease Control, Atlanta, GA as well as a human isolate from the Kenya 2006/7 outbreak provided by Health Canada. At KSU, a mosquito isolate (SA01-1322) from the Saudi Arabia outbreak in 2001 was provided by Barry Miller CDC Fort Collins, CO through Richard Bowen Colorado state university and the Kenya 2006 strain was also a mosquito isolate (13); both isolates were propagated twice in Vero cells and twice in on mosquito C6/36 cells (8).

South African researchers have used two other strains of RVFV in a vaccine efficacy trial (82). The first RVFV strain was Buffalo/99/MB/CER, isolated from an aborted fetus from an outbreak in 1998, and the second strain was the reference strain RVF 35-74, isolated from a sick sheep from an outbreak in 1974. Both virus strains were isolated using intra-cranial inoculation of mice plus one passage in BHK cells. Subcutaneous infection of sheep with 10^6 pfu of the Zimbabwean strain of RVF produced viremia by 4 days post-infection (dpi) and caused transient fever, viraemia, leucopaenia, relative thrombocytopaenia, haemoconcentration and raised serum enzyme levels that indicated the development of necrotic hepatitis and virulence (139).

In another study evaluating an adenovirus-based vaccine in Kenya, researchers used the RVF 56/74IN strain propagated in C6/36 cells and purified before challenge. This virus strain caused clinical disease and viremia following subcutaneous inoculation of a rather high dose of 10^7 pfu per animal (115). In addition, a variety of field isolates have been compared in sheep including RVFV strains 56/74, 252/75, AN-1830 and AR-20368 (107), 1678/78 and Lunyo (139) and Zinga (108).
- (c) Cell line: Perhaps one of the most intriguing aspects of arbovirus infections is that the virus's pathogenicity can be changed depending on whether the inoculum is produced in a mammalian or insect host cell. This phenomenon was first characterized *in vitro* for alphaviruses (145) and then also for RVFV (146); importantly, we were able to demonstrate that these source effects also apply to RVFV infections *in vivo* (112). In addition, we could show that RVFV which is grown in mosquito cells incorporates the viral P78 protein into its virions, i.e., P78 is a structural protein of RVF viruses produced in insect cells. In contrast, P78 is not found in the virion when the virus is grown in mammalian cells (58). We proposed that the p78 present in virions of mosquito-grown viruses may function as a type I interferon antagonist, which may allow for a productive infection of initial target cells (58), as was shown earlier for alphaviruses (145).
- (d) Route of inoculation: In addition to the experimental infections performed at NCFAD and KSU, other studies have shown that viremia can be induced by a wide variety of different routes including intramuscular (IM), intravenous (IV), intraperitoneal (IP), intracerebral (IC), subcutaneous (SQ), conjunctival, and oral inoculations (112, 137, 147–149). Subcutaneous injection consistently produced clinical responses and is easy to administer under BSL-3Ag conditions thus is a common method of administration (113, 125).

FUTURE DIRECTIONS AND UNANSWERED QUESTIONS

In the field, the infecting dose of RVFV is widely variable ranging from a single mosquito bite which may contain a low level of infectious virions to animals being fed on repeatedly by numerous RVFV-infected mosquitoes, potentially resulting in

inoculation with high levels of infectious virions. Understanding the minimal infectious dose required for infection in ruminants and humans is currently an area requiring further investigation. In addition, it is not well-understood what the effect of mosquito saliva has on *in vivo* RVFV infection or pathogenesis, especially in ruminants. In containment and field studies, inoculations such as subcutaneous injections are used to mimic a mosquito bite; however, the differences between a natural infection and a subcutaneous (or intradermal) injection are not well-characterized.

Moreover, it has recently been shown that goats (117) and cattle (126) produce robust viremia when experimentally inoculated through the intranasal route. This is not necessarily unexpected since humans can be infected by inhaling aerosols produced during animal slaughtering of infected livestock (91), but the differences between an intranasal and subcutaneous infection are not well-understood. While intranasal RVFV infection can produce severe encephalitis in rodents (102), NHPs (150) and people, it did not create any neurological disease in goats (117). However, the neurological effects of intranasal infection in cattle are still unknown, as the study was terminated at peak infection at 4 dpi. Therefore, it would be interesting to also characterize the intranasal infection in cattle over a longer period of time.

The transmission of RVFV in livestock in the absence of mosquitoes is also not fully understood and there have been conflicting reports. Although it was demonstrated in sheep that transmission could occur through contact exposure (98, 109), more recent studies have not demonstrated transmission from subcutaneously infected lambs to naïve or immunosuppressed lambs (105). One possible explanation for these conflicting results is that transmission between animals requires a minimum virus dose which likely varies between different virus strains. Alternatively, upper respiratory infections with parainfluenza 3 virus, adenoviruses, reoviruses, infectious bovine rhinotracheitis virus, maedi-visna virus, sheepox virus, goatpox virus, peste des petits ruminants virus or *Mycoplasma spp.* could cause perturbations in the nasal mucosa which could possibly enhance transmission efficiency in the field but are usually absent in laboratory experiments. The potential role of urine in transmission or RVFV has recently been highlighted by a study that isolated RVFV from the urine of an infected person (151), and it is possible that milk may serve as a source of RVFV transmission (152) to animal offspring and human consumers; both of these areas require further investigation. Mosquito transmission has been considered the primary route of exposure for livestock and wildlife, but not for humans. Additional investigations of alternative routes of exposure will provide further insights into the infectability of different RVFV strains and might allow correlation of phenotype with genotype.

The selection of mosquito cell line propagated virus for livestock inoculation studies at KSU was based on the observation of more consistent viremia in our early studies. The importance of the p78 protein in the insect vs mammalian hosts has been reported (57, 58, 60) but mechanistic understanding of how this might affect virulence in the vertebrate host has

not been determined yet. Studies are also needed to examine whether the individual host animal or cell selects for specific genotypes, and how that might affect viral maintenance and virulence in both the vertebrate and invertebrate host. For example, the basis of the increased virulence of RVFV in recent outbreaks is yet unknown. So far, Sanger sequencing of many RVFV isolates has demonstrated a surprisingly high stability of RVFV (140, 142) however, there are no studies on the quasi-species variation of RVFV within a specific virus population either over a period of several days in a target animal or during and after several passages in the same or different animals. Such information would be important to understand the relative fitness and overall replication ability of RVF viruses.

It was recently determined that there is low level transmission during the inter-epidemic period (81); whether this is the only mechanism of viral maintenance is not clear yet. Also, how low level transmission restricts viral evolution is not known. Similarly, there is empirical evidence that various animal breeds have different susceptibilities to RVFV infection. However, well-designed controlled studies are needed to substantiate this observation. If confirmed, this could change husbandry techniques in RVFV endemic areas, and not only improve animal health but also have a significant effect on public health.

CONCLUSION

The renewed interest in RVFV since the 2006/7 outbreak in Kenya has resulted in many advances in our basic knowledge about RVFV replication strategies and molecular pathogenesis in small animal and livestock models. It also has resulted in novel vaccine candidates and novel experimental challenge models as discussed in this review. In addition, novel tools for the detection of viral nucleic acids and antibodies, both for laboratory and point-of need use have been developed. These recent advances in RVFV mitigation strategies will allow a more rapid and effective control of the disease; unfortunately, the availability of these tools in endemic areas is rather limited (84). There are still many questions about the mechanisms and factors affecting viral maintenance and virulence. The models described here provide a good basis for developing studies to investigate these factors. There are still many questions to be addressed in RVF biology and epidemiology as discussed above in this review. We hope that the RVFV livestock models described here in detail will provide a sound basis for the design of studies to investigate these yet unknown questions.

AUTHOR CONTRIBUTIONS

WW, JR, and AK conceived the review topic. AK and WW prepared and wrote the manuscript. JR, SB, and BP critically reviewed the manuscript.

FUNDING

NBAF Transition Funds from the State of Kansas. DHS Grant Award Number: DHS-2010-ST-061-AG0001. USDA-ARS CRIS Project 3020-3200-009-00D.

REFERENCES

- Gubler DJ. The global emergence/resurgence of arboviral diseases as public health problems. *Arch Med Res.* (2002) 33:330–42. doi: 10.1016/S0188-4409(02)00378-8
- Bonnet P, Tibbo M, Workalemahu A, Gau M. *Rift Valley Fever - an Emerging Threat to Livestock Trade and Food Security in the Horn of Africa: a Review.* Ethiopian Society of Animal Production. (2001).
- Chevalier V, Rakotondrafara T, Jourdan M, Heraud JM, Andriamanivo HR, Durand B, et al. An unexpected recurrent transmission of Rift Valley fever virus in cattle in a temperate and mountainous area of Madagascar. *PLoS Negl Trop Dis.* (2011) 5:e1423. doi: 10.1371/journal.pntd.0001423
- Rolin AI, Berrang-Ford L, Kulkarni MA. The risk of Rift Valley fever virus introduction and establishment in the United States and European Union. *Emerg Microbes Infect.* (2013) 2:e81. doi: 10.1038/emi.2013.81
- Daubney R, Hudson JR, Garnham PC. Enzootic hepatitis or rift valley fever. An undescribed virus disease of sheep, cattle and man from east africa. *J Pathol Bacteriol.* (1931) 34:545–79. doi: 10.1002/path.1700340418
- Meegan JM. The Rift Valley fever epizootic in Egypt 1977–78. 1. Description of the epizootic and virological studies. *Trans R Soc Trop Med Hyg.* (1979) 73:618–23. doi: 10.1016/0035-9203(79)90004-X
- Siam AL, Meegan JM, and Gharbawi KF. Rift Valley fever ocular manifestations: observations during the 1977 epidemic in Egypt. *Br J Ophthalmol.* (1980) 64:366–74. doi: 10.1136/bjo.64.5.366
- Miller BR, Godsey MS, Crabtree MB, Savage HM, Al-Mazrao Y, Al-Jeffri MH, et al. Isolation and genetic characterization of Rift Valley fever virus from *Aedes vexans arabiensis*, Kingdom of Saudi Arabia. *Emerg Infect Dis.* (2002) 8:1492–4. doi: 10.3201/eid0812.020194
- Faye O, Diallo M, Diop D, Bezeid OE, Ba H, Niang M, et al. Rift Valley fever outbreak with East-Central African virus lineage in Mauritania, 2003. *Emerg Infect Dis.* (2007) 13:1016–23. doi: 10.3201/eid1307.061487
- Hassan OA, Ahlm C, Sang R, Evander M. The Rift Valley fever outbreak in Sudan. *PLoS Negl Trop Dis.* (2007) 5:e1229. doi: 10.1371/journal.pntd.0001229
- Jost CC, Nzietchueng S, Kihu S, Bett B, Njogu G, Swai ES, et al. Epidemiological assessment of the Rift Valley fever outbreak in Kenya and Tanzania in 2006 and 2007. *Am J Trop Med Hyg.* (2010) 83:65–72. doi: 10.4269/ajtmh.2010.09-0290
- Munyua P, Muriithi RM, Wainwright S, Githinji J, Hightower A, Mutonga D, et al. Rift Valley fever outbreak in livestock in Kenya, 2006–2007. *Am J Trop Med Hyg.* (2010) 83:58–64. doi: 10.4269/ajtmh.2010.09-0292
- Sang R, Kioko E, Lutomia J, Warigia M, Ochieng C, O'Guinn M, et al. Rift Valley fever virus epidemic in Kenya, 2006/2007: the entomologic investigations. *Am J Trop Med Hyg.* (2010) 83:28–37. doi: 10.4269/ajtmh.2010.09-0319
- El Mamy AB, Baba MO, Barry Y, Isselmou K, Dia ML, El Kory MO, et al. Unexpected Rift Valley fever outbreak, northern Mauritania. *Emerg Infect Dis.* (2011) 17:1894–6. doi: 10.3201/eid1710.110397
- Monaco F, Pinoni C, Cosseddu GM, Khaibse S, Calistri P, Molini U, et al. Rift Valley fever in Namibia, 2010. *Emerg Infect Dis.* (2013) 19:2025–7. doi: 10.3201/eid1912.130593
- Archer BN, Weyer J, Paweska J, Nkosi D, Leman P, Tint KS, et al. Outbreak of Rift Valley fever affecting veterinarians and farmers in South Africa, 2008. *S Afr Med J.* (2011) 101:263–6. doi: 10.7196/SAMJ.4544
- Fafetine JM, Coetzee P, Mubemba B, Nhambirre O, Neves L, Coetzer JA, et al. Rift valley fever outbreak in livestock, Mozambique, 2014. *Emerg Infect Dis.* (2016) 22:2165–67. doi: 10.3201/eid2212.160310
- Lagare A, Fall G, Ibrahim A, Ousmane S, Sadio B, Abdoulaye M, et al. First occurrence of Rift Valley fever outbreak in Niger, 2016. *Vet Med Sci.* (2019) 5:70–78. doi: 10.1002/vms3.135
- Andayi F, Charrel RN, Kieffer A, Richet H, Pastorino B, Leparco-Goffart I, et al. A sero-epidemiological study of arboviral fevers in Djibouti, Horn of Africa. *PLoS Negl Trop Dis.* (2014) 8:e3299. doi: 10.1371/journal.pntd.0003299
- Nanyingi MO, Munyua P, Kiama SG, Muchemi GM, Thumbi SM, Bitek AO, et al. A systematic review of Rift Valley Fever epidemiology 1931–2014. *Infect Ecol Epidemiol.* (2015) 5:28024. doi: 10.3402/iee.v5.28024
- Dar O, Hogarth S, and McIntyre S. Tempering the risk: Rift Valley fever and bioterrorism. *Trop Med Int Health.* (2013) 18:1036–41. doi: 10.1111/tmi.12108
- Mandell RB, Flick R. Rift Valley fever virus: an unrecognized emerging threat? *Hum Vaccin.* (2010) 6:597–601. doi: 10.4161/hv.6.7.11761
- Baba M, Masiga DK, Sang R, Villinger J. Has Rift Valley fever virus evolved with increasing severity in human populations in East Africa? *Emerg Microbes Infect.* (2016) 5:e58. doi: 10.1038/emi.2016.57
- Bird BH, Nichol ST. Breaking the chain: Rift Valley fever virus control via livestock vaccination. *Curr Opin Virol.* (2012) 2:315–23. doi: 10.1016/j.coviro.2012.02.017
- Faburay B. The case for a 'one health' approach to combating vector-borne diseases. *Infect Ecol Epidemiol.* (2015) 5:28132. doi: 10.3402/iee.v5.28132
- Chevalier V, Pepin M, Plee L, Lancelot R. Rift Valley fever—a threat for Europe? *Euro Surveill.* (2010) 15:19506. doi: 10.2807/ese.15.10.19506-en
- Kortekaas J. One Health approach to Rift Valley fever vaccine development. *Antiviral Res.* (2014) 106:24–32. doi: 10.1016/j.antiviral.2014.03.008
- Linthicum KJ, Davies FG, Kairo A, Bailey CL. Rift Valley fever virus (family Bunyaviridae, genus Phlebovirus). Isolations from Diptera collected during an inter-epizootic period in Kenya. *J Hyg (Lond).* (1985) 95:197–209. doi: 10.1017/S0022172400062434
- Linthicum KJ, Britch SC, Anyamba A. Rift Valley Fever: an emerging mosquito-borne disease. *Annu Rev Entomol.* (2016) 61:395–415. doi: 10.1146/annurev-ento-010715-023819
- Bergren NA, Kading RC. The Ecological Significance and Implications of Transovarial Transmission among the Vector-Borne Bunyaviruses: a review. *Insects.* (2018) 9:173. doi: 10.3390/insects9040173
- Mwaengo D, Lorenzo G, Iglesias J, Warigia M, Sang R, Bishop RP, et al. Detection and identification of Rift Valley fever virus in mosquito vectors by quantitative real-time PCR. *Virus Res.* (2012) 169:137–43. doi: 10.1016/j.virusres.2012.07.019
- Gargan T. P., 2nd, Clark GG, Dohm DJ, Turell MJ, Bailey CL. Vector potential of selected North American mosquito species for Rift Valley fever virus. *Am J Trop Med Hyg.* (1988) 38:440–6. doi: 10.4269/ajtmh.1988.38.440
- Turell MJ, Dohm DJ, Geden CJ, Hogsette JA, Linthicum KJ. Potential for stable flies and house flies (Diptera: Muscidae) to transmit Rift Valley fever virus. *J Am Mosq Control Assoc.* (2010) 26:445–8. doi: 10.2987/10-6070.1
- Turell MJ, Wilson WC, Bennett K. E. Potential for North American mosquitoes (Diptera: Culicidae) to transmit rift valley fever virus. *J Med Entomol.* (2010) 47:884–9. doi: 10.1093/jmedent/47.5.884
- Balkema-Buschmann A, Rissmann M, Kley N, Ulrich R, Eiden M, and Groschup MH. Productive Propagation of Rift Valley Fever Phlebovirus Vaccine Strain MP-12 in *Rousettus aegyptiacus* Fruit Bats. *Viruses.* (2018) 10:681. doi: 10.3390/v10120681
- Imam IZ, El-Karamany R, Darwish MA. An epidemic of Rift Valley fever in Egypt. 2. Isolation of the virus from animals. *Bull World Health Organ.* (1979) 57:441–3.
- Kading RC, Kityo RM, Mossel EC, Borland EM, Nakayiki T, Nalikka B, et al. Neutralizing antibodies against flaviviruses, Babanki virus, and Rift Valley fever virus in Ugandan bats. *Infect Ecol Epidemiol.* (2018) 8:1439215. doi: 10.1080/2008686.2018.1439215
- Oelofsen MJ, Van der Ryst E. Could bats act as reservoir hosts for Rift Valley fever virus? *Onderstepoort J Vet Res.* (1999) 66:51–4.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2020.00238/full#supplementary-material>

39. Swanepoel R, Blackburn NK, Efstratiou S, Condy JB. Studies on Rift Valley fever in some African murids (Rodentia: Muridae). *J Hyg (Lond)*. (1978) 80:183–96. doi: 10.1017/S0022172400053535
40. Youssef BZ, Donia HA. The potential role of *Rattus rattus* in enzootic cycle of Rift Valley Fever in Egypt. 1-Detection of RVF antibodies in *R. rattus* blood samples by both enzyme linked immuno sorbent assay (ELISA) and immuno-diffusion technique (ID). *J Egypt Public Health Assoc*. (2001) 76:431–41.
41. Beechler BR, Bengis R, Swanepoel R, Paweska JT, Kemp A, van Vuren PJ, et al. Rift valley Fever in Kruger national park: do buffalo play a role in the inter-epidemic circulation of virus? *Transbound Emerg Dis*. (2015) 62:24–32. doi: 10.1111/tbed.12197
42. Gaudreault NN, Indran SV, Bryant PK, Richt JA, Wilson WC. Comparison of Rift Valley fever virus replication in North American livestock and wildlife cell lines. *Front Microbiol*. (2015) 6:664. doi: 10.3389/fmicb.2015.00664
43. Olive MM, Goodman SM, Reynes JM. The role of wild mammals in the maintenance of Rift Valley fever virus. *J Wildl Dis*. (2012) 48:241–66. doi: 10.7589/0090-3558-48.2.241
44. Rostal MK, Liang JE, Zimmermann D, Bengis R, Paweska J, Karesh WB. Rift Valley Fever: does wildlife play a role? *ILAR J*. (2017) 58:359–70. doi: 10.1093/ilar/ilx023
45. Walsh MG, Mor SM. Interspecific network centrality, host range and early-life development are associated with wildlife hosts of Rift Valley fever virus. *Transbound Emerg Dis*. (2018) 65:1568–75. doi: 10.1111/tbed.12903
46. Britch SC, Binpal YS, Ruder MG, Kariithi HM, Linthicum KJ, Anyamba A, et al. Rift Valley fever risk map model and seroprevalence in selected wild ungulates and camels from Kenya. *PLoS ONE*. (2013) 8:e66626. doi: 10.1371/journal.pone.0066626
47. Muller R, Saluzzo JF, Lopez N, Dreier T, Turell M, Smith J, et al. Characterization of clone 13, a naturally attenuated avirulent isolate of Rift Valley fever virus, which is altered in the small segment. *Am J Trop Med Hyg*. (1995) 53:405–11. doi: 10.4269/ajtmh.1995.53.405
48. Collett M. S. Messenger RNA of the M segment RNA of Rift Valley fever virus. *Virology*. (1986) 151:151–6. doi: 10.1016/0042-6822(86)90114-5
49. Kakach LT, Suzich JA, Collett MS. Rift Valley fever virus M segment: phlebovirus expression strategy and protein glycosylation. *Virology*. (1989) 170:505–10. doi: 10.1016/0042-6822(89)90442-X
50. Suzich JA, Collett MS. Rift Valley fever virus M segment: cell-free transcription and translation of virus-complementary RNA. *Virology*. (1988) 164:478–86. doi: 10.1016/0042-6822(88)90562-4
51. Suzich JA, Kakach LT, Collett MS. Expression strategy of a phlebovirus: biogenesis of proteins from the Rift Valley fever virus M segment. *J Virol*. (1990) 64:1549–55. doi: 10.1128/JVI.64.4.1549-1555.1990
52. Huiskonen JT, Overby AK, Weber F, Grunewald K. Electron cryo-microscopy and single-particle averaging of Rift Valley fever virus: evidence for GN-GC glycoprotein heterodimers. *J Virol*. (2009) 83:3762–9. doi: 10.1128/JVI.02483-08
53. Lozach PY, Kuhbacher A, Meier R, Mancini R, Bitto D, Bouloy M, et al. DC-SIGN as a receptor for phleboviruses. *Cell Host Microbe*. (2011) 10:75–88. doi: 10.1016/j.chom.2011.06.007
54. Riblett AM, Blumen VA, Jie LT, Altamura LA, Doms RW, Brummelkamp TR, et al. A haploid genetic screen identifies heparan sulfate proteoglycans supporting Rift Valley Fever Virus infection. *J Virol*. (2016) 90:1414–23. doi: 10.1128/JVI.02055-15
55. Won S, Ikegami T, Peters CJ, Makino S. NSm protein of Rift Valley fever virus suppresses virus-induced apoptosis. *J Virol*. (2007) 81:13335–45. doi: 10.1128/JVI.01238-07
56. Bird BH, Albarino CG, Nichol ST. Rift Valley fever virus lacking NSm proteins retains high virulence *in vivo* and may provide a model of human delayed onset neurologic disease. *Virology*. (2007) 362:10–5. doi: 10.1016/j.virol.2007.01.046
57. Kreher F, Tamietti C, Gommel C, Guillemot L, Ermonval M, Failloux AB, et al. The Rift Valley fever accessory proteins NSm and P78/NSm-GN are distinct determinants of virus propagation in vertebrate and invertebrate hosts. *Emerg Microbes Infect*. (2014) 3:e71. doi: 10.1038/emi.2014.71
58. Weingartl HM, Zhang S, Marszal P, McGreevy A, Burton L, Wilson WC. Rift Valley fever virus incorporates the 78 kDa glycoprotein into virions matured in mosquito C6/36 cells. *PLoS ONE*. (2014) 9:e87385. doi: 10.1371/journal.pone.0087385
59. Crabtree MB, Kent Crockett RJ, Bird BH, Nichol ST, Erickson BR, Biggerstaff BJ, et al. Infection and transmission of Rift Valley fever viruses lacking the NSs and/or NSm genes in mosquitoes: potential role for NSm in mosquito infection. *PLoS Negl Trop Dis*. (2012) 6:e1639. doi: 10.1371/journal.pntd.0001639
60. Kading RC, Crabtree MB, Bird BH, Nichol ST, Erickson BR, Horiuchi K, et al. Deletion of the NSm virulence gene of Rift Valley fever virus inhibits virus replication in and dissemination from the midgut of *Aedes aegypti* mosquitoes. *PLoS Negl Trop Dis*. (2014) 8:e2670. doi: 10.1371/journal.pntd.0002670
61. Vialat P, Billecocq A, Kohl A, and Bouloy M. The S segment of rift valley fever phlebovirus (Bunyaviridae) carries determinants for attenuation and virulence in mice. *J Virol*. (2000) 74:1538–43. doi: 10.1128/JVI.74.3.1538-1543.2000
62. Le May N, Gauliard N, Billecocq A, and Bouloy M. The N terminus of Rift Valley fever virus nucleoprotein is essential for dimerization. *J Virol*. (2005) 79:11974–80. doi: 10.1128/JVI.79.18.11974-11980.2005
63. Jansen van Vuren P, and Paweska JT. Laboratory safe detection of nucleocapsid protein of Rift Valley fever virus in human and animal specimens by a sandwich ELISA. *J Virol Methods*. (2009) 157:15–24. doi: 10.1016/j.jviromet.2008.12.003
64. Billecocq A, Spiegel M, Vialat P, Kohl A, Weber F, Bouloy M, et al. NSs protein of Rift Valley fever virus blocks interferon production by inhibiting host gene transcription. *J Virol*. (2004) 78:9798–9806. doi: 10.1128/JVI.78.18.9798-9806.2004
65. Habjan M, Pichlmair A, Elliott RM, Overby A. K., Glatte T, Gstaiger M, et al. NSs protein of rift valley fever virus induces the specific degradation of the double-stranded RNA-dependent protein kinase. *J Virol*. (2009) 83:4365–75. doi: 10.1128/JVI.02148-08
66. Ikegami T, Narayanan K, Won S, Kamitani W, Peters CJ, Makino S. Dual functions of Rift Valley fever virus NSs protein: inhibition of host mRNA transcription and post-transcriptional downregulation of protein kinase PKR. *Ann N Y Acad Sci*. (2009) 1171(Suppl. 1):E75–85. doi: 10.1111/j.1749-6632.2009.05054.x
67. Ikegami T, Peters CJ, Makino S. Rift valley fever virus nonstructural protein NSs promotes viral RNA replication and transcription in a minigenome system. *J Virol*. (2005) 79:5606–15. doi: 10.1128/JVI.79.9.5606-5615.2005
68. Bird BH, Albarino CG, Hartman AL, Erickson BR, Ksiazek TG, Nichol ST. Rift valley fever virus lacking the NSs and NSm genes is highly attenuated, confers protective immunity from virulent virus challenge, and allows for differential identification of infected and vaccinated animals. *J Virol*. (2008) 82:2681–91. doi: 10.1128/JVI.02501-07
69. Bird BH, Maartens LH, Campbell S, Erasmus BJ, Erickson BR, Dodd KA, et al. Rift Valley fever virus vaccine lacking the NSs and NSm genes is safe, nonteratogenic, and confers protection from viremia, pyrexia, and abortion following challenge in adult and pregnant sheep. *J Virol*. (2011) 85:12901–9. doi: 10.1128/JVI.06046-11
70. Hightower A, Kinkade C, Nguku PM, Anyangu A, Mutonga D, Omolo J, et al. Relationship of climate, geography, and geology to the incidence of Rift Valley fever in Kenya during the 2006–2007 outbreak. *Am J Trop Med Hyg*. (2012) 86:373–80. doi: 10.4269/ajtmh.2012.11-0450
71. Drolet BS, Weingartl HM, Jiang J, Neufeld J, Marszal P, Lindsay R, et al. Development and evaluation of one-step rRT-PCR and immunohistochemical methods for detection of Rift Valley fever virus in biosafety level 2 diagnostic laboratories. *J Virol Methods*. (2012) 179:373–82. doi: 10.1016/j.jviromet.2011.11.025
72. Turell M, Dave K, Mayda M, Parker Z, Coleman R, Dave S, et al. Wicking assay for the rapid detection of Rift Valley fever viral antigens in mosquitoes (Diptera: Culicidae). *J Med Entomol*. (2011) 48:628–33. doi: 10.1603/ME10219
73. van Vuren PJ, Paweska JT. Comparison of enzyme-linked immunosorbent assay-based techniques for the detection of antibody to Rift Valley fever virus in thermochemically inactivated sheep sera. *Vector Borne Zoonotic Dis*. (2010) 10:697–9. doi: 10.1089/vbz.2009.0213
74. Wilson WC, Romito M, Jasperson DC, Weingartl H, Binpal YS, Maluleke MR, et al. Development of a Rift Valley fever real-time RT-PCR assay that

- can detect all three genome segments. *J Virol Methods*. (2013) 193:426–31. doi: 10.1016/j.jviromet.2013.07.006
75. Wilson WC, Weingartl HM, Drolet BS, Dave K, Harpster MH, Johnson P. A, et al. Diagnostic approaches for Rift Valley fever. *Dev Biol (Basel)*. (2013) 135:73–8. doi: 10.1159/000276828
 76. Anyamba A, Linthicum KJ, Small J, Britch SC, Pak ES, et al. Prediction, assessment of the Rift Valley fever activity in East and Southern Africa 2006–2008 and possible vector control strategies. *Am J Trop Med Hyg*. (2010) 83:43–51. doi: 10.4269/ajtmh.2010.09-0289
 77. Gaff HD, Gross LJ. Modeling tick-borne disease: a metapopulation model. *Bull Math Biol*. (2007) 69:265–88. doi: 10.1007/s11538-006-9125-5
 78. Xue L, Cohnstaedt LW, Scott HM, Scoglio C. A hierarchical network approach for modeling Rift Valley fever epidemics with applications in North America. *PLoS ONE*. (2013) 8:e62049. doi: 10.1371/journal.pone.0062049
 79. Xue L, Scott HM, Cohnstaedt LW, Scoglio C. A network-based metapopulation approach to model Rift Valley fever epidemics. *J Theor Biol*. (2012) 306:129–44. doi: 10.1016/j.jtbi.2012.04.029
 80. LaBeaud AD, Ochiai Y, Peters CJ, Muchiri EM, King CH. Spectrum of Rift Valley fever virus transmission in Kenya: insights from three distinct regions. *Am J Trop Med Hyg*. (2007) 76:795–800. doi: 10.4269/ajtmh.2007.76.795
 81. Lichoti JK, Kihara A, Orikio AA, Okutoyi LA, Wauna JO, Tchouassi DP, et al. Detection of rift valley Fever virus interepidemic activity in some hotspot areas of kenya by sentinel animal surveillance, 2009–2012. *Vet Med Int*. (2014) 2014:379010. doi: 10.1155/2014/379010
 82. Dungu B, Louw I, Lubisi AP, Hunter von Teichman BF, Bouloy M. Evaluation of the efficacy and safety of the Rift Valley Fever Clone 13 vaccine in sheep. *Vaccine*. (2010) 28:4581–7. doi: 10.1016/j.vaccine.2010.04.085
 83. von Teichman B, Engelbrecht A, Zulu G, Dungu B, Pardini A, Bouloy M. Safety and efficacy of Rift Valley fever Smithburn and Clone 13 vaccines in calves. *Vaccine*. (2011) 29:5771–7. doi: 10.1016/j.vaccine.2011.05.055
 84. Dungu B, Lubisi BA, Ikegami T. Rift Valley fever vaccines: current and future needs. *Curr Opin Virol*. (2018) 29:8–15. doi: 10.1016/j.coviro.2018.02.001
 85. Ikegami T. Rift Valley fever vaccines: an overview of the safety and efficacy of the live-attenuated MP-12 vaccine candidate. *Expert Rev Vaccines*. (2017) 16:601–11. doi: 10.1080/14760584.2017.1321482
 86. Mansfield KL, Banyard AC, McElhinney L, Johnson N, Horton DL, Hernandez-Triana LM, et al. Rift Valley fever virus: A review of diagnosis and vaccination, and implications for emergence in Europe. *Vaccine*. (2015) 33:5520–31. doi: 10.1016/j.vaccine.2015.08.020
 87. Gaudreault NN, Indran SV, Balaraman V, Wilson WC, Richt JA. Molecular aspects of Rift Valley fever virus and the emergence of reassortants. *Virus Genes*. (2019) 55:1–11. doi: 10.1007/s11262-018-1611-y
 88. Davies FG. The historical and recent impact of Rift Valley fever in Africa. *Am J Trop Med Hyg*. (2010) 83:73–4. doi: 10.4269/ajtmh.2010.83s2a02
 89. Coetzer JA. The pathology of Rift Valley fever. I Lesions occurring in natural cases in new-born lambs. *Onderstepoort J Vet Res*. (1977) 44:205–11.
 90. Coetzer JA. The pathology of Rift Valley fever. II. Lesions occurring in field cases in adult cattle, calves and aborted foetuses. *Onderstepoort J Vet Res*. (1982) 49:11–7.
 91. WHO. *Rift Valley Fever* (2019). Available online at: <https://www.who.int/emergencies/diseases/rift-valley-fever/en/>
 92. Nyakarahuka L, de St Maurice A, Purpura L, Ervin E, Balinandi S, Tumusiime A, et al. Prevalence and risk factors of Rift Valley fever in humans and animals from Kabale district in Southwestern Uganda, 2016. *PLoS Negl Trop Dis*. (2018) 12:e0006412. doi: 10.1371/journal.pntd.0006412
 93. Sow A, Faye O, Ba Y, Diallo D, Fall G, Faye O, et al. Widespread Rift Valley Fever Emergence in Senegal in 2013–2014. *Open Forum Infect Dis*. (2016) 3:ofw149. doi: 10.1093/ofid/ofw149
 94. Brand RF, Rostal MK, Kemp A, Anyamba A, Zwieggers H, Van Huyssteen CW, et al. A phytosociological analysis and description of wetland vegetation and ecological factors associated with locations of high mortality for the 2010–11 Rift Valley fever outbreak in South Africa. *PLoS ONE*. (2018) 13:e0191585. doi: 10.1371/journal.pone.0191585
 95. Nabeth P, Kane Y, Abdalahi MO, Diallo M, Ndiaye K, Ba K, et al. Rift Valley fever outbreak, Mauritania, 1998: seroepidemiologic, virologic, entomologic, zoologic investigations. *Emerg Infect Dis*. (2001) 7:1052–4. doi: 10.3201/eid0706.010627
 96. OIE. *Rift Valley Fever, South Africa* (2010). Available online at: https://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapEventSummary&reportid=8967
 97. Barnard BJ. Rift Valley fever vaccine–antibody and immune response in cattle to a live and an inactivated vaccine. *J S Afr Vet Assoc*. (1979) 50:155–7.
 98. Harrington DG, Lupton HW, Crabbs CL, Peters CJ, Reynolds JA, Slone TW, et al. Evaluation of a formalin-inactivated Rift Valley fever vaccine in sheep. *Am J Vet Res*. (1980) 41:1559–64.
 99. Morrill JC, Mebus CA, Peters CJ. Safety of a mutagen-attenuated Rift Valley fever virus vaccine in fetal and neonatal bovines. *Am J Vet Res*. (1997) 58:1110–4.
 100. Morrill JC, Mebus CA, Peters CJ. Safety and efficacy of a mutagen-attenuated Rift Valley fever virus vaccine in cattle. *Am J Vet Res*. (1997) 58:1104–9.
 101. Oymans J, Wichgers Schreur PJ, van Keulen L, Kant J, Kortekaas J. Rift Valley fever virus targets the maternal-foetal interface in ovine and human placentas. *PLoS Negl Trop Dis*. (2020) 14:e0007898. doi: 10.1371/journal.pntd.0007898
 102. Ross TM, Bhardwaj N, Bissel SJ, Hartman AL, Smith DR. Animal models of Rift Valley fever virus infection. *Virus Res*. (2012) 163:417–23. doi: 10.1016/j.virusres.2011.10.023
 103. Kortekaas J, Oreshkova N, van Keulen L, Kant J, Bosch BJ, Bouloy M, et al. Comparative efficacy of two next-generation Rift Valley fever vaccines. *Vaccine*. (2014) 32:4901–8. doi: 10.1016/j.vaccine.2014.07.037
 104. Oreshkova N, van Keulen L, Kant J, Moormann RJ, Kortekaas J. A single vaccination with an improved nonspreading Rift Valley fever virus vaccine provides sterile immunity in lambs. *PLoS One*. (2013) 8:e77461.
 105. Wichgers Schreur PJ, Kant J, van Keulen L, Moormann RJM, Kortekaas J. Four-segmented Rift Valley fever virus induces sterile immunity in sheep after a single vaccination. *Vaccine*. (2015) 33:1459–64. doi: 10.1016/j.vaccine.2015.01.077
 106. Kortekaas J, Antonis AF, Kant J, Vloet RP, Vogel A, Oreshkova N, et al. Efficacy of three candidate Rift Valley fever vaccines in sheep. *Vaccine*. (2012) 30:3423–9. doi: 10.1016/j.vaccine.2012.03.027
 107. Busquets N, Xavier F, Martin-Folgar R, Lorenzo G, Galindo-Cardiel I, del Val BP, et al. Experimental infection of young adult European breed sheep with Rift Valley fever virus field isolates. *Vector Borne Zoonotic Dis*. (2010) 10:689–96. doi: 10.1089/vbz.2009.0205
 108. Olaleye OD, Tomori O, Fajimi JL, Schmitz H. Experimental infection of three Nigerian breeds of sheep with the Zinga strain of the Rift Valley Fever virus. *Rev Elev Med Vet Pays Trop*. (1996) 49:6–16.
 109. Busquets N, Lorenzo G, Lopez-Gil E, Rivas R, Solanes D, Galindo-Cardiel I, et al. Efficacy assessment of an MVA vectored Rift Valley Fever vaccine in lambs. *Antiviral Res*. (2014) 108:165–172. doi: 10.1016/j.antiviral.2014.05.020
 110. Lorenzo G, Lopez-Gil E, Ortego J, Brun A. Efficacy of different DNA and MVA prime-boost vaccination regimens against a Rift Valley fever virus (RVFV) challenge in sheep 12 weeks following vaccination. *Vet Res*. (2018) 49:21. doi: 10.1186/s13567-018-0516-z
 111. Chrun T, Lacote S, Urien C, Jouneau L, Barc C, Bouguyon E, et al. A Rift Valley fever virus Gn ectodomain-based DNA vaccine induces a partial protection not improved by APC targeting. *NPJ Vaccines*. (2018) 3:14. doi: 10.1038/s41541-018-0052-x
 112. Weingartl HM, Miller M, Nfon C, Wilson WC. Development of a Rift Valley fever virus viremia challenge model in sheep and goats. *Vaccine*. (2014) 32:2337–44. doi: 10.1016/j.vaccine.2014.02.066
 113. Faburay B, Gaudreault NN, Liu Q, Davis AS, Shivanna V, Sunwoo SY, et al. Development of a sheep challenge model for Rift Valley fever. *Virology*. (2016) 489:128–40. doi: 10.1016/j.virol.2015.12.003
 114. Nguku PM, Sharif SK, Mutonga D, Amwayi S, Omolo J, Mohammed O, et al. An investigation of a major outbreak of Rift Valley fever in Kenya: 2006–2007. *Am J Trop Med Hyg*. (2010) 83:5–13. doi: 10.4269/ajtmh.2010.09-0288
 115. Warimwe GM, Gesharisha J, Carr BV, Otieno S, Otingah K, Wright D, et al. Chimpanzee adenovirus vaccine provides multispecies protection against Rift Valley Fever. *Sci Rep*. (2016) 6:20617. doi: 10.1038/srep20617
 116. Nfon CK, Marszal P, Zhang S, Weingartl HM. Innate immune response to Rift Valley fever virus in goats. *PLoS Negl Trop Dis*. (2012) 6:e1623. doi: 10.1371/journal.pntd.0001623

117. Kroeker AL, Smid V, Embury-Hyatt C, Moffat E, Collignon B, Lung O, et al. RVFV Infection in Goats by Different Routes of Inoculation. *Viruses*. (2018) 10. doi: 10.20944/preprints201811.0022.v1
118. Conway MJ, Watson AM, Colpitts TM, Dragovic SM, Li Z, Wang P, et al. Mosquito saliva serine protease enhances dissemination of dengue virus into the mammalian host. *J Virol*. (2014) 88:164–75. doi: 10.1128/JVI.02235-13
119. Le Coupanec A, Babin D, Fiette L, Jouvion G, Ave P, Misse D, et al. Aedes mosquito saliva modulates Rift Valley fever virus pathogenicity. *PLoS Negl Trop Dis*. (2013) 7:e2237. doi: 10.1371/journal.pntd.0002237
120. Pinggen M, Bryden SR, Pondeville E, Schnettler E, Kohl A, Merits A, et al. Host inflammatory response to mosquito bites enhances the severity of arbovirus infection. *Immunity*. (2016) 44:1455–69. doi: 10.1016/j.immuni.2016.06.002
121. Pinggen M, Schmid MA, Harris E, McKimmie CS. Mosquito biting modulates skin response to virus infection. *Trends Parasitol*. (2017) 33:645–57. doi: 10.1016/j.pt.2017.04.003
122. Schmid MA, Glasner DR, Shah S, Michlmayr D, Kramer LD, and Harris E. Mosquito saliva increases endothelial permeability in the skin, immune cell migration, and dengue pathogenesis during antibody-dependent enhancement. *PLoS Pathog*. (2016) 12:e1005676. doi: 10.1371/journal.ppat.1005676
123. Wichit S, Diop F, Hamel R, Talignani L, Ferraris P, Corneli S, et al. Aedes Aegypti saliva enhances chikungunya virus replication in human skin fibroblasts via inhibition of the type I interferon signaling pathway. *Infect Genet Evol*. (2017) 55:68–70. doi: 10.1016/j.meegid.2017.08.032
124. Ayari-Fakhfakh E, Ghram A, Albina E, Cetre-Sossah C. Expression of cytokines following vaccination of goats with a recombinant capripoxvirus vaccine expressing Rift Valley fever virus proteins. *Vet Immunol Immunopathol*. (2018) 197:15–20. doi: 10.1016/j.vetimm.2018.01.001
125. Wilson WC, Davis AS, Gaudreault NN, Faburay B, Trujillo JD, Shivanna V, et al. Experimental infection of calves by two genetically-distinct strains of Rift Valley Fever Virus. *Viruses*. (2016) 8:145. doi: 10.3390/v8050145
126. Kroeker AL, Smid V, Embury-Hyatt C, Collignon B, Pinette M, Babiuk S, et al. Increased susceptibility of cattle to intranasal RVFV infection. *Front Vet Sci*. (2020). doi: 10.3389/fvets.2020.00137
127. Campagnolo ER, Tewari D, Farone TS, Livengood JL, Mason KL. Evidence of Powassan/deer tick virus in adult black-legged ticks (*Ixodes scapularis*) recovered from hunter-harvested white-tailed deer (*Odocoileus virginianus*) in Pennsylvania: a public health perspective. *Zoonoses Public Health*. (2018) 65:589–94. doi: 10.1111/zph.12476
128. Hollis-Etter KM, Anchor CL, Chelsvig JE, Dubey JP, Warner RE. Suburban white-tailed deer seropositive for *Toxoplasma gondii* from Chicago, Illinois. *Parasitol Res*. (2019) 118:2271–76. doi: 10.1007/s00436-019-06347-1
129. Ruder MG, Stallknecht DE, Allison AB, Mead DG, Carter DL, Howerth EW. Host and potential vector susceptibility to an emerging orbivirus in the United States: epizootic hemorrhagic disease virus serotype 6. *Vet Pathol*. (2016) 53:574–84. doi: 10.1177/0300985815610387
130. Kasari TR, Carr DA, Lynn TV, Weaver JT. Evaluation of pathways for release of Rift Valley fever virus into domestic ruminant livestock, ruminant wildlife, and human populations in the continental United States. *J Am Vet Med Assoc*. (2008) 232:514–29. doi: 10.2460/javma.232.4.514
131. Kakani S, LaBeaud AD, King CH. Planning for Rift Valley fever virus: use of geographical information systems to estimate the human health threat of white-tailed deer (*Odocoileus virginianus*)-related transmission. *Geospat Health*. (2010) 5:33–43. doi: 10.4081/gh.2010.185
132. Hartley DM, Rinderknecht JL, Nipp TL, Clarke NP, Snowden GD. Animal National Center for Foreign, and Fever Zoonotic Disease Defense Advisory Group on Rift Valley. Potential effects of Rift Valley fever in the United States. *Emerg Infect Dis*. (2011) 17:e1. doi: 10.3201/eid1708.101088
133. Golnar AJ, Kading RC, Hamer GL. Quantifying the potential pathways and locations of Rift Valley fever virus entry into the United States. *Transbound Emerg Dis*. (2018) 65:85–95. doi: 10.1111/tbed.12608
134. Wilson WC, Kim JJ, Trujillo JD, Sunwoo SY, Noronha LE, Urbaniak K, et al. Susceptibility of White-Tailed Deer to Rift Valley Fever Virus. *Emerg Infect Dis*. (2018) 24:1717–19. doi: 10.3201/eid2409.180265
135. Van der Lugt JJ, Coetzer JA, Smit MM. Distribution of viral antigen in tissues of new-born lambs infected with Rift Valley fever virus. *Onderstepoort J Vet Res*. (1996) 63:341–7.
136. Coetzer JA, Ishak KG, Calvert RC. Sequential development of the liver lesions in new-born lambs infected with Rift Valley fever virus. II Ultrastructural findings. *Onderstepoort J Vet Res*. (1982) 49:109–22.
137. Easterday B. C., Gavran Mh, M.c, Rooney JR, Murphy LC. The pathogenesis of Rift Valley fever in lambs. *Am J Vet Res*. (1962) 23:470–9.
138. Wichgers Schreur PJ, van Keulen L, Kant J, Kortekaas J. Four-segmented Rift Valley fever virus-based vaccines can be applied safely in ewes during pregnancy. *Vaccine*. (2017) 35:3123–8. doi: 10.1016/j.vaccine.2017.04.024
139. Swanepoel R, Struthers JK, Erasmus MJ, Shepherd SP, McGillivray GM, Shepherd AJ, et al. Comparative pathogenicity and antigenic cross-reactivity of Rift Valley fever and other African phleboviruses in sheep. *J Hyg (Lond)*. (1986) 97:331–46. doi: 10.1017/S0022172400065426
140. Bird BH, Khristova ML, Rollin PE, Ksiazek TG, Nichol ST. Complete genome analysis of 33 ecologically and biologically diverse Rift Valley fever virus strains reveals widespread virus movement and low genetic diversity due to recent common ancestry. *J Virol*. (2007) 81:2805–16. doi: 10.1128/JVI.02095-06
141. Bird BH, Githinji JW, Macharia JM, Kasiiti JL, Muriithi RM, Gacheru SG, et al. Multiple virus lineages sharing recent common ancestry were associated with a Large Rift Valley fever outbreak among livestock in Kenya during 2006–2007. *J Virol*. (2008) 82:11152–66. doi: 10.1128/JVI.01519-08
142. Grobelaar AA, Weyer J, Leman PA, Kemp A, Paweska JT, Swanepoel R. Molecular epidemiology of Rift Valley fever virus. *Emerg Infect Dis*. (2011) 17:2270–6. doi: 10.3201/eid1712.111035
143. Ikegami T, Balogh A, Nishiyama S, Lokugamage N, Saito TB, Morrill JC, et al. Distinct virulence of Rift Valley fever phlebovirus strains from different genetic lineages in a mouse model. *PLoS ONE*. (2017) 12:e0189250. doi: 10.1371/journal.pone.0189250
144. Anderson GW Jr, Peters CJ. Viral determinants of virulence for Rift Valley fever (RVF) in rats. *Microb Pathog*. (1988) 5:241–50. doi: 10.1016/0882-4010(88)90096-4
145. Shabman RS, Morrison TE, Moore C, White L, Suthar MS, Hueston L, et al. Differential induction of type I interferon responses in myeloid dendritic cells by mosquito and mammalian-cell-derived alphaviruses. *J Virol*. (2007) 81:237–47. doi: 10.1128/JVI.01590-06
146. Moutailler S, Roche B, Thiberge JM, Caro V, Rougeon F, Failloux AB. Host alternation is necessary to maintain the genome stability of rift valley fever virus. *PLoS Negl Trop Dis*. (2011) 5:e1156. doi: 10.1371/journal.pntd.0001156
147. Easterday BC. Rift valley fever. *Adv Vet Sci*. (1965) 10:65–127.
148. Findlay GM. The relation between dengue and rift valley fever. *Trans Royal Soc Trop Med Hygiene*. (1932) 26:157–60. doi: 10.1016/S0035-9203(32)90115-1
149. Wallace DB, Ellis CE, Espach A, Smith SJ, Greyling RR, Viljoen GJ. Protective immune responses induced by different recombinant vaccine regimes to Rift Valley fever. *Vaccine*. (2006) 24:7181–9. doi: 10.1016/j.vaccine.2006.06.041
150. Hartman AL, Powell DS, Bethel LM, Caroline AL, Schmid RJ, Oury T, et al. Aerosolized rift valley fever virus causes fatal encephalitis in african green monkeys and common marmosets. *J Virol*. (2014) 88:2235–45. doi: 10.1128/JVI.02341-13
151. Li M, Wang B, Li L, Wong G, Liu Y, Ma J, et al. Rift Valley Fever Virus and yellow fever virus in urine: a potential source of infection. *Virol Sin*. (2019) 34:342–45. doi: 10.1007/s12250-019-00096-2
152. Grossi-Soyster EN, Lee J, King CH, LaBeaud AD. The influence of raw milk exposures on Rift Valley fever virus transmission. *PLoS Negl Trop Dis*. (2019) 13:e0007258. doi: 10.1371/journal.pntd.0007258

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Kroeker, Babiuk, Pickering, Richt and Wilson. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers



OPEN ACCESS

Articles are free to read
for greatest visibility
and readership



FAST PUBLICATION

Around 90 days
from submission
to decision



HIGH QUALITY PEER-REVIEW

Rigorous, collaborative,
and constructive
peer-review



TRANSPARENT PEER-REVIEW

Editors and reviewers
acknowledged by name
on published articles

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org

Contact us: info@frontiersin.org | +41 21 510 17 00



REPRODUCIBILITY OF RESEARCH

Support open data
and methods to enhance
research reproducibility



DIGITAL PUBLISHING

Articles designed
for optimal readership
across devices



FOLLOW US

[@frontiersin](https://twitter.com/frontiersin)



IMPACT METRICS

Advanced article metrics
track visibility across
digital media



EXTENSIVE PROMOTION

Marketing
and promotion
of impactful research



LOOP RESEARCH NETWORK

Our network
increases your
article's readership