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# Sentinels of health: advancements in monitoring and surveillance of vector-borne diseases in domestic and wild animals and vectors

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# Sentinels of health: advancements in monitoring and surveillance of vector-borne diseases in domestic and wild animals and vectors

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# Editorial: Sentinels of health: advancements in monitoring and surveillance of vector-borne diseases in domestic and wild animals and vectors

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**vector-borne diseases, surveillance, zoonotic pathogens, One Health, vector ecology, integrated control strategies**

Editorial on the Research Topic
Sentinels of health: advancements in monitoring and surveillance of vector-borne diseases in domestic and wild animals and vectors

Vectors and vector-borne diseases in domestic and wild animals are undergoing significant shifts in distribution and impact due to environmental and climate changes, globalization, and human encroachment on natural habitats. Formerly localized, many of these pathogens are now spreading across new regions, with several acquiring zoonotic potential and posing public health risks. These diseases also impose substantial economic burdens through veterinary costs, reduced productivity, and control efforts.

Recognizing the complexity of these challenges, *Frontiers in Veterinary Science* launched the Research Topic "*Sentinels of health: advancements in monitoring and surveillance of vector-borne diseases in domestic and wild animals and vectors*" in February 2024. This initiative aimed to consolidate current knowledge on surveillance, vector ecology, pathogen detection, and control strategies, emphasizing a multidisciplinary and One Health approach.

The contributions highlight advances in molecular diagnostics, phylogenetics, ecological modeling, and field surveillance, demonstrating how science is improving our capacity to the early detection and to monitor and respond to emerging threats.

Strengthening epidemiological systems and understanding the ecological drivers of vector dynamics remain essential for early outbreak prediction and effective intervention. This Research Topic underscores the urgent need for coordinated, adaptive strategies to manage the evolving landscape of vector-borne and zoonotic diseases.

A multi-year surveillance study conducted by Ivović et al. in Slovenia provided critical insights into the ecology of sandflies, including *Phlebotomus papatasi*, *P. neglectus*, *P. perniciosus*, and *P. mascittii*. The survey, covering 43 sampling sites from 2020 to 2022, recorded peak sandfly activity in July and showed a strong correlation between sandfly distribution and climatic variables such as temperature and precipitation. Although *Leishmania* spp. and *Phleboviruses* were not detected, predictive habitat models underlined the significance of ongoing monitoring in the face of climate change. Continuous

surveillance remains vital for early detection of shifts in vector populations and for assessing future pathogen emergence.

Complementing ecological investigations, Vaselek and Alten explored the gut microbiome of *Ph. papatasi*, emphasizing the crucial role of microbial communities in larval development. Their results showed that larvae reared in microbiome-depleted conditions had higher mortality rates and delayed development compared to those raised on media with live, diverse microbiota. These findings suggest that manipulating sandfly microbiota could potentially be employed in biological control strategies. Additionally, the influence of the gut microbiome on *Leishmania* development within the sandfly vector has been previously demonstrated (1), underscoring the microbiome's role in vector competence.

Surveillance of zoonotic pathogens in domestic ruminants was addressed by Barbic et al., who conducted a seroprevalence study in sheep from endemic areas of Croatia. Historically, chickens and horses were used as sentinels for detecting seasonal WNV incursions, correlating with human outbreaks (2). This study expanded sentinel species to include sheep, revealing exposure to TBEV (9.7%), WNV (3.0%), *Borrelia burgdorferi* s.l. (2.7%), and USUV (1.3%). Significant associations were observed with micro-location, sheep breed, and farm distance from households. The data support the inclusion of sheep in integrated surveillance networks and call for targeted prevention strategies based on local epidemiological factors.

In a comprehensive study from Vietnam, Do et al. reported a 29% prevalence of *Rhipicephalus sanguineus* infestation in client-owned dogs. Pathogen screening of tick pools revealed high infection rates with *Mycoplasma* spp. (78.5%), *Anaplasma* spp. (37.3%), and *Rickettsia felis* (5.1%). Seasonal patterns and host factors such as age, breed, and lifestyle were associated with tick infestation risk. These results underscore the zoonotic potential of tick-borne pathogens in companion animals and the need for integrated tick control, owner education, and surveillance.

Addressing inaccuracies in host-vector records, Millot et al. critically reevaluated the literature on *Culicoides* biting midges in France. While previous data suggested that 92 species fed on cattle, their molecular and indirect investigations confirmed feeding behavior in only 45 species. This correction highlights the importance of combining morphological and molecular tools in determining vector-host interactions, which is essential for accurate risk assessments of vector-borne diseases.

In the field of veterinary diagnostics, Li et al. developed and validated a multiplex quantitative PCR assay capable of simultaneously detecting six major pathogens associated with the bovine respiratory disease complex: BRSV, BPIV3, BVDV, BAV3, *Mycoplasma bovis*, and IBRV. The assay demonstrated high specificity, sensitivity, and reproducibility, with detection limits ranging from 4.99 to 74.4 copies/μl. Among 224 clinical samples, a 25% mixed infection rate was observed, illustrating the assay's utility in complex diagnostic scenarios.

Refinements in environmental sampling for ASFV surveillance were investigated by Kwon et al., who compared multiple swabbing devices and preservation techniques on clean stainless steel surfaces. Their results showed that pre-moistened gauze, sponge sticks, and nucleic acid preservatives significantly improved ASFV DNA detection compared to dry swabs. These optimized methods can enhance early outbreak detection and inform disinfection protocols on farms.

While Glišić et al., (3) showed that the human stands for the main risk of disease spreading, Vasić et al. examined alternative ASFV transmission routes during the 2023 outbreak in Serbia. While no viral DNA was detected in soil, feed, or barn swabs, positive results were obtained from fly larvae and adults (*Lucilia sericata*, *Stomoxys calcitrans*) collected from carcasses. Experimental infections confirmed short-term ASFV persistence in *S. calcitrans*. These findings suggest a potential mechanical transmission pathway and highlight the role of insect vectors in outbreak dynamics.

Šolaja et al. focused on the genetic evolution of WNV in the Western Balkans through whole-genome sequencing of field strains. Phylogenetic analysis revealed two major sublineages within the E clade (E1 and E2), distinct from previously defined D and E clades (4). Variants in E159, NS1, and NS3 proteins were identified, with potential roles in virulence and immune evasion. These findings demonstrate the continued evolution of WNV and underscore the need for molecular surveillance to track viral adaptation.

In a separate case of emerging viral threat, Hu et al. documented an outbreak of recombinant LSDV among yaks on the Qinghai-Tibet Plateau. The strain, classified within Cluster 1.2 recombinant subclade, showed high similarity to Southeast Asian LSDV isolates and induced systemic lesions and 46.7% mortality. Histopathology and immunohistochemistry confirmed viral replication in epithelial and immune cells. The study illustrates how recombinant viruses can overcome ecological barriers and emphasizes the necessity of high-altitude-specific vaccination and surveillance strategies.

In geographically isolated ecosystems, Zanella et al. screened 411 equids in the Galapagos Islands for WNV, USUV, and EIAV. All samples tested negative, indicating the current absence of these pathogens. However, the presence of competent vectors and increasing human and animal movement necessitates continued surveillance to prevent pathogen introduction in these ecologically sensitive environments.

Warm climates in Africa, southern Europe, and Southeast Asia support year-round *Culicoides* activity, enabling BTV overwintering. In the past 20 years, climate change has driven BTV spread beyond 50° latitude and led to the emergence of new vector species (5). Beyan et al. and Zhugunissov et al. provided critical data on the spread of bluetongue virus (BTV) in Ethiopia and Kazakhstan, respectively. In Ethiopia, individual-level seroprevalence reached 84.5%, with age identified as a significant risk factor. In Kazakhstan, a sharp increase in BTV RNA detection occurred between 2023 and 2024, especially in goats and cattle. Infection in multiple *Culicoides* species, including *C. obsoletus*, which is recognized as a primary European vector, was confirmed by rRT-PCR. These studies reflect the dynamic expansion of BTV and the importance of regional vector competence assessments.

Despite growing interest in species distribution modeling, conceptual and methodological uncertainties are often underappreciated. While previous studies (6, 7) employed

virtual species to assess the impact of sample size at comparable scales, they reported general patterns without identifying an optimal threshold. To address this gap, Mitchel et al. investigated the optimal sample size using simulated data and the Random Forest algorithm. Their findings suggest that a minimum of 750–1,000 presence/absence records is required to ensure reliable model performance, offering practical guidance for vector mapping and field sampling strategies.

Although mechanistic models have been used to examine the effects of abiotic factors on tick life cycles and population dynamics, none have explicitly modeled the mechanisms of diapause regulation (8). To fill this gap, Erguler et al. developed a structured population model that simulates tick development and diapause responses under varying photoperiod and temperature conditions. This approach improves predictions of seasonal tick activity and supports the design of targeted control measures.

Expanding the discussion to wildlife reservoirs, Han et al. identified multiple zoonotic pathogens in long-tailed ground squirrels and their ectoparasites in Xinjiang, China. DNA from *Rickettsia sibirica*, *R. raoultii*, *R. slovaca*, *R. felis*, and *Coxiella burnetii* was detected in both rodents and fleas/lice. This study suggests a previously underappreciated transmission cycle and emphasizes the importance of including small wild mammal reservoirs in zoonotic disease surveillance.

Patouillat et al. conducted a systematic review of zoonotic pathogen surveillance in wild Asian primates. From 152 articles, they cataloged 183 pathogens across 39 primate species. The analysis revealed strong geographic and taxonomic biases, with most studies focused on a few well-known primates and using narrow diagnostic approaches. These gaps hinder a comprehensive understanding of zoonotic spillover risks and call for a broader, One Health–oriented surveillance strategy utilizing advanced molecular techniques.

Wu et al. addressed vector control innovation through the field evaluation of a novel sugar bait device (NSBD) in four residential sites in Zhejiang, China. Combining sugar bait with adhesive capture, the NSBD trap achieved up to 86.3% reduction of adult mosquitoes and 57.8% reduction of larvae. The 10% sugar concentration yielded the most effective results. This eco-friendly, insecticide-free approach offers a promising alternative for mosquito management, particularly in urban settings with rising resistance to chemical insecticides.

Collectively, these studies highlight significant progress in vector surveillance, pathogen detection, and disease modeling, underscoring the growing importance of integrated, region-specific strategies to address the complex challenges posed by vector-borne and zoonotic diseases. The integration of high-throughput diagnostics, ecological modeling, and field epidemiology demonstrates the potential of multidisciplinary approaches to enhance early detection and response capacities.

Despite these advancements, critical gaps remain, that require sustained research investment and coordinated policy support. Adopting a One Health framework that unites human, animal, and environmental health is essential for mitigating emerging disease risks, especially in ecologically sensitive and vulnerable regions.

Moving forward, regionally adapted surveillance systems, predictive modeling, and innovative, environmentally conscious control strategies will be key components in a robust global response to evolving infectious disease threats.

## Author contributions

VM: Validation, Writing – original draft. FM: Validation, Writing – review & editing. MK: Validation, Writing – review & editing.

## 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.

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The author(s) declare that no Gen AI was used in the creation of this manuscript.

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# Development and field evaluation of a novel sugar bait device for controlling residential vector mosquitoes in Zhejiang Province, China

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**Background:** Mosquito-borne diseases pose serious public health threats in Zhejiang Province, China, and vector control is believed to be the primary method for reducing transmission. Due to severe resistance problems, effective and sustainable methods without chemical insecticides are urgently required to control mosquito vectors. Attractive toxic sugar baits (ATSB) are newly developed methods to control mosquitoes in recent decades with the core element sugar bait, which was invented according to the sugar-feeding behavior of mosquitoes. In this study, we developed a Novel Sugar Bait Device (NSBD) trap by combining sugar bait and physical adhesive capture technology. The study aimed to evaluate the effect of the NSBD trap on controlling mosquitoes in residential environments and to identify the optimal sugar solution concentration in the sugar bait of the NSBD for real use.

**Methods:** Four residential villages in Ningbo City with similar geographic environments and mosquito densities were selected for field trials in 2022. One village (site 1) was designated as the control group, and three villages (sites 2–4) served as the test groups to assess the effectiveness of NSBD traps with different sugar solution concentrations (6, 8, and 10%) in the sugar bait. Larval and adult mosquito densities were monitored monthly before and semi-monthly after the trials using the CDC light trap and larval pipette method.

**Results:** Before the trials, we monitored mosquito density for 3months to confirm the baseline mosquito density among the four sites, and no statistical differences in adult and larval mosquitoes were found (adult, $F$ =3.047, $p$ >0.05; larvae, $F$ =0.436, $p$ >0.05). After the trials, all NCBD traps effectively controlled larval and adult mosquito densities, with the highest standard decrease rates of larval and adult mosquito densities at 57.80 and 86.31%, respectively, observed in site 4. The most suitable sugar solution concentration in the sugar bait was 10%.

**Conclusion:** NSBD traps effectively controlled mosquitoes in residential environments during field trials. Without the use of insecticides, this may be a promising choice for mosquito vector control to prevent mosquito-borne diseases.

KEYWORDS

**sugar bait, vector control, a novel sugar bait device, mosquito-borne diseases, dengue fever**

# 1 Introduction

Mosquito-borne diseases, such as dengue fever, malaria, and Japanese encephalitis, have imposed a significant disease burden in Zhejiang Province, located on the south-eastern coast of China. Due to its suitable climate, robust tourism, and active commercial exchanges, Zhejiang has emerged as one of the provinces with the highest incidence of dengue fever. In the last century, dengue fever outbreaks occurred continuously in Hangzhou City (1928–1929, 2017), Ningbo City (1928, 2004, 2018), Yiwu City (2009), Shaoxing City (2015), and Wenzhou City (2019). According to a study, dengue fever cases were reported in 95.55% (86/90) of towns in Zhejiang province between 2015 and 2019 (1). In 2018, dengue virus genotype I was identified from *Aedes albopictus* sampled in Wenzhou City (2, 3). The Japanese encephalitis virus was detected in Zhejiang Province in 2023 via integrated vector surveillance of arthropod vectors, with a positive rate of 0.491‰ (unpublished data). Arboviral illnesses have become a public health threat in Zhejiang Province.

Mosquito control is believed to be a more feasible method for preventing mosquito-borne diseases than commercial vaccines and drugs (4, 5). However, traditional control measures rely heavily on chemical insecticides such as pyrethroids, organophosphates, and carbamates. Excessive and repeated use of chemical insecticides has caused serious drug resistance in field populations of mosquitoes in Zhejiang. According to the results of insecticide susceptibility tests conducted in 2020 via the Zhejiang integrated vector surveillance, almost all *Ae. albopictus* field populations exhibit resistance to pyrethroids (3). Knockdown resistance (kdr) mutations F1534S were detected in all *Ae. albopictus* field populations sampled from northern, southern, western, and central Zhejiang, with the highest mutation frequency at 88.37% (Yiwu city) (6). Resistance has become a major challenge for current mosquito control strategies in Zhejiang. Additionally, using large amounts of chemicals in residential environments may pollute the environment and harm human health. Thus, there is an urgent need to devise effective and environmentally friendly methods for controlling vector mosquitoes.

In contrast to traditional chemical control methods, attractive toxic sugar baits (ATSB) have been developed as a new mosquito control method. ATSB is based on the feeding behavior of mosquitoes. Adult mosquitoes feed on sugars such as nectar and honeydew as energy sources (7). Sugars and insecticides have been developed to attract and kill mosquitoes (8, 9). In 1965, Lea prepared a solution by mixing sugar bait with malathion in a laboratory and tested its effect on killing *Aedes aegypti*. An 85.2% mortality rate of the *Ae. aegypti* has been observed in the laboratory tests (7). ATSB has been proven effective in controlling adult mosquitoes (8). However, ATSB also uses chemical insecticides to kill mosquitoes, and insecticidal resistance cannot be avoided. Based on this consideration, we combined sugar baits and physical adhesive capture technology to develop a novel sugar bait device (NSBD) trap. The sugar bait used in the NSBD was identified via laboratory tests. Semi-field trials on NSBD have been conducted previously, showing that NSBD effectively killed *Ae. albopictus* and *Culex quinquefasciatus* (unpublished data). In this study, we selected four residential villages to test the effect of NSBD on controlling mosquitoes in a real environment, aiming to identify the effect of NSBD and clarify the most suitable sugar bait components for use in real residential environments. Finally, we evaluated the potential of NSBD for actual applications in controlling mosquitoes in residential environments.

# 2 Materials and methods

## 2.1 Ethics statement

This study was approved by the Ethics Committee of the Zhejiang Provincial Center for Disease Control and Prevention (approval number: 2019-048).

## 2.2 The novel sugar bait device trap

The novel sugar bait device (NSBD) trap is shown in Figure 1. It consisted of five parts. Part 1 exhibited a cover that protected the trap from rainwater to maintain a stable sugar bait concentration. Part 2 was a mesh support with holes allowing the mosquitoes to fly into the trap. Part 3 comprised a black plastic bucket. Part 4 represented the sugar bait. Part 5 featured a black sticky insect paper adhering to the inwall of the plastic bucket for mosquitoes to land on. The sugar bait consisted of different concentrations (6–10%) of sugar solution, 1 g/L sodium benzoate, and 100 mg/L ammonium sulfate hydrochloride, and it was put inside the bucket with the volume reaching half of the bucket capacity. The relationships between the different sugar baits and NSBD traps are shown in Table 1.

## 2.3 Study sites

Field trials were conducted between July and November 2022 in Ningbo City, Zhejiang Province, China. Before the trials, we selected four villages (sites 1–4) in Ningbo with similar geographic environments (area size ranges from 4,000 to 9,000 square meters) and mosquito densities. One village was randomly chosen as the control group and named site 1, and the other three villages (sites 2–4) were designated as the test group. The villages were at least 200 m apart (Table 1). To confirm whether mosquito density was comparable among the four sites, we monitored the mosquito larvae and adults monthly from April to June 2022 before the trials began. For mosquito larvae, 30 residential yards in each site were randomly chosen and all larval breeding sites there were monitored using the larval pipette method (Container Index, CI, CI = number of containers with living mosquito larvae/number of ponding containers *100%) recommended by the national standard for surveillance methods for vector density (10). For mosquito adults, one CDC light trap was set in each site for one-overnight monitoring (female adults per trap night) according to Chinese national vector monitoring programme (11).

## 2.4 Study design

Based on previous laboratory studies and semi-field trials, we selected a sugar (glucose) concentration of 8% as the baseline. To identify the most suitable sugar bait concentration for use in real residential environments, we fluctuated a set of sugar concentrations (6, 8, and 10%) as sugar bait in NSBD traps by two percentage points.

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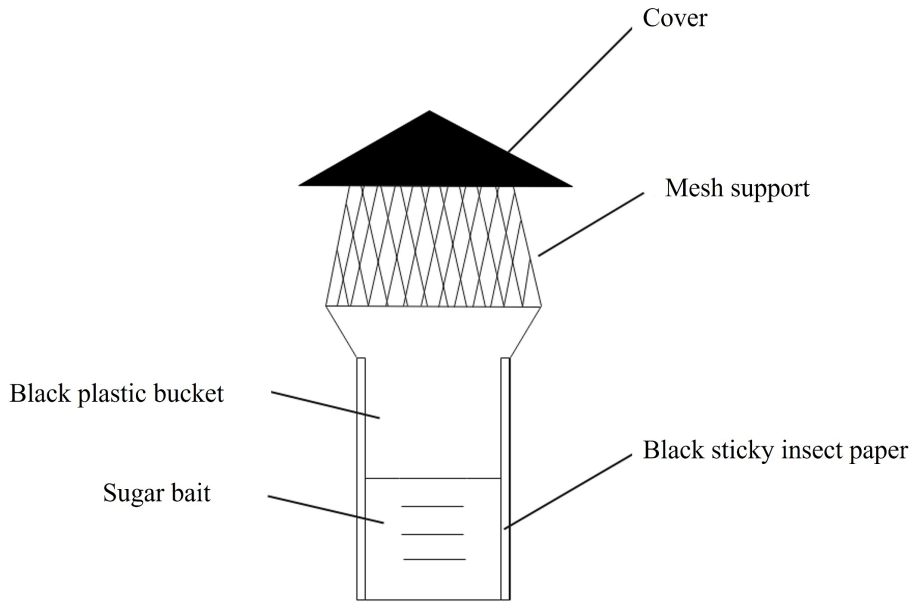


FIGURE 1
The overall schematic diagram of the novel sugar bait device (NSBD) trap.

TABLE 1 The geographical information for the four villages in field trials.

| Sites | Coordinates | Sugar solution concentrations (glucose) in sugar bait | NSBD |
|---|---|---|---|
| Site 1 | 121.474812, 29.669169 | Control group | None |
| Site 2 | 121.399339, 29.622876 | 6% | NSBD-1 |
| Site 3 | 121.490556, 29.687967 | 8% | NSBD-2 |
| Site 4 | 121.411018, 29.582785 | 10% | NSBD-3 |

Site 1 was designated the control group, and no mosquito control measures were performed. In sites 2–4, NSBD traps were positioned on flat ground in places away from direct sunlight, rainfall, and wind, with one NSBD trap every 30 square meters. The relationships between the different sites and NSBD traps are shown in Table 1. The NSBD traps were replaced with new ones containing fresh sugar bait (the same sugar bait components) every 2 weeks. Adult and larval mosquitoes were monitored every 2 weeks in each site using CDC light traps and larval pipette method (CI), respectively, during field trials, and the sampling method was the same to the monitoring before trials during April to June, with totally two trap-nights per site per month for adult mosquitoes and twice larval breeding sites monitoring per site per month for larval mosquitoes.

## 2.5 Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 23.0). The adult mosquito and mosquito larval densities among the four villages before the field trials were compared using nonparametric tests to determine whether the baseline mosquito density among villages was similar. Generalized linear mixed models (GLMMs) were employed to assess differences in larval and adult mosquito density monitored in different test groups during trials. Mosquito larvae and adult density were used as dependent variables, NSBD traps (control group, NSBD-1, NSBD-2 and NSBD-3) and collection date as fixed independent variables (negative binomial regression model). The means and standard errors associated with GLMMs were calculated. The rates of decrease and the standard decrease in mosquito density (adult and larval mosquitoes) were calculated for all four villages before and after the trials.

The decrease rates of adult mosquito density = (the average density of adult mosquito before trials- the average density of adult mosquito after trials)/ the average density of adult mosquito before trials × 100%.

The standard decrease rates of adult mosquito density = (the decrease rates of adult mosquito density in the test group- the decrease rates of adult mosquito density in the control group)/ (1- the decrease rates of adult mosquito density in the control group) × 100%.

The decrease rates of mosquito larvae density = (the average density of mosquito larvae before trials- the average density of mosquito larvae after trials)/ the average density of mosquito larvae before trials × 100%.

The standard decrease rates of mosquito larvae density = (the decrease rates of mosquito larvae density in the test group- the decrease rates of mosquito larvae density in the control group)/ (1- the decrease rates of mosquito larvae density in the control group) × 100%.

TABLE 2 The density of mosquito larvae[b] among four sites before and after the trials.

| Field trials | Month | Site 1[a] | Site 2 | Site 3 | Site 4 |
|---|---|---|---|---|---|
| Before trials | April | 11.54 | 12.24 | 10.81 | 9.38 |
| | May | 22.73 | 28.57 | 21.43 | 22.22 |
| | June | 57.50 | 40.63 | 53.85 | 57.14 |
| After trials | Early July | 27.03 | 22.22 | 10.00 | 27.78 |
| | Late July | 30.00 | 14.29 | 20.00 | 11.76 |
| | Early August | 25.45 | 44.44 | 23.68 | 10.00 |
| | Late August | 24.66 | 18.18 | 13.79 | 6.67 |
| | Early September | / | / | / | / |
| | Late September | 40.54 | 13.64 | 20.75 | 10.53 |
| | Early October | 29.73 | 21.05 | 20.83 | 13.33 |
| | Late October | 24.32 | 20.00 | 18.60 | 17.24 |
| | Early November | 20.29 | 12.50 | 11.43 | 9.68 |
| | Late November | 4.84 | 0.00 | 6.90 | 0.00 |
| The decrease rates of mosquito larvae density (%) | | −6.03 | 31.71 | 45.69 | 55.25 |
| The standard decrease rates of mosquito larvae density (%) | | / | 35.59 | 48.78 | 57.80 |

[a]Site 1 is control group.
[b]The density of mosquito larvae was represented by Container index (CI).

TABLE 3 The density of mosquito adults[a] among four sites before and after the trials.

| Field trials | Month | Site 1 | Site 2 | Site 3 | Site 4 |
|---|---|---|---|---|---|
| Before trials | April | 0 | 1 | 2 | 5 |
| | May | 8 | 3 | 8 | 12 |
| | June | 14 | 5 | 15 | 18 |
| After trials | Early July | 5 | 3 | 2 | 5 |
| | Late July | 8 | 2 | 2 | 4 |
| | Early August | 9 | 4 | 2 | 1 |
| | Late August | 12 | 4 | 4 | 2 |
| | Early September | 16 | 4 | 7 | 2 |
| | Late September | 18 | 7 | 11 | 2 |
| | Early October | 17 | 4 | 8 | 3 |
| | Late October | 12 | 1 | 3 | 2 |
| | Early November | 3 | 0 | 3 | 1 |
| | Late November | 1 | 0 | 1 | 0 |
| The decrease rates of mosquito adult density (%) | | -37.73 | 3.33 | 48.40 | 81.14 |
| The standard decrease rates of mosquito adult density (%) | | / | 29.81 | 62.53 | 86.31 |

[a]The density of mosquito adults was represented by mosquitoes per CDC light trap per night.

# 3 Results

## 3.1 General information

Before the field trials, we monitored the density of adult and larval mosquitoes monthly for 3 months to confirm whether the density baseline among the four villages was comparable. A median of 58 (Interquartile Range, IQR: 46, 59), 63 (IQR: 56, 63.5), 42 (IQR: 39.5, 47) and 45 (IQR: 38.5, 50.5) of larval breeding sites per month were monitored in four sites, respectively. The densities are listed in Tables 2, 3. No statistically significant differences in adult and larval mosquitoes were found among the four villages before the trials (adults, $F = 3.047$, $p = 0.384 > 0.05$; larvae, $F = 0.436$, $p = 0.933 > 0.05$).

## 3.2 Effects of NSBD traps on controlling mosquito larvae

A median of 62 (IQR: 42, 73), 54 (IQR: 45, 57), 58 (IQR: 53, 60) and 58 (IQR: 54, 60) of larval breeding sites per half-month were monitored in four sites, respectively. The density of mosquito larvae after 5 months of field trials is shown in Table 2. Natural fluctuations

in the mosquito larval density were observed in the control group, with the mosquito larval density increased by 6.03% after the trials. However, different levels of larval density were found in the test groups (sites 2–4) after the trials. The rates of decrease in mosquito larval density among the three test villages were 31.71, 45.69, and 55.25%. After correction using the control group, the standard decrease rates of mosquito larvae density were 35.59, 48.78, and 57.80% at sites 2, 3, and 4, respectively (Table 2). Significant differences in larval mosquito densities were observed among the four sites after the trials (GLMM, $F = 5.808$, $p < 0.05$). Compared with the control group (site 1), both sites 3 and 4 showed significantly lower larval densities after the trials (GLMM, site 3 vs. site 1, $F = 2.755$, $p < 0.05$; site 4 vs. site 1, $F = 4.083$, $p < 0.01$). NSBD-3 traps at site 4 showed the best effect in controlling mosquito larvae though no statistical differences were found, with a sugar concentration (glucose) of sugar bait at 10%.

## 3.3 Effects of NSBD traps on controlling mosquito adults

During mosquito surveillance, a total of 202 adult mosquitoes had been captured by CDC light traps in site 2, 3, and 4. Five species were identified; *Cx.pipiens pallens* was the most abundant species (73.27%), followed by *Cx. tritaeniorhynchus* (10.89%) and *Ae. albopictus* (7.43%). The effect of NSBD traps on controlling adult mosquitoes was similar to that on larvae. The densities of adult mosquitoes in the four villages before and after the trials are shown in Table 3 and Figure 2. An increase in density was also observed in adult mosquitoes in the control group (site 1), with the density increased by 37.73% after the trials compared to that before. In the test groups (sites 2-site 4), the density of adult mosquitoes decreased significantly in all three villages. The rates of decrease in adult mosquito density among the three test villages were 3.33, 48.40, and 81.14%. After correction using the control group, the standard decrease rates of adult mosquito density were 29.81, 62.63, and 86.31% at sites 2, 3, and 4, respectively (Table 3). A significant difference in adult mosquito density was observed among the four sites after the trials (GLMM, $F = 25.495$, $p < 0.01$). Compared to the control group (site 1), sites 2, 3, and 4 showed significantly lower larval densities after the trials (GLMM, site 2 vs. site 1, $F = 4.919$, $p < 0.01$; site 3 vs. site 1, $F = 6.213$, $p < 0.01$; site 4 vs. site 1, $F = 6.795$, $p < 0.01$). NSBD-3 traps at site 4 showed statistically the best effect in controlling mosquito adults, with a sugar concentration (glucose) of sugar bait at 10%.

# 4 Discussion

Sugar bait has a mosquito-control history of over 60 years (8). Based on the results of previous studies, we developed a novel sugar bait device (NSBD) trap by combining sugar bait and physical adhesive capture technology to develop an effective and environmentally friendly method to control mosquitoes around human residents and prevent mosquito-borne diseases. This study evaluated the effects of NSBD traps with different sugar baits by controlling larval and adult mosquitoes in residential environments through field trials. The results showed that the NSBD traps could effectively reduce larval and adult mosquito densities, and the most suitable sugar solution concentration in the sugar bait was 10%.

In this study, four residential villages with similar geographical environments were selected. To better demonstrate the effectiveness of the NSBD, the basic mosquito densities in the four villages were assumed to be the same. Thus, 3 months of mosquito monitoring was conducted before the field trials, and no statistical differences were found in the densities of larval and adult mosquitoes among the four villages. NSBD traps were proven effective in controlling mosquitoes in residential environments, with the highest standard decrease rates of larval and adult mosquito densities of 57.80 and 86.31%, respectively. The mosquito densities at sites 3 (8% sugar solution concentration in sugar bait) and 4 (10% sugar solution concentration in sugar bait) were significantly lower than those at site 1 (control group). Similar effect had been found in some ATSBs with sugar concentrations ranging from 5 to 20% and insecticides on killing different species of mosquitoes in laboratory and field studies, which

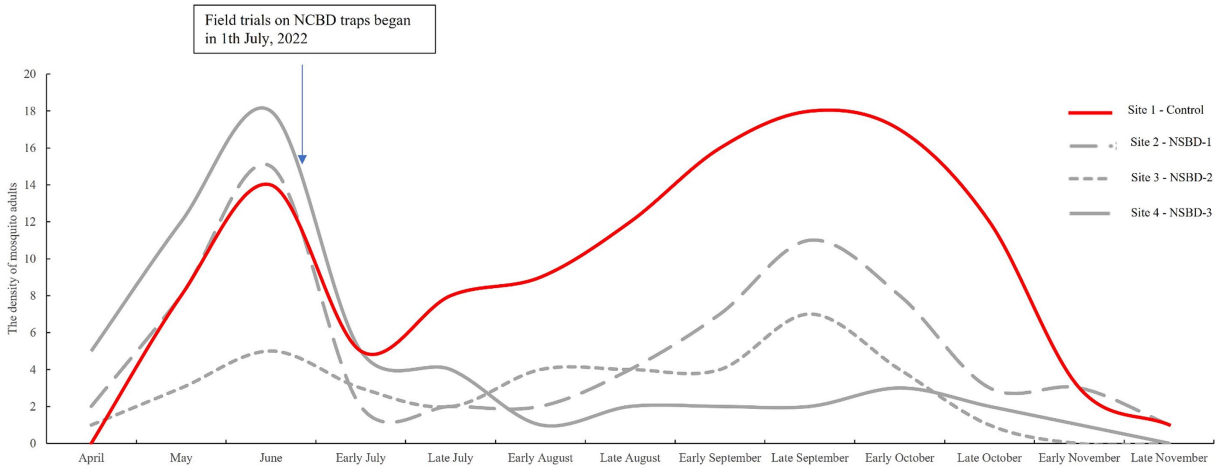


FIGURE 2
The trend of density changes in adult mosquitoes in four sites before and after trials.

had reduced the density of mosquitoes by 52 to 96% after exposure to ATSBs (8, 12, 13).

The concept of insect baiting is not novel, it was first described in 77 AD in Historia Naturalis (14). Subsequently, an increasing number of insect-baiting attempts have been made to dissuade behavior and induce mortality (8). Sugar feeding is a basic biological habit of mosquitoes. Almost all adult mosquitoes feed on sugary meals to supplement their energy throughout their lives (12, 15–17). This behavior has always been used to control mosquitoes. In this research, sugar bait was combined with sticky black insect paper in a black bucket to lure and stick the mosquitoes, and it proved to be effective on controlling mosquitoes. Similar results had been found in MosHouse traps by Pattamaporn Kittayapong et al. After adding a sugar stick and sticky flags, its effect on capturing mosquitoes significantly increased (18). However, while NSBD traps attract and kill mosquitoes, ensuring that the provided sugar bait solution does not become a new breeding place for mosquito larvae is crucial, especially when we should use methods without chemical insecticides. Thus, many preliminary experiments have been conducted in the laboratory, and it has been shown that sugar solution concentrations above 5% could effectively prevent larval survival (unpublished data). After considering the luring effect and unsuitability for larval survival, a sugar bait with a sugar solution concentration of 8% was chosen as the baseline to explore the effect of controlling mosquitoes in real residential environments. Throughout the field trials, we found no live mosquito larvae in the NSBD traps. Black was chosen as the color of sticky insect papers and buckets because of the black preference characteristics of *Ae. albopictus*, which is one of the most advantageous mosquito populations in Zhejiang Province (19).

To control vector mosquitoes, several traps had been developed such as BG- Sentinel traps and backpack aspirators, which are chemically independent and effectively on collecting mosquitoes especially *Aedes* mosquitoes (20–22). However, they both require power source and are not cost-effective in mosquito collection (23, 24). So more economical traps had been developed to captured gravid mosquitoes such as Stick ovitraps, AedesTrap, both of which attract and capture gravid mosquitoes by creating suitable oviposition environments (23, 25). MosHouse trap was developed using odor created by hay infusion and oviposition environment to attract gravid and non-gravid mosquitoes (18). However, all of them use *Bacillus thuringiensis israelensis* (Bti) as substances to killing mosquito larvae, which is also one of insecticides and might induce drug resistance. The advantage of NSBD traps is low-cost, easy to use, without use of chemical insecticides and requires no power sources for its usage, it could be considered as an appropriate alternative trapping method to control mosquitoes around residential areas, then to reduce mosquito infestation and to prevent mosquito-borne diseases.

The limitation of this research is that within this investigation, only three concentration gradients of sugar solutions in the sugar bait were tested in the field trials; thus, caution should be exercised when extrapolating the results. However, according to the results of the laboratory experiments, the luring effect of sugar bait with a sugar solution concentration of 15% on *Ae. albopictus* and *C. quinquefasciatus* was much lower than that of 8%. Therefore, it can be inferred that the effect of sugar solution concentrations above 10% on mosquito control might not be better than that of concentrations below 10%. On the other hand, in the present study we focus more on the overall decrease in adult and larval mosquito density than on some specific mosquito species by considering that sugar bait could attract many mosquito species (8). And no comparison had been made on the control effect of NSBD traps on different mosquito species. In the future, more researches like the long-term efficacy of NSBD traps and mosquito species that are more likely to be controlled by NSBD traps, the variability in different environmental conditions and geographical locations would be conducted to clarify the application potential of NSBD.

In conclusion, a novel sugar bait device (NSBD) trap, developed by combining sugar bait and physical adhesive capture technology, can effectively control larval and adult mosquitoes in residential environments. Without the use of chemical insecticides, this could be an effective and environmentally friendly method for controlling mosquitoes. The most suitable sugar concentration for the sugar bait of the NCBD traps was 10%.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## Author contributions

YW: Investigation, Methodology, Writing – original draft, Writing – review & editing. CZ: Investigation, Writing – original draft. WF: Investigation, Resources, Writing – original draft. SF: Project administration, Resources, Writing – original draft. WD: Methodology, Resources, Writing – original draft. JW: Investigation, Writing – original draft. QL: Investigation, Methodology, Writing – original draft. TL: Investigation, Writing – original draft. ML: Investigation, Writing – original draft. ZG: Writing – original draft, Writing – review & editing.

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## 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.

## Publisher's note

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# Undetection of vector-borne viruses in equids of Galapagos Islands

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Domestic species, including equids, were introduced in the Galapagos Islands in the XIX century. Equine vector-borne diseases are circulating in South America but their occurrence in the Galapagos Island was unknown. The objective of this study was to detect the occurrence of West Nile virus (WNV), Usutu virus (USUV) and equine infectious anemia virus (EIAV) in the four Galapagos Islands raising equids if they were present at a prevalence >1%. Serum samples were collected from 411 equids belonging to 124 owners from April to July 2019. All the results were negative to the ELISA tests used suggesting that WNV, USUV and EIAV are not circulating in the equine population of the Galapagos Islands.

KEYWORDS

**WNV, USUV, EIAV, West Nile, Usutu, equine infectious anemia, Galapagos Islands, Ecuador**

## Introduction

The Galapagos Islands are a set of insular territories 960 km off the coast of Ecuador and an Ecuadorian province. They have been listed as a UNESCO World Heritage Site. Only 3% of their territory is colonized while the remaining 97% belongs to the Galapagos National Park. The introduction of livestock into the islands dates back from the beginning of the XIX century. In 1832, there was a massive introduction of productive domestic species, including equids, which may have carried parasites or pathogens (1). In 2003, animal importation from mainland Ecuador was banned, except for day-old chicks. Equids are present among the four colonized islands: Santa Cruz, Isabela, San Cristóbal and Floreana. Equid movements are allowed between the Galapagos Islands for animal genetic improvement. Surveillance of viral equid disease through laboratory diagnosis had never been conducted. Considering the movement of animals between mainland Ecuador and the Galapagos Islands in the past, the likelihood of the occurrence of viruses affecting the equids into the islands could not be excluded. Vector-borne viruses could have also been introduced by vectors coming from mainland.

West Nile virus (WNV) and Usutu virus (USUV) are vector–borne viruses that can affect equids and humans (2). They belong to the family *Flaviviridae*, genus *Flavivirus*, and are

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TABLE 1 Number of farms and equids sampled in the Galapagos Islands.

| Island | No. of farms sampled | No. of equids sampled | Number of equids sampled per farm (min.–max.) | Age (min.–max.) |
|---|---|---|---|---|
| Santa Cruz | 64 | 140 | 1–10 | 1 year–30 years |
| Isabela | 25 | 171 | 1–36 | 3 months–30 years |
| San Cristóbal | 29 | 80 | 1–10 | 3 months–20 years |
| Floreana | 6 | 20 | 1–11 | 3 months–19 years |
| Total | 124 | 411 | 1–36 | 3 months–30 years |

responsible for multiple outbreaks of disease in different countries of the world. WNV and USUV transmission cycles include wild birds as amplifying hosts and ornithophilic mosquitoes as vectors but can also infect and cause disease in horses and humans, which serve as incidental dead-end hosts (3, 4). WNV is one of the pathogenic agents that can lead to equine neurological clinical signs, although the infection is not usually accompanied by presentation of clinical illness (5). WNV is endemic in parts of Africa, Europe, the Middle East, and Asia, and since 1999 has spread to North America, Mexico, South America, and the Caribbean (6). WNV has been detected in mainland Ecuador through serological surveys in equids (7) and also in Colombia, an Ecuadorian neighboring country (8). USUV is particularly pathogenic in a few species of birds and has been detected in many mammalian species, including equids, considered to be dead-end hosts (9). USUV has not been reported in Latin America but in view of the emergence of different arboviral diseases, such as chikungunya and Zika, it can be considered as a new emerging threat (10).

Equine infectious anemia (EIA) is caused by a lentivirus and can be transmitted mechanically by biting flies (the virus does not replicate in the vector) (11). EIA virus (EIAV) infection leads to recurring episodes of fever in equids, thrombocytopenia, and wasting symptoms. EIA has been detected in different regions in Brazil (12–14) and Argentina (15) and several outbreaks were reported in different regions in mainland Ecuador (16).

The present study aimed to detect the occurrence of WNV, USUV and EIAV in the Galapagos Islands where equids are raised.

# Method

## Study population

The Galapagos veterinary services had estimated that around 650 equids are present in the islands of Santa Cruz (~ 230 equids), Isabela (~ 270 equids), San Cristóbal (~ 100 equids) and Floreana (~ 30 equids). The equids often belong to farmers who raise other species, mainly cattle, and are used for farm work, transportation or leisure purposes. There are approximately 132 equid owners in the four islands (63 in Santa Cruz, 34 in Isabela, 26 in San Cristobal, 9 in Floreana) and between one and 15 equids per farm.

## Sampling

For each island, the sample size was calculated to detect a 1% seroprevalence with a 95% level of confidence at the level of the equid population using Epitools.[1] This yielded animal sample sizes of 144 in Santa Cruz, 171 in Isabela, 79 in San Cristobal and 24 in Floreana. A mean number of three equids per farm was used to estimate the total number of farms to be included in the study. A minimum of 10 animals were sampled per farm and in farms with less than 10 animals, all animals were tested. The farms and the animals were randomly selected.

## Laboratory tests

Sera were tested using the commercial ID Screen West Nile competition multi-species ELISA kit (Innovative Diagnostics, Montpellier, France) according to manufacturer's instructions. This method, that uses pre-coated plates with the envelop (E) protein of WNV, estimates the competition between antibodies present in the animal serum and monoclonal anti-WNV E antibody conjugated to horseradish peroxidase (HRP). This ELISA, through cross-reactions, allows the detection of other flaviviruses belonging to the Japanese encephalitis virus serocomplex as USUV (17). Assays were interpreted according to Beck et al. (18).

To detect antibodies against EIAV, serum samples were screened using a commercial ELISA, Equine Infectious Anemia Virus Antibody Test Kit, ELISA v2 (VMRD, Pullman, USA). Assays were performed according to the manufacturer's instructions.

# Results

Serum samples were collected from 411 equids (367 horses, 20 donkeys and 24 mules) in 124 farms in the four islands (Table 1; Figure 1) from April to July 2019. The sampled animals were between 3 months and 30 years old.

All animals had negative results in the serological tests for the Flavivirus genus and EIAV.

# Discussion

The negative serological results obtained in this study suggest that the flavivirus that could affect equids, such WNV or USUV, and EIAV were not circulating in the Galapagos Islands above the prevalence value used to calculate the sample size for this study. This absence could go up to 30 years considering the age of some of the sampled equids.

1 https://epitools.ausvet.com.au/

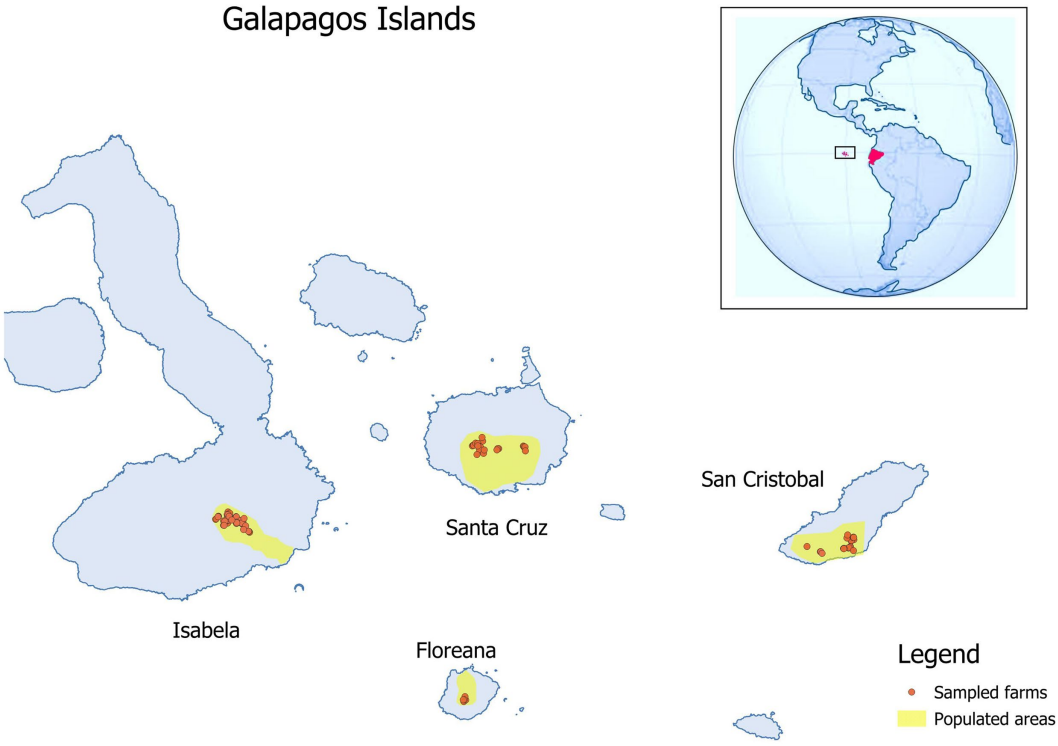


FIGURE 1
Location of the farms where equids where sampled to detect West Nile virus, Usutu virus and equine infectious anemia virus in 2019 in the Galapagos Islands. The georeferenced map was obtained in shape file format from the Geoportal of the Ecuadorian Military Geographic Institute and processed in Qgis 3.6.0 Noosa (QGIS.org, 2020).

The Galapagos Islands fill the climatic conditions favorable for the circulation of flaviviruses or EIAV provided the presence of competent vectors. The islands has three mosquito vectors capable of transmitting WNV and USUV (*Culex quinquefasciatus, Aedes aegypti, and Aedes taeniorhynchus*) (19, 20), bird species related to competent avian hosts (*Dendroica petechia*, *Mimus* spp) (21) and horseflies that can transmit EIAV (20). Since the climatic and vector conditions are filled, it could be argued that the prevalence viruses could be <1% in the equid population. However, in territories where those viruses are circulating, the prevalence values were higher than 1%. For example, WNV has been reported to be at seroprevalence values of 11% in horses in equids in Algeria (serums collected from 2015 to 2017) (22), 44.9% in Spain further to an outbreak in 2020 (23) or 31.6% in asymptomatic horses in Mexico (24). Analysis of 28,089 equids included in a systematic review and meta-analysis of seroprevalence studies of WNV in equids from 16 European countries between 2001 and 2018 revealed a pooled seroprevalence of 8% (25). In Morocco, prevalence of USUV was estimated to be 4% in a survey conducted in military working horses (26). In Spain, of 341 feral horses tested from 2010 to 2020, 9% were found seropositive to USUV (27). Regarding EIAV, in countries where this virus is circulating, prevalence values can be as high as 44% [survey conducted in Argentina (28)] but has also be found at lower values [2% in horses of the Para State in Brazil (14)]. On the other hand, according to the local veterinary services, there had been no reports of disease in equids in the Galapagos Islands consistent with WNV, USUV or EIAV or mortalities linked with WNV in birds.

The fact that the Galapagos Islands have semi-isolated conditions and the ban on animal importation do not provide sufficient guarantees to prevent the introduction of these viruses. Their introduction could arise by windborne transportation of infected vectors, infected migratory birds or infected vectors carried by airplanes or ships. These introduction routes were explored by Kilpatrick et al. (29) through a quantitative risk assessment to predict the WNV introduction into Galapagos Islands. These authors also included day-old chicks imports and mosquitoes in the larval stage present in shipment of tires as likely sources of WNV infection. They found that mosquitoes transported on airplanes carrying tourists represented the highest risk of WNV reaching the Galapagos Islands by a vector pathway and that the risk of introduction through migratory birds was lower but non-negligible. Moreover, it has been estimated that 107 Diptera species have arrived on the Galapagos Islands through human introductions and that 42 species have naturally colonized the islands from mainland Americas (20). Bataille et al. (30) showed from the monitoring of aeroplanes and genetic analysis that *C. quinquefasciatus* was regularly introduced via aircraft into the Galapagos islands. Therefore, effective control methods should be implemented by the national authorities to minimize the likelihood of introduction of insects through these routes that could put in danger endemic wildlife of Galapagos as well as domestic animal populations. Those measures could be combined with surveillance measures to detect any possible introduction. As the equid population is naïve to WNV and EIAV it is possible that that they could display clinical signs and, hence, equid owners should

be made aware of the importance to report the occurrence of unexpected clinical cases. Future serum sampling carried out for other diseases in equids could also be used to detect flavivirus or EIAV. Hence, equids could act as sentinels to provide early warning of virus circulation.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal studies were approved by Agencia de Regulación y Control de la Bioseguridad y Cuarentena para Galápagos (ABG), Puerto Ayora, Ecuador. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

GZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. CB: Formal analysis, Resources, Writing – review & editing. J-CV-C: Formal analysis, Resources, Writing – review & editing. MA: Formal analysis, Writing – review & editing. MC: Resources, Supervision, Validation, Writing – review & editing. AV: Resources, Supervision, Validation, Writing – review & editing. RV: Data curation, Validation, Writing – review & editing. GG: Formal analysis, Resources, Validation, Writing – review & editing.

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## 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.

## Publisher's note



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# Zoonotic pathogens in wild Asian primates: a systematic review highlighting research gaps

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**Introduction:** Ongoing global changes, including natural land conversion for agriculture and urbanization, modify the dynamics of human–primate contacts, resulting in increased zoonotic risks. Although Asia shelters high primate diversity and experiences rapid expansion of human–primate contact zones, there remains little documentation regarding zoonotic surveillance in the primates of this region.

**Methods:** Using the PRISMA guidelines, we conducted a systematic review to compile an inventory of zoonotic pathogens detected in wild Asian primates, while highlighting the coverage of primate species, countries, and pathogen groups surveyed, as well as the diagnostic methods used across the studies. Moreover, we compared the species richness of pathogens harbored by primates across diverse types of habitats classified according to their degree of anthropization (i.e., urban vs. rural vs. forest habitats).

**Results and discussion:** Searches of Scopus, PubMed, and the Global Mammal Parasite Database yielded 152 articles on 39 primate species. We inventoried 183 pathogens, including 63 helminthic gastrointestinal parasites, two blood-borne parasites, 42 protozoa, 45 viruses, 30 bacteria, and one fungus. Considering each study as a sample, species accumulation curves revealed no significant differences in specific richness between habitat types for any of the pathogen groups analyzed. This is likely due to the insufficient sampling effort (i.e., a limited number of studies), which prevents drawing conclusive findings. This systematic review identified several publication biases, particularly the uneven representation of host species and pathogen groups studied, as well as a lack of use of generic diagnostic methods. Addressing these gaps necessitates a multidisciplinary strategy framed in a One Health approach, which may facilitate a broader inventory of pathogens and ultimately limit the risk of cross-species transmission at the human–primate interface. Strengthening the zoonotic surveillance in primates of this region could be realized notably through the application of more comprehensive diagnostic techniques such as broad-spectrum analyses without *a priori* selection.

KEYWORDS

**human-primate interface, habitat type, infection diagnostics, pathogen specific richness, gastrointestinal parasites, virus, bacteria, protozoa**

## 1 Introduction

The expansion of human populations, coupled with natural habitat degradation, land-use change, and illegal hunting, have broken down the natural barriers between humans and

non-human primates (hereafter, primates), forcing the latter to increasingly live in human-modified environments (1, 2). Even though humans have always shared habitats with primates in some regions, the dynamics of human–primate interactions are radically changing and intensifying. As a result, increasing contacts and conflicts occur, representing a growing risk for zoonotic transmission and wildlife conservation (3, 4). More specifically, the zoonotic risk increases with changes in the dynamics of interactions following (i) the loss and fragmentation of natural habitats for agricultural and industrialization purposes, (ii) the expansion of road networks, and (iii) the greater urban demands for bushmeat and exotic pets, which exacerbate wildlife exploitation (1, 5, 6). Zoonotic pathogens can be transmitted naturally from vertebrate animals to humans, as opposed to reverse zoonotic agents, which are transmitted from humans to animals (7). A large proportion of the major human infectious diseases like measles, plague, or yellow fever, originate in animals, notably in domestic animals within temperate regions, or in non-human primates, the closest evolutionary relatives to humans, found in tropical regions (8). Nowadays, pathogens can spread more rapidly to new regions through international travel and commerce, and be transmitted to new susceptible hosts (9). The alteration of historical distribution patterns of pathogens, associated with the increasing spatial proximity between species, has allowed novel species to come into contact with new specific infectious agents, thus increasing the risk of epidemics (10, 11).

Emerging infectious diseases (EIDs), of which ~75% are of animal origin and are caused by infectious organisms, are characterized by a very large increase of new infections in a host population over a given period of time. This surge in infections likely leads to epidemics or pandemics (12). Recent epidemics such as coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and the Ebola virus disease, have stressed how knowledge about human–animal interactions and ecosystem health is essential to control the emergence and spread of zoonotic diseases (13). Thus, it is necessary to assess the factors influencing the risks for human and animal health (14). The process of disease emergence appears to be driven by recent changes in human demographics and behavior and by ecological disruption (15, 16). While habitat alteration typically leads to biodiversity loss (17, 18), the risk of pathogen transmission is strongly linked to the diversity of host species in an ecosystem (19). More specifically, the likelihood of the emergence of zoonotic pathogens depends on several factors, including the prevalence of zoonotic pathogens in wildlife reservoirs, the frequency and intensity of interspecies contacts, the effects of environmental changes on these reservoirs and vectors (e.g., modified geographic range of diseases following climate change), and the type of habitat (6, 20, 21). Currently, the influence of habitat degradation on the prevalence and diversity of infectious pathogens in wildlife is still debated, with conflicting findings reported (22, 23). Some studies have shown that habitat anthropization is negatively correlated with wildlife health, while other studies support that urban environments have no negative effect or even positive effects on animal health. Regarding primates, a study on parasite infection in toque macaques (*Macaca sinica*) and lion-tailed macaques (*Macaca silenus*) (24) living in (sub)urban habitats showed a higher parasite richness and prevalence compared with the populations living in undisturbed natural habitats. Conversely, in long-tailed macaques (*Macaca fascicularis*), it was found a lower prevalence and diversity of gastrointestinal (GI) parasites and protozoa in anthropogenic landscapes (25).

Two contrasting concepts in disease ecology describe the influence of biodiversity loss on the prevalence of pathogens in an ecosystem. The dilution effect implies that biodiversity may act as a barrier to the spread of infectious diseases. Indeed, in a diverse ecosystem, high species diversity may dilute the pool of host species that are competent for pathogens, including many poor reservoirs, thus reducing the persistence and transmission of some pathogens (26). Conversely, the amplification effect represents the scenario in which high biodiversity with diverse competent zoonotic reservoirs or vectors promotes the prevalence of more diverse pathogens and their transmission to humans (27). The predominance of those effects depends on many ecological factors, including the host community and the specific diseases. Nonetheless, it has been suggested that some synanthropic animal species that proliferate in human-dominated environments are more likely to be competent hosts for EIDs than others and, therefore, increase the risk of pathogen transmission to humans (28). Conversely, in less disturbed habitats, competent zoonotic reservoir hosts are less prevalent, and non-reservoir species predominate. Therefore, biodiversity loss in human-modified environments appears to increase the risk of human exposure to new or established zoonotic pathogens (26).

Humans tend to share a greater proportion of pathogens with primates compared with others animals, due to their genetic, physiological, and sometimes social similarities (29). Ebola virus and human immunodeficiency virus (HIV) are textbook examples of epidemic viruses that originated from primates (30–32). These epidemics illustrate how primates can be potential reservoirs of zoonotic infectious agents (33, 34). Zoonotic pathogens (viruses, bacteria, parasites, and fungi) can be transmitted between primates and humans via (in)direct contacts and several pathways (30). They may spread rapidly via direct host-to-host contacts (e.g., respiratory viruses) or by exchange of body fluids such as blood, urine, or saliva (e.g., herpesvirus B and simian foamy virus). GI parasites may enter hosts via exposure to shared contaminated environmental sources such as food, water, and soil. Pathogens can also be transmitted by vectors such as arthropods (e.g., *Plasmodium knowlesi* transmitted through the mosquitoes *Anopheles latens* and *A. hackeri*) (35).

The ongoing biodiversity crisis has taught us that primates are a particularly vulnerable group. Two thirds of primate species are threatened with extinction mostly due to anthropogenic pressures driving habitat loss, species exploitation, and emerging threats including zoonotic diseases (34, 36, 37). A recent interest has developed regarding the transmission mechanisms and the prevalence of zoonotic pathogens in primates that interact with humans (13, 38). The term "human–primate interface" encapsulates all aspects of the socio-ecological relationships linking humans and other primates together, that is, their dynamic interactions in shared environments (39). This interface is diverse. There are different degrees of habitat anthropization, such as urban settings, rural landscapes, and forest habitats, where multiple social and environmental factors may influence the likelihood of interspecies transmission of zoonotic pathogens (22, 40). Therefore, adopting a One Health transdisciplinary approach by recognizing the interconnected links between human, animal, and environmental health is particularly relevant in such interfaces (11, 41).

Asia represents a critical hotspot for zoonotic EIDs (42) given the high human population density combined with a large primate

community and frequent human–primate contacts (28). Especially in South and Southeast Asia, humans and primates increasingly overlap spatially and ecologically in cities, temples, and recreational parks. Generalist primate species such as the rhesus macaque (*Macaca mulatta*) or the long-tailed macaque (*M. fascicularis*) are synanthropic species frequently encountered in anthropogenic habitats due to their ecological and behavioral flexibility (2, 43). Notwithstanding the critical significance of the cross-species transmission risk at the human–primate interface (44), lacunae persist in the comprehension of host–pathogen dynamics and the compilation of zoonotic pathogens across primate species, notably within specific regions such as Southeast Asia (45). Few studies have sought to review zoonotic pathogens detected in free-ranging Asian primates. Balasubramaniam et al. (40) focused on gastrointestinal parasites in Asian macaques. In another recent review, Liu et al. (46) surveyed viral infections among primates worldwide, encompassing studies involving both captive and wild individuals. Yet, a holistic review requires the inclusion of diverse types of pathogens and all Asian primate species. In addition, there is still a need to better understand how the risk of infections is influenced by environmental factors, such as the type of habitat.

Thus, the primary objective of this systematic review is to compile an inventory of the zoonotic pathogens reported from free-ranging Asian primates, exploring the diversity of pathogens found across diverse habitat types (i.e., forest vs. rural vs. urban habitat) and their routes of transmission. Through this updated overview, we aim to investigating potential disparities in the current knowledge about pathogen groups surveyed among primate species, Asian countries, and diagnostic methods employed.

# 2 Methods

## 2.1 Data compilation

Using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) methods, we conducted a systematic literature search of papers up to December 2023 on zoonotic pathogens (i.e., bacteria, protozoa, viruses, fungi, and metazoan parasites) found in non-captive primates living in and native to Asia. Because our goal was to inventory zoonotic pathogens in free-ranging Asian primates and living in different habitat types, we did not include studies on captive or laboratory primates. We searched Scopus, PubMed, and the Global Mammal Parasite Database (47) with the following keywords and Boolean operators in abstract, title and keywords: "primate* OR monkey* AND pathogen* OR disease* OR zoono* OR infect* AND virus* OR parasite* OR bacteria OR fungi AND NOT captive* OR experimental OR zoo AND NOT Afric* OR Neotropic* AND NOT chimpanzee* OR gorilla* OR capuchin* OR baboon*." We also included additional records identified through other sources (based on article reading or the reference lists of the included studies). After identifying the articles, we screened them by eliminating studies using the following exclusion criteria: (1) The study was not performed on wild primates and native to Asia (papers included referred specifically to primate species ranging in three regions: South Asia, East Asia, and Southeast Asia). (2) The study did not search for at least one zoonotic pathogen (a pathogen was considered to be zoonotic if it is explicitly defined as zoonotic in the article or if it has been listed at least once as infecting humans in the literature). (3) The study did not provide information on the habitat type where the screened primates lived. (4) The study was written in a language other than English. (5) The study was a duplicate, not an original research article or reported same database. As for eligibility, we included all records that clearly indicated the species of the zoonotic pathogen, the host species, and the type of habitat where the host lives (Figure 1). In the end, we included a total of 152 studies in this review.

For each included study, we recorded the following information: (1) primate host species, (2) zoonotic pathogen taxa recorded, (3) main pathogen transmission route (i.e., respiratory, body fluid contact, vector borne, and fecal-oral route, determined according to the literature), (4) country where the study was performed, (5) type of habitat in which the host lives, and (6) diagnostic methods used to identify zoonotic pathogens. We distinguished between generic detection methods, which include microscopy, metagenomics, spectrometry and culture; and specific detection methods which encompass polymerase chain reaction (PCR), sequencing, serology, and isolation. As regard microscopy, our typology included three categories: (a) direct optic examination with staining, (b) direct optic examination without staining (only flotation and/or sedimentation), and (c) direct electron microscopy examination. Finally, we divided the zoonotic pathogens into six groups: viruses, fungi, bacteria, protozoa, and gastrointestinal metazoan parasites (hereafter, GI parasites) including Platyhelminthes (Cestoda and Trematoda) and Nematoda, and blood-borne parasites.

To compare pathogen diversity between habitat types, we classified the studies according to the degree of anthropization of the habitat in which the primates live. This level of anthropization was determined by relying on the habitat descriptions provided in the articles. Based on a simple system of landscape classification according to anthropogenic disturbance and land use, we distinguished between urban, rural, and forest habitats (48). Urban habitats, characterized by the highest anthropization degree, are defined as zones where human infrastructures prevail, such as towns, villages, temples, and gardens. Urban habitats are also characterized by the highest degree of spatial overlap between humans and primates. Rural habitats correspond to an intermediate degree of anthropization, including open areas (cropland and pastures), tree plantations, agroforestry, and small villages. In rural habitats, crop-feeding by primates is frequently observed. Finally, forest habitats include secondary forests that have undergone human disturbances such as fragmentation or logging, and more preserved forests in protected areas where human impact remains limited. Forest habitats have the least spatial overlap between humans and primates, and primates mainly feed on natural resources.

## 2.2 Data analysis

When a single study investigated several elements belonging to the same variable of interest (i.e., different taxa of zoonotic pathogens, different types of habitats, different species of host primates, different types of transmission routes, or different types of diagnostic methods), we considered each element as a separate study in the analysis. For example, we counted a study having screened protozoa and GI parasites as two separate studies in the analysis.

We used extrapolation of accumulation curves of species richness (49, 50) to quantify and statistically measure the differences across habitat types in the diversity of zoonotic agent species, while accounting for uneven sampling efforts (50). We used sample-based species accumulation curves to model the rarefaction curves, that is, the expectation of the

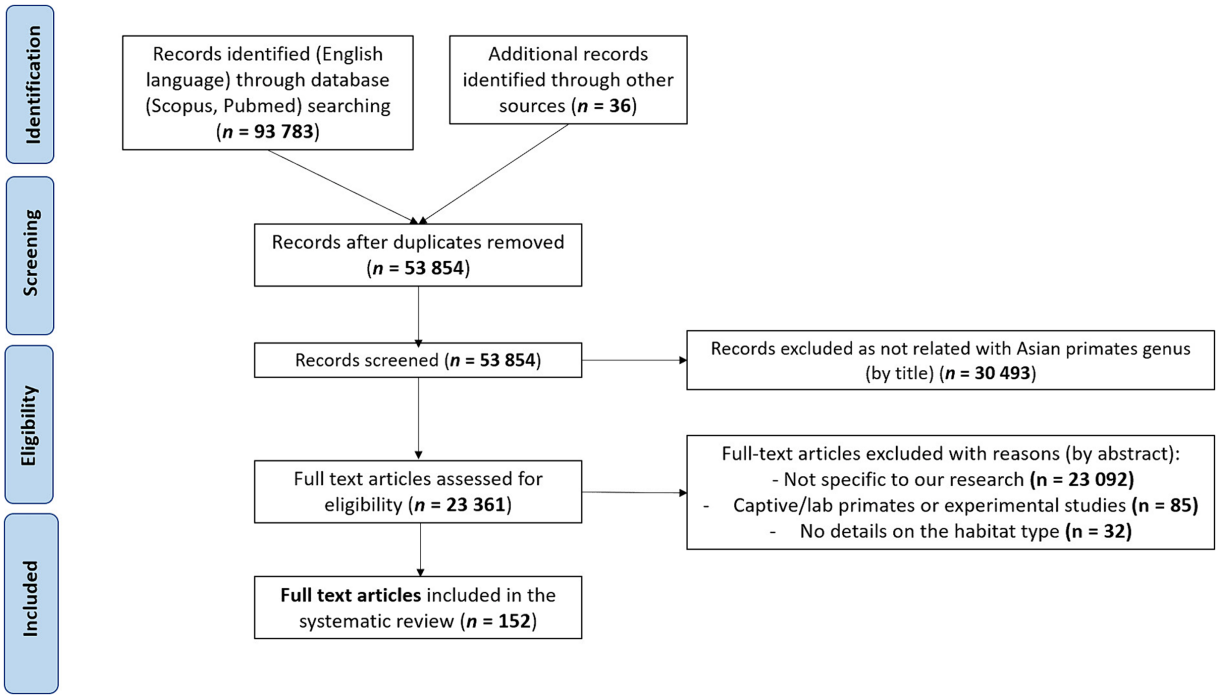


FIGURE 1
Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart describing the literature search and the selection of articles.

cumulative number of species for a given number of samples. The extrapolation of the rarefaction curve is based on a Bernoulli product model including a non-parametric estimator of total species richness and provides exhaustive species richness and confidence intervals (51). We performed this procedure for each type of habitat and each group of pathogens using the EstimateSWin.8.2 software (52). Due to limited number of studies for fungi ($N=3$ studies) and blood-borne parasites ($N=1$ study), we only conducted this analysis for GI parasites ($N=57$ studies), protozoa ($N=68$ studies), bacteria ($N=23$ studies), and viruses ($N=35$ studies).

# 3 Results

## 3.1 Overview of zoonotic pathogen reported in Asian primates

### 3.1.1 Sampling effort by country

Our search of the literature yielded 152 articles dating from 1965 to 2023 that studied zoonotic pathogens in free-ranging primates living in 15 Asian countries (Figure 2). Thailand and Indonesia were the countries with the highest number of studies ($N=25$ for each). Laos and Cambodia had the lowest number of studies ($N=1$ for each country). Finally, there were no studies for several primate-range Asian countries, including Pakistan, Bhutan, Afghanistan, Timor-Leste, and Vietnam (Figure 2).

### 3.1.2 Primate species

We identified an uneven distribution of studies on zoonotic pathogens across primate genera and species. Although 119 species of primates, belonging to 18 genera, are found in Asia (34), only 11 genera (61%) have been screened for zoonotic pathogens, for a total of 39 species (i.e., only 33% of the Asian species). The distribution of Asian primate species (34) screened across genera is as follows: *Macaca* sp. ($N=16$ species among 22), *Pongo* sp. ($N=2$ species among 3), *Presbytis* sp. ($N=5$ species among 17), *Semnopithecus* sp. ($N=3$ species among 8), *Trachypithecus* sp. ($N=5$ species among 20), *Nasalis* sp. ($N=1$ species among 1), *Nycticebus* sp. ($N=1$ species among 8), *Cephalopachus* sp. ($N=1$ species of 1), *Rhinopithecus* sp. ($N=1$ species among 5), *Tarsius* sp. ($N=1$ species among 13 species), and *Hylobates* sp. ($N=2$ species among 9). The genus *Macaca* has been the most studied primate genus, covering about 75% of the included studies (Figure 3). Among the macaque species, more than half of the studies (65%) were carried out on *M. fascicularis* and *M. mulatta*.

### 3.1.3 Diagnostic modalities

Regarding the methods used to detect pathogens, PCR (41% of studies, $N=73$ studies) and microscopy (32%, $N=56$) were the most frequent, followed by serology (16%, $N=28$). Among studies using microscopy to identify pathogens, different techniques were employed including (a) direct optic examination with staining (36%, $N=20$), (b) direct optic examination without staining (61%, $N=34$), and (c) direct electron microscopy examination, which was used in a few studies (4%, $N=2$). Conversely, other detection methods such as bacteriological culture/isolation, spectrometry, and metagenomics were rare (11%, $N=20$, including five studies using metagenomics). Generic detection methods were predominantly used in the detection of GI parasites (87%, $N=52$) and blood-borne parasites (100%, $N=1$). Conversely, specific detection methods were primarily employed for protozoa (60%, $N=50$), fungi (100%, $N=3$), bacteria (66%, $N=11$), and viruses (91%, $N=39$) (Figure 4). When examining the methods used to detect each group of pathogen, the results show a

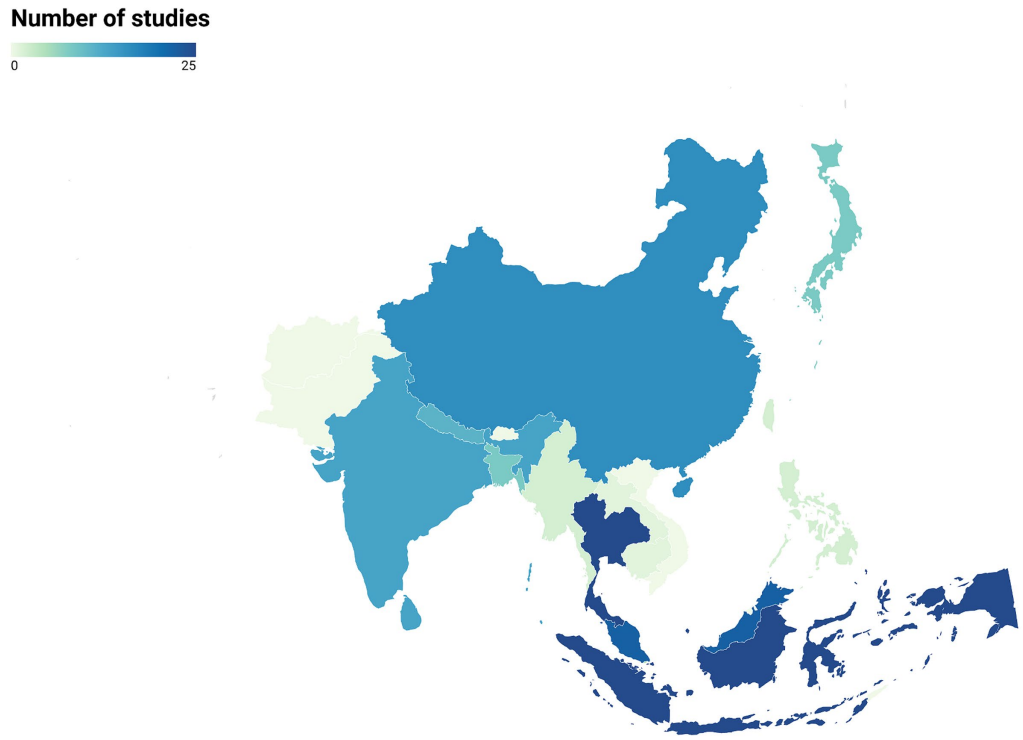


FIGURE 2
Distribution of studies on zoonotic pathogens in free-ranging primates across Asian countries ($N = 152$ articles).

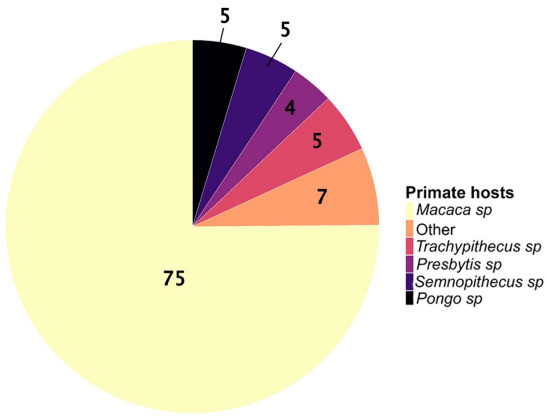


FIGURE 3
The proportions of wild Asian primate genera studied for zoonotic pathogens ($N$=193 studies). The "other" category corresponds to the sum of the proportions for the genera *Nasalis* sp., *Hylobates* sp., *Cephalopachus* sp., *Nycticebus* sp., *Rhinopithecus* sp., and *Tarsius* sp.

predominance of PCR in detection of fungi (100%, $N=3$), bacteria (41%, $N=13$), and protozoa (48%, $N=40$). Viral infections were primarily detected indirectly through serology (56%, $N=24$) or directly by PCR (33%, $N=14$). Microscopy, a generic method, remains essential for the identification of GI parasites (83%, $N=50$). Among these studies, 67% used direct optic examination without staining, 31% used direct optic examination with staining, and only 2% used direct electron microscopy examination. In microscopy-based studies on protozoa (38%, $N=32$), both direct optic examination with and without staining were equally utilized. By contrast, advanced generic methods such as metagenomics were used in very few cases, with only 2% of studies on GI parasites ($N=1$ study), 1% of studies on protozoa ($N=1$), and 7% of studies on viruses ($N=3$) (Figure 4).

### 3.1.4 Groups of infectious agents and transmission pathways

The review enabled us to highlight unequal sampling efforts between the groups of pathogenic agents screened (Figure 5A). Protozoa ($N=68$ studies) and GI parasites ($N=57$ studies) together represented more than two thirds of the pathogens screened in the studies (36 and 30%, respectively), while bacteria ($N=23$ studies) and viruses ($N=35$ studies) were less studied (12 and 19%, respectively). We found only three studies that screened for fungi and one study for blood-borne parasites. Regarding the transmission pathways, the most common route of transmission of the zoonotic pathogens screened in the studies was the fecal-oral route (62%, $N=104$) followed by the vector-borne route (23%, $N=38$), body fluid contact (11%, $N=19$), and the respiratory route (4%, $N=6$) (Figure 5B).

Gastrointestinal parasites and protozoa were found in the largest number of primate genus (in $N=10$ and $N=8$ genera, respectively). Conversely, fungi, blood-borne parasites, and bacteria were screened and reported in very few primate genera ($N=1$, 1, and 2, respectively). For example, *Nycticebus* sp., *Cephalopachus* sp., and *Tarsius* sp. have only been studied for GI parasites, with no other pathogen groups reported. In contrast, *Macaca* sp. has been extensively studied on various groups of pathogens: protozoa (37%, $N=58$ studies), GI parasites (26%, $N=40$), viruses (19%, $N=30$), bacteria (15%, $N=24$), and fungi (2%, $N=3$). Regarding the diagnostic methods used for pathogen detection, the results show a predominance of microscopy and PCR, although this varies by primate genus. For example, microscopy was the sole method used in studies on *Hylobates* sp.,

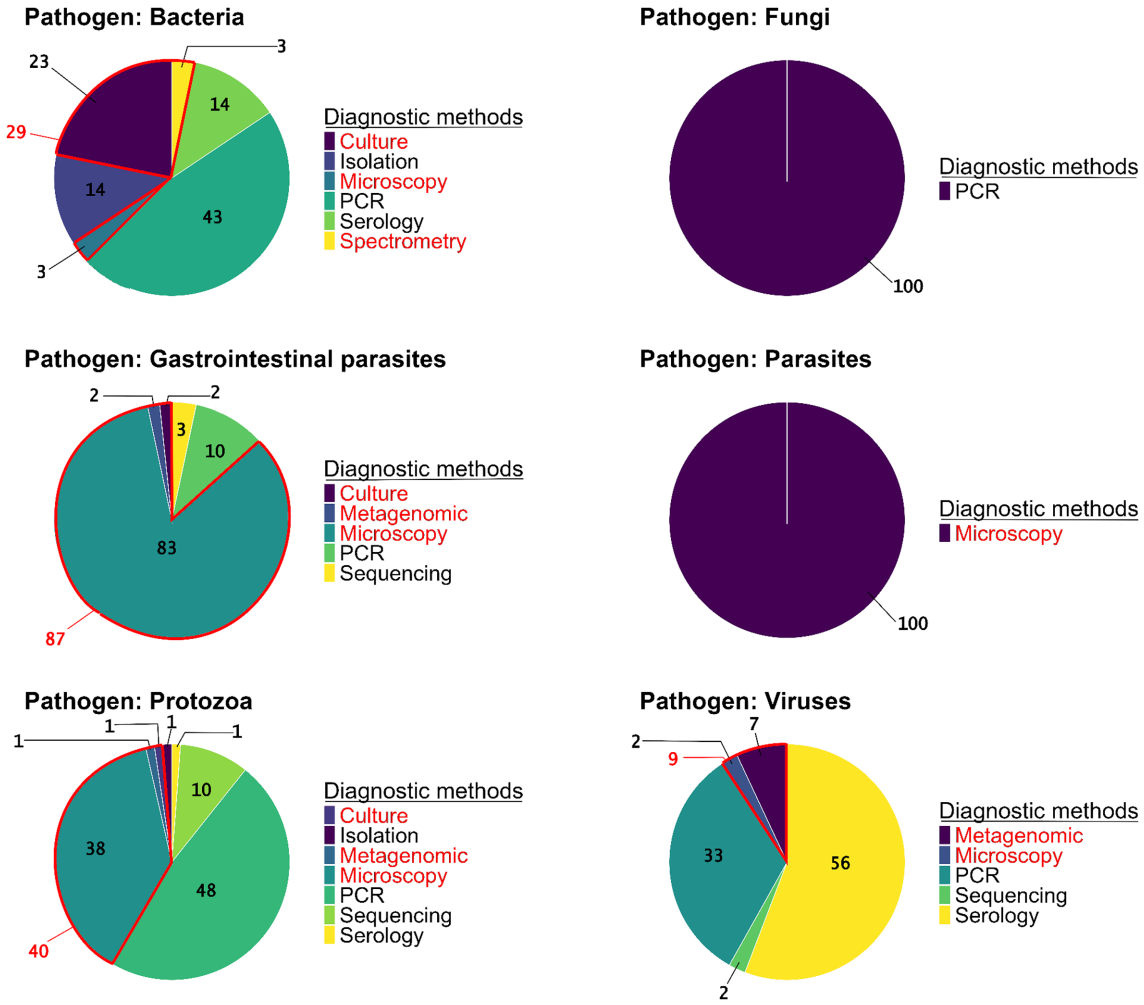


FIGURE 4
The proportions of pathogen detection methods used in the studies per pathogen group (*N* = 223 studies). Diagnostic methods are specified as generic (red) or specific (black).

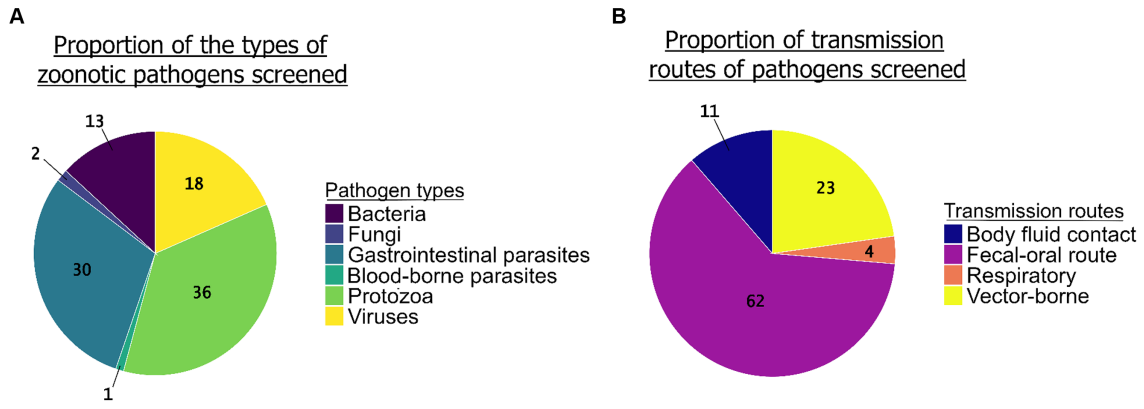


FIGURE 5
The proportion of groups of zoonotic pathogens screened (*N* = 190 studies) (**A**, left graph) and the proportion of their transmission routes (**B**, right graph) (*N* = 167 studies).

*Tarsius* sp., *Nycticebus* sp., and *Cephalopachus* sp. Microscopy was also the most commonly method used with *Nasalis* sp. (71%, *N* = 5 studies), *Pongo* sp. (36%, *N* = 4 studies), *Presbytis* sp. (60%, *N* = 3 studies), and *Trachypithecus* sp. (56%, *N* = 5 studies). Finally, PCR was also prevalent in studies on *Macaca* sp. (39%, *N* = 65 studies) and *Rhinopithecus* sp. (67%, *N* = 4 studies) (Table 1).

## 3.2 Inventory of zoonotic pathogens

### 3.2.1 Protozoa

Protozoa were the most studied zoonotic agents reported (*N* = 68 studies). Forty-two species of protozoa were identified, including 35 species transmitted by the fecal-oral route, which was the most common route (*N* = 47 studies), and seven species transmitted by the vector-borne route (*N* = 20 studies) (Supplementary Table S1). Among the vector-borne protozoa, the most common diagnostic method was PCR (*N* = 18 studies). The two genera of vector-borne protozoa reported were *Hepatocystis* sp. (*N* = 2 studies) and *Plasmodium* sp. (*N* = 18 studies). Studies mainly reported species belonging to the genus *Plasmodium* (*N* = 6 species). More specifically, *Plasmodium cynomolgi* and *Plasmodium inui* were detected in the largest number of host primate species, including *Macaca fascicularis*, *M. nemestrina*, *M. leonina*, *M. arctoides*, *M. sinica* (only for *P. cynomolgi*), *M. radiata*, and *Presbytis entellus* (only for *P. cynomolgi*) (53–66). In addition, *Plasmodium falciparum* was detected in *M. radiata* and *M. mulatta* (56), while *P. knowlesi* was detected in *M. fascicularis*, *M. nemestrina*, and *M. arctoides* (53, 54, 57, 59–61, 64–69). These two zoonotic *Plasmodium* species are known to cause severe cases of malaria in humans. In fact, *Plasmodium falciparum* is responsible for the most severe and deadly forms of malaria, with complications such as severe anemia, coma, and multi-organ failure (70). *Plasmodium knowlesi*, which has recently been recognized as a human pathogen, can also cause severe clinical symptoms including respiratory and renal failure. However, most cases respond well to prompt treatment (71).

### 3.2.2 GI parasites

Helminthic GI parasites were the second most studied pathogens (*N* = 57 studies), with a total of 63 species that have been reported in all habitat types. Nematodes were the most detected helminthic GI parasites: indeed, of the 63 species described, 42 were nematodes (Supplementary Table S2). Certain species of nematodes were found in many host primates and reported in several studies. This is the case for *Strongyloides* sp., detected in 19 primate species in 33 studies; *Trichostrongylus* sp., detected in 17 primate species in 23 studies; and *Trichuris* sp., reported in 19 primate species in 32 studies (24, 25, 72–112). Concerning the other species of helminthic GI parasites, eight species of cestodes (i.e., *Diphyllobothrium* sp., *Dipylidium caninum*, *Echinococcus* sp., *Hymenolepsis* sp., *Hymenolepsis diminuta*, *Hymenolepsis nana*, *Moniezia* sp., and *Taenia* sp.) have been reported (24, 25, 85, 86, 91, 92, 95, 97, 99, 100, 102, 106, 108, 109, 113–115) (Supplementary Table S3), and a total of 13 species of trematodes were detected in all different habitat types (Supplementary Table S4).

### 3.2.3 Viruses

In total, 45 species of zoonotic viruses were reported in Asian primates of the included studies (Supplementary Table S5). Viruses represented the third most studied type of zoonotic pathogens (*N* = 35 studies). The majority of viruses found in urban habitats are transmitted by body fluid contact (*N* = 9 viruses) (116–123). Regarding diagnostic modalities, most studies on viruses used serological methods (*N* = 24 studies) or PCR (*N* = 14 studies). Simian foamy virus (116–120, 123), Japanese encephalitis virus, and dengue virus (74, 124–130) were the most studied viruses (*N* = 6, *N* = 5, and *N* = 5 studies, respectively). However, dengue and chikungunya were the viruses reported in the largest number of primate species (five primate species for each virus).

### 3.2.4 Bacteria, fungi, and blood-borne parasites

We found few studies screening for bacteria, fungi, and blood-borne parasites (*N* = 23 studies, *N* = 3 and *N* = 1, respectively). Regarding zoonotic fungi, only *Enterocytozoon bieneusi* was detected by PCR in *M. mulatta and M. assamensis* (131–133). Two genera of blood-borne pathogens *Brugia* sp. and *Wuchereria* sp. were found by microscopic analyses in *Presbytis cristatus* (112). For bacteria, a total of 30 species were reported, but in only three primate genera, i.e., *Pongo* sp. (*N* = 1 study), *Trachypithecus* sp. (*N* = 1 study), and *Macaca* sp. (*N* = 24 studies) (Supplementary Table S6). The bacterium *Escherichia coli* found in *M. mulatta, M. fascicularis*, and *M. fuscata* (38, 88, 134–136), along with *Staphylococcus aureus* found in *M. mulatta*, *M. fascicularis*, and *Trachypithecus cristatus* (80, 137–140) were the most common bacteria (*N* = 5 and *N* = 5 studies, respectively). Apart from the typical oral-fecal route, there were two bacterial species transmitted via the vector-borne route—*Bartonella quintana*, a zoonotic bacterium that causes fever in humans and was found in the Japanese macaque (*M. fuscata*) and *M. fascicularis* (141, 142), and *Candidatus Mycoplasma haemomacaque* in *M. fascicularis* (143)—and two bacterial species transmitted by the respiratory route, namely *Mycobacterium tuberculosis* in *M. mulatta* and *M. fascicularis* (144) *and Streptococcus* sp. in *M. fascicularis* (145–147).

## 3.3 Comparison of pathogen specific richness between habitat types

Most studies were conducted in forest habitats (*N* = 107 studies), followed by urban habitats (*N* = 75 studies) and rural habitats (*N* = 43 studies). From a descriptive perspective, comparison of the accumulation curves and the associated rarefaction curves highlighted a lower species accumulation trend in urban habitats for GI parasites, and in forest habitats for protozoa (Figure 6). A higher exhaustive species richness of GI parasites was found in forest habitat compared with the other two habitats. We also found a reduced protozoan richness in forest habitat and the highest richness in urban habitat (Figure 6). Regarding bacteria, species richness was the highest in urban habitat and the lowest in rural habitat (Figure 7). Finally, viruses were unfrequently reported in rural habitat (Figure 7). Despite these trends, the confidence intervals of the rarefaction curves showed broad overlap among the habitat types for all pathogen groups, which suggest no statistical difference in the predominance of zoonotic species between urban, rural, and forest habitats. Additionally, all pathogen groups (except Protozoa in forest habitat) displayed non-asymptotic accumulation and rarefaction curves (Figures 6, 7). This indicates that the saturation level (i.e., exhaustivity) for pathogen taxa was not reached in any habitat type, supporting that the sampling effort was insufficient to extrapolate differences, and calling for further surveillance to identify more pathogens.

TABLE 1 Percentage (and number) of studies relative to each zoonotic pathogen group, and each diagnostic method, per Asian primate genus.*

| Primate genus | Pathogen group % (no.) | | | | | | Diagnostic method % (no.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PGI | Protozoa | Viruses | Bacteria | Fungi | Blood-borne parasites | Culture | Metagenomic | Microscopy (a, b, c) | Spectrometry | Isolation | Serology | PCR | Sequencing |
| *Macaca sp.* | 26 (40) | 37 (58) | 19 (30) | 15 (24) | 2 (3) | 0 | 7 (11) | 2 (3) | 27 (44) | 1 (1) | 4 (6) | 15 (25) | 39 (65) | 6 (10) |
| *Presbytis sp.* | 44 (4) | 33 (3) | 11 (1) | 0 | 0 | 11 (1) | 0 | 20 (1) | 60 (3) | 0 | 20 (1) | 0 | 0 | 0 |
| *Trachypithecus sp.* | 60 (6) | 20 (2) | 10 (1) | 10 (1) | 0 | 0 | 0 | 0 | 56 (5) | 0 | 11 (1) | 0 | 22 (2) | 11 (1) |
| *Semnopithecus sp.* | 43 (3) | 29 (2) | 29 (2) | 0 | 0 | 0 | 0 | 0 | 57 (4) | 0 | 0 | 0 | 43 (3) | 0 |
| *Pongo sp.* | 50 (7) | 36 (5) | 14 (2) | 0 | 0 | 0 | 9 (1) | 0 | 36 (4) | 0 | 0 | 18 (2) | 27 (3) | 9 (1) |
| *Rhinopithecus sp.* | 0 | 50 (2) | 50 (2) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 17 (1) | 67 (4) | 17 (1) |
| *Nasalis sp.* | 86 (6) | 14 (1) | 0 | 0 | 0 | 0 | 0 | 0 | 71 (5) | 0 | 0 | 0 | 14 (1) | 14 (1) |
| *Hylobates sp.* | 50 (1) | 50 (1) | 0 | 0 | 0 | 0 | 0 | 0 | 100 (2) | 0 | 0 | 0 | 0 | 0 |
| *Tarsius sp.* | 100 (1) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 (1) | 0 | 0 | 0 | 0 | 0 |
| *Nycticebus sp.* | 100 (1) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 (1) | 0 | 0 | 0 | 0 | 0 |
| *Cephalopachus sp* | 100 (1) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 (1) | 0 | 0 | 0 | 0 | 0 |

Diagnostic methods are specified as generic (blue) or specific (red). Microscopy diagnostic methods include: (a) direct optic examination with staining, (b) direct optic examination without staining (only flotation and/or sedimentation), and (c) direct electron microscopy examination. Asian primate genera for which no studies have been found on zoonotic pathogens include *Pygathrix* sp., *Hoolock* sp., *Nomascus* sp., *Symphalangus* sp., *Simias* sp., *Loris* sp. and *Carlito* sp.





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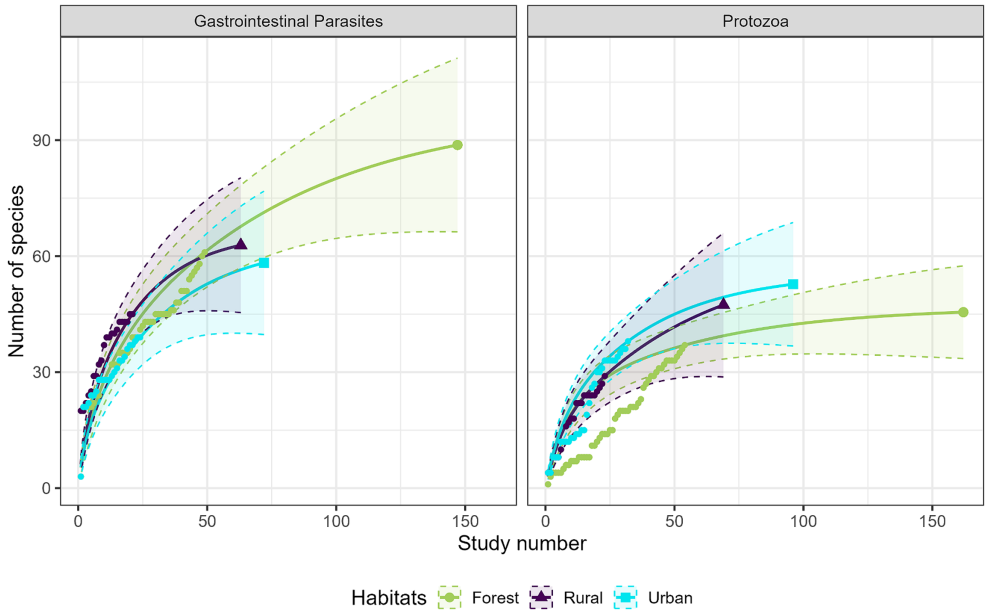


FIGURE 6
Comparison of the species accumulation curves (dotted line) and the extrapolated species rarefaction curves (solid line with confidence intervals) between urban, rural, and forest habitats for gastrointestinal parasites (left graph) and protozoa (right graph).

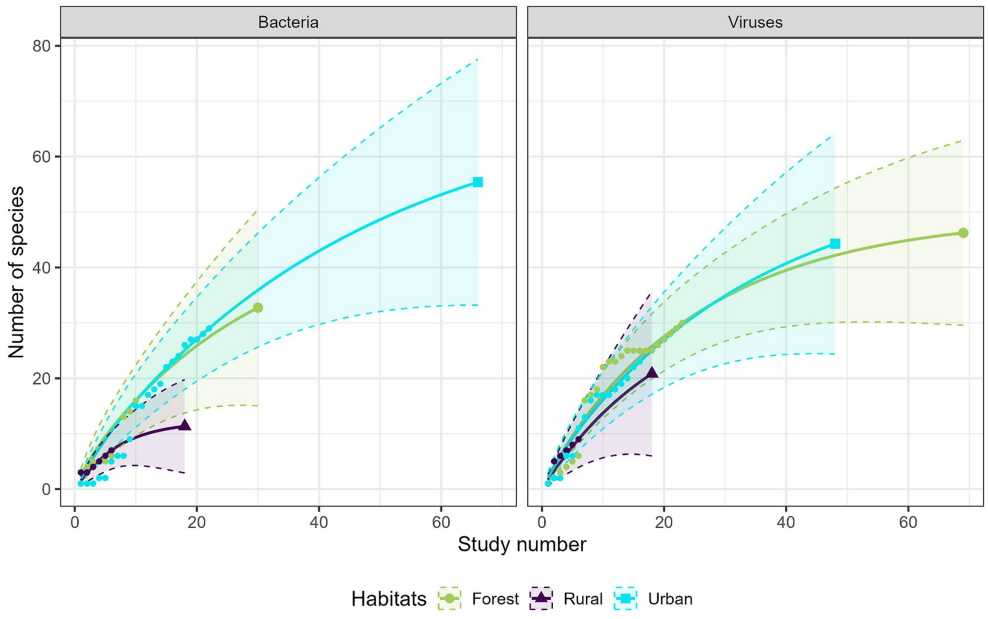


FIGURE 7
Comparison of the species accumulation curves (dotted line) and the extrapolated species rarefaction curves (solid line with confidence intervals) between urban, rural, and forest habitats for bacteria (left graph) and viruses (right graph).

## 4 Discussion

The COVID-19 pandemic has been a reminder of the paramount importance of zoonotic diseases for global health (148). Many zoonoses originate from or have as reservoirs non-human primates (30, 149). Asia has been the origin of several suspected zoonotic transmission events over the past decades (e.g., previous SARS outbreaks in the 2000s, Nipah virus in 1998, and H5N1 avian influenza virus in the 2000s); underscoring the critical role of the region in the transmission dynamics and the emergence of zoonotic diseases (150). Surveillance of wildlife hosts and potential reservoirs is a crucial initial step to mitigate the risk of future pandemics (13). Given their genetic similarity to humans, non-human primates are important potential reservoirs of zoonotic infections (151). To limit the risk of

transmission, it is essential to identify and document the zoonotic pathogens carried by free-ranging primates that often lead to interact with humans. In this review, we conducted the first comprehensive inventory of the various groups of zoonotic pathogens identified in non-captive Asian primates in urban, rural, and forest habitats. By doing so, we have highlighted current research gaps regarding zoonotic pathogens in wild Asian primates, focusing on coverage in primate taxonomic hosts, pathogen groups, and diagnostic methods. As an example, we could not draw a robust conclusion about the potential differences in pathogen-specific richness across habitat types due to insufficient research efforts.

Our review included 152 studies on zoonotic pathogens encompassing reports on 39 primate species from 15 Asian countries. This sampling represents only 33% of the extant primate species found in Asia (34). Hence, a small number of species, especially those of the *Macaca* genus, are oversampled in infectious disease studies, while many others are disregarded such as the doucs (*Pygathrix* sp.) or some gibbons (*Hoolock* sp. and *Nomascus* sp.). This result confirms a significant taxonomic bias of sampling in the scientific literature that has been raised previously. In their 2007 review, Hopkins and Nunn (45) examined research on infectious agents in primates throughout the world, and found that African primates were sampled twice as much as Asian primates. The disparities we found across Asian primate taxa could be explained by several factors. Sampling in infectious disease research is influenced by the geographic range and the locomotion mode of the primate species (152). Widespread and semi-terrestrial species are sampled more frequently than geographically restricted and strictly arboreal species. Consistently, the overrepresentation of *M. fascicularis* and *M. mulatta* may be ascribed to their extensive distribution range across Asia compared with other primate species (153). Moreover, *M. fascicularis* and *M. mulatta* are conspicuous and often found in anthropogenic habitats where they are more terrestrial, making their access and sample collection easier compared with elusive species in remote areas (154). Interestingly, while *Semnopithecus entellus*, a terrestrial species that often inhabits human-modified environments (2, 155) would represent an easy and relevant candidate for sampling and assessing zoonotic risks, it was underrepresented in our dataset, comprising only 2% ($N = 5$ studies) of the studies. Conversely, survey effort has been greater for emblematic and threatened species such as orangutans (*Pongo* sp.) ($N = 10$ studies, 5%). Furthermore, since this review primarily examined English-language publications, it could be advisable for future bibliographic searches to encompass literature published in Oriental languages, with careful consideration of potential publication biases.

The under-sampling of many primate taxa in surveillance studies may pose a public health risk as these primates are potential unknown reservoirs of zoonotic pathogens. This risk is exacerbated by the context of the growing demand for bushmeat and wildlife products, which is also observed in Asia (156, 157). The growing threat of illegal hunting has implications for primate conservation and human health as it intensifies the potential for the circulation of zoonotic pathogens (158, 159). As evidenced by several examples in Africa, the manipulation and consumption of primate meat facilitates the transmission of zoonotic diseases to humans, resulting in dire consequences, such as the emergence of HIV or Ebola virus outbreaks (30, 149). In Asia, although lorises (*Loris* sp. and *Nycticebus* sp.) and tarsiers (*Tarsius* sp., *Cephalopachus* sp., and *Carlito* sp.) are widely traded as pets, presenting risks of zoonotic transmission (160, 161), there are still very few infectious disease studies on these species, with the exception of a small fraction screening for GI parasites.

Another publication bias underscored by this review concerns the uneven allocation of sampling efforts regarding the types of screened pathogenic agents and studied transmission routes. Overall, we found that the focus of most empirical studies that examined zoonotic pathogens in free-ranging Asian primates was on protozoa and GI parasites, with most of the identified agents transmitted through the fecal-oral route. Concerning GI parasites, nematodes such as *Strongyloides* sp., *Trichostrongylus* sp., and *Trichuris* sp. were the most reported infectious agents. Viruses, bacteria, blood-borne parasites and fungi have been documented less frequently in the literature on Asian primates. These results are consistent with Hopkins and Nunn (45) and Cooper and Nunn (152) studies, who showed that helminths are the most commonly studied pathogens in primates, while bacteria, viruses, and fungi are the least investigated infectious agents. However, it is important to note that most GI parasites have frequent asymptomatic carriage in wildlife, which may not always reflect a significant health risk for animals or humans (162, 163). The oversampling of pathogens transmitted by the fecal-oral route, such as *Strongyloides* sp. and *Entamoebas* sp., could be explained by logistic and ethical constraints related to the sample matrix necessary for diagnosis. Indeed, fecal samples collected noninvasively from the ground are an easy and conventional tool for evaluating zoonotic pathogens in primates, in particular GI parasites (164). Given the vulnerable status of many primate species and ethical restrictions, it may be difficult to obtain authorizations to collect blood or other body fluid samples in the wild (165). Therefore, although molecular techniques with fecal samples can be used to identify diverse types of agents such as blood-borne pathogens (166), microscopy allowing researchers to identify macroparasites and protozoa are commonly used in the field. In future studies, it would be beneficial to expand surveillance strategies through other types of non-invasively collected sample matrices such as saliva, hairs or urine, which can also be gathered without harming the animals (167). These alternative samples could provide valuable insights into a broader range of pathogens, including viruses and bacteria or those difficult to detect through fecal sample analysis, thus enhancing our understanding and management of zoonotic diseases.

So far, PCR, microscopy, and serology have been the most prevalent methods used in studies on Asian primate infections. For all pathogens, except GI parasites and blood-borne parasites, more than half of the studies used pathogen-specific detection methods requiring an *a priori* selection of the pathogens potentially present in the population. Even though microscopy is a generic detection method (that is sometimes supplemented by more specific detection methods such as PCRs to allow the identification beyond the genus level), it is mainly used for the detection of GI parasites and protozoa. The predominance of those pathogen-specific methods likely skewed the true representation of the infectious agents. Indeed, while sensitive, specific, and efficient methods such as real-time polymerase chain reaction (qPCR) are routinely used for known pathogens, the identification of emerging or unknown pathogens is more challenging (168). In this regard, the *de novo* metagenomics approach has proved to be a powerful new tool with infinite fields of application (169). For example, many novel and divergent viruses can be detected simultaneously and genetically characterized for the first time (170).

In addition, metagenomic analyses of the microbial community also provide important insights and tools to monitor the health and nutritional status of primates and thus contribute to primate conservation (171). Generic next-generation sequencing approaches, through a wide variety of samples (i.e., feces, blood, nasal swab, saliva, and biological tissues), are likely to shed light on little known or novel zoonotic pathogens in primates (169) such as the ChiSCVs virus detected in stool samples of wild chimpanzees (172) or the Primate Bocaparvovirus Species 3 discovered in wild rhesus macaques (173). However, the relatively limited use of generic methods can likely be attributed to the high cost associated with next-generation sequencing techniques. Primate conservation research in developing countries is often a low priority (174), given the growth needs of local populations and the lack of technical resources and funding (175). Despite improved efforts in recent years, there remains a lack of international collaboration, which reduces opportunities for local research, capacity building, and access to cutting-edge technologies needed to improve the detection of zoonotic EIDs and the underpinning mechanisms (175, 176).

Based on the existing literature, we were unable to confidently determine whether the type of habitat influences the diversity of zoonotic agents that infect wild Asian primates. With respect to protozoa, bacteria, viruses, and GI parasites, even though their specific richness did not show significant variations across forest, rural, and urban habitats, the inadequacy of sampling effort is apparent from the absence of asymptotes in the rarefaction curves. Yet, anthropogenic disturbances such as forest degradation and land-use conversion are suspected to deeply interact with infectious diseases in primates, notably through a multiplication of direct and indirect contacts with humans and domestic animals (30). In rhesus macaques, habitat attributes correlated with host density and appeared to be a significant determinant of GI parasite infections. Parasitic richness was higher in large macaque groups interacting with human communities and livestock in (peri-)urban habitats, although parasitic prevalence was higher in rural habitats (95). Consistently, the prevalence of *Salmonella* sp. and *E. coli* was higher in provisioned groups of rhesus macaques interacting with humans in anthropogenic habitats (38). Conversely, another study in Indonesia demonstrated that anthropogenic landscape components decreased the prevalence and intensity of GI parasites in long-tailed macaques, probably due to good nutritional conditions following heavy food provisioning near human settlements (25). The prevalence and risk of transmission of viruses transmitted through physical contact or aerosols are expected to be higher in urban habitats such as at touristic sites and temples in Asia (116, 118, 119), where close and frequent human–primate contacts are common (101). In rural landscapes, agricultural practices, such as the use of antibiotics, can also contribute to drug resistance of bacteria and therefore increase their prevalence in primates (135). Conversely, other several studies on African primates have shown a higher richness and prevalence of GI parasites in populations from disturbed forests compared with more preserved habitats (177, 178). Finally, the prevalence of vector-borne pathogens, such as protozoa responsible for malaria, may be increased by forest degradation and associated changes in vector (anopheline mosquitoes) and host (*Macaca* sp.) density (179, 180).

Nevertheless, it is important to acknowledge that the descriptive categorization of habitats based on site descriptions we used in this review to delineate three categories (i.e., urban, rural, and forest), may entail certain limitations in the results. A more empirical approach using satellite images of land cover could potentially provide a more accurate representation of the environmental complexity by considering finer variations in land use and thereby capturing a broader spectrum of anthropogenic influences on primate habitats. In sum, the influences of anthropogenic components on primate infections appear complex. Urgent additional comparative studies are needed to investigate changes in primate-pathogen dynamics in rapidly changing environments, particularly among primate populations inhabiting habitats with varying degrees of human disturbances (30, 40).

## 5 Conclusion

The different biases highlighted in this literature review warrant further investigation, particularly on the under-screened primate species and on a wider range of etiological agents by using generic diagnostic methods. Primates are good candidates as sentinels for the surveillance of zoonotic diseases, particularly in Asia, where their close spatial proximity to humans is rapidly increasing. This endeavor requires researchers to address knowledge gaps regarding the risks and mechanisms associated with zoonotic transmissions. For example, it would be promising to improve our understanding of the behavior and socio-ecology of synanthropic primates. So far, few studies have focused on the risk factors of disease transmission associated with primate social dynamics, personality traits, and risk-taking behaviors promoting contacts with humans, domestic animals, or shared resources (40). It is worth emphasizing that such knowledge into primate health and the mechanisms of disease transmission also has substantial implications for primate conservation (171).

A One Health conceptual approach grounded in multidisciplinary collaborations is crucial for conducting action research on the emergence and transmission of zoonoses (181, 182). Establishing effective preventive measures requires a targeted surveillance of potential zoonotic reservoirs to identify mechanisms and risk factors of EIDs, and to raise awareness among populations about zoonotic risks. Today, considering previous sanitary crises associated with wildlife reservoirs [e.g., Ebola, Middle East respiratory syndrome (MERS), and COVID-19], it is essential to draw on the lessons that have been learned to make informed decisions. Prioritizing preventive measures, such as identifying infection reservoirs, implementing surveillance, and communicating risks, is advised over reactive measures like implementing physical barriers and restricting human populations in response to zoonotic outbreaks.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

## Author contributions

LP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft. AH: Formal analysis, Methodology, Software, Supervision, Validation, Writing – review & editing. SA: Validation, Writing – review & editing. MG: Conceptualization, Funding acquisition, Methodology, Project

administration, Supervision, Validation, Writing – review & editing. FB: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

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## 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.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1386180/full#supplementary-material

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# Development and optimization of sampling techniques for environmental samples from African swine fever virus-contaminated surfaces with no organic contaminants

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African swine fever (ASF) is a highly contagious diseases in domestic pigs and wild boars with up to 100% mortality. ASF virus (ASFV) is a causative agent responsible for ASF and highly resistant in environments, which creates a significant challenge for the control and eradication of the virus. Despite the geographical expansion of ASFV and international movement of products to sustain the swine production system, there is limited knowledge on the use of environmental samples to perform surveillance to prevent the introduction of ASFV into ASFV-free areas and for control of transmission in affected areas. Therefore, this study aimed to develop and optimize sampling techniques for environmental samples for ASFV detection. The stainless steel surfaces were contaminated with ASFV-infected blood, swabbed using different devices, and then processed through different techniques. The environmental samples were processed and tested using qPCR analysis. The results showed that the use of pre-moistened gauze surgical sponges, sweeping pads, and sponge sticks resulted in increased sensitivity, when compared to either dry sampling devices or Dacron swab. In particular, the combination of the sponge stick and the commercial nucleic acid preservative supported the best detection of ASFV DNA on the clean stainless steel surfaces evaluated. Pre-incubation for the short period of time and centrifugation at low speed were sufficient to provide satisfactory diagnostic sensitivity of ASFV detection using qPCR for environmental samples. Our findings contribute to the development of techniques for environmental samples for ASFV surveillance to prevent the introduction and dissemination of ASFV.

KEYWORDS

**African swine fever, ASFV, environmental sample, fomite, surface**

# 1 Introduction

African swine fever is a WOAH-reportable disease and fatal disease with up to 100% mortality in domestic pigs and wild boars. African swine fever virus (ASFV) is a causative agent and only member of family Asfaviridae. It is a double-stranded DNA virus, and its genome size is 150–180 kbs in length, encoding 150–180 proteins (1). Since its first identification in Kenya in the early 1900's, the virus exists in the wild in Africa and is maintained in the sylvatic cycle where the virus is transmitted between ticks and warthogs (2). In addition, modes of ASFV transmission to domestic pigs include direct contact to infected pigs or biological vectors, the consumption of ASFV-contaminated meat and/or carcasses and the exposure to ASFV-containing fomites. After the introduction of the genotype II ASFV into the Caucasian region (3), possibly through swill feedings in 2007, wild boars have been responsible for spreading ASFV gradually to Europe over the decade (4). Once the virus was first detected in China in 2018, the virus spread rapidly to the neighboring Asian countries. Given the geographical expansion in the short period of time, ASFV transmission could be mediated by human activities including the movement of humans, live domestic pigs, and/or fomites (5). In 2021, ASFV was detected in the Dominican Republic and spread to nearly all provinces in the country. Eventually, the virus was officially confirmed in the neighboring country, Haiti (6). This epidemiological situation poses a major threat to the US swine production system given the close geographical proximity to the mainland of North America.

Early detection is crucial to prevent the introduction and further spread of ASFV. The most common type of diagnostic samples includes animal-origin samples, such as whole blood, serum, swab and tissues, thus, the protocol for these types of samples has been well-established to ensure high sensitivity and specificity (7, 8). In contrast, despite the prolonged persistence in environments and the potential role of fomites in ASFV transmission (9), our knowledge of environmental samples remains still limited. Therefore, this study aimed to develop and optimize the sampling and processing of the environmental samples for better understanding prevention of ASFV introduction and control of ASFV transmission.

# 2 Materials and methods

## 2.1 Virus

The Georgia07 strain of ASFV was used in this study. Whole blood was collected from an experimentally infected pig in a previous animal study using an ETDA tube and stored at −80°C until the experiment was conducted. The virus titer of EDTA blood was $1.36 \times 10^8$ $TCID_{50}$/mL.

## 2.2 Experiment 1

A total of 16 drops of EDTA blood were added onto 10 × 10 cm stainless steel surfaces and dried for 30 min. Each drop was 6.25 μL and total inoculum was 100 μL per each surface. Each sampling strategy was applied to a separate stainless steel coupon, each with no organic material present, with a total of 5 replications for each sampling strategy. In total, eight different sampling strategies were tested: (1) 10 × 10 cm of dry cotton gauze (Dynarex Corporation, Orangeburg, NY, USA), (2) 10 × 10 cm of wet cotton gauze premoistened with 5 mL of phosphate buffered saline (PBS), (3) 10 × 10 cm of dry sweeping pad (Swiffer, P&G, OH, USA), (4) 10 × 10 cm of wet sweeping pad premoistened with 7.5 mL of PBS, (5) sponge stick containing 10 mL neutralizing buffer (Cat. #SSL10NB, 3M, St. Paul, MN, USA), (6) sponge stick (Cat. #SSL100, 3M, MN, USA) premoistened with 10 mL DNA/RNA shield (Zymo Research, Irvine, CA), (7) dry Dacron swab (Puritan Medical Products, Guilford, ME, USA), and (8) wet Dacron swab premoistened with 2 mL of PBS. A disposable tweezer was used to hold the cotton gauze and sweeping pad when swabbing. After swabbing on surface, the cotton gauze and the sweeping pad were placed in the 50 mL conical tube, the sponge stick in a sample bag, and the Dacron swab in a 2 mL cryovial. PBS was added into the tubes; 25 mL for (1) dry cotton gauze, 20 mL for (2) wet cotton gauze, 25 mL for (3) dry sweeping pad, 17.5 mL for (4) wet sweeping pad, and 2 mL for (7) dry Dacron swab. PBS volume for pre-moistening and elution was determined based on our routine sampling strategy for cotton gauze and sweeping pad (10) and Dacron swab (11), or manufacturers' instruction for the sponge sticks. After the tube was vortexed or the bag was massaged for 15 s, the supernatant was transferred into a new cryovial. The equal volume of supernatant and AL lysis buffer (Qiagen, Germantown, MD, USA) was mixed and stored at −80°C until further experiment.

## 2.3 Experiment 2

Experiment 2 aimed to compare different premoistened sampling devices in ASFV detection in environmental samples. The triplicates of ASFV-contaminated stainless steel surface for each sampling strategy were prepared as described in experiment 1. In experiment 2.1, five different sampling devices were tested: (1) the wet cotton gauze premoistened with 5 mL PBS, (2) the wet sweeping pad premoistened with 7.5 mL of PBS (3) the sponge stick containing 10 mL neutralizing buffer, (4) the sponge stick premoistened with 10 mL DNA/RNA shield, and (5) the wet Dacron swab premoistened with 2 mL of PBS. After placing in the tube or bag, PBS was added to the cotton gauze, the sweeping pad and the Dacron swab to normalize the total volume of liquid to 10 mL. After the tube was vortexed or the bag was massaged for 15 s, the supernatant was transferred into a new cryovial. The equal volume of supernatant and AL lysis buffer was mixed and stored at −80°C until further experiment. A 100 microliter of blood was mixed with 9.9 mL of PBS for positive control. PBS was used for negative control. In experiment 2.2, four different sampling approaches were evaluated for swabbing the ASFV-contaminated surface: (1) the wet cotton gauze premoistened with 5 mL PBS, (2) the wet cotton gauze premoistened with 5 mL DNA/RNA shield, (3) the sponge stick premoistened with 10 mL DNA/RNA shield, and (4) the sponge stick premoistened with 10 mL PBS. After placing the cotton gauze in the tube, either 5 mL PBS or DNA/RNA shield was added to the tube. The sponge stick was placed in the sample bag. The tube and bag were vortexed and massaged, respectively,

for 15 s, and supernatant was transferred into the new cryovial. The AL lysate was generated as mentioned above and stored at −80°C until further experiment.

## 2.4 Experiment 3

In experiment 3, we determined the effect of pre-incubation on ASFV DNA in environmental samples. The triplicates of ASFV-contaminated surface for each pre-incubation condition were prepared and swabbed using (1) the wet cotton gauze premoistened with 5 mL PBS and (2) the sponge stick premoistened with 10 mL DNA/RNA shield as mentioned above. The gauze was placed in the 50 mL conical tube, and PBS was added to the tube. The stick was placed in the bag. The swab samples were incubated for (1) 5 min at room temperature (RT), (2) 1 h at RT, (3) 1 h at 4°C, (4) 24 h at RT, and (5) 24 h at 4°C. After incubation, the swab samples were vortexed or massaged, and supernatant was collected to make the AL lysate, which was stored at −80°C until further experiment.

## 2.5 Experiment 4

In order to determine the effect of centrifugation and filtration on ASFV DNA detection on environmental samples from stainless steel surface. For experiment 4, similar procedures were used as previously evaluated which included the inoculation of the stainless steel coupon with the mixture of 100 μL of ASFV-infected blood and 5 mL of PBS and dried for 30 min. The steel surface was swabbed by using the wet cotton gauze premoistened with 5 mL PBS, the gauze was placed in the 50 mL conical tube, and 5 mL of PBS was added to the tube. After vortexing for 15 s, the supernatant was aliquoted into three tubes, and each tube was subjected to no centrifugation, centrifugation for 5 min at 700 × g for 5, 10, and 15 min, or centrifugation at 10,000 × g for 5, 10, and 15 min. After the centrifugation, the supernatant was transferred into the new cryovial, and the AL lysate was generated and stored at −80°C until further experiment. Next, the swab samples were generated as mentioned previously: swabbed the contaminated steel surface with 100 μL blood and 5 mL PBS, and added 5 mL PBS into tube, and vortexed for 15 s and aliquoted into four microtubes. Each tube was subjected to (1) no processing, (2) centrifugation for 5 min at 700 × *g*, (3) filtration using a 0.45 μm syringe filter, or (4) centrifugation for 5 min at 700 × *g* and then filtration through 0.45 μm syringe filter. After treatment, the supernatant was collected to make the AL lysate and stored at −80°C until further experiment.

## 2.6 Quantitative PCR

ASFV DNA was extracted from the AL lysate using a magnetic-based extraction system as described previously (12). Briefly, the AL lysate was heat-inactivated at 70°C for 10 min, and 200 μL of the lysate and 200 μL of isopropyl alcohol were added into a pre-loaded extraction plate, and extraction was performed on the automatic extractor. Extracted DNA was mixed with the forward and reverse primers, probe, and PerfeCTa® FastMix® II (Quanta Biosciences; Gaithersburg, MD, USA), in a total of 20 μL reaction. PCR reaction was performed on CFX machine. The Cq value was converted to copy numbers/mL using the standard curve.

## 2.7 Statistical analysis

ASFV DNA copy number was log-transformed and analyzed in GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). For experiment 1, *t*-tests were performed to compare dry and wet device conditions. Analysis of variance was used for experiments 2, 3, and 4. Within all statistical analysis, the positive control treatment was excluded.

# 3 Results

To compare the dry and wet sampling devices for ASFV detection, three different samples devices were used to swab ASFV-contaminated surfaces and supernatant was tested for ASFV detection by qPCR. All samples tested were positive for ASFV detection (Figure 1). The highest ASFV detection was found in the samples from the dry and wet Dacron swabs, and there was

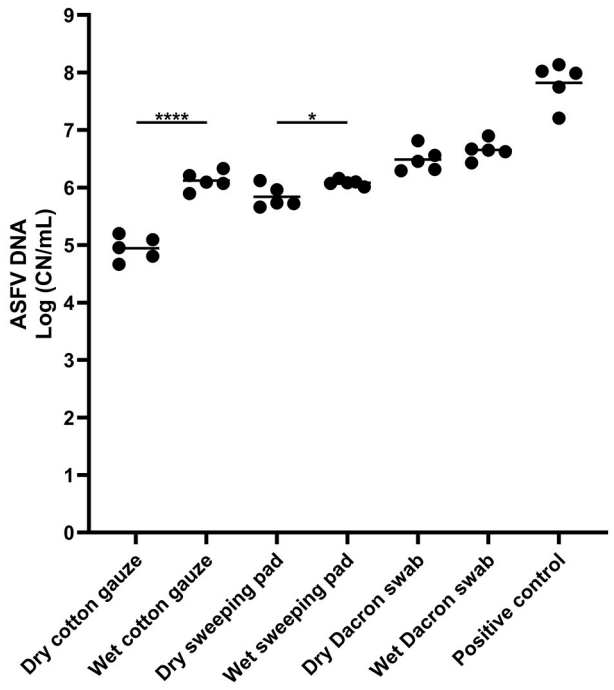


FIGURE 1
African swine fever virus (ASFV) DNA detection in environmental samples. Stainless steel surfaces were inoculated with 100 μL of ASFV-infected blood and swabbed using different types of sampling devices. The supernatant was subjected to quantitative PCR detecting ASFV DNA. A 100 microliter of blood was used for positive control. The amount of ASFV DNA (copy numbers per mL) was log-transformed for statistical analysis and the central tendency was represented mean of log-transformed values. Statistical differences between each dry and wet devices were assessed by Student *t*-test (*p*-value <0.05: * and <0.0001: ****).

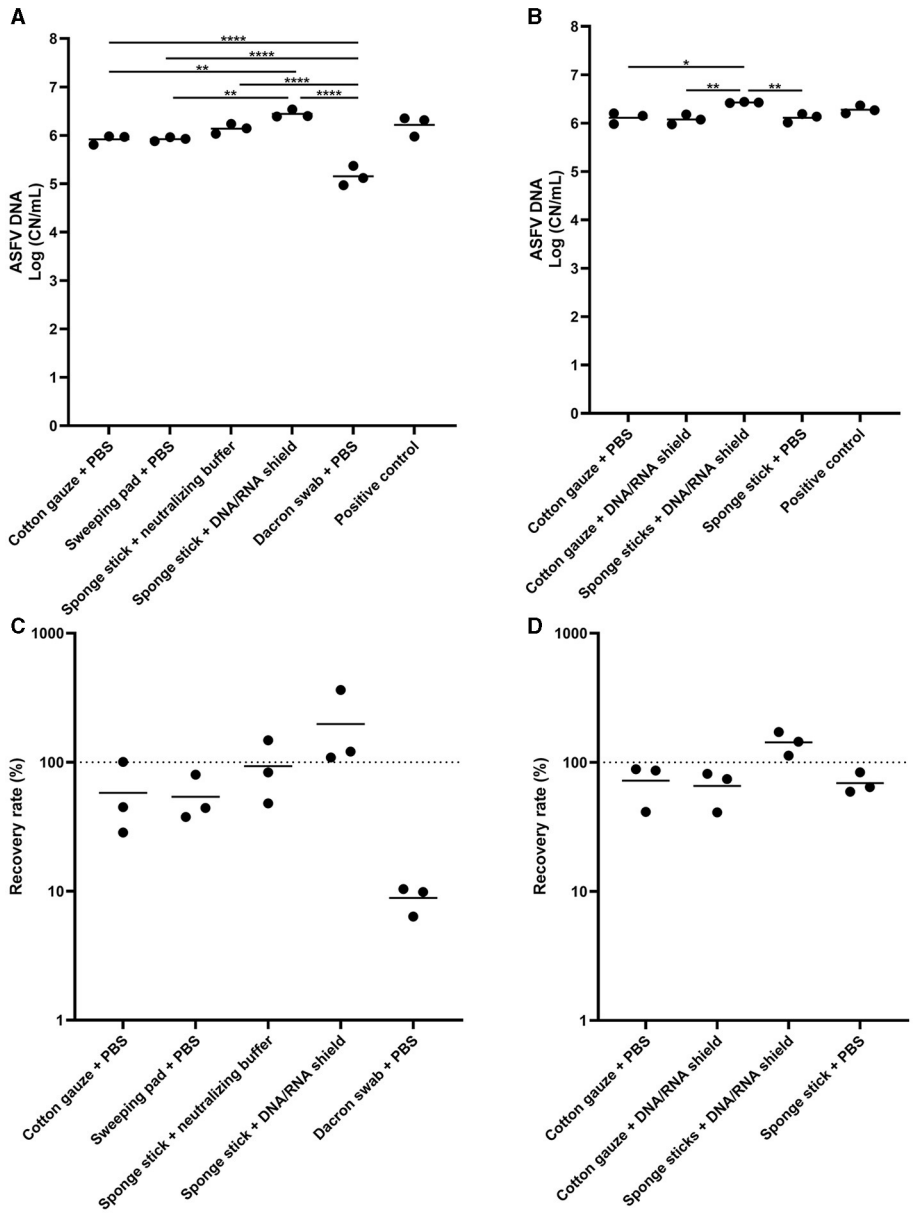


FIGURE 2
African swine fever virus (ASFV) DNA detection in environmental samples. Stainless steel surfaces were inoculated with 100 μL of ASFV-infected blood and swabbed using five different types of sampling devices **(A, C)** or four combinations of devices and buffers **(B, D)**. After normalization to 10 mL, the supernatant was subjected to quantitative PCR detecting ASFV DNA. Positive control was 100 of μL blood in 10 mL of PBS. The amount of ASFV DNA (copy numbers per mL) was log-transformed for statistical analysis and the central tendency was represented mean of log-transformed values **(A, B)**. Recovery rate (%) was calculated by dividing the amount of ASFV DNA of the sample by that of the positive control and central tendency was represented mean **(C, D)**. Statistical differences among sampling devices were assessed by ANOVA ($p$-value <0.05: *, <0.01: **, and <0.0001: ****).

no significant difference in ASFV detection between dry and wet swabs. In contrast, we found significantly higher detection of ASFV in wet cotton gauze and sweeping pad when compared to dry sampling devices, respectively.

Next, we tested the several sampling devices and different buffers for pre-moistening devices. In the first experiment, we compared the five different conditions and normalized to 10 mL of the buffer. The highest ASFV DNA detection was found in the sponge stick pre-moistened with DNA/RNA shield, and the amount of DNA was significantly higher than those in wet cotton gauze, wet sweeping pad, and wet Dacron swab (Figure 2A). In the samples of the Dacron swabs, we found the lowest ASFV DNA detection. Two sampling devices, cotton gauze and sponge stick, and two buffers, PBS and DNA/RNA shield, were selected for further analysis and a total of four different combinations of sampling devices were tested. We observed the highest virus detection when the ASFV-contaminated surfaces were swabbed using the sponge stick containing DNA/RNA shield (Figures 2B–D).

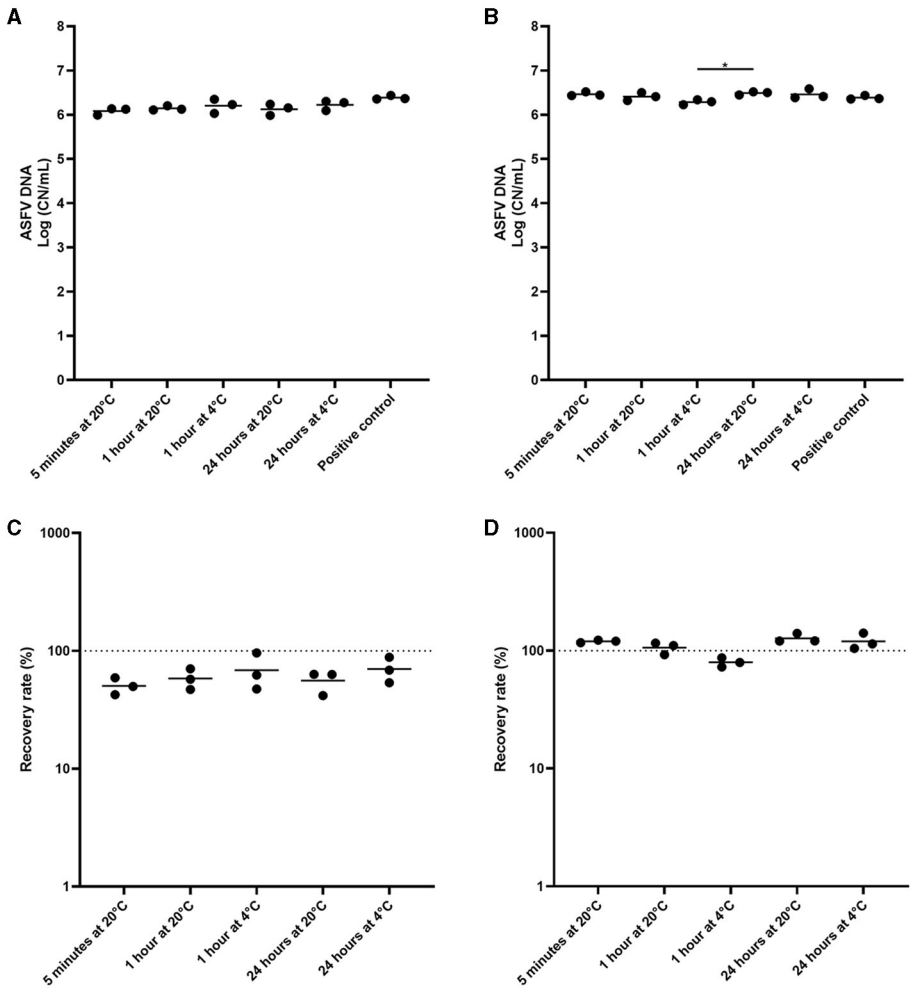


FIGURE 3
Comparison of pre-incubation conditions of environmental samples from African swine fever virus (ASFV)-contaminated surfaces for ASFV detection. Stainless steel surfaces were inoculated with 100 μL of ASFV-infected blood, swabbed using the cotton gauze premoistened with PBS **(A, C)** or the sponge stick with DNA/RNA shield **(B, D)**, and incubated at 4°C or room temperature (RT) for different time periods. After normalization to 10 mL, the supernatant was subjected to quantitative PCR detecting ASFV DNA. Positive control was 100 of μL blood in 10 mL of PBS. The amount of ASFV DNA (copy numbers per mL) was log-transformed for statistical analysis and the central tendency was represented mean of log-transformed values **(A, B)**. Recovery rate (%) was calculated by dividing the amount of ASFV DNA of the sample by that of the positive control and central tendency was represented mean **(C, D)**. Statistical differences among sampling devices were assessed by ANOVA ($p$-value <0.05: *).

In experiment 3, we incubated the environmental samples under different conditions to determine the most effective pre-extraction incubation conditions prior to analysis by qPCR. The environmental samples were prepared and incubated under five different conditions. There was no significant difference when cotton gauze was used (Figure 3A). In the sponge stick, the detection of ASFV DNA was similar across all conditions evaluated, with the exception of a small increase in detection when incubating samples at 24 h at RT compared to 1 h at 4°C (Figures 3B–D).

Lastly, we determined the effect of centrifugation and filtration of the environmental samples on ASFV DNA detection. The low-speed centrifugation did not affect ASFV detection in the environmental samples (Figure 4A). In contrast, we found a significant reduction in the level of ASFV DNA after centrifugation at 10,000 × g. Furthermore, filtration had a negative impact on ASFV DNA detection in the environmental samples (Figures 4B–D).

## 4 Discussion

Because there is no available commercial vaccine against ASFV in domestic pigs and wild boars, the current strategy to control and prevent ASF outbreaks relies on biosecurity at individual farm level as well international boundaries to prevent introduction. Biosecurity involves restricting the movement of anything potentially causing disease, such as humans, live animals, animal products and/or fomites and eliminating them. One of the key points in ASFV preparedness and response is early detection of ASFV, which enables rapid detection and isolation of the site or object to prevent further spread to other areas. In contrast to the biological fluids or tissue samples that are common

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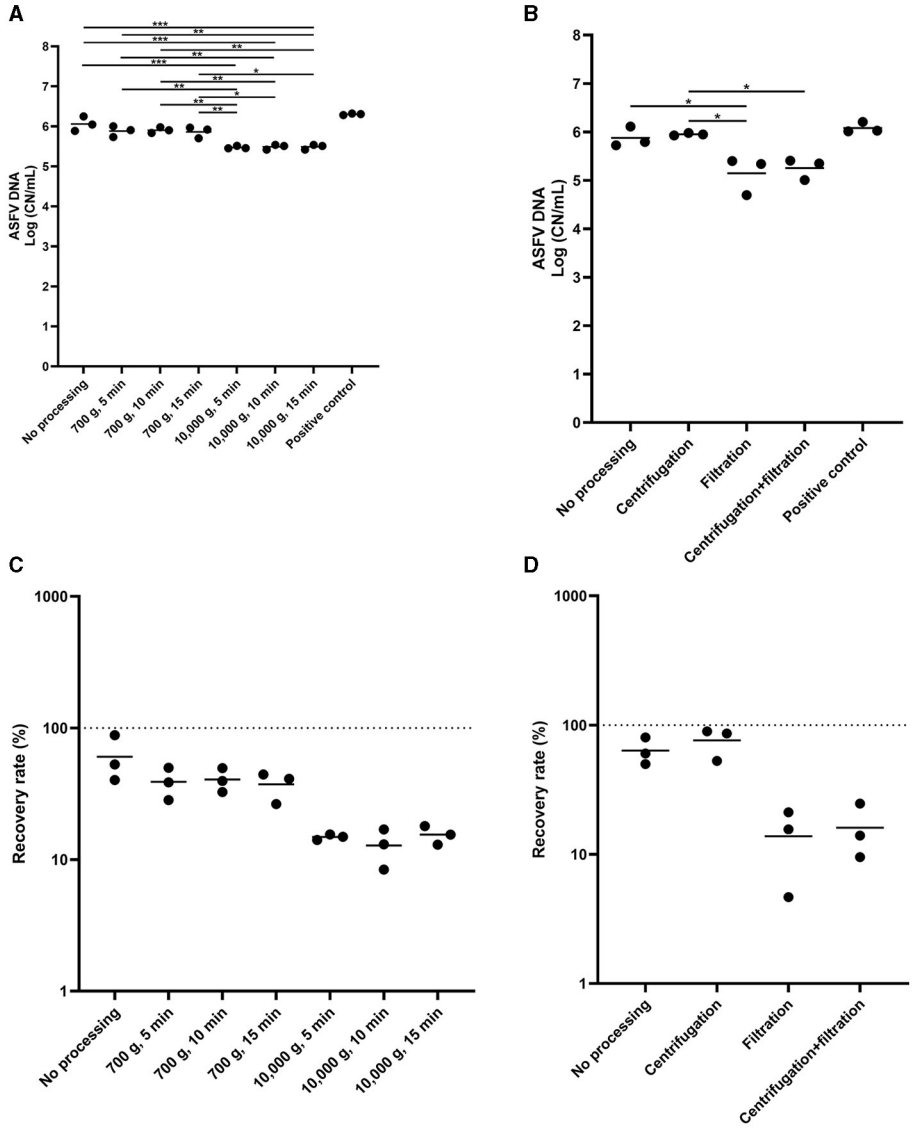


FIGURE 4
The effect of centrifugation and filtration on African swine fever virus (ASFV) detection in environmental samples. Stainless steel surfaces were inoculated with the mixture of 100 μL of ASFV-infected blood and 5 mL of PBS and swabbed using the cotton gauze premoistened with PBS. After normalization to 10 mL, the supernatant was aliquoted into several tubes and subjected to centrifugation **(A, C)** or combination of centrifugation and filtration **(B, D)** for ASFV detection. Positive control was 100 of μL blood in 10 mL of PBS. The amount of ASFV DNA (copy numbers per mL) was log-transformed for statistical analysis and the central tendency was represented mean of log-transformed values **(A, B)**. Recovery rate (%) was calculated by dividing the amount of ASFV DNA of the sample by that of the positive control and central tendency was represented mean **(C, D)**. Statistical differences among sampling devices were assessed by ANOVA ($p$-value <0.05: *, <0.01: **, and <0.001: ***).

sample types for clinical diagnostic testing, environmental samples contain a variety of PCR inhibitors, resulting in the reduction of PCR sensitivity and false-negative results. In addition, despite the extended survival of ASFV in environments, the processing techniques of environmental samples guaranteeing high sensitivity have not been evaluated. Therefore, this study aimed to develop and establish the protocol for testing the environmental samples from the common surface, stainless steel, while not contaminated with any organic material such as dirt or fecal material.

First, we found that wet sampling devices resulted in better detection of ASFV DNA in the environmental samples. This result was consistent with the general protocol of microbiology for environmental sampling (13), in which moisture from either surfaces or sampling devices is required for effective sampling of surfaces. However, it should be noted that all samples from dry devices were also positive for ASFV detection. In some scenarios, immediate actions might be required to response and control urgent situations, when the appropriate reagents are not prepared in the field. Under this situation, dry sampling devices could be an alternative for environmental sampling to prevent potential cross-contamination in the preparation of sampling devices.

Next, we tested several sampling devices from practical devices which can be easily accessible in the field to specialized sampling tools. Sterile synthetic fiber swabs with plastic shafts have been commonly used to collect environmental samples from surfaces, however, the lowest recovery rate was identified in this study. This is because the limited contact area of the swab was not sufficient to transfer the contaminants from large surfaces used in this study. While these sampling devices are considered the industry standard for nasal, oropharyngeal, and rectal swabs or samples from tissues, the limited contact area limits their utility as environmental sampling devices. In contrast, ASFV detection was satisfactory in the environment samples using two practical devices containing the common buffer; cotton gauze and sweeping pad pre-moistened with PBS. This technique has been widely used to detect various bacterial and viral diseases in environmental samples in pig industries because of its easy accessibility and cost-effectiveness (14, 15). In particular, the materials have been successfully used to determine the level of ASFV contamination within a feed manufacturing and swine production system in the regions of active ASFV circulation as well as under the experimental conditions (10, 16–18). The highest detection was identified in the sponge stick with DNA/RNA shield, therefore, we decided to further determine the best combination of the sampling device and buffer. Interestingly, the significance was found only in the combination of the sponge stick and commercial nucleic acid preservative, implying the synergetic effect of them on ASFV detection in the environmental samples. The sponge stick is widely used in environmental microbiology because it is able to sample larger surface areas than standard swabs, giving more chances to capture microorganisms, and it contains the variety of buffers in the product for subsequent enrichment of bacteria (19). This efficient capacity of transferring contaminant to the testing samples might contribute to better detection of ASFV DNA in the presence of the DNA preservative, which would prevent the degradation of ASFV in the environment. A recent study showed that the use of the sponge sticks pre-hydrated with a surfactant liquid also resulted in similar sensitivity, when compared to the traditional sampling method using a cotton swab, with effective viral inactivation (20). In this study, the virucidal effect of the commercial nucleic acid preservative on environmental samples from ASFV-contaminated surfaces has not been evaluated, but other studies showed the efficient inactivation of several different viruses (21–23). Given that ASFV is not only a WOAH-reportable disease but also a select agent in US, the combination of the sponge stick and DNA/RNA shield could be one of the sampling methods ensuring the high sensitivity of ASFV detection without risk of potential exposure of ASFV samples during transportation and sample processing.

The key point of environmental sampling is to remove the contaminants from the surface and then release them from the sampling devices to the buffer for subsequent cultivation or quantitative analysis. Several different techniques allow the efficient release of contaminants to the buffer: incubation overnight in elution buffer (24), mechanical mixing using a vortex mixer, shaking with beads, ultrasonication, and/or stomaching (13). Given its biosecurity level, we excluded the methods that have potential risk of spills in the processing of samples and compared the different incubation conditions for better ASFV DNA detection. Our results showed that incubation for the short period of time at RT and subsequent vortexing was sufficient to release viral DNA from the sampling devices. It is worth noting that immediate responses are crucial to control and prevent ASF outbreaks, thus, this shortened sample processing technique would contribute to early detection of ASFV.

Lastly, one of the important findings in this study was that centrifugation at high speed and filtration reduced the sensitivity of ASFV detection in environmental samples. In contrast, low speed centrifugation of the environmental samples had no impact on ASFV DNA detection. These results were consistent with the previous findings in which centrifugation at low speed had no impact on diagnostic sensitivity for porcine epidemic diarrhea virus, but the reduced sensitivity was found after filtration (25). In this study, the effect of centrifugation and filtration on environmental samples containing organic matters was not evaluated, but it would improve our understanding of ASFV diagnostics for environmental samples which mimic the real-world situation where organic matters are present.

In the present study, the sampling devices and processing techniques for environmental samples have been evaluated for diagnostic purposes. The use of pre-moistened gauzes, sweeping pads and sponge sticks resulted in the greatest sensitivity. In particular, the combination of the sponge stick and the commercial nucleic acid preservative supported the best detection of ASFV DNA from clean stainless steel surfaces. Pre-incubation for the short period of time and centrifugation at low speed were sufficient to provide the sensitivity of ASFV qPCR for environmental samples under the conditions of this study.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

## Author contributions

TK: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. JG: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. EL: Formal analysis, Investigation, Writing – review & editing. NG: Funding acquisition, Methodology, Writing – review & editing. JT: Investigation, Methodology, Writing – review & editing. JW: Funding acquisition, Writing – review & editing. CP: Funding acquisition, Writing – review & editing. CJ: Funding acquisition, Writing – review & editing. JR: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

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## 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.

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# The detection of zoonotic microorganisms in *Rhipicephalus sanguineus* (brown dog ticks) from Vietnam and the frequency of tick infestations in owned dogs

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Dog owners are greatly concerned about tick infestations in their pets. The prevalence and dispersion of ticks and their disease-causing microorganisms have been limited from the viewpoint of dog owners in Vietnam. This study investigated the presence of tick infestation and the pathogens associated with it in canines that were brought to veterinary hospitals in Vietnam. In the survey, 1,423 dogs participated from February to October 2022. Molecular and morphological methods were utilized to identify ticks and the associated pathogens. In addition,risk variables linked to tick infestation were documented and analyzed using statistical methods. The total exposure to the brown dog tick (*Rhipicephalus sanguineus sensu lato*) was 29.01%. Nam Dinh has the highest tick prevalence among the research areas. Tick infestation reached its highest point between June and September in the northern region of the country, with distinct seasons showing a strong correlation with tick infestation in dogs. Out of 177 tick pools examined, 146(82.49%) tested positive for at least one infection. *Mycoplasma* spp. (78.53%) was the most common, followed by *Anaplasma* spp. (37.29%), *Rickettsia felis* (5.08%), *Babesia vogeli*, and *Hepatozoon canis* (2.82%). In the current study, there was a statistically significant link between tick infestation and characteristics such as age, breed, body size, lifestyle, and bathing frequency. Understanding the seasonal behavior of vector ticks is crucial for identifying individuals or animals susceptible to tick-borne diseases. Studying the distribution of ticks and their ability to carry and disseminate zoonotic germs in specific places could assist veterinarians and policymakers in implementing effective strategies to manage zoonotic infections.

KEYWORDS

**dogs, ticks, tick-borne pathogens, risk factors, Vietnam**

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# 1 Introduction

Ticks are significant blood-feeding carriers of disease-causing agents that impact both pets and humans worldwide. Ticks can transfer many zoonotic pathogens such as viruses (tick-borne encephalitis virus), bacteria (*Anaplasma, Ehrlichia, Rickettsia*), and protozoa (*Babesia* and *Hepatozoon*) to vertebrate hosts during blood feeding, which might damage their health (1). More than seven tick species have been recently found in dogs in Asia, such as *Rhipicephalus haemaphysaloides, Rhipicephalus sanguineus, Haemaphysalis longicornis, Haemaphysalis campanulata, Haemaphysalis wellingtoni, Haemaphysalis hystricis,* and species within the genus *Ixodes* (2). Among these, *R. sanguineus*, also known as the brown dog tick and taxonomically designated as *R. sanguineus sensu lato* (s.l.) of tropical lineage, was the predominant tick species infesting dogs in numerous nations (2). *Rhipicephalus sanguineus* is believed to transmit many infections that cause babesiosis, hepatozoonosis, ehrlichiosis, mycoplasmosis, and rickettsiosis in dogs throughout Southeast Asia (3, 4). Apicomplexan protozoa parasites often found in dogs worldwide include species of *Babesia* (*Babesia canis*, *Babesia gibsoni*, and *Babesia vogeli*) and species of *Hepatozoon* (*Hepatozoon canis* and *Hepatozoon americanum*) (5, 6). Anaplasmataceae bacteria, including *Ehrlichia canis* and *Anaplasma platys*, are the most common bacterial tick-borne diseases found in dogs in Asia. All these infections are transferred through the vector's bite during blood feeding, except for *Hepatozoon*, which is transmitted through the ingestion of *R. sanguineus* (7).

Vietnam is one of the rapidly developing economies in Asia, leading to an increase in the number of companion animals. Vietnam is geographically organized into three primary regions: Northern, Central, and Southern. It features both tropical and temperate temperature zones. In contrast to the South, which has two different seasons—dry and rainy—the North experiences four distinct seasons: spring, summer, autumn, and winter. The northern regions experience summertime averages of 22°C–27.5°C and wintertime averages of 15°C–20°C. Southern regions see consistently high temperatures ranging from 26°C to 29°C year-round.[1] So far, the only type of tick found on dogs during studies on ticks in dogs in Vietnam was the brown dog tick (2, 8, 9). Protozoa and bacterial tick-borne organisms were recently found in dogs in Vietnam, with a detection rate ranging from 0.8% to 25.8% (2). The presence of a large dog population, favorable weather conditions, and close contact between humans and pet dogs facilitate the spread of zoonotic tick-borne diseases in the area. There have been few surveillance efforts to quantify tick abundance in dogs and to comprehend their frequency and spatial distribution in Vietnam. This study aimed to survey ticks infesting privately owned dogs at veterinary hospitals in Vietnam and detection of selective tick-borne pathogens that are reported commonly in dogs and ticks in Asia. It involved morphological and genetic species characterization of the collected ticks. Factors contributing to tick infestation were documented and examined.

1 https://climateknowledgeportal.worldbank.org/country/vietnam/climate-data-historical (Accessed 22 February 2024).

# 2 Materials and methods

## 2.1 Ethical consent

The Ethics Committee of Obihiro University of Agriculture and Veterinary Medicine accepted the protocol for using animal samples in this work (Permit for animal experiment: 21-25; DNA experiment: 1725-5).

## 2.2 Area of study, collection of samples, and tick identification

A survey on tick infestation in dogs was conducted from February to October 2022 with voluntary participation from veterinary clinics in Hanoi (21°01′N, 105°51′E), Ho Chi Minh City (10°46'N; 106°42′E), Nam Dinh (20°25′N; 106°10′E), and Dak-Lak (12°4′N, 108°3′E) in Vietnam (Figure 1). Veterinarians conducted a comprehensive inspection of the animals to detect ticks by inspecting the entire body surface for more than 5 min. The examinations involved assessing the animals' physical condition and noting any abnormalities in the skin/haircoat and mucous membranes such as jaundice, paleness, or hemorrhaging. A questionnaire was created to collect data about the sampled dogs' location, breed, sex, age, and body size for analyzing several categories related to tick infestation. Further inquiries were made regarding the living environment (indoor, outdoor, or semi-outdoor) and bathing frequency (weekly, monthly, every 2 months, or annually) if applicable.

From one to five ticks attached to the dog were collected and stored in 1.5 mL tubes containing 70% ethanol. The specimens were subsequently transferred to the National Research Center for Protozoan Diseases in Obihiro, Hokkaido, Japan for identification using morphological and molecular analysis. A total of 555 ticks were submitted for species identification and detection of microorganisms. Ticks were examined using a stereomicroscope (Olympus SZX16) to determine their species and life stage (larva, nymph, or adult) by utilizing specific physical characteristics (10). Following the initial morphological identification, the ticks were rinsed extensively with phosphate-buffered saline. Afterwards, ticks in the identical life stage and obtained from the identical dog were pooled for the DNA extraction using an DNeasy Blood and Tissue Kits (Quiagen, Germany) as per the manufacturer's guidelines. Following the pooling process, a total of 177 pools was obtained, with each pool containing between one and five individual ticks for DNA extraction. Following DNA extraction, the sequences aimed at the species-specific gene were amplified to molecularly confirm the tick species and their associated microorganism (Table 1).

## 2.3 Molecular detection and phylogenetic analysis of microorganism

The DNA samples underwent screening for apicomplexan protozoa (such as *Babesia* spp., *Hepatozoon* spp.), bacteria of Anaplasmataceae family (such as *A. platys*, *E. canis*), *Mycoplasma* spp., *Rickettsia* spp., and *Bartonella* spp. using primer sets by conventional PCR (cPCR). The cPCR amplified targeted sequences of *Ehrlichia/Anaplasma* spp. (*16S rRNA* gene), *Babesia/Hepatozoon* spp. (*18S rRNA* gene), and *Rickettsia* spp. from the spotted fever group (*OmpA*). Positive samples for the

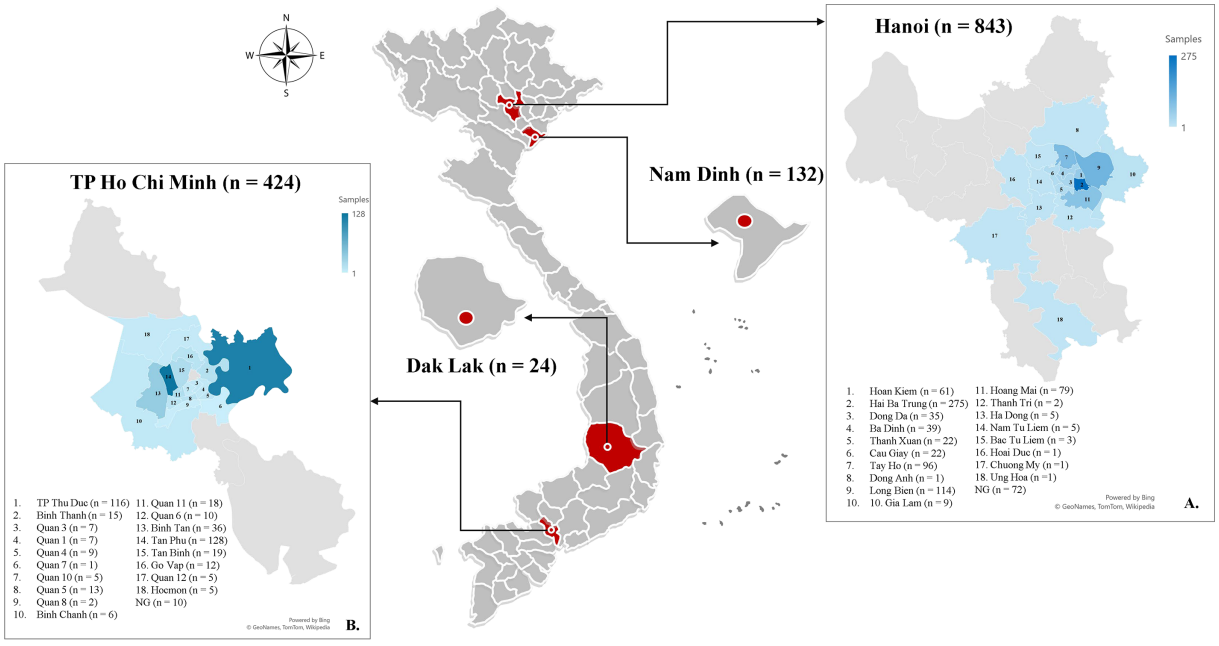


FIGURE 1
Map showing the locations where tick infestation on dogs is being studied in Vietnam, with red circles indicating the sampling sites in each province. The blue highlighted districts indicate the specific geographic areas where samples were gathered in Hanoi **(A)** and Ho Chi Minh City **(B)**.

genus of the stated species were then analyzed using primers that target species-specific sequences by cPCR or nested PCR. All primers and the target genes utilized for pathogen detection were outlined in Table 1. Pathogen-positive samples and distilled deionized water were utilized as the positive and negative controls, respectively, for all experiments. The PCR amplicons were examined using electrophoresis in a 1.5% agarose gel (LE agarose, Thermo Fisher Scientific, Waltham, MA, United States) and seen under a UV transilluminator (ATTO, Tokyo, Japan). DNA was extracted and purified from the positive amplicon by excising it from a gel using a Gel Extraction Kit from QIAGEN, Germany. The result, purified and having the desired sequences, underwent sequencing using the BigDye v3.1 Terminator Cycle Sequencing Kit in an ABI PRISM 3100 Genetic Analyzer by Applied Biosystem, United States.

The sequences' chromatograms were evaluated with BioEdit software (version 7.5.2) and compared to sequences in the Genbank database using the Basic Local Alignment Search Tool (BLAST) on the U.S. National Center for Biotechnology Information (NCBI) website. This work evaluated the genetic variation of *Mycoplasma* spp. and *Rickettsia felis* by examining *16S rRNA* gene-based *Mycoplasma* spp. sequences (618 bp) and *gltA* gene-based *R. felis* sequences (654 bp) using phylogenetic analysis with the MEGA X tool. Phylogenetic trees were created using the maximum likelihood method and the most suitable substitution model. A bootstrap analysis with 1,000 replications was performed to evaluate the reliability of the branching patterns in the trees. Every sequence is provided with Genbank accession numbers, isolation sources, and countries of origin.

## 2.4 Statistical analysis

The OpenEpi application was utilized to calculate the detection rate of tick infestation, determine the percentage of discovered diseases, and estimate 95% confidence intervals (95% CI).[2] A chi-square test was used to investigate the statistical relationship between the prevalence of tick infestation and the host independent factors. The odds ratio (OR) was calculated to assess the strength of the relationship between each category and tick infestation, determining if there was a significant association between them. The level of statistical significance was established at $p \leq 0.05$. The study utilized a confidence level of 95%. The Statulator tool was utilized for data analysis.[3]

# 3 Results

## 3.1 Sample data and tick morphological identification

A total of 1,423 dogs participated in the survey, with 843 from Hanoi (59.24%), 424 from Ho Chi Minh City (29.8%), 132 from Nam Dinh (9.28%), and 24 from Dak-lak (1.68%; Figure 1). The enrolled dogs included: 630 females (44.27%), 729 males (51.23%), and 64 with unreported data (4.5%). The animals' ages varied from 2 months to 20 years, with around 43.5% (619) of the population falling between one and 5 years old. The dogs were of more than 20 breeds, which were divided into three groups. Details of different breeds of dogs observed in the current study were presented in Figure 2; of which, 1,145 (80.46%) were of the exotic breed, 161 (11.31%) were of the domestic breed. The majority were indoor lifestyle (662, 46.52%),

2 https://www.openepi.com/Proportion/Proportion.htm
3 https://statulator.com/stat/chisq.html

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TABLE 1 Primer sequences used for pathogen detection in ticks in this study.

| Target pathogens (target gene) | Primer sequences (F/R) 5′-3′ | Product size (bp) | Annealing temperature (°C) | References |
|---|---|---|---|---|
| Tick species (*Cox1*) | GGTCAACAAATCATAAAGATATTGG | 710 | 55 | (11) |
| | TAAACTTCAGGGTGACCAAAAAATCA | | | |
| *Rickettsia* spp. spotted fever group (*OmpA*) | GCTTTATTCACCACCTCAAC | 212 | 55 | (12) |
| | TR(G/A)A TCACCACCGTAAGTAAAT | | | |
| *Rickettsia felis* (*gltA*) | F1 GCAAGTATTGGTGAGGATGTAATC | 654 | 58 & 55 | (13) |
| | R1 CTGCGGCACGTGGGTCATAG | | | |
| | F2 GCGACATCGAGGATATGACAT | | | |
| | R2 GGAATATTCTCAGAACTACCG | | | |
| *Mycoplasma* spp. (*16S rRNA*) | ATACGGCCCATATTCCTACG | 595–618 | 60 | (14) |
| | TGCTCCACCACTTGTTCA | | | |
| Anaplasma/Ehrlichia (*16S rRNA*) | GGTACCTACAGAAGAAGTCC | 345 | 52 | (15) |
| | TAGCACTCATCGTTTACAGC | | | |
| *Anaplasma platys* (*groEL*) | AAGGCGAAAGAAGCAGTCTTA | 724 | 54 | (16) |
| | CATAGTCTGAAGTGGAGGAC | | | |
| *Ehrlichia canis* (*gltA*) | TTATCTGTTTATGTTATATAAGC | 1,372 | 50 | (17) |
| | CAGTACCTATGCATATCAATCC | | | |
| *Bartonella* spp. (*16S-23S rRNA*) | (C/T)CTTCGTTTCTCTTTCTTCA | 260 | 55 | (18) |
| | AACCAACTGAGCTACAAGCC | | | |
| Babesia/Hepatozoon (18S rRNA) | CCAGCAGCCGCGGTAATTC | 350–400 | 57 | (19) |
| | CTTTCGCAGTAGTTYGTCTTTAACAAATCT | | | |
| *Babesia canis* (*18S rRNA*) | GCW(A/T)TTTAGCGATGGACCATTCAAG | 208 | 60 | (20) |
| | CCTGTATTGTTATTTCTTGTCACTACCTC | | | |
| *Hepatozoon canis* (*18S rRNA*) | ATACATGAGCAAAATCTCAAC | 666 | 57 | (21) |
| | CTTATTATTCCATGCTGCA | | | |

F, Forward; R, Reverse; Cox1, cytochrome c oxidase subunit I; ompA, outer membrane protein A; groEL, heat shock protein gene; gltA, citrate synthase gene.

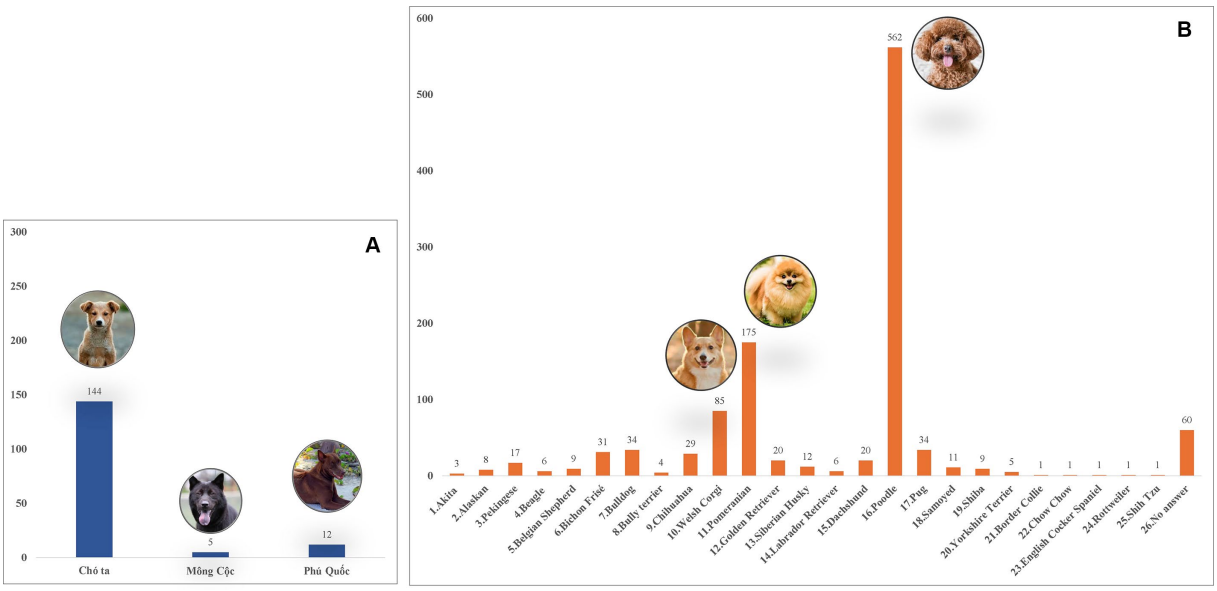


FIGURE 2
Different dog breeds surveyed in Vietnam during the study. **(A)** Domestic breeds. **(B)** Exotic breeds.

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small size (≤3.5 kg; 462, 32.46%), and bathed at least once a week (707, 49.68%). Clinical symptoms such as skin and mucous membrane abnormalities were found to be statistically linked to the presence of tick infestation in dogs (Table A1). A total of 555 ticks were submitted for species identification and detection of microorganisms. In particular, based on the morphological typical traits, 33 larvae, 75 nymphs, and 447 adults (195 males and 252 females) are identified as belonging to the species complex *R. sanguineus* s.l. (Figure 3).

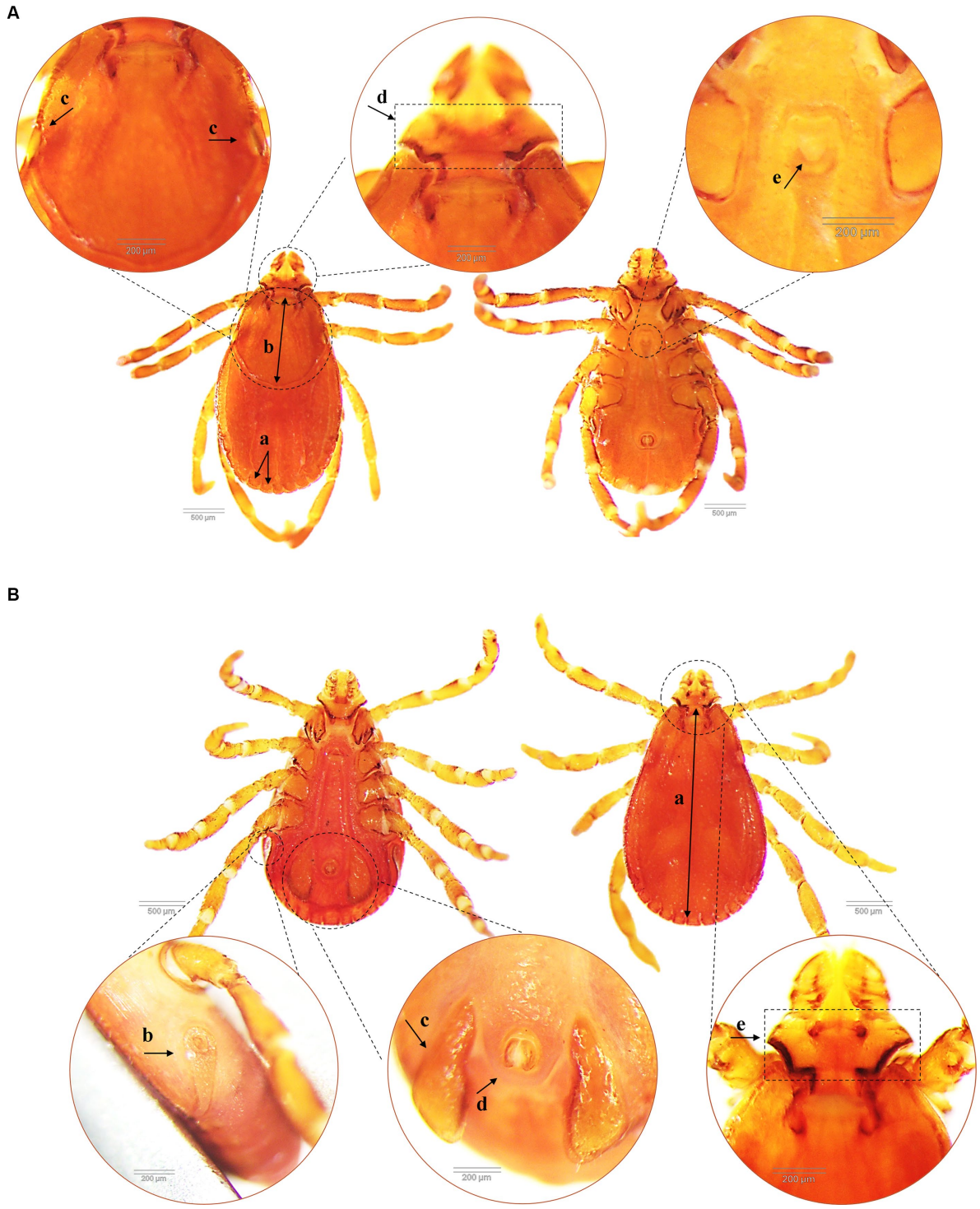


FIGURE 3
Morphological characteristics of *Rhipicephalus sanguineus* tick collected from dogs (dorsal and ventral side). **(A)** Female *R. sanguineus* with the festoon (similar for male) **(a)**, the scutum covers approximately 1/3 of the dorsal surface **(b)**, flat eyes **(c)**, hexagonal angulate basis capituli **(d)** and U-shaped genital aperture **(e)**. **(B)** Male *R. sanguineus* with the complete scutum covering most of the dosal surface **(a)**, coma-shaped spiracular plates (similar for female) **(b)**, subtriangular adanal plates **(c)**, anus groove posteriorly **(d)** and hexagonal angulate basis capituli **(e)**.

## 3.2 Geographical and tick seasonal distribution

Of 1,423 dogs examined, 414 (29.01, 95% CI: 26.79–31.51%) were carrying at least one tick at the time of sampling. According to statistical analysis, there was a significant difference determined between geographical areas and tick infestation rate in dogs. Tick infestation rate was highest in dogs sampled in Nam Dinh at 55.3% (73/132, 95% CI: 46.79–63.52) followed by Ho Chi Minh City at 39.86% (169/424, 95% CI: 35.31–44.59) and Hanoi 20.7% (170/843, 95% CI: 17.6–23.01). In Dak-Lak, tick presence was found in two dogs (8.33, 95% CI, 2.3–25.85). Besides, dogs residing in Nam Dinh and Ho Chi Minh City were 4.9 times ($p < 0.001$, 95% CI: 3.34–7.18%) and 2.62 times ($p < 0.001$, 95% CI: 2.03–3.39%) more likely to get tick infestation compared to those in Hanoi. Samples collected from the north of Vietnam were subjected to tick seasonal distribution analysis. During different seasons, more dogs were tick-infested from May to September and the number of tick-infested dogs declined between February and April (Figure 4). Additionally, the detection rate of tick infestation in dogs was statistically associated with the different seasons in the north. Specifically, the majority of tick-infested dogs in the study were detected in the warmer months of summer (21.94%, 113/515) and autumn (30.79%, 121/393) compared to spring (9.89%, 9/91). In summer and autumn, dogs had 2.56 ($p = 0.008$) and 4.05 times ($p < 0.001$) the odds of getting tick infestation compared to those in spring, respectively (Table 2).

## 3.3 Risk factors associated with tick infestation in dogs in Vietnam

The relationship of tick infestation with different host attributes was statistically assessed in the present study and reported in Table 2 along with the number and percentage of tick infestation. Samples with missing information were excluded from the analysis. Majority of tick-infested dogs detected were juveniles (35.38%, 219/619), domestic breed (56.52%, 91/161), medium size (42.7%, 114/267), outdoor lifestyle (72.84%, 118/162) and bathed less than once a month (36.83%, 242/657), compared to the other groups of accordingly category. There was no statistically significant association between tick positivity and sex. The juveniles and adults had 1.95 times ($p < 0.001$) and 1.78 times ($p < 0.001$) the odds of getting tick infestation than the puppies, respectively. Domestic dogs were 3.71 times more likely to get tick infestation than exotic dogs ($p = 0.001$, 95% CI: 2.65–5.21). Dogs in small sizes were 2 times less likely to get tick infestation than those in medium ($p = 0.001$, 95% CI: 1.99–3.85) or large size ($p = 0.001$, 95% CI: 1.61–3.7). Dogs with outdoor lifestyle were significantly correlated with tick detection and had 10 times the risk of getting tick infestation compared to those living in-house in this study ($p < 0.001$, 95% CI: 7.19–15.83).

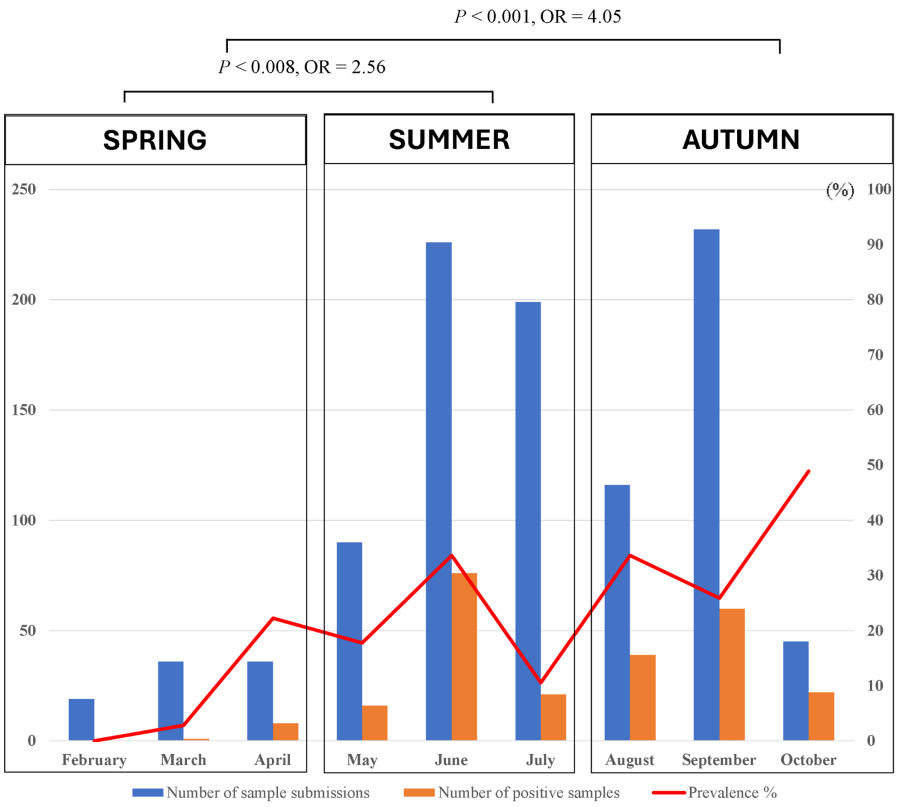


FIGURE 4
Monthly seasonal dynamics of the *Rhipicephalus sanguineus* tick found on owned dogs in northern Vietnam (2022).

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TABLE 2 Risk factors associated with tick infestation in dogs in Vietnam.

| Variables ($N = 1,423$) | No. tested dogs | No. tick-infested dogs | Df ($\chi^2$) $P$-value | OR ($P$-value) | 95% CI |
|---|---|---|---|---|---|
| **Seasons** | | | | | |
| Spring (Feb/Mar/April) | 91 | 9 (9.89) | 2 (14.55) 0.0007 | Ref | |
| Summer (May/June/July) | 515 | 113 (21.94) | | 2.56 (0.008) | 1.25–5.26 |
| Autumn (Aug/Sep/Oct) | 393 | 121 (30.79) | | 4.05 (0.001) | 1.97–8.33 |
| NG | 424 | 171 (40.33) | | | |
| **Age (year)** | | | | | |
| Puppy (≤1) | 560 | 123 (21.96) | 2 (26.89) 0.0001 | Ref | |
| Juvenile (1–5) | 619 | 219 (35.38) | | 1.95 (0.001) | 1.50–2.52 |
| Adult (>5) | 198 | 66 (33.33) | | 1.78 (0.001) | 1.24–2.54 |
| NG | 46 | 6 (13.04) | | | |
| **Breed** | | | | | |
| Domestic | 161 | 91 (56.52) | 2 (63.64) 0.0001 | 3.71 (0.001) | 2.65–5.21 |
| Exotic | 1,145 | 297 (25.94) | | Ref | |
| Mix | 74 | 20 (27.03) | | 1.06 (0.836) | 0.62–1.80 |
| NG | 43 | 6 (13.95) | | | |
| **Sex** | | | | | |
| Female | 630 | 184 (29.21) | 1 (0.25) 0.617 | 0.94 (0.617) | 0.75–1.19 |
| Male | 729 | 222 (30.45) | | Ref | |
| NG | 64 | 8 (12.50) | | | |
| **Body size (kg)** | | | | | |
| X-Small (≤3.5) | 462 | 98 (21.21) | 3 (44.22) 0.0001 | Ref | |
| Small (3.6–7.5) | 489 | 138 (28.22) | | 1.46 (0.012) | 1.08–1.97 |
| Medium (7.6–15) | 267 | 114 (42.70) | | 2.77 (0.001) | 1.99–3.85 |
| Large (≥15) | 131 | 52 (39.69) | | 2.44 (0.001) | 1.61–3.70 |
| NG | 74 | 12 (16.22) | | | |
| **Life-style** | | | | | |
| Indoor | 662 | 133 (20.09) | 2 (173) 0.0001 | Ref | |
| Outdoor | 162 | 118 (72.84) | | 10.67 (0.001) | 7.19–15.83 |
| Semi-outdoor | 558 | 162 (29.03) | | 1.63 (0.001) | 1.25–2.12 |
| NG | 41 | 1 (2.44) | | | |
| **Bathing frequency** | | | | | |
| Every week | 707 | 165 (23.34) | 3 (30) 0.0001 | Ref | |
| Every month | 144 | 56 (38.89) | | 2.09 (0.001) | 1.43–3.05 |
| Every 2, 3 months | 193 | 70 (36.27) | | 1.87 (0.001) | 1.33–2.63 |
| Once a year | 320 | 116 (36.25) | | 1.87 (0.001) | 1.40–2.49 |
| NG | 59 | 7 (11.86) | | | |

NG, Not given; Df, Degrees of freedom; $\chi^2$, Chi-square test; OR, Odds ratio; CI, confidence intervals; Ref., reference used; No., number.

## 3.4 Microorganism detection in tick samples collected from dogs in Vietnam

Among the 177 tick pools that were examined, 146 (82.49%) had at least one pathogen found, with *Mycoplasma* spp. being the most common (78.53%, CI: 71.91–83.94), followed by *Anaplasma* spp. (37.29%, CI: 30.51–44.61), *R. felis* (5.08%, CI: 2.69–9.38), *B. vogeli*, and *H. canis* (2.82%, CI: 1.04–6.82). In 79 pools, a single pathogen was discovered (44.63%, CI: 37.5–51.99). Multiple pathogens were detected in 67 pools (37.85%, CI: 31.04–45.18; Table 3). In terms of tick stages, out of 151 adult-stage tick pools, 121 (80.13%) pools had *Mycoplasma* spp. infections. These were followed by pools infected with *Anaplasma* spp. (39.07%, 59/151), *R. felis* (5.3%, 8/151), *B. vogeli* (2.65%, 4/151), and *H. canis* (0.66%, 1/151). In this investigation, nymphs and adults were found to have the same infections, while larvae primarily had *Mycoplasma* spp. (87.5%; Table 4).

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TABLE 3 The occurrence rate of pathogens in tick pools collected from dogs.

| Pathogen detected (*N* = 177) | No. positive samples | Detection rate (%) | Confidence intervals 95% |
|---|---|---|---|
| **TBP total** | 146 | 82.49 | 76.21–87.38 |
| *Babesia vogeli* | 5 | 2.82 | 1.04–6.82 |
| *Hepatozoon canis* | 5 | 2.82 | 1.04–6.82 |
| *Mycoplasma* spp. | 139 | 78.53 | 71.91–83.94 |
| *Rickettsia felis* | 9 | 5.08 | 2.69–9.38 |
| *Anaplasma* spp. | 66 | 37.29 | 30.51–44.61 |
| **Single infection** | 79 | 44.63 | 37.5–51.99 |
| *Babesia vogeli* | 2 | 1.13 | 0.31–4.02 |
| *Mycoplasma* spp. | 71 | 40.11 | 62.94–76.32 |
| *Anaplasma* spp. | 6 | 3.39 | 1.56–7.2 |
| **Co-infection** | 67 | 37.85 | 31.04–45.18 |
| *B. vogeli* + *Mycoplasma* spp. | 2 | 1.13 | 0.57–4.86 |
| *Anaplasma* spp. + *Mycoplasma* spp. | 53 | 29.94 | 23.68–37.06 |
| *Mycoplasma* spp. + *R. felis* | 5 | 2.82 | 1.92–7.93 |
| *B. vogeli* + *Anaplasma* spp. + *Mycoplasma* spp. | 1 | 0.56 | 0.31–4.02 |
| *H. canis* + *Anaplasma* spp. + *Mycoplasma* spp. | 3 | 1.69 | 0.57–4.86 |
| *H. canis* + *Mycoplasma* spp. + *R. felis* | 1 | 0.56 | 0.31–4.02 |
| *Anaplasma* spp. + *Mycoplasma* spp. + *R. felis* | 2 | 1.13 | 0.57–4.86 |
| *H. canis* + *Anaplasma* spp. + *Mycoplasma* spp. + *R. felis* | 1 | 0.56 | 0.31–4.02 |

TABLE 4 Screening of pathogens in different life stages of *Rhipicephalus sanguineus* tick.

| Stage of ticks | No. pools | No. positive pools for pathogens (%) | No. positive pools (%) | | | | |
|---|---|---|---|---|---|---|---|
| | | | *B. vogeli* | *H. canis* | *Mycoplasma* spp. | *R. felis* | *Anaplasma* spp. |
| Adult | 151 | 121 (80.13) | 4 (2.65) | 1 (0.66) | 114 (75.5) | 8 (5.3) | 59 (39.07) |
| Nymph | 18 | 18 (100) | 1 (5.56) | 4 (22.22) | 18 (100) | 1 (5.56) | 7 (38.89) |
| Larva | 8 | 7 (87.5) | 0 | 0 | 7 (87.5) | 0 | 0 |
| Total | 177 | 141 (79.66) | 5 (2.82) | 5 (2.82) | 139 (78.53) | 9 (5.08) | 66 (37.92) |

## 3.5 Sequencing identities and phylogenetic analysis

The nucleotide sequences for tick and each identified pathogen showed 99–100% similarity with sequences in the Genbank database. Specimens of *R. sanguineus* s.l. were genetically identified as part of the tropical lineage with 100% nucleotide identity (GenBank accession numbers MF425992–MF425994). The *18S rRNA* gene sequences of *B. vogeli* matched MT386936 with 100% identity, while *H. canis* showed 99.85% identity with MG050161. The *gltA* gene sequences of *R. felis* showed a high level of similarity, ranging from 99.84% to 100%, with sequences OM936910–MT019627. The *16S rRNA* gene sequences of *Mycoplasma* spp. demonstrated a nucleotide identity of 99.51% to 99.68% with *Mycoplasma wenyonii* sequences MF377464–MG948627. Whereas, *16S rRNA* gene sequences of *Anaplasma* spp. showed a high level of similarity, ranging from 99.03% to 99.67%, with sequences of the different species within the genus *Anaplasma* and *Ehrlichia* (EU090184–MN481611). However, none of the *Anaplasma/Ehrlichia* positive samples tested positive to *E. canis* or *A. platys* assays.

The molecular identification of particular sequences for *Mycoplasma* spp. and *R. felis* was confirmed by the clear separation of species-specific groups determined through phylogenetic analysis. The phylogenetic tree of the *16S rRNA* gene-based *Mycoplasma* spp. indicated that all *M. wenyonii* sequences (618 bp in length) grouped together in a distinct clade with strong support (99% bootstrap value). This clade included sequences from the same species found in various geographic locations, while excluding other *Mycoplasma* spp. species (Figure 5). Phylogenetic analysis of incomplete *gltA* gene sequences (654 bp in length) showed that all *R. felis* from dogs grouped together in a clade with reference sequences of the same species, supported by a bootstrap value of 70% (Figure 6).

The representative sequences produced in this research have been deposited in Genbank database with the following accession numbers: PP389595 and PP389596 for *R. sanguineus*, PP377903–PP377905 for

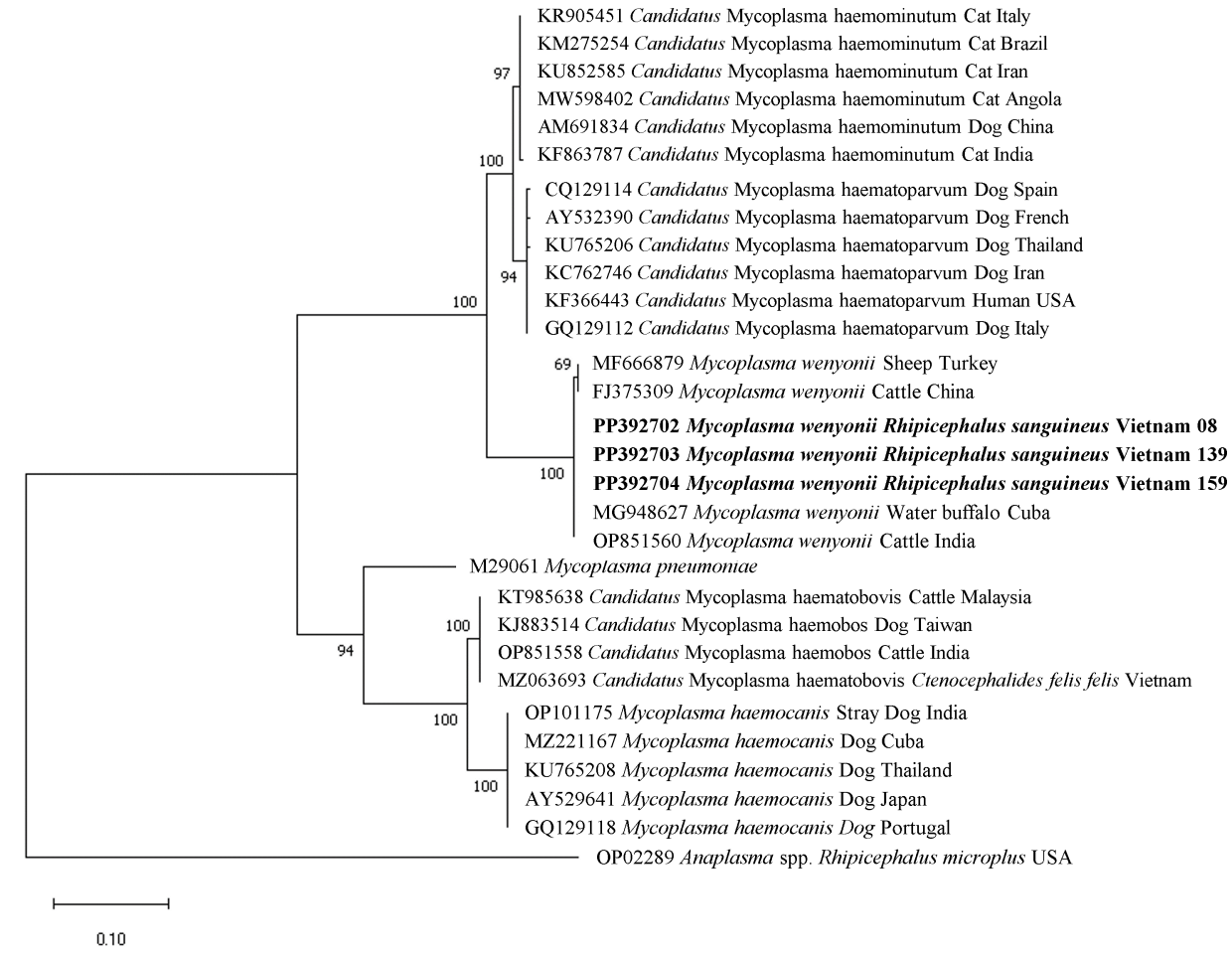


FIGURE 5
Phylogenetic analysis of *Mycoplasma wenyonii* based on the nucleotide sequences of a 618 bp fragment of *16S rRNA* gene using Tamura 2 parameter model. Isolates obtained from this study are presented in bold text.

*B. vogeli*, PP373785 for *H. canis*, PP392702–PP392704 for *M. wenyonii*, and PP393500–PP393501 for *R. felis*.

## 4 Discussion

The survey assessed the occurrence of ticks and the microorganisms they harbor on 1,423 dogs owned by clients, mainly in the rapidly expanding Vietnamese cities of Hanoi and Ho Chi Minh City, which house the majority of the country's human and animal population, along with two other provinces (Nam Dinh and Dak Lak). The overall exposure to brown dog ticks was 29.01%. This study represents the initial systematic examination of tick distribution and frequency on privately owned dogs in Vietnam, utilizing voluntary enrollment in veterinarian care. The study analyzed seasonal tick dynamics and risk factors associated with tick infestation in the canines studied for the first time. The findings and data will provide valuable information to epidemiologists, veterinarians, and legislators to develop treatment and prevention measures for vectors and their related infections.

The tick species found in dogs in this study was *R. sanguineus* s.l. from the tropical lineage. Previous research in various regions of Vietnam has also confirmed that dogs are commonly infested by *R. sanguineus* s.l. (2, 8, 9). *Rhipicephalus sanguineus* is the predominant tick species that targets domestic animals in Southeast Asia (2). The study found that most dogs with ticks were taken to hospitals throughout the summer and autumn months in the Northern region, characterized by hot, humid weather and high precipitation (22). During these periods, ectoparasites such as ticks are more active due to climate conditions that support the survival and reproduction of blood-feeding arthropods. Previous research has indicated that *R. sanguineus* ticks prefer to lay their eggs at temperatures ranging from 20 to 30°C (23). Optimal parameters for nymph survival include a temperature of 20°C and a relative humidity of 85%. Engorged larvae and nymphs of brown dog ticks successfully underwent molting under various combinations of constant temperature ranging from 10 to 35°C and relative humidity from 15% to 95% (24). The higher incidence of tick infestation in dogs in Ho Chi Minh City compared to Hanoi may be due to the ticks being consistently active throughout the year because of the favorable climate in the region, with an average temperature ranging between 26°C and 29°C annually, resulting in a greater risk of dogs being exposed to ticks.

This study identified variations in the likelihood of tick infection in dogs based on age, breed, body size, lifestyle, and bathing frequency

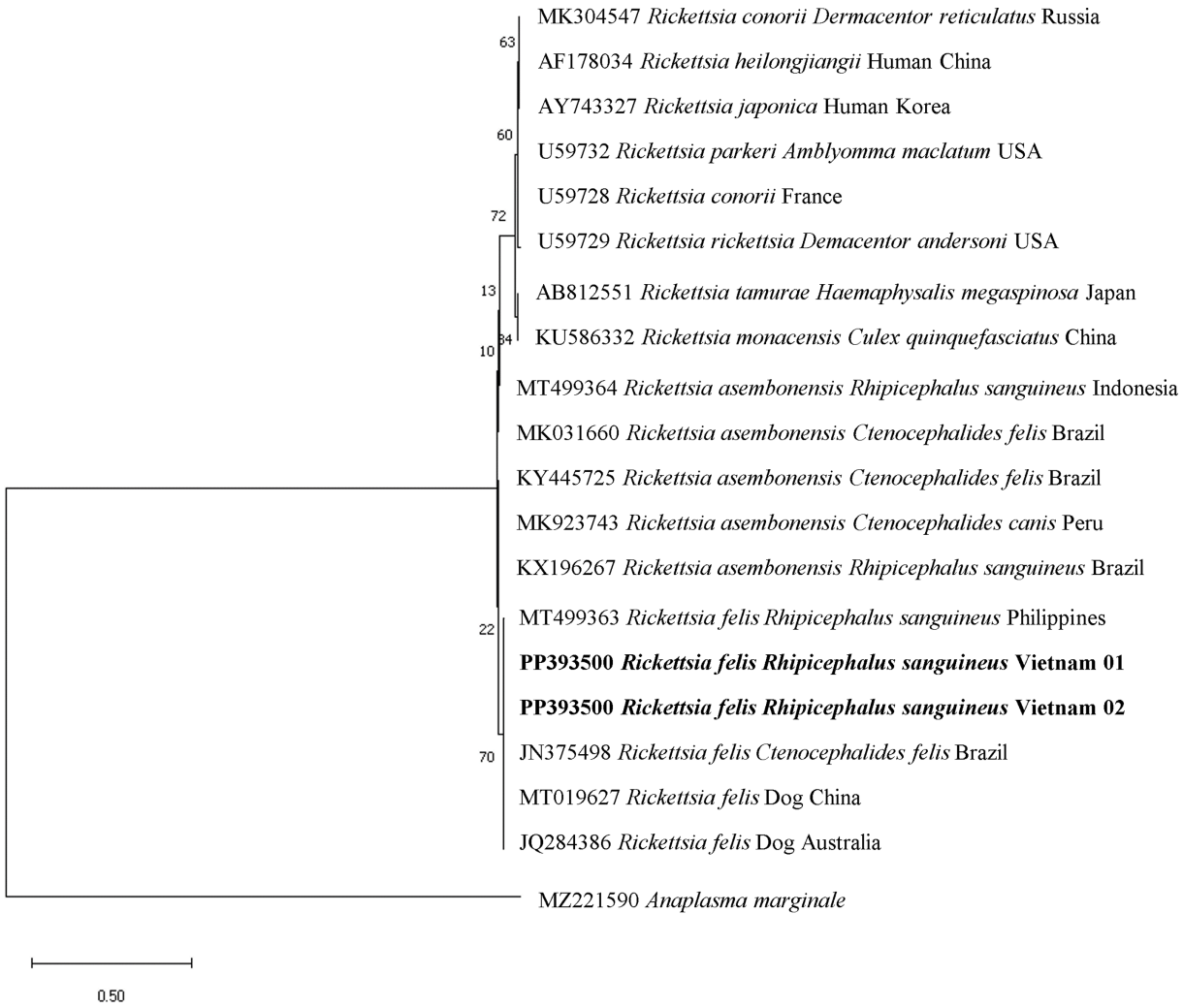


FIGURE 6
Phylogenetic analysis of *Rickettsia felis* based on the nucleotide sequences of a 654 bp fragment of *gltA* gene using Kimura 3 parameter model. Isolates obtained from this study are presented in bold text.

(Table 2). The lifestyle group was the primary variable related with tick infestations. The study found that dogs allowed to roam freely were more prone to tick infestation compared to those kept indoors. Dogs that roam outdoors freely are more likely to encounter ticks due to greater exposure to the environment and contact with tick-infested wild animals nearby. Studies undertaken in ASEAN nations have revealed tick incidence exceeding 80% in stray animals (25, 26). If the outdoor activity of dogs is the mechanism by which host-seeking tick activity is increasingly successful, then the pattern of association between rural areas and the risk of tick infestation is understandable in Vietnam; since dogs in rural regions reported often semi-domiciled and have free access to outside environment, predisposing them to ticks (27). Our research supported the claim that the rate of tick infestation in dogs in Nam Dinh, a rural location, was notably greater than in Hanoi and Ho Chi Minh City, metropolitan areas. Tick infestation is notably higher in domestic dogs than in exotic breeds. The various reasons for owning a dog may have been the cause. Most domestic breed dogs in Vietnam are typically raised outdoors for guarding purposes and are kept in kennels, making them more susceptible to tick infestations compared to exotic breeds that are usually kept as pets for companionship and may even share a bed with their owners. Older dogs, including juveniles and adults, are more susceptible to tick infestation in this study, maybe because they spend more time outdoors than pups. Small dogs who are bathed frequently may have a reduced chance of tick attachment, according to the study. Small dogs may be easier to bathe than larger dogs due to their size, making them more approachable and manageable. Previous research on the relationship between tick infestation and gender found that male dogs had a much higher risk of infestation (28); however, this relationship was not seen in the current study.

The molecular testing revealed that the majority of tick samples were infected with *Mycoplasma* spp. (78.53%) rather than *Anaplasma* spp., *Ehrlichia* spp., *H. canis*, *B. vogeli*, or *R. felis*. The transmission pathways of hemoplasmas in dogs are under discussion, with blood-feeding arthropods including fleas and ticks proposed as potential vectors (29). Mycoplasmosis is not considered to be a tick-borne pathogen, however some hemotrophic mycoplasms that are significant in veterinary and public health can sometimes be found in ticks and co-infect hosts with established tick-borne pathogens. *Mycoplasma* spp. were found alongside other microorganisms, including

*Anaplasma* spp., *B. vogeli*, *H. canis*, and *R. felis* in tick samples collected from the same host. The findings suggest that vertebrate hosts can be infected with several tick-borne diseases either by a single tick carrying multiple pathogens or by distinct ticks carrying individual pathogens. Hemotropic mycoplasma species, specifically *Mycoplasma haemocanis* and *Candidatus* Mycoplasma haematoparvum, have been recently identified in ticks and dogs in Vietnam and various regions of Southeast Asia, causing canine mycoplasmosis (3, 30, 31). A significant detection rate of *M. wenyonii* was unexpectedly detected in brown dog ticks and all three different stages of ticks (adults, nymph, larva) in this study, despite it being regularly reported to infect cattle and sheep after phylogenetic analysis. The role of ticks in the epidemiology of this pathogen remains uncertain.

The second most prevalent bacterium detected in tick samples in this study was *Ehrlichia/Anaplasma* spp. (37.29%). *Anaplasma platys* was the species found in dogs in Vietnam (30) and *E. canis* commonly detected in ticks and canines in different countries in Southeast Asia such as Thailand (3), Cambodia (32), or Philippines (33). However, all tick pools in the current study tested negative for *A. platys* and *E. canis,* when further analyzed for *A. platys* and *E. canis* identification using species-specific primers. These findings indicate other species belonging to the genus *Ehrlichia* and *Anaplasma* might be circulating in ticks in the studied areas. A further investigation on this bacteria in ticks with an increased number of samples is suggested to identify and investigate the phylogeny of these bacteria in Vietnam. *Rickettsia felis* is an emerging insect-borne rickettsial pathogen causing flea-borne spotted fever, which can be found in mammalian hosts and arthropods worldwide (34), and *Ctenocephalides felis* fleas—known as the main vector and reservoir for this pathogen (35). This pathogen has been recently detected in *C. felis* from dogs and cats in several parts of Vietnam (36–38). Our study found *R. felis* for the first time in both adult and nymph stages of *R. sanguineus* from owned dogs in Vietnam. The discovery of *R. felis* in both adult and nymph stages of *R. sanguineus*, as well as the high frequency of this tick species parasitizing dogs in the study, raises concerns about the potential transmission of *R. felis* to residents in the areas under investigation. Human cases of febrile sickness caused by *R. felis* have been reported in Thailand (39), Indonesia (40), and Laos (41). In Vietnam, *R. felis* was recently discovered and detected in humans by a molecular assay (42). The agent was reported to cause a severe undifferentiated fever in patients who displayed an eschar, a common clinical indicator that sometimes appeared at the location of the tick or mite bite (43, 44). *Rhipicephalus sanguineus* can transmit other species of the spotted fever group Rickettsiae, including *Rickettsia rickettsii*, which causes Rocky Mountain spotted fever, a severe and potentially fatal tick-borne disease; however, *R. rickettsii* has not been reported yet in Southeast Asia (43). The presence of *R. felis* and other pathogens found in *R. sanguineus* in this study suggests that these pathogens may also be present in the canines under investigation, posing a risk of infection to the host. This emphasizes the significance of performing epidemiological investigations on tick-borne infections in canines in the future. Moreover, *H. canis* and *B. vogeli* were discovered in a substantially lower percentage, which showed similar to the previous studies (9, 38). In summary, the identification of the above mentioned pathogens in ticks highlights the potential of this ectoparasite to transmit harmful organisms, posing a threat to both human and animal health (1, 4).

## 5 Conclusion

The study found that the brown dog tick (*R. sanguineus* s.l.) is common in dogs in the studied areas of Vietnam. This study validated multiple parameters that contribute to the probability of tick infestation in dogs, essential for successful veterinary therapies. The data indicate the presence of *R. felis*, a causative agent of spotted fever, in *R. sanguineus* ticks in Vietnam for the first time, which poses substantial health concerns to animals.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

## Ethics statement

The animal studies were approved by the Ethics Committee of Obihiro University of Agriculture and Veterinary Medicine accepted the protocol for using animal samples in this work (Permit for animal experiment: 21-25; DNA experiment: 1725-5). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

TD: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing – review & editing. LKB: Funding acquisition, Resources, Writing – original draft, Writing – review & editing. RU-S: Methodology, Writing – original draft, Writing – review & editing. TI: Methodology, Writing – original draft, Writing – review & editing. TH: Methodology, Writing – original draft, Writing – review & editing. IZ: Methodology, Writing – original draft, Writing – review & editing. ZM: Methodology, Writing – original draft, Writing – review & editing. LH: Methodology, Writing – original draft, Writing – review & editing. UKM: Methodology, Writing – original draft, Writing – review & editing. MA: Methodology, Writing – original draft, Writing – review & editing. SAE-S: Methodology, Writing – original draft, Writing – review & editing. XX: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. KK: Funding acquisition, Writing – review & editing.

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## 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.

## Publisher's note



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# Appendix

TABLE A1 Clinical signs associated with the detection of tick infestation in dogs.

| Clinical signs | Tested samples | Tick-infested samples (%) | Df ($\chi^2$) *p*-value | OR (*p*-value) | CI 95% |
|---|---|---|---|---|---|
| **Mucus membrane** | | | | | |
| Normal | 1,285 | 343 (26.69) | 3 (97.14) 0.0001 | Ref | |
| Jaundice | 14 | 14 (100) | | Invalid | |
| Pale | 68 | 49 (72.06) | | 7.08 (0.001) | 4.11–12.20 |
| Hemorrhage | 3 | 1 (33.33) | | Invalid | |
| NG | 53 | 7 (13.21) | | | |
| **Skin and hair status** | | | | | |
| Normal | 1,120 | 262 (23.39) | 1 (118.62) 0.001 | Ref | |
| Abnormal | 251 | 146 (58.17) | | 4.55 (0.001) | 3.42–6.06 |
| NG | 52 | 6 (11.54) | | | |

NG, Not given; Df, Degrees of freedom; $\chi^2$, Chi-square test; OR, Odds ratio; CI, confidence intervals; Ref., reference used; No., number.

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# *Culicoides* biting midges among cattle in France: be wary of data in the literature

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*Culicoides* are vectors that can transmit many different pathogens to mammals — including humans, and domestic and wild animals — and birds. In order to take preventive measures against any vector-borne disease, it is important to gather information on both the host and vector species. *Culicoides* species are mainly mammalophilic, ornithophilic or ornithophilic/mammalophilic, but females have also been found to occasionally feed on engorged insects. A recent systematic review based on three groups of key words investigated *Culicoides* on farms, and asserted that 92 species (including four not present species) have been reported among cattle in mainland France and Corsica. We have re-evaluated the presence of *Culicoides* species in cattle in France using the same data of the review. Our data show that only 18 species are reported among cattle. Furthermore, our research used molecular and indirect investigations to analyse *Culicoides* species that had been feeding on cattle. Our results demonstrate that 45 species feed on cattle out of 92 species present in France. The paper discusses the relevance of data in the literature when investigating hosts of *Culicoides* species.

KEYWORDS

**France, cattle, *Culicoides*, vectors, pathogen**

## Introduction

Vector-borne diseases, transmitted by a variety of arthropods, cause health problems for humans, livestock and wild animals. Currently, more than 1,400 *Culicoides* species have been described worldwide (1). *Culicoides* biting midges have been incriminated in the transmission of viruses, protozoa and filarial worms (2). Their economic impact in Europe is due to their transmission of the bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV), Schmallenberg virus (SBV) and African horse sickness virus (AHSV). In order to take preventive measures against a vector-borne disease, it is important to gather information on both the host and vector species. Identifying the feeding patterns of *Culicoides* biting midges is an essential step in pathogen circulation in order, for example, to break the transmission chain by vaccinating animals at greatest risk of infection (3).

Generally speaking, *Culicoides* midges have a wide host spectrum and tend to feed on blood opportunistically (4–7); they are classified as mammalophilic, ornithophilic or ornithophilic & mammalophilic species (8–10). *Culicoides* species are more attracted by cattle than sheep (11), and are found in natural areas and on farms. Indeed, the same species feed on both wild and domestic animals, so a switch is possible and could facilitate the circulation of pathogens between these two communities (12). In this context, an accurate

knowledge of *Culicoides* species present on farms is essential for anticipating diseases. In France, 92 species of *Culicoides* are reported (13–18). A recent study examining *Culicoides* species on farms in mainland France and Corsica suggested that 94 species (including two invalid species and four not present species) were present among cattle. The study was based on a systematic review using three groups of key words (19). In this context, do these species play an epidemiological role in the transmission of pathogens among cattle? The first step in answering this question is to prove that there is a relationship between the host and the insect. *Culicoides* species-host pairing is a key factor in anticipating and understanding the transmission of vector-borne pathogens. Here, we investigate *Culicoides* species and cattle based on a review in order to research bibliographic references with traditional approach (PCR analysis of blood meals taken from the host by female *Culicoides* in the field) and indirect investigations (animal baits, direct aspiration, light traps and glue strips). We compare the checklist of *Culicoides* species reported in France with their cattle preference. Prudhomme et al. (19) reported that all *Culicoides* species in France are present among cattle and this number of species is superior on other references reported. So, a second objective is to re-evaluate the presence of *Culicoides* spp. in cattle in France.

## Materials and methods

First, in 2012, a list of *Culicoides* present in France was drawn up by Venail et al. (16). The list of *Culicoides* species identified 88 species in all, 83 for mainland France and 61 for Corsica.

Four species have since been added to this preliminary list: (i) *C. paradoxalis* Ramilo and Delécolle, 2013 was identified in mainland France and Corsica (13); (ii) C. *boyi* Nielsen, Kristensen and Pape, 2015 was reported in mainland France (14) on a farm with cattle (20); (iii) *C. bysta* Sarvašová and Mathieu, 2017 was reported in mainland France (15) and (iv) *C. cryptipulicaris* Talavera, Muñoz-Muñoz, Verdún and Pagès, 2017 (13, 17, 18).

The distribution of species is not homogeneous in these four groups: for example Mediterranean littoral and Corsica present the same climate but a species present in Corsica is no necessary present in Mediterranean littoral and *vice-versa* (i.e., *C. corsicus* Kremer, Leberre and Beaucournu-Saguez, *C. derisor* Callot and Kremer, *C. heteroclitus* Kremer and Callot, *C. impunctatus* Goetghebuer, *C. indistinctus* Khalaf, *C. jamaicensis* Edwards, *C. maritimus paucisensillatus* Callot, Kremer and Rioux, *C. minutissimus* (Zetterstedt), *C. montanus* Shakirzjanova, *C. nubeculosus* (Meigen), *C. paradisionensis* Boorman, *C. reconditus* Campbell and Pelham-Clinton, *C. riebi* Delécolle, Mathieu and Baldet, *C. saevus* Kieffer, *C. sahariensis* Kieffer, *C. salinarius* Kieffer, *C. segnis* Campbell and Pelham-Clinton, *C. simulator* Edwards, *C. tauricus* Gutsevich, *C. vexans* (Staeger)). All articles including *Culicoides* spp. and host preference were selected. All publications have been reading. Our study used a traditional approach (PCR analysis of blood meals taken from the host by female *Culicoides*) and indirect investigations (animal baits, direct aspiration, light traps and glue strips) in the Palaeartic region to research the feeding preferences of *Culicoides* spp. The data we found in the literature were from various countries, including the country that first described the type and species in the Palaeartic region (Table 1). The feeding preferences of *Culicoides* biting midge species present in France (Table 1) has been linked to cattle (Table 2).

Secondly, documents, selected by Prudhomme et al. (19), are analysed in Supplementary Table S1. Publications selected (focus on biting midges) were reviewed in full text for inclusion of cattle (cattle OR livestock OR bovine OR cow OR beef OR calf OR calves OR heifer).

## Results

In all, we found 45 species that take blood meals from cattle (Table 2). Four species (*C. fagineus* Edwards *C. haranti* Rioux, Descours and Pech, *C. montanus* Shakirzjanova and *C. semimaculatus* Clastrier) have been reported to feed on cattle without information as to the method of collection (noted ¥ in Table 2). Finally, 14 species (*C. albicans* (Winnertz), *C. cataneii* Clastrier, *C. fascipennis* (Staeger), *C. festivipennis* Kieffer, *C. gejgelensis* Dzhafarov, *C. heliophilus* Edwards, *C. impunctatus* *C. longipennis* Khalaf, *C. maritimus* Kieffer, *C. minutissimus*, *C. shaklawensis* Khalaf, *C. stigma* (Meigen). *C. subfagineus* Delécolle and Ortega and *C. subfasciipennis* Kieffer) have been reported to feed on cattle based only on indirect investigations.

Second, based on 62 number of articles reviewed, only 4 articles, were used to compile the current species distribution list among the cattle (21–24). Overall, 18 species were reported in cattle: *C. achrayi* Kettle and Lawson, *C. brunnicans* Edwards, *C. chiopterus* (Meigen), *C. deltus* Edwards, *C. dewulfi* Goetghebuer, *C. duddingstoni* Kettle and Lawson, *C. festivipennis*, *C. furcillatus* Callot, Kremer and Paradis, *C. lupicaris* Downes and Kettle, *C. minutissimus*, *C. newsteadi* Austen, *C. obsoletus* (Meigen), *C. pallidicornis* Kieffer, *C. pictipennis* (Staeger), *C. pulicaris* (Linnaeus), *C. punctatus* (Meigen), *C. scoticus* Downes and Kettle, *C. vexans* Obsoletus group and *C. obsoletus/C. scoticus*.

## Discussion

The epidemiology of vector-borne diseases is linked to the preferred host and feeding behaviour of the arthropod vector. Our results reveal that 45 species feed on cattle (Table 2). The vertebrate hosts of 31 (9) and 37 (10) *Culicoides* species have previously been identified by molecular means. Indirect and molecular biology methods are in fact compatible (Table 2). There are more reports on the preferred hosts of *Culicoides* spp. by indirect methods than molecular tools (Table 2). Finally, research using proteomics to identify host proteins reveals a more diverse host pool than research using a traditional molecular approach (24). For *C. imicola* Kieffer, for example, the number of hosts varies between 1.5 and 5. Future studies on blood meal sources (i.e., hosts) could use a combination of PCR and proteomics-based methods to better characterise the *Culicoides* host spectrum.

Prudhomme et al. (19) proposed applying a step with three items (France OR Corsica OR French) AND (cattle OR livestock OR bovine OR cow OR beef OR calf OR calves OR heifer) AND (haematophag* OR hematophag* OR vector* OR arthropod* OR insect* OR tick* OR mite* OR acari*) to search for literature on the presence of *Culicoides* among cattle. They reported 94 *Culicoides* species recorded on different types of cattle farms in mainland France and Corsica. However, their review contains many mistakes: (i) many of the references are based on larval ecology (25–30) and these papers did not describe either the animals or any farms in the vicinity of the muds collected; (ii) references

TABLE 1 Checklist of *Culicoides* species reported in mainland France and Corsica with the countries where types were first identified.

| Species name | Country of identification of *Culicoides* type | Distribution | |
|---|---|---|---|
| | | Mainland | Corsica |
| *C. abchazicus* Dzhafaraov, 1964 | Georgia | ☒ | |
| *C. achrayi* Kettle and Lawson, 1955 | Ukraine | ☒ | ☒ |
| *C. alazanicus* Dzhafaraov, 1961 | Azerbaijan | ☒ | ☒ |
| *C. albicans* (Winnertz), 1852 | Germany | ☒ | |
| *C. albihalteratus* Goetghebuer, 1935 | France | ☒ | |
| *C. begueti* Clastrier, 1957 | Algeria | ☒ | ☒ |
| *C. boyi* Nielsen, Kristensen and Pape, 2015 | Denmark | ☒ | |
| *C. brunnicans* Edwards, 1939 | Great Britain | ☒ | ☒ |
| *C. bysta* Sarvašová and Mathieu, 2017 | Slovakia | ☒ | |
| *C. cameroni* Campbell and Pelham-Clinton, 1960 | Great Britain | ☒ | ☒ |
| *C. cataneii* Clastrier, 1957 | Algeria | ☒ | ☒ |
| *C. caucoliberensis* Callot, Kremer, Rioux and Descours 1967 | France | | ☒ |
| *C. chiopterus* (Meigen), 1830 | Europe | ☒ | ☒ |
| *C. circumscriptus* Kieffer, 1918 | Tunisia | ☒ | ☒ |
| *C. clastrieri* Callot, Kremer and Déduit, 1962 | France | ☒ | ☒ |
| *C. clintoni* Boorman, 1984 | Great Britain | ☒ | |
| *C. comosioculatus* Tokunaga, 1956 | Japan | ☒ | |
| *C. corsicus* Kremer, Leberre and Beaucournu-Saguez, 1971 | France | ☒ | ☒ |
| *C. cryptipulicaris* Talavera, Muñoz-Muñoz, Verdún and Pagès, 2017 | Spain | | |
| **C. delta** Edwards, 1939 (=*C. lupicaris* Downes and Kettle, 1952 in Borkent 2022) | Great Britain | | |
| *C. derisor* Callot and Kremer, 1965 | France | ☒ | ☒ |
| *C. dewulfi* Goetghebuer, 1936 | Belgium | ☒ | ☒ |
| *C. duddingstoni* Kettle and Lawson, 1955 | Great Britain | ☒ | ☒ |
| *C. dzhafarovi* Remm, 1967 | Azerbaijan | ☒ | |
| *C. fagineus* Edwards, 1939 | Great Britain | ☒ | ☒ |
| *C. fascipennis* (Staeger), 1839 | Denmark | ☒ | ☒ |
| *C. festivipennis* Kieffer, 1914 | Germany | ☒ | ☒ |
| *C. flavipulicaris* Dzhafarov, 1964 | Azerbaijan | ☒ | ☒ |
| *C. furcillatus* Callot, Kremer and Paradis, 1962 | France | ☒ | ☒ |
| *C. gejgelensis* Dzhafarov, 1964 | Azerbaijan | ☒ | ☒ |
| *C. griseidorsum* Kieffer, 1918 | Algeria | ☒ | ☒ |
| *C. grisescens* Edwards, 1939 | Great Britain | ☒ | |
| *C. haranti* Rioux, Descours and Pech, 1959 | France | ☒ | ☒ |
| *C. heliophilus* Edwards, 1921 | Great Britain | ☒ | |
| *C. helveticus* Callot, Kremer and Déduit, 1962 | Switzerland | ☒ | |
| *C. heteroclitus* Kremer and Callot, 1965 | France | ☒ | |
| *C. ibericus* Dzhafarov, 1963 | Azerbaijan | ☒ | |
| *C. imicola* Kieffer, 1913 | Kenya | ☒ | ☒ |
| *C. impunctatus* Goetghebuer, 1920 | Belgium | ☒ | |
| *C. indistinctus* Khalaf, 1961 | Iraq | ☒ | ☒ |
| *C. jamaicensis* Edwards, 1922 (=*C. paolae* Boorman 1996) | Jamaica | | ☒ |
| *C. jumineri* Callot and Kremer, 1969 | Tunisia | ☒ | ☒ |
| *C. jurensis* Callot, Kremer and Déduit, 1962 | France | ☒ | |

*(Continued)*

TABLE 1 (Continued)

| Species name | Country of identification of *Culicoides* type | Distribution | |
|---|---|---|---|
| | | Mainland | Corsica |
| *C. kibunensis* Tokunaga, 1937 | Japan | ☒ | ☒ |
| *C. kurensis* Dzhafarov, 1960 | Azerbaijan | ☒ | ☒ |
| *C. longipennis* Khalaf, 1957 | Iraq | ☒ | ☒ |
| **C. lupicaris** Downes and Kettle, 1952 (= *C. delta* Edwards, 1939 in Borkent, 2022) | Great Britain | ☒ | ☒ |
| *C. malevillei* Kremer and Coluzzi, 1971 | Italy | ☒ | ☒ |
| *C. manchuriensis* Tokunaga, 1941 | China | ☒ | |
| *C. maritimus* Kieffer, 1924 (=*C. submaritimus* Dzhafarov, 1962 in Borkent, 2022) | Germany | ☒ | ☒ |
| **C. maritimus pauscisensillatus** Callot, Kremer and Rioux, 1963 | France | ☒ | |
| *C. minutissimus* (Zetterstedt), 1855 | Sweden | ☒ | ☒ |
| *C. montanus* Shakirzjanova, 1962 | Kazakhstan | | ☒ |
| *C. newsteadi* Austen, 1921 | Israel | ☒ | ☒ |
| *C. nubeculosus* (Meigen), 1830 | Europe | ☒ | |
| *C. obsoletus* (Meigen), 1818 | Europe | ☒ | ☒ |
| *C. odiatus* Austen, 1921 | Israel | ☒ | ☒ |
| **C. pallidicornis/subfasciipennis Kieffer, 1919** | Hungary | ☒ | ☒ |
| *C. paradisionensis* Boorman, 1988 | Greece | | ☒ |
| *C. paradoxalis* Ramilo and Delécolle, 2013 | France | ☒ | ☒ |
| *C. parroti* Kieffer, 1922 | Algeria | ☒ | ☒ |
| *C. pictipennis* (Staeger), 1839 | Denmark | ☒ | ☒ |
| *C. picturatus* Kremer and Déduit, 1961 | France | ☒ | ☒ |
| *C. poperinghensis* Goetghebuer, 1953 | Belgium | ☒ | ☒ |
| *C. pseudopallidus* Khalaf, 1961 | Iraq | ☒ | |
| *C. pulicaris* (Linnaeus), 1758 | Europe | ☒ | ☒ |
| *C. punctatus* (Meigen), 1804 | Europe | ☒ | ☒ |
| *C. puncticollis* (Becker), 1903 | Egypt | ☒ | ☒ |
| *C. reconditus* Campbell and Pelham-Clinton, 1960 | Great Britain | ☒ | |
| *C. riebi* Delécolle, Mathieu and Baldet, 2005 | France | ☒ | ☒ |
| *C. riethi* Kieffer, 1914 | Germany | ☒ | |
| *C. riouxi* Callot and Kremer, 1961 | France | ☒ | ☒ |
| *C. saevus* Kieffer, 1922 | Algeria | ☒ | |
| *C. sahariensis* Kieffer, 1923 | Algeria | ☒ | |
| *C. salinarius* Kieffer, 1914 | Germany | ☒ | ☒ |
| *C. santonicus* Callot, Kremer, Rault and Bach, 1966 | France | ☒ | ☒ |
| *C. scoticus* Downes and Kettle, 1952 | Great Britain | ☒ | ☒ |
| *C. segnis* Campbell and Pelham-Clinton, 1960 | Great Britain | ☒ | |
| *C. semimaculatus* Clastrier, 1958 | Algeria | ☒ | ☒ |
| *C. shaklawensis* Khalaf, 1957 | Iraq | ☒ | ☒ |
| *C. simulator* Edwards, 1939 | Great Britain | ☒ | |
| *C. sphagnumensis* Williams, 1955 | USA (Michigan) | ☒ | |
| *C. stigma* (Meigen), 1818 | Europe | ☒ | |
| *C. subfagineus* Delécolle and Ortega, 1998 | Spain | ☒ | ☒ |
| **C. subfasciipennis/pallidicornis Kieffer, 1919** | Hungary | ☒ | ☒ |
| **C. submaritimus** Dzhafarov, 1962 (=*C. maritimus* Kieffer, 1924 in Borkent, 2022) | Azerbaijan | ☒ | ☒ |
| *C. tauricus* Gutsevich, 1959 | Ukraine | ☒ | |
| *C. tbilisicus* Dzhafarov, 1964 | Azerbaijan, Georgia | ☒ | ☒ |

*(Continued)*

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TABLE 1 (Continued)

| Species name | Country of identification of *Culicoides* type | Distribution | |
|---|---|---|---|
| | | Mainland | Corsica |
| *C. truncorum* Edwards, 1939 | Great Britain | ☒ | ☒ |
| *C. univittatus* Vimmer, 1932 | Israel | ☒ | ☒ |
| *C. vexans* (Staeger), 1839 | Denmark | ☒ | |
| *C. vidourlensis* Callot, Kremer, Mollet and Bach, 1968 | France | ☒ | ☒ |

The species name is based on the catalogue compiled by Borkent and Dominiak (1). In bold: discrepancies between the catalogue and Venail et al. (16).

(25, 31–33) do not comply with the item "cattle OR livestock OR bovine OR cow OR beef OR calf OR calves OR heifer"; (iii) *C. cameroni* Campbell and Pelham-Clinton and *C. clintoni* Boorman were collected in natural areas and not on farms (34), so *C. clintoni* was not found with cattle; (iv) many *Culicoides* have been collected through the national surveillance programme in France (35). Traps were implemented among several types of livestock in both Corsica [sheep, cattle, horses (36); sheep (37); sheep, cattle (16)] and the French mainland [sheep (37); sheep and cattle (16, 21, 38, 39)]. Thus, the references using results of *Culicoides* trapped during national surveillance programme activities did not separate farms with cattle only from farms with other animals too. Consequently, the resulting list, which is said to focus only on cattle, is unreliable; (v) at the beginning of the *Culicoides* national surveillance programme, the composition of the Pulicaris group included three species (*C. pulicaris, C. lupicaris* Downes and *C. flavipulicaris* Dzhafarov) (16). Nowadays, in France the Pulicaris group includes six species (*C. boyi, C. bysta, C. cryptipulicaris*, *C. pulicaris, C. lupicaris* and *C. flavipulicaris*) (see Material and Methods and Table 1) but this new group has not been incorporated in the last publication (40); (vi) *Culicoides ibericus* Dzhafarov, *C. manchuriensis* Tokunaga, *C. sahariensis*, *C. tauricus* and *C. vexans* are found in mainland France but not Corsica (16); (vii) *C. pseudopallidus* Khalaf 1961 is present in France but *C. pseudoheliophilus* is not (16); (viii) finally, *C. accraensis* Carter, Ingram and Macfie, *C. albipennis* Kieffer, *C. musicola* (invalid species), *C. pumilus* (Winnertz), *C. sergenti* (Kieffer) and *C. sigrosignatus* (invalid species) have not been reported in France (14, 16). In our opinion, the *Culicoides* species presented in this systematic review (19) are not aligned with field reality. Our data show that 18 species are present among cattle in France (mainland without Corsica). The review of Prudhomme et al. (19) uses much results obtained by the national surveillance. But publications did not separate farms with cattle only from farms with other animals (See above). A great deal of vigilance must be exercised when we elaborate a review (based on requests in databases), in particular to choose of items.

The references used by Prudhomme et al. (19) include four techniques for characterising the presence of *Culicoides* biting midges among cattle: animal baits, larval ecology, light traps and the blood meals of *Culicoides* females. While light traps offer a good description of diversity (7), they do not accurately reflect the proportions of biting midges in an area (41). The light trap is not suited for midges feeding on an animal (42). Trapped engorged females with a blood meal in their abdomen can be used to accurately reveal host preferences by identifying the origin of the blood meal, but there is a flagrant bias because only night species are caught, and the attractiveness of light traps is limited. Animal baits can be used to identify host preferences and attack rates by counting and identifying the numbers of biting midges that can be captured from a specific host. Larval ecology offers knowledge of substrates suitable for *Culicoides* larval development. This method is not suitable for investigating relationships between a species and a host [in Prudhomme et al. (19) there is only one reference (23)]. A study on Belgian cattle farms has shown that 13 *Culicoides* species were obtained by incubation of soil samples (43) with 11 species presenting on cattle. In contrast, Uslu and Dik (44) describes the breeding sites of 18 *Culicoides* species in Turkey without describing the environmental sites. Future studies on larval development in the immediate surroundings of cattle farms will shed light on the microhabitats of *Culicoides* biting midges.

Five criteria are used to consider an arthropod as a biological vector of arboviruses (45, 46), including the presence of virus (es) and abundance of the suspected vector. Light traps underestimate the numbers of *Culicoides* present in an area (41), so *C. chiopterus* (Meigen) had not been seriously considered as a potential vector of BTV (41). The French national surveillance programme (35) has reported species in "groups or complex" to show species distribution and seasonal dynamics (16). Epidemiological studies can use wing characteristics to group together species with similar markings without needing to mount the specimens (47). But in this case, the results are not suitable for characterising the abundance of specific species. But, surveillance programme allows to model the abundance of *Culicoides* spp. in order to identify risk periods in France (48).

Contacts between competent vertebrate hosts and insect vectors are vital for vector-borne pathogens to successfully complete their transmission cycle (49). Cattle are very attractive to *Culicoides*, with 45 species feeding on them (Table 2) out of 92 species present in France. Among the species that feed on cattle, *C. chiopterus, C. dewulfi, C. imicola, C. obsoletus, C. pulicaris, C. punctatus, C. scoticus* are confirmed or probable vectors of BTV (50) and SBV (51). In addition, two species of the *newsteadi* complex and two species (*C. nubeculosus C. lupicaris*) are recorded or suspected of being involved in BTV and SBV transmission (50, 51), respectively. Most *Culicoides* species are opportunistic and may change host depending on availability (9, 10). For example, *C. scoticus* can switch from its preferential (predominant) mammal host to other hosts according to site and host availability (20). Many *Culicoides* species are known to feed on birds and to transmit the avian *Haemoproteus* parasite (52, 53). The ornithophilic *Culicoides* list includes 18 species (*C. alazanicus* Dzhafaraov, *C. cataneii, C. chiopterus, C. circumscriptus* Kieffer, *C. clastrieri* Callot, Kremer and Déduit, *C. festivipennis, C. griseidorsum* Kieffer, *C. impunctatus C. kibunensis* Tokunaga, *C. obsoletus, C. pallidicornis, C. pictipennis*, *C. pulicaris, C. punctatus, C. scoticus, C. segnis*, *C. seminaculatus* and *C. univittatus* Vimmer) (9, 52–54). Fourteen of these species may occasionally switch

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TABLE 2 Cattle preference of *Culicoides* species in France based on molecular analysis of engorged *Culicoides* females and other techniques (animal baits, direct aspiration, light traps, glue strips).

| Species | Indirect methods | Modern tools | Molecular and indirect studies |
|---|---|---|---|
| | References (2, 3, 4, 5, 6, 7, 8, 10, 11, 19, 24) | References (1, 8, 9, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23) | Reference (1) |
| *C. achrayi* | ☒ | ☒ | ☒ |
| *C. albicans* | ☒ | | ☒ |
| *C. brunnicans* | | ☒ | ☒ |
| *C. cataneii* | ☒ | | ☒ |
| *C. chiopterus* | ☒ | ☒ | ☒ |
| *C. circumscriptus* | ☒ | ☒ | ☒ |
| *C. deltus* | | ☒ | ☒ |
| *C. dewulfi* | ☒ | ☒ | ☒ |
| *C. fagineus* | ☒ ¥ | | ☒ |
| *C. fascipennis* | ☒ | | ☒ |
| *C. festivipennis* | ☒ | | ☒ |
| *C. furcillatus* | | ☒ | ☒ |
| *C. gejgelensis* | ☒ | | ☒ |
| *C. griseidorsum* | ☒ | ☒ | ☒ |
| *C. griescens* | ☒ | ☒ | ☒ |
| *C. haranti* | ☒ ¥ | | ☒ |
| *C. heliophilus* | ☒ | | ☒ |
| *C. imicola* | ☒ | ☒ | ☒ |
| *C. impunctatus* | ☒ | | ☒ |
| *C. jumineri* | | ☒ | ☒ |
| *C. kibunensis* | ☒ | ☒ | ☒ |
| *C. longipennis* | ☒ | | ☒ |
| *C. lupicaris* | ☒ | ☒+L2 | ☒ |
| *C. maritimus* | ☒ | | ☒ |
| *C. minutissimus* | ☒ | | |
| *C. montanus* | ☒ ¥ | | ☒ |
| *C. newsteadi* | ☒ | ☒ | ☒ |
| *C. obsoletus* | ☒ | ☒ | ☒ |
| *C. pallidicornis* | ☒ | ☒ | ☒ |
| *C. pictipennis* | | ☒ | ☒ |
| *C. picturatus* | | ☒ | ☒ |
| *C. poperinghensis* | | ☒ | ☒ |
| *C. pulicaris* | ☒ | ☒ | ☒ |
| *C. punctatus* | ☒ | ☒ | ☒ |
| *C. puncticollis* | ☒ | ☒ | ☒ |
| *C. riethi* | | ☒ | ☒ |
| *C. scoticus* | ☒ | ☒ | ☒ |
| *C. segnis* | ☒ | ☒ | ☒ |
| *C. semimaculatus* | ☒ ¥ | | ☒ |
| *C. shaklawensis* | ☒ | | ☒ |
| *C. stigma* | ☒ | | ☒ |
| *C. subfagineus* | ☒ | | ☒ |
| *C. subfasciipennis* | ☒ | | ☒ |

*(Continued)*

TABLE 2 (Continued)

| Species | Indirect methods | Modern tools | Molecular and indirect studies |
|---|---|---|---|
| | References (2, 3, 4, 5, 6, 7, 8, 10, 11, 19, 24) | References (1, 8, 9, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23) | Reference (1) |
| *C. submaritimus* | | ☒ | |
| *C. vexans* | ☒ | ☒ | ☒ |
| *C. obsoletus/scoticus* | ☒ | ☒ | |
| *C. subfascipennis/pallidicornis* | | ☒ | |
| *C.* sp. *nr. Newsteadi* | ☒ | | |

¥: Unspecified. References (see Supplementary Table S2).

to cattle (Table 2). In contrast, several species usually take their blood meals from mammals or birds indifferently, and can switch from one to another (9, 10). The relationship between insects and their host is thus a key factor in the transmission of pathogens, including host availability (attractiveness and acceptability) (55). *Culicoides* species feed disproportionately on different host species (11, 42). For example, *Culicoides* spp. have been reported as feeding on cattle nine times more than on sheep (42), showing their attraction to cattle over sheep (11). Nevertheless, host diversity impacts *Culicoides* species diversity (7, 11, 22, 34, 42). *Culicoides* use hosts differently according to their preferential feeding locations on the body. Moreover, in one study *Culicoides* species switched between hosts (a cow and ewe) and between parts of the body attacked (42). More biting midges were collected from the head than the back, belly/flank and legs (11). One possible explanation could be the adaptation of *Culicoides* to a preferential (predominant) host through specific morphology of antennae, palpi and the number and/or distribution of sensilla (9, 10). This means that the sensory structures can be used to distinguish between ornithophilic, mammalophilic, or ornithophilic & mammalophilic species. *Culicoides* blood meals are not systematically included in *Culicoides* research literature (only 24 references in the Palaeartic region). As discussed, it is nonetheless a key factor for identifying the host spectrum of a species in order to anticipate/understand the transmission of pathogens. Hence, further studies that include both molecular approaches and indirect investigations and that focus on host preference by *Culicoides* species with nocturnal and diurnal activities would provide greater insights into *Culicoides* feeding patterns and vector-host interactions.

In conclusion, our study investigated the relationship between cattle and biting midges based on direct and indirect investigations. Data in the literature reports that all *Culicoides* species in France are present among cattle but our results reveal that only 18 species are present among cattle and 45 species feed on cattle out of the 92 species present. It could be misleading to state that species found near cattle interact with those cattle through blood meals, and research should focus on proving the midge vector-host relationship through both modern and traditional means in order to identify both the species involved in viral transmission and the viruses transmitted.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

## Author contributions

CM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. LH-H: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. DA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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## 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.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1451442/full#supplementary-material

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# The transmission routes of African swine fever during an outbreak in Serbia July–August 2023: African swine fever virus detections in environmental samples and insects

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African swine fever (ASF) is a highly contagious viral disease of domestic pigs and wild boar (*Sus scrofa*) caused by an arbovirus- African swine fever virus (ASFV), which is classified into the family *Asfarviridae*. Even though the main transmission route of ASFV is direct contact between animals and carcasses releasing ASFV into the environment, the role of other transmission routes such as via environmental contamination or insects remains in great part unclear. During an epidemic f ASF in Serbia in 2023, environmental samples (soil, feed, water and swabs from the pig barns), and insects [resulting in collection of adult and/or larval stages of non-biting flies (*Diptera*: *Calliphoridae* and *Muscidae*)] were collected in four locations in South Banat district of Serbia. To assess the possibility that insects carry the ASFV in infected courtyards, sticky fly traps and open Petri dishes containing meat mixed with humid cotton wads were offered in three locations during the five days of the experiment in the Belgrade area. Furthermore, to confirm the role of flies in ASF in mechanical transmission pathway in Serbia, L2 and L3 larvae of flies (*Lucilia sericata* Meigan, 1826; *Stomoxys calcitrans* Linnaeus, 1758) were collected from the pig carcasses from infected farms, bred to adults in the Laboratory and an ASFV spiked meat meal was placed into cages with three-day-old flies ($n = 52$) to estimate positivity of flies and duration of mechanical transmission of ASFV. The results from the environmental samples showed no positive ASFV DNA detection, the same was true for the samples from meat-based traps and sticky fly traps, while ASFV DNA was detected in three samples containing eggs, L1 and L3 fly larvae collected from carcasses and adult flies (*L. sericata*). In experimental conditions, only one *S. calcitrans* fly tested positive on day 1 post-infection. The results implicate the possible role of *Lucilia* sp. flies in the mechanical transmission of ASFV as well as *S. calcitrans* in Serbia during an outbreak, while ASFV DNA was not detected in environmental samples in this study.

KEYWORDS

**African swine fever virus, flies, environment, *Lucilia sericata*, *Stomoxys calcitrans*, Serbia**

## Introduction

African swine fever (ASF) is a highly contagious hemorrhagic disease of domestic pigs and wild boar (*Sus scrofa*) caused by an arbovirus classified into the family *Asfarviridae* and genus *Asfivirus*. In Europe, the presence of African swine fever virus (ASFV) genotype II was first detected in 2007 in Georgia (1), and in the following years it spread in the direction of Western Europe reaching Serbia in 2019 (2). In later years ASFV has been detected in Serbia occurring in both domestic pigs and wild boar (3). Furthermore, multiple ASFV strains of genotype II have been circulating in Serbia (4). During 2022 ASFV was detected in different locations, in western Serbia (5) in domestic pigs enhanced by specific structure of swine holdings in rural areas with low biosecurity measures (6).

According to Serbian pig holding categorization given by the Veterinary Directorate, Ministry of Agriculture, there are five production types: commercial farms (high biosecurity); family farms type A (over 10 animals, high biosecurity); family farms type B (over 10 animals, low biosecurity); backyards (few pigs for self-consumption, minimal biosecurity); and free-range systems with no biosecurity. Backyard production in Serbia focuses on home consumption and local sales for extra income, often involving home slaughtering, swill feeding, and little biosecurity (7). Furthermore, this widespread type of pig production in villages in Serbia mostly does not meet even the minimum biosecurity standards. In a study, it was shown that backyard pig production is usually consisting of up to five pigs per holding, aimed at production of fatteners (59.5%), located within a hunting area or within 1,000 meters of a hunting ground (61.8%), and approximately 60.7% of holdings were situated close to other pig holdings within 100 meters distance with contact between different animal species (such as dogs, cats) in 76.4% of holdings and with. Complete fencing in 57.3% of holdings (8).

In the natural cycle in Africa where ASFV originated, soft ticks *(Acari: Argasidae)* of the genus *Ornithodoros* are reservoirs and vectors, through the population of several species of wild pigs [such as the common warthog (*Phacochoerus africanus*) and bushpigs (*Potamochoerus larvatus*)] (9). The main transmission routes of ASFV in Europe include wild boar population and human activities (10) since established population of the only competent tick vector species present in Europe, *Carios erraticus* (Lucas, 1849), formerly *Ornithodoros erraticus,* is found only in restricted areas of Iberian Peninsula (11). Possibilities of ASFV transmission via different insect species as mechanical vectors are well established for several families such as flies (*Diptera: Muscidae*) (12, 13), but in large part the role of insects as vectors in the epidemiology of ASF in Europe is unclear and under debate (13–15).

The ASFV in highly resistant to environmental factors (such as pH) and can last over a year in blood, a few months in boneless meat and even a few years in frozen carcasses (1). Experiments confirmed excretion of ASFV in nasal, rectal and oral fluids as well as faces and urine of infected animals, thus causing contamination of feed, water, soil and environment (16). Contaminated pig feed was responsible for at least two independent introductions of ASFV in China in 2018 (17). Furthermore, ASFV is not sensitive to the process of decomposition of carcasses and survives up to 112 days in forest soil (1). In a study from Germany, soil pH, soil structure, and ambient temperature played a role in the stability of infectious ASFV. Infectious ASFV was demonstrated in specimens originating from sterile sand for at least three weeks, from beach sand for up to two weeks, from yard soil for one week, and from swamp soil for three days while ASFV was not recovered from two acidic forest soils (18). In Romania, contamination of water from Danube river is a suspected cause of ASF outbreak in a large farm with high biosecurity measures (19). Possibilities for infection of swine via ingestion of contaminated feed and water have been assessed, showing that liquid diet enhance ASFV infection (20).

For a better understanding and exploration of more effective prophylactic and reactive management actions against ASFV in Serbia, an individual-based approach is recommended to comprehensively investigate and consider all the local patterns of the disease, including characteristic outbreaks in wild boars, potential amplifying spots, virus sources, and other potential drivers such as habits of the human population, and vectors. The implementation of measures based on the knowledge is urgently needed (21).

In Serbia, it is shown that outbreaks and ASFV eradication processes in domestic pigs in poor, low-biosecurity are not dependent on ASF occurrence in wild boar whose involvement in ASFV transmission needs further investigation (3). Additionally, during the outbreaks in Serbia, observations revealed instances where ASF spread rapidly under epidemiologically unclear circumstances, despite the implementation of timely and comprehensive epidemiological investigations and measures. It was suspected that potential alternative pathways or drivers, facilitating disease transmission between locations, might be present including the role of environmental contamination and/or arthropod vectors. The role of these two potential ASFV transmission paths in Serbia is unclear and understudied. Hence, our study aimed to give further insight into transmission pathways of ASFV in Serbia through ASFV DNA detection in an environment and insects- flies during an outbreak. Furthermore, the role of adult flies (*Lucilia sericata* (*Diptera: Calliphoridae*), *S. calcitrans*) in the mechanical transmission of ASFV was experimentally assessed in order to determinate a potential timeframe in real-time setting and laboratory conditions.

## Materials and methods

### Study area and description of sampling localities

This study was performed in backyard pig production units and commercial farms consistent with aforementioned characteristics in two neighboring districts in Serbia (Belgrade city area and Southern Banat district) (Figure 1). All samples were collected during July and August 2023 in backyard pig production units (which in our study consisted of up to 5 pigs per unit), where ASF was previously detected by the National Reference Laboratory (NRL) at the Scientific Institute of Veterinary Medicine of Serbia (up to 48 h) before implementation of culling and disinfection measures (Figure 1). Established morbidity rate in households was between 50 and 70% depending on timepoint between the onset of the disease and the day of sampling.

The environmental samples (soil, feed, water, and environmental swabs from the pig barns) were taken from four localities, two in Belgrade city area (Duboka bara-Palilula 44.875905, 20.612613 and Borča- Put za Crvenku 44.861237, 20.438505) and two in Southern Banat district (Jabuka 44.943056, 20.593056 and Glogonj 44.983333, 20.533333). The soil samples ($n = 6$) were taken from three backyard

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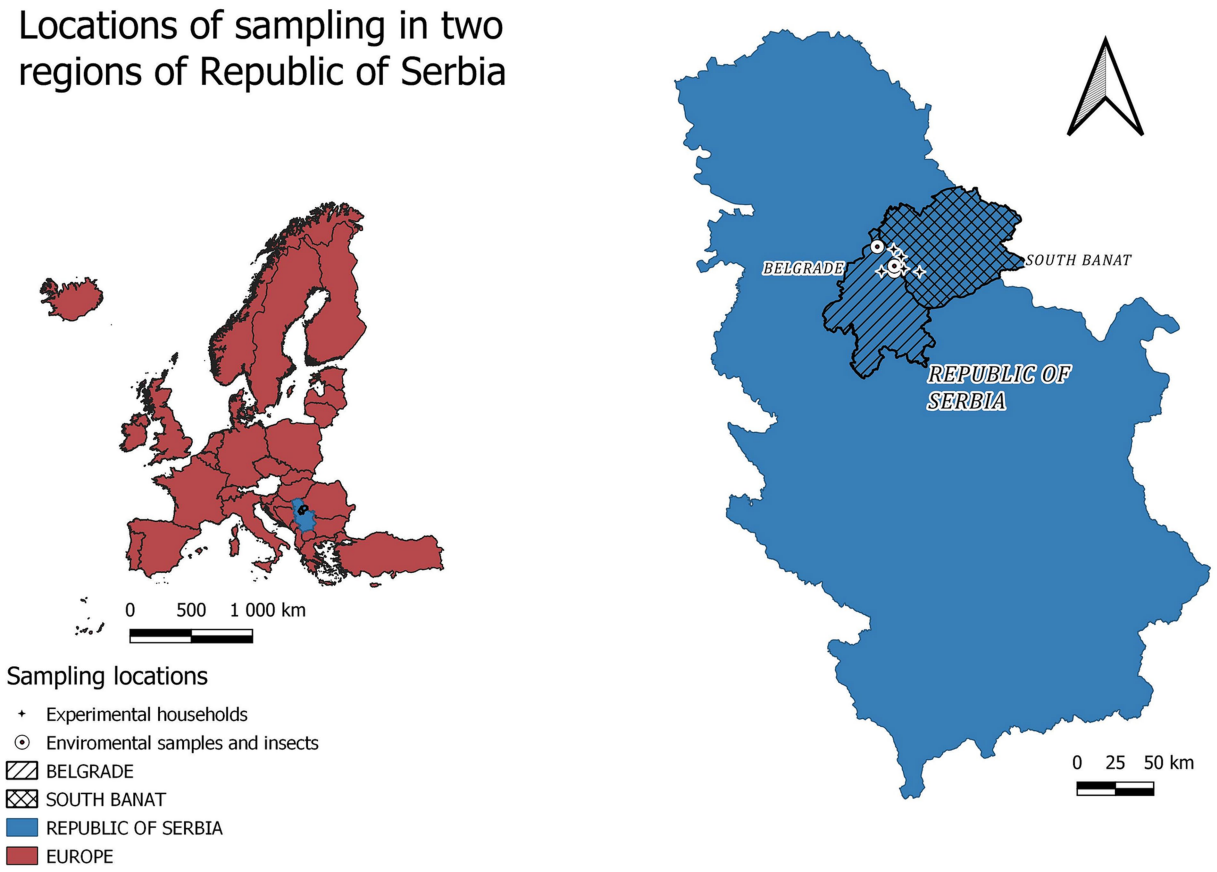


FIGURE 1
Locations of sampling in two regions of Serbia. Figure was produced using QGIS Version 3.28.3 Firence [QGIS.org (2024). QGIS Geographic Information System. Open Source Geospatial Foundation Project, "http://qgis.org"].

pig production units, in the radius of 10 meters from barn where the affected animals stayed, based on the hypothesis of active contamination due to sewage leakage. From one household in the Borca locality, feed samples ($n = 3$) were taken from full feeders where a traces of commercial feed, grains and swill existed. Drinking water ($n = 3$) was sampled at two backyard pig production units in Glogonj and one in Jabuka village. Environmental swabs were taken directly at the pig pen with outdoor access ($n = 12$), and 30 m from the place where diseased animals lived ($n = 3$) in all four localities. Insect samples-pools up to 20 flies ($n = 9$) were taken at two localities (Glogonj and Duboka bara-Palilula), while pools up to 100 flies were sampled at a commercial pig farm, where ASF was confirmed (44.858669, 20.736289) directly near the entrance to the pig production objects ($n = 3$) and up to 30 m away ($n = 3$). Additionally, the *Muscidae* eggs ($n = 2$) and larval stages ($n = 5$) were collected from the carcass of swine improperly disposed by the road in Borča-Put za Crvenku.

The households hosting sticky fly traps and open Petri dishes containing minced meat mixed with humid cotton wads to prevent desiccation at high summer temperatures were offered at three locations in Belgrade area namely Reva (44.861237, 20.538505), Ovča (44.890923, 20.538998) and Besni fok (44.998121, 20.405613) during five days of field experiment in August 2023. These localities were chosen based on the intensive ASFV activity nearby and accessibility with consent of the owners.

Larvae of the L2 and L3 instar for ASFV transmission experiments were sampled at two backyard pig production units (45.015525, 20.385289; 44.861237, 20.438505), in Besni fok, Belgrade location.

Average summer temperature for wider Belgrade area in July 2023 was 24.7°C, while maximal temperatures during the peak of the day were up to 36°C based on the data of Republic Hydrometeorological Service of Serbia.

## Sample collection and analysis

Environmental samples of soil were collected by direct sampling of a superficial layer (up to 5 cm in depth) with a spatula in a quantity of 50 g to the plastic containers from the location near the stables where ASFV-infected animals were bred. The soil samples were thoroughly mixed in 10% suspension in a minimal essential medium (MEM, Gibco, ThermoFisher Scientific, Waltham, MA, USA) enriched with antibiotics and antimycotics (MycoZap, Lonza Bioscience, Basel, Switzerland) and centrifuged at 2000 × g for 30 min. Supernatant was decanted and used for further analysis.

Feed was probed from a feeder in 500 g quantity. In the laboratory, the feed was thoroughly mixed, then from each mixed sample, one gram of feed was further mixed in 10% suspension in MEM (MEM, Gibco, ThermoFisher Scientific, Waltham, MA, USA) with antibiotics and antimycotics (MycoZap, Lonza Bioscience, Basel, Switzerland),

centrifuged at 2000 x g for 30 min, decanted, and frozen (at −80°C) until further use.

Drinking water was taken from the drinking troughs, from which the pigs drank, in 500 mL sterile containment. Water samples were collected from three locations, and brought to the Institute. The water samples were centrifuged at 5000 × g for 10 min, decanted, and frozen (at −80°C) until further use.

Equipment and metal surfaces, in the direct environment of ASFV-infected animals in pig barns, were swabbed using pre-wetted swabs with 0.9% natrium-chloride solution Swabs were submerged in 1 mL of phosphate-buffered saline (PBS) and subjected to homogenization. Subsequently, the samples underwent centrifugation at 1500 × g for 10 min. The resulting supernatants were carefully decanted and preserved at −80°C for subsequent analysis, following the protocol by Gallardo et al. (22).

Adult flies were collected manually using entomological nets, while on a farm sticky traps were set for the collection purpose. The flies were transferred to the NRL at the Scientific Institute of Veterinary Medicine of Serbia in cold chain. Representative subsample ($n = 5$ flies per sample selected by an entomologist) was taken for morphological insect identification. After morphological identification, the flies were pooled up to 100 flies based on observed morphological characteristics and stored at −80°C for subsequent analysis. *Muscidae* eggs and larval stages were collected using entomological tweezers, brought to the NRL, subsampled for confirmation of the fly species and stored at −80°C for subsequent analysis. Homogenization was performed using Tissuelyser II (Qiagen, Hilden Germany) at 30 Hz for 1 min.

## ASFV transmission trials in households which encountered ASFV outbreak

The attractant for flies for transmission trials in households which encountered ASFV outbreak consisted of Petri dishes containing minced beef meat (for human consumption, bought on the day when the traps were set), wads and water. Samples were left for three days exposed to contact with flying insects. Afterwards, Petri dishes were collected and transferred to the laboratory in hand freezers at +4°C. These samples were divided in three 1 g samples, homogenized in Tissuelyser II (Qiagen, Hilden Germany) at 30 Hz for 1 min.

## Preparation of the first-generation flies for ASFV transmission experiments

Larval instars L2 and L3 were taken from the carcasses of found dead pigs which tested positive for ASF. The collected larvae for the Besni fok location were split into two groups, one was tested for ASF, and the other was collected for rearing at the Insitute of Veterinary Medicine of Serbia. Larvae were selected based on the instars and morphological characteristics and placed in double containment. Larvae, pupae and adults were kept at 24–26°C, 60–70% relative humidity (RH). Larvae were fed with feed composed of 50% humid paper wads, and 50% minced cattle meat replaced every second day, while adults were offered 5% glucose solution *ad libitum*. Larvae L3 ($n = 3$) were tested for the presence of ASFV DNA using qPCR (23). A total of 42 pupae in the first experiment and 21 pupae in the second experiment were transferred into modified trial cages- with slight modifications to described in [(24); Figure 2]. Prior to ASFV transmission trials, adult flies ($n = 3$ per trial) were taken for morphological identification and molecular confirmation of species.

## ASFV transmission experiments

ASFV transmission trials were done in two separate consecutive trials. Three-day-old adult flies were deprived of glucose solution 24 h prior to experiments. They were offered a pork meat meal (1 $cm^3$) spiked with 100 µL ASFV titre $6\log_{10}HAD_{50}$/mL. Before and after feeding, control samples of viral stock were taken and controlled for ASFV titre using qPCR quantification. The meat meal was kept for 24 h, then replaced on a daily basis with non-ASFV spiked meat to mimic transmission to non-infected animals up to 5 days after infection, depending on the available number of flies. Flies ($n = 3$) were taken for morphological insect identification to confirm identity. Day-0 samples ($n = 3$) for control purposes were taken after 1 h exposure to ASFV spiked meat.

## Identification of insect species

Morphological identification of third instar larvae and adult flies was performed using morphological identification keys (25–27).

For confirmation of morphological identification, a representative number of samples was analyzed using molecular biology methods. Flies were kept in 70% ethanol which was withdrawn prior to DNA extraction and insects were left in open 2.0 mL tubes to dry out, and homogenized in Tissuelyser II (Qiagen, Hilden Germany) at 30 Hz for 1 min. DNA extraction was performed using a Tissue DNA purification kit, EURX, Poland following the manufacturer's instructions. Isolated DNA was kept at −20\u00B0C. Molecular identification of flies was performed using the protocol described in Folmer et al. (1994), which established "universal" DNA primers to amplify a fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI) of invertebrates from 11 phyla.

Sanger sequencing was performed using the commercial service of the Macrogen company (Amsterdam, The Netherlands) and the Faculty of Biology, University of Belgrade (Belgrade, Serbia).

## Isolation of ASFV DNA from environmental samples and qPCR for ASFV detection in environmental samples and insects

Viral DNA extraction was performed utilizing the IndiSpin Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany), adhering strictly to the protocols specified by the manufacturer. To identify the ASFV genome, the study employed the primer and probe set delineated in the work by King et al. (23). The quantitative PCR (qPCR) reaction was formulated using 12.5 µL of Luna Universal Probe qPCR Master Mix (New England BioLabs, Ipswich, MA, USA), 1 µL of each primer at a concentration of 10 mM, 0.5 µL of a 10 mM probe, and 5 µL of the extracted DNA template. Nuclease-free water was added to adjust the total volume of the reaction mixture to 25 µL. The qPCR thermocycling

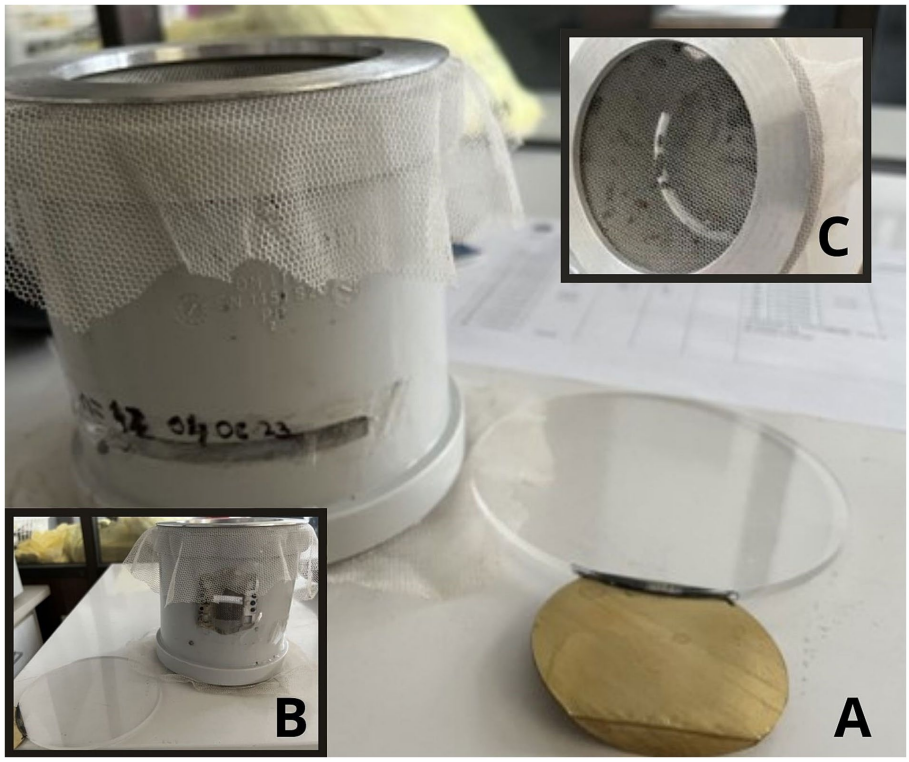


FIGURE 2
ASFV transmission experimental set-up: modified infection traps for adult flies – (A) front, (B) back, (C) above perspective. Figure was produced using Canva (2024), https://www.canva.com/design/DAGLIXnv1U4/EL8tjI6kDIVMqHwdWryjrQ/edit.

conditions commenced with an initial denaturation at 95°C for 1 min, followed by a series of 40 cycles, each consisting of a denaturation phase at 95°C for 15 s and an annealing/extension phase at 60°C for 30 s.

# Results

## Morphological identification of flies and molecular confirmation of fly species

At two localities (Glogonj and Duboka bara-Palilula), subsamples of non-biting flies caught using entomological nets were morphologically identified as *L.sericata, Lucilia silvarum* (Diptera: Calliphoridae), and *Chrysomya albiceps* (Diptera: Calliphoridae) while flies in pools up to 100 that were sampled at the near of Stari Tamiš pig farm were identified as *M. domestica* and *S. calcitrans*. The molecular insect identification confirmed the presence of *L. sericata, C. albiceps* and *S. calcitrans*. Additionally, the collected muscidae eggs were identified as *C. albiceps and L. sericata,* while larval stages collected from the carcass of swine in Borča-Put za Crvenku were identified as *M. domestica* and *L. caesar* (Diptera: Calliphoridae) using molecular biology methods.

The adult flies taken for morphological identification prior to two transmission experiments were identified as *L. sericata*. After molecular insect species identification, a presence of *S. calcitrans* was confirmed in one sample.

## Detection of ASFV DNA

The environmental samples (soil, water, feed and swabs) were all tested negative for the presence of ASFV DNA.

The pool of Muscidae eggs (*C. albiceps*), and a pool of L1 instar larvae (*L. sericata*) collected at the carcass of diseased swine at the location Borča-Put za Crvenku tested positive for the presence of ASF DNA (Ct = 29.3 and 33.4).

All flies taken for control testing prior to experiments were tested negative.

All Petri dishes containing meat at three locations were tested negative for the presence of ASFV DNA.

One *S. calcitrans* fly sampled on day 1 post-infection tested positive (Ct-34.2) in the first experimental trial group.

# Discussion

Initial outbreaks of ASF in Serbia were detected in the domestic pig population (2). The main transmission paths in backyard pig production units seemed to be mostly independent of contact with wild boar population and related to human activities and unclear factors. Nevertheless by 2023 ASFV became present in eastern part of Serbia and had tendency of spreading westwards.

The data from the field suggest that adult flies have a role in the mechanical transmission of ASFV, but the knowledge of transmission dynamics or exact fly species involved is insufficient A recent EFSA entomological study in Romania identified 62.96% of *Stomoxys*

*calcitrans* (*Diptera: Muscidae*) and 42.02% collected hematophagous biting midges (*Diptera: Ceratopogonidae*) pools as positive for ASFV DNA presence emphasizing the need of vector competence and virus transmission studies in different insect species (14). In Estonia, on a ASFV infected farm, viral DNA was detected in the housefly *M. domestica (Diptera: Muscidae)* and one *Drosophila* spp. (*Diptera: Drosophilidae*) (13). In another study *M. domestica*, *Calliphoridae (Diptera: Calliphoridae)* and stable fly *S. calcitrans (Diptera: Muscidae)* also tested positive for ASFV DNA presence (28). Mechanical transmission of ASFV by *S. calcitrans* has been experimentally shown to transmit ASFV to domestic pigs 1 and 24 h after feeding on infected material (29). However, in the same experimental work, transmission failed if the interval between feeding on infected material and uninfected pigs increased. Stable flies were also shown under experimental conditions to be able to transmit ASFV if ingested by naive domestic pigs (30). ASFV has been detected in various body parts of *S. calcitrans* for up to three days following experimental infection (31). Furthermore, the role of the common green bottle fly, *L. sericata (Diptera: Calliphoridae),* and the blue bottle fly, *C. vicina (Diptera: Calliphoridae), w*as experimentally shown when larvae experimentally bred on ASFV-infected spleen tissue. It was concluded that immature blowfly stages do not play a relevant role as reservoirs or mechanical vectors of ASFV unlikely adults (32). Recent study demonstrated dynamics of ASFV transmission in mosquitoes as representative of small blood feeding insects, tabanids and *S. calcitrans* at three different incubation temperatures (10°C, 20°C, 30°C) (33). It was proven that ASFV is viable in *S. calcitrans* at 48 h post infection sampling point at the temperature of 30°C, but not later (33), which is similiar to summer temperatures in Serbia. Since our result showed presence of ASFV DNA in *S. calcitrans* only at 24 h post infection at the temperature of 27 ± 1°C, we hypothesize that this result is in agreement with results of (33) and that the higher the temperature, the less viable ASFV is when transmitted via *S.calcitrans*. Nevertheless, our result comes from infection trial, and not from the field-collected insects and therefore further investigation is needed in the field conditions.

In our study, we were able to confirm ASFV DNA in field-collected eggs and larvae from carcasses of swine tested positive for ASF which is in correlation with results of (32). Subsequent sequencing proved these positive samples to originate from *C. albiceps* and *L. sericata* flies, respectively. These results are in correlation with Balmoş et al. (12) where among different fly species which were tested positive, most classified as belonging to *Calliphoridae* family. The adult flies are known to be able to mechanically spread ASFV in areas of up to 2–3 km between pig farms (28), and therefore might have an influence on ASFV transmission. However, in our study the samples (meat traps) from nearby households which encountered ASFV outbreak tested negative for ASFV DNA, highlighting the complexity of environmental transmission of ASFV. Nevertheless, ASFV transmission via flies as mechanical vectors could not be ruled out based on these results, since environmental conditions (e.g., high summer temperatures, placement of samples in yards) might have had influence on them results.

The role of *L. sericata* and *C. vicina* flies as mechanical vectors has been previously proven starting on larval feeding on ASFV infected tissue (32). In our approach, after the initial results of ASFV DNA positivity in field-collected *L. sericata* larvae, we used negative larvae morphologically identified as *L. sericata* to develop adult flies to be exposed to the known titre of ASFV with a goal to get insight into transmission dynamics in adult flies, i.e., duration of ASFV positivity which could imply the role in local ASFV transmissions observed in the field. Our results showed that no *L. sericata* flies tested positive for ASFV DNA, while the only fly identified as *S. calcitrans* by molecular insect identification was positive only on day 1 of the transmission experiment 24 h after the exposure to the ASFV. No further positivity was observed in both experiments. This result highlights again the potential role of *S. calcitrans* as a mechanical vector of ASFV. Nevertheless, the limitations of this study are that we were not able to prove if the ASFV was located on the fly or ingested, since no dissections were done, nor if the ASFV was live or not, while only molecular biology methods were used in viral detection. Therefore we could not establish whether ASFV transmission would be possible through ingestion of whole insect or by piercing the susceptible animal.

In our study, we were not able to detect ASFV DNA in environmental samples of soil, water, feed and swabs taken directly where the diseased animals were present. This might be due to the limited number of samples in this study [soil samples ($n = 6$), feed samples ($n = 3$), environmental swabs ($n = 15$)], but also might be due to the low ASFV contamination in the environment where sampling took place. It was shown that the applied study methodology is a critical issue when conducting stability testing (33). Furthermore, the time frame of sampling (from 48 to 96 h from the onset of clinical signs- during the culling procedure) might have influenced the outcome of this study since high viral loads in blood of ASFV infected animals start to be present from day five post infection (34, 35). Finally, sampling was performed during period of high summer temperatures in Serbia reaching 40°C, which might have also influenced the outcome, since it is shown that ASFV persistence is declining with the rise of temperature in feces and urine of ASFV infected pigs (36). The negative results obtained in this study do not exclude the possibility of ASFV transmission via contaminated surfaces or fomites. Olsen et al. found that naive pigs were not infected, if they were put in the pens where ASF-pos pigs had been staying 3, 5, or 7 days earlier, while naive pigs did get infected if inserted 24-h after removal of ASF-pos pigs. These pens were not cleaned and disinfected, while visible blood was removed in between (37). Environmental samples are effectively used for detection of ASFV in wild boars (38).

Effective management of ASFV outbreaks requires a comprehensive approach considering local disease patterns, wild boar outbreaks, potential amplifying spots, and human activities. In Serbia, low-biosecurity settings in rural areas with backyard production have been particularly challenging (39). In Serbia dominant transmission route in domestic pigs seems to be direct contact between infected and susceptible swine, together with human activity (40).

## Conclusion

ASF remains a significant threat to swine populations, with its persistence in the environment and potential vector-mediated transmission complicating control efforts. Continuous monitoring,

genetic characterization of virus strains, and comprehensive management strategies are essential for effective ASF control. Understanding the roles of various vectors and environmental factors will be crucial in developing more targeted and effective interventions to prevent and mitigate ASF outbreaks.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

## Author contributions

AV: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft. BM: Investigation, Visualization, Writing – review & editing. DG: Investigation, Writing – review & editing. MK: Conceptualization, Investigation, Methodology, Supervision, Writing – original draft. JK: Investigation, Writing – review & editing. AŽ: Investigation, Writing – review & editing. BK: Investigation, Writing – review & editing. VM: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing.

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## 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 FDF declared a past co-authorship with the author BK.

## Publisher's note



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# Complex patterns of WNV evolution: a focus on the Western Balkans and Central Europe

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**Introduction:** West Nile Virus, an emerging zoonotic pathogen, has been circulating in Serbia for over a decade, with its first detection in mosquitoes in 2010. Since then, the virus has led to increasing cases in both animals and humans, peaking in 2018 with 415 human cases and 36 fatalities. This study aimed to explore the phylogenetic relationships between previously sequenced West Nile virus strains from Serbia and those sequenced in this study, while also identifying possible virulence factors.

**Materials and methods:** Whole genome sequencing was conducted using a targeted approach on the MinION Mk1C platform, following a two-step process involving cDNA synthesis and amplification. Bioinformatics analysis included demultiplexing, primer trimming, and sequence mapping using tools such as iVar, Minimap2, and Samtools. Phylogenetic analysis was performed using MAFFT alignment and the Maximum Likelihood method with the Tamura Nei model in MEGA X software. Virulence factors were assessed in both structural and nonstructural proteins, focusing on key glycosylation motifs and specific mutations. Homology modeling of the E protein was also performed to evaluate potential structural changes due to mutations.

**Results:** Phylogenetic analysis revealed two major sublineages within the E subclade, representing the majority of strains from Western and Central Europe. These sublineages likely originated from Austria, Serbia, and Hungary between 2008 and 2012. The study also identified three distinct sublineages within the D subclade, which includes more diverse strains from Southern Europe. The E protein exhibited significant variations, particularly at the E159 site, which is crucial for virulence. The EI159T aa change has become dominant in recent years, replacing the previously prevalent EI159M. Additionally, changes in the NS1 glycoprotein and NS3 protein, both of which are involved in immune modulation and viral replication, were identified, with potential implications for the virus's virulence.

**Conclusion:** The study's findings highlight the Western Balkans and Central Europe as key regions for the mixing and dissemination of West Nile virus strains from both Western-Central and Southern Europe. These results underscore the importance of continuous surveillance and phylogenetic analysis to monitor the evolution and spread of West Nile virus, particularly in light of the frequent mutations observed in virulence-associated sites.

KEYWORDS

**West Nile virus, phylogenetics, molecular characterization, phylogeography, Serbia**

# 1 Introduction

West Nile Virus (WNV) is an arbovirus primarily transmitted by mosquitoes to susceptible hosts. It belongs to the species *Orthoflavivirus nilense*, the genus *Orthoflavivirus* within the family *Flaviviridae* (1). While a wide range of hosts can be infected, birds are considered the primary, amplifying hosts. Other vertebrates are regarded as dead-end hosts since they do not contribute significantly to the transmission cycle (2). The viral genome consists of a single-stranded, positive-sense RNA molecule, which encodes one polyprotein. This polyprotein is subsequently cleaved by structural proteases into structural proteins (Capsid (C), Membrane (prM), and Envelope (E)) and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (1). The polyprotein is flanked by untranslated regions (UTRs) at both the 3′ and 5′ ends, consisting of approximately 300–600 nucleotides (nt) and 90–92 nt, respectively (2). The prM is a glycoprotein essential for the proper folding and rearrangement of the E protein. Cleavage of prM by furin-like proteases induces global conformational changes, leading to the formation of mature virus particles (2, 3). The E protein is a structural protein composed of 500 amino acids (aa) and is divided into three domains: DI, DII, and DIII. DI, consisting of 127 aa, forms eight-stranded $\beta$-barrels that comprise the protein's central region (4). DII, comprising 170 aa, has its proximal end within DI and its distal end forming a fusion loop, which facilitates contact with the host cell (4). The DIII section forms an ectodomain composed of seven $\beta$-stranded barrels (4). Within the E protein, there is a conserved N-linked glycosylation site that has been associated with the neurovirulence of the virus in vertebrates (2, 4). Certain changes in the E protein have also been linked to increased virulence, such as the EI159V mutation. Under experimental conditions, this mutation showed higher replication in brain tissue compared to the EI159 strain (5). Changes in the NS1 and NS3 proteins, which are involved in immunomodulation of the host response and cleaving junctions in the WNV polyprotein, respectively, have also been associated with increased virulence. A significant number of potential virulence factors have been described for WNV, with different virulence factors depending on the host (2). The virus is genetically unstable, exhibiting high genetic diversity with nine currently described lineages (6).

The earliest description of WNV dates back to 1937 in Uganda, Africa, from where it is believed the virus spread across the continent. In Europe, the earliest recorded instance of WNV was in Albania in 1958, with a resurgence in the 1990s in Romania and Russia, resulting in a high number of human cases (6). These early outbreaks of West Nile Fever (WNF) in the 1990s and early 2000s were primarily connected to WNV lineage 1 (7). An increase in cases occurred in 2004 when a goshawk was found dead in Hungary, and it was later determined that the cause was an infection with WNV lineage 2 (6). This lineage is now the predominant lineage in Europe, with cases reported in Western, Central, Southern, and Southeastern Europe, while lineage 1 has primarily been described in southwestern Spain and Italy (7–13). Other lineages (3, 4, 7, 8, and 9) have also been detected in Europe, although they have not been connected with major outbreaks (6). The risk of introducing novel exotic lineages of WNV into Europe is primarily associated with migratory birds transiting through the Palaearctic-African flyway (14). In contrast, the local spread of the virus is not influenced by bird migration but relies on local transmission through mosquitoes (6, 14).

The first case of WNV in Serbia was recorded in 2010 when viral nucleic acid was detected in a mosquito pool, while the first case in birds was detected in 2012 from samples of both live and dead birds (12). Since then the number of cases has increased each year and reached its peak in 2018 with 415 human cases and 36 fatalities (12), while in 2022, the second-highest peak with 226 human cases was recorded (15). Additionally, a high seroprevalence of WNV has been detected in wild animals, highlighting an important but often overlooked circulation pathway of the virus (16). Considering the threat posed by WNV, an integrated government surveillance program is in place. This program aims to detect WNV nucleic acid in birds and mosquitoes, along with conducting serological surveillance of horses and calves as an early warning system. Since WNV has been circulating in Serbia for over a decade and is prone to frequent mutations, it is crucial to monitor its genetic evolution to understand its epidemiology and potential impacts on public health. In this study, we aimed to assess the phylogenetic relationships between previously sequenced strains from the country and those sequenced in this study. Additionally, we sought to identify possible virulence factors in Serbian strains that could influence the virus's transmission dynamics, pathogenicity, and the effectiveness of control measures. By comparing the genetic makeup of different strains, we can gain insights into the evolutionary pressures acting on the virus and track the emergence of any new, potentially more virulent variants.

# 2 Materials and methods

## 2.1 Samples

The samples used in this study are from the Institute of Veterinary Medicine's sample bank. Originally the samples were collected as a part of the West Nile virus surveillance program, funded by the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management and included samples from mosquitos and tracheal swabs from birds. In the study, only samples previously characterized as positive by real-time qPCR with a Ct value below 25 were used for sequencing. In total 12 samples were selected, 1 from a mosquito, and 11 from birds (Supplementary material 1).

## 2.2 Extraction of nucleic acid and real-time qPCR detection

The viral nucleic acid was isolated using the IndiSpin Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany) following the instructions provided by the manufacturer. To confirm the successful extraction of nucleic acid, each sample included an external VetMax Xeno Internal Positive RNA Control (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA). For the detection of nucleic acid of WNV, the following master mix for rtRT-PCR was prepared: 10 µL of Luna Universal Probe One-Step Reaction Mix (New England BioLabs, Ipswich, MA, USA), 0.8 µL of each 10 mM primer, 0.4 µL of 10 mM probe, 1 µL of RT Enzyme Mix, 5 µL of template, and 1 µL of Xeno Internal Positive RNA Control Primer. The remaining volume, up to 20 µL, was filled with nuclease-free water (RT-PCR Grade Water, Thermo Fisher Scientific, Massachusetts, USA). The primers and probe used were previously published by Eiden et al. (17). The thermal

profile used was programmed on the QIAquant 96 5plex (Qiagen, Les Ulis, France), according to the manufacturer's instructions.

## 2.3 WNV amplification

Whole genome sequencing was done by utilizing the previously published targeted approach for amplicon sequencing of the WNV (18). Sequencing was performed on a MinION (Mk1C device) sequencing platform. The approach used can be divided into two major steps.

The first step involved first-strand cDNA synthesis and amplification using the following procedures: Initially, the cDNA synthesis mix was prepared, which included 1 µL of 50 ng/µl Random Hexamers, 1 µL of 10 mM dNTPs mix (both from the SuperScript™ III First-Strand Synthesis System, Invitrogen - Thermo Fisher Scientific, Waltham, Massachusetts, USA), and 11 µL of 1 µg/mL viral RNA. This mixture was incubated at 65°C for 5 min, then quickly transferred to −80°C for 1 min.

For the cDNA amplification step, the mix included the following components: 4 µL of 5X SSIV Buffer, 1 µL of 100 mM DTT, 1 µL of 200 U/µl SuperScript™ IV Reverse Transcriptase (all from the SuperScript™ IV Reverse Transcriptase kit, Invitrogen - Thermo Fisher Scientific, Waltham, Massachusetts, USA), and 1 µL of RNase OUT Recombinant RNase Inhibitor (Invitrogen - Thermo Fisher Scientific, Waltham, Massachusetts, USA). The cDNA synthesis mix (with viral RNA) and the cDNA amplification mix were combined in a single tube, resulting in a total volume of 20 µL. This mixture was then subjected to the following thermal profile: 42°C for 50 min, followed by 70°C for 10 min. The resulting cDNA was stored at −80°C for future use.

The second step involved the primer-specific amplification of WNV cDNA. Previously published primers were used for this step (18). The primers were divided into two pools (odd and even primers), each with a volume of 10 µL and a concentration of 100 µM. These pools were further diluted in a 1:10 ratio to achieve a final concentration of 10 µM. Each sample was then amplified in two parallel reactions, each containing a different primer pool in the following mix: 12.5 µL of Q5 High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, MA, USA), 0.77 µL of each primer pool, 2.5 µL of cDNA, and PCR-grade water to a final volume of 25 µL. The thermal profile used for amplification included: initial activation at 98°C for 30 s, followed by 35 cycles of 98°C for 15 s, and annealing at 65°C for 5 min. The amplified pools from each step were then combined in a single tube.

## 2.4 Sample barcoding

The sample concentration was optimized to 200 fmol. The amount of DNA was measured by using a Qubit dsDNA HS (High Sensitivity) Assay Kit on a Qubit™ 4 device (Thermo Fisher Scientific, Massachusetts, USA) as per the manufacturers' instructions. Samples with excessive amounts of DNA were diluted with RNA pure water. Barcoding of the samples was performed using the Native Barcoding Kit 24 V14 (SQK-NBD114.24) from Oxford Nanopore, UK, following the Ligation Sequencing Amplicons protocol with v14 chemistry. The prepared library was then loaded onto a 10.4.1 flow cell and sequenced using the MinION Mk1C device. Sequencing lasted for 12 h, with basecalling conducted using Guppy v. 24.02.16 in the MinKNOW GUI v. 5.9.18, and an initial quality score set to 9.

## 2.5 Bioinformatics analysis

Demultiplexing was performed using MinKNOW GUI v. 5.9.18. The additional quality check was performed with Seqkit v2.8.2[1], and the primers were removed from the raw sequences using the iVar trim option (iVar v1.4.2)[2]. The resulting reads were mapped to the reference genome with Minimap2 v2.24-r1122[3]. The files generated were subsequently checked and indexed using Samtools 1.19.2[4]. Finally, consensus sequences were created with iVar v1.4.2 (see text footnote 2). The resulting consensus sequences were annotated using Geneious Prime (Dotmatics, Boston, MA, USA) and submitted to the GenBank NCBI.

## 2.6 Sequence and phylogenetic analysis

The sequences were aligned using the MAFFT alignment algorithm with 205 WGS (Supplementary material 1) from the GenBank (NCBI) and analyzed with Geneious Prime (Dotmatics, Boston, MA, USA) software. The aligned sequences were then used for phylogenetic analysis in the Molecular Evolutionary Genetics Analysis (MEGA X) software (19). The Maximum Likelihood method and Tamura Nei model employed in the study were selected based on the results from the "Find Best DNA/Protein Models" feature in MEGA X. Previously described nomenclature for WNV was used for classification of strains (20).

Virulence factors were assessed in both structural and nonstructural proteins as was previously described (2).

In the structural proteins, the analysis focused on the 15–17 N-linked glycosylation motif in the prM protein, the 154–156 glycosylation motif, and the E159 mutation in the E protein.

For the nonstructural proteins:

- NS1 Protein: Glycosylation sites were analyzed using the N-X-S/T motif via the NetNglyc server[5]. Additionally, the presence of the E154S mutation was evaluated.
- NS3 Protein: The 249th position was evaluated for the presence of proline, which is associated with virulence.
- NS4B Protein: The conserved positions D35, P38, W42, and Y45, as well as the C102 and E249th positions, were evaluated for their connection to virulent strains.
- NS5 Protein: The K at 61st, 128th, 218th, and 804th positions were assessed for the presence of virulence factors.

1 https://bioinf.shenwei.me/seqkit/
2 https://github.com/andersen-lab/ivar
3 https://github.com/lh3/minimap2/releases
4 https://github.com/samtools/samtools/releases/
5 https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0

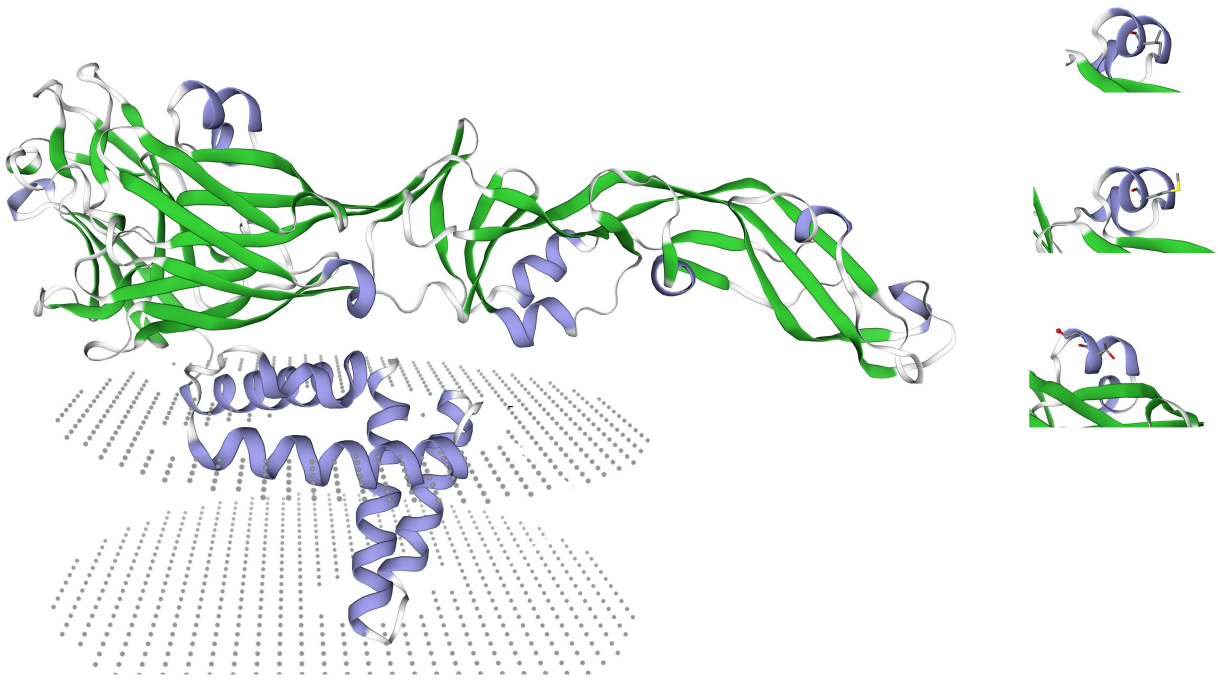

FIGURE 1
A presentation of a 3D homology model of the E protein created using the SWISS-MODEL online tool (https://swissmodel.expasy.org/). Labeled on the right side of the figure are the three aa changes at the E159th position, while on the left side, the entire E protein can be seen with the labeled glycolisation site and the transmembrane domain.

## 2.7 E protein homology model

The molecular structure of the E protein was evaluated by creating a homology three-dimensional model using the SWISS-MODEL online tool[6]. To assess potential structural changes caused by mutations at the E159th position, three models were created with a focus on the E159th site (Figure 1).

Based on the phylogenetic trees obtained, a map of Europe was created using QGIS. Representatives from each sublineage were selected for mapping based on publicly available geolocation data from NCBI. Sequences that only listed the country of origin without specific location details were excluded. The coordinates for these representatives were approximated using the publicly available website Maps.ie[7]. It is important to note that the obtained coordinates represent the approximate locations of the sequenced strains.

# 3 Results

Sequencing was terminated after 12 h. The average quality score of 1,8 million generated reads was 14.78, the average base length was 552 bp, and the average sequencing depth was 4,300x. Out of 12 sequenced samples, good-quality sequences were obtained from 10 (83.3%). Four (33.3%) strains including SRB/WNF/6569/Belgrade 2018, SRB/WNF/8505-3/Belgrade 2018, SRB/WNF/8505/Belgrade 2018, SRB/WNF/8805-58846/Belgrade 2018 had gaps from the 8,909–9,505 nucleotide position, while the strain SRB/WNF/8805-58846/Belgrade 2018 had additional gaps in the 5,715–6,035 and the 6,877–7,395 positions. Excluding these 4 strains, the obtained genome length was 10,305 bp. High-quality sequences were processed and submitted to GenBank (NCBI) using the following accession numbers PQ053322 - PQ053331. Based on the BLAST similarity results the highest percent identity was recorded with Lineage 2 sequences PP212881.1 and PP212882.1 (Hungary) with 99.49 and 99.51%, respectively.

## 3.1 Phylogenetic analysis

Phylogenetic analysis revealed the existence of 4 major clades within WNV lineage 2 (A, B, C, and F). Clade F can be considered the European strain clade and was further divided into 2 subclades: E and D (Figure 2 and Supplementary Image 1). Clade E consisted of Western and Central European strains and was divided into two sublineages: E1 (Western-Central European strains from 2010 to 2018) and E2 (Western-Central European strains from 2015 to 2018). Within the E sublineage, 2 strains, MH244513 (West Nile strain 200.B/2013/Secovce/SVK) and OK129333 (West Nile strain 4,693 HUN2017), formed a distinct branch. Clade D comprised Central European, Western Balkan, and Southeast European strains, and was further divided into three sublineages: D1, D2, and D3. Sublineage D1 includes strains from the Western Balkans and Southeast Europe from 2010 to 2013, along with an additional strain, KC496015 (West Nile strain 578/10). These were further divided into D1.1 and D1.2. Sublineage D2 was further subdivided into 2 Southeast Europe sub-sublineages: D2.1, which includes Western Balkan strains from

6 https://swissmodel.expasy.org/
7 https://www.maps.ie/coordinates.html

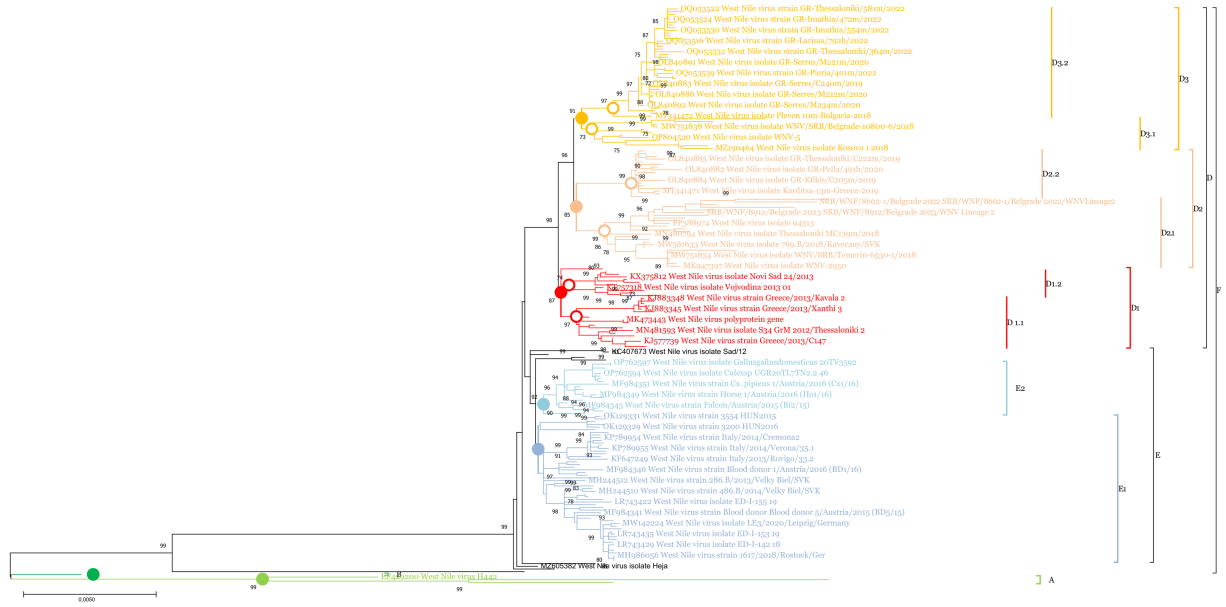


FIGURE 2
A condensed phylogenetic tree showing the genetic relationships between Serbian strains (PQ053322-PQ053331) and strains from the NCBI. Utilizing MEGA X software, the study employed the Maximum Likelihood Method alongside the Tamura Nei model for its investigation. To increase the accuracy 1,000 bootstrap replicates were included, a Gamma distribution to account for varying rates across sites (+G) across five rate categories, and the assumption that certain sites remain unchanged over time (+I). Branches not supported in at least 70% of the bootstrap replicates were condensed. To identify the most suitable model for analysis, the software's "Find best DNA/Protein Models" functionality was utilized. Clades, and sublineages were labeled with the following colors with HTML color notations in brackets: Clade A light green (#91cf4f), Clade B dark green (#00b04f), sublineage E1 blue (#94b2d6), sublineage E2 light blue (#91ccdb), sublineage D1 red (#ff0000), sublineage D2 peach (#fabf8f), sublineage D3 orange (#ffbf00).

2018 to 2023, and D2.2, which includes Southeast Europe strains from 2018. Sublineage D3 encompassed both Western Balkan and Southeast European strains, with the D3.1 sub-sublineage comprising strains from 2018 to 2019, while the D3.2 clade consisted entirely of Southeast European strains (Figure 3. Map). The circles were created to illustrate the potential overlapping of different strains.

## 3.2 Nonsynonimus mutation analysis

Of the 122 recorded aa changes, 30 were unique to our sequenced samples. Recorded changes in the sequenced samples are in Supplementary material 1.

No changes were observed in the N-linked 15–17 glycosylation site of the prM protein, nor at the M-36 or M-43 residues. In the E protein, the NYS motif at the 154–156 glycosylation site is preserved, while an EI159T mutation was evident in all sequenced strains. Additionally, no changes were detected in the 107 virulence motif across the sequenced strains. The E154S change was not observed in the sequenced samples. Based on the E protein homology model no changes in the folding of the three domains in the E protein were observed, besides the changes in the side chains of the aa at the E159th position.

For the NS1 nonstructural protein, glycosylation analysis revealed preserved N-glycosylation sites (NNTF/NTTE/NDTW) at the 135, 175, and 207 positions in all sequenced strains, except for the SRB/WNF/8870-10/Belgrade 2022/WNV Lineage 2 (PQ053330) strain, which had preserved glycosylation sites only at the 135th and 175th positions. Additionally, this strain showed changes at the 208th and 205th positions, specifically the D208G and T209P mutations. In the SRB/WNF/8805-58846/Belgrade 2018/WNV Lineage 2 (PQ053328) strain, the 207 glycosylation site experienced a change at the 208th position, with an NS1 D208H mutation, but the glycosylation site remained preserved (Supplementary material 1).

For the Serine protease/Helicase NS3 protein, proline was present at the 249th position, which has been described in highly virulent strains.

For the NS4B protein, no changes were observed at the D35, P38, W42, Y45, 102nd, or 249th positions.

For the NS5 protein, no changes were observed at the K61, K128, or K804 positions. However, a K218G mutation was observed at the 218th position in the PQ053331 strain, while no changes were observed in other strains.

# 4 Discussion

In this study, we report on the phylogenetic and molecular changes observed in strains sequenced directly from clinical samples, following a previously published protocol (18). Whole genome sequencing provides deeper insights into the evolution of WNV and valuable data for molecular epidemiology. Srihi et al. (20) described the existence of six different clades (A-F) within lineage 2 of WNV, with clades A, B and C being of primarily African strains. In Europe, there were two active subclades, D and E, which were also labeled as the southeastern European and Central/Eastern European clades, respectively (20).

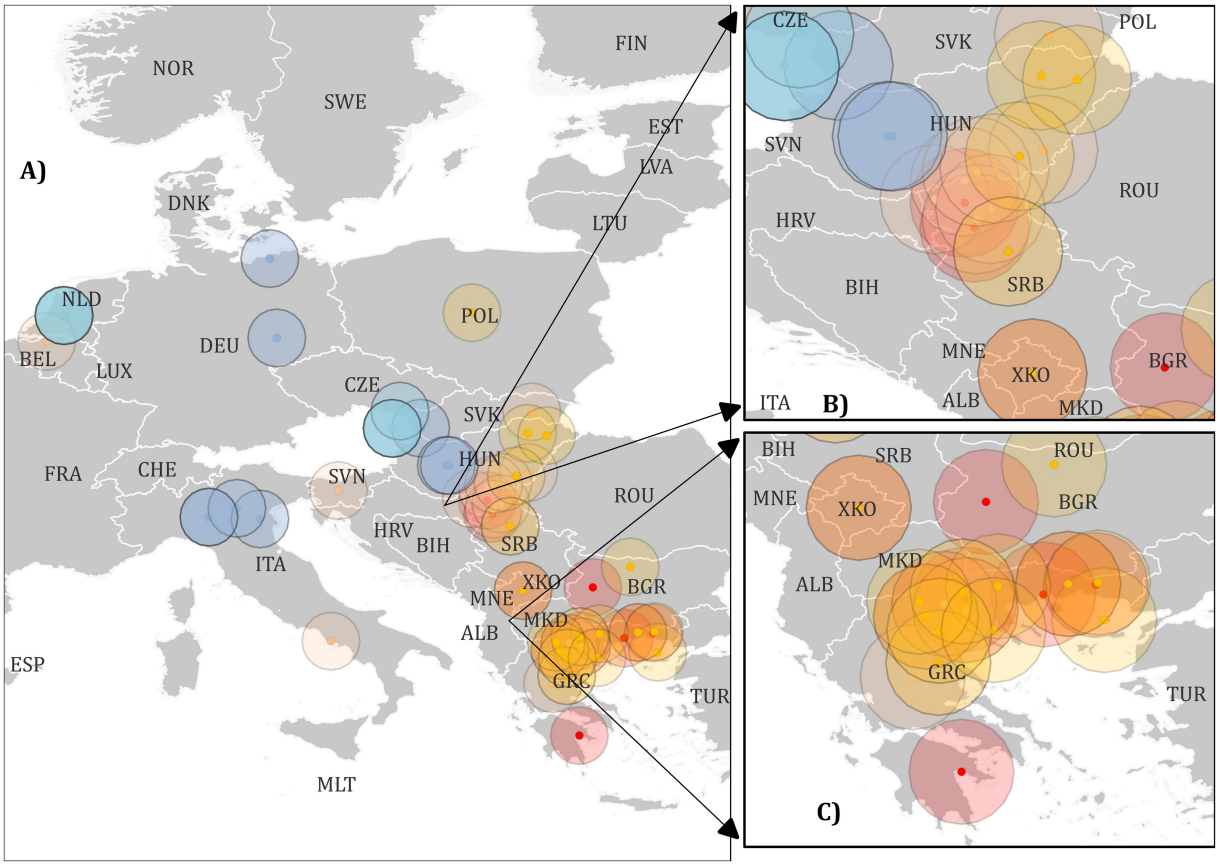


FIGURE 3
A map of Europe showing labelled locations of West Nile Virus (WNV) strains representing various sublineages used in the study. The dots and circles are color-coded as per the scheme below. Label A) indicates all European strains, label B) represents strains from Central Europe and the Balkans, and label C) represents strains from Southern Europe. Clades, and sublineages were labelled with the following colours with HTML colour notations in brackets: Clade A light green (#91cf4f), Clade B dark green (#00b04f), sublineage E1 blue (#94b2d6), sublineage E2 light blue (#91ccdb), sublineage D1 red (#ff0000), sublineage D2 peach (#fabf8f), sublineage D3 orange (#ffbf00).

In our study, we identified two major sublineages within the E subclade, designated as E1 and E2. These sublineages represented the majority of strains from Western and Central Europe between 2013 and 2018. Notably, three strains have separated from the others: WNV/SRB/Curug-5871/2018 (KC407673) and West Nile virus isolate Novi Sad/12 (KF179640) from Serbia, dated 2018 and 2012 respectively, and the West Nile virus strain Austria/2008 gh from an Austrian goshawk (KF179640). The earliest detection of strains from this E subclade can be traced back to 2008 in Austria, as well as strains from Serbia and Hungary from 2012. These early detections may represent the origin of these sublineages.

Sublineage D1 comprises strains from 2010 to 2015, originating from Greece and the Western Balkans. Our analysis indicated that sublineage D1 was no longer dominant in this region, suggesting other sublineages have supplanted it. Sub-sublineage D2.1, which includes strains from Greece dated 2018–2019, may represent a localized and self-limiting circulation of this sub-sublineage. In contrast, sub-sublineage D2.2 includes strains from the Western Balkans, Hungary, and a single strain from Italy, all collected between 2018 and 2023. Additionally, this sub-sublineage includes a strain from Belgium, identified in a previous study by Srihi et al. (20), which represents the earliest instance of this sub-sublineage, dating back to 2017. The majority of the strains sequenced in this study belong to sub-sublineage D2.2, suggesting its ongoing circulation in the Western Balkan region and potential for further spread, as evidenced by a strain from Italy identified in the fall of 2023. Sub-sublineage D3.1 includes strains from Western Balkan countries, Hungary, a single strain from Greece dated 2018–2019, and a strain from Poland dated to 2022. On the other hand, sub-sublineage D3.2 consists exclusively of strains from Greece, spanning from 2018 to 2022. This pattern suggests that D3.2 may represent a currently circulating sublineage within Greece.

The highest diversity of sublineages can be observed in southern Greece and the Western Balkans, where three different sublineages have circulated and overlapped since 2010. This study highlights that the Western Balkan and Central European countries are important mixing grounds for both Western-Central European strains (Clade E) and Southern European strains (Clade D). This finding aligns with the proposal by Srihi et al. (20), which identified Austria and Hungary as significant dissemination centers for WNV in Europe. This is further evidenced by the detection of strains from these sublineages in more northern locations, such as the D2.2 strain West Nile virus isolate WNV/Belgium/2017/Antwerpen (MH021189) from Belgium and the D3.1 strain West Nile virus isolate WNV-5 (OP804520.1) from Poland. These findings underscore the widespread dissemination and the complex epidemiology of WNV in Europe.

Protein characterization revealed no aa differences in the Capsid protein C, PROPEP, and Protein prM genomic regions in sequences from Serbia.

Virulence factors for WNV have been previously described, with major factors found primarily in the E protein. Variations in virulence have previously been linked to changes in the E protein particularly at the E154-156 (NYS) glycosylation site and the EI159V aa change. Based on the study by Davis et al. (21) the EV159 strains have been described as more virulent. A later study by Kobayashi et al. (5) suggested that the E159 site plays a crucial role in modulating pathogenicity, viral replication, and T-cell recruitment. The E154-156 glycosylation site remains preserved in our sequenced samples, while the E159th position exhibits an EI159T aa change. Chronologically, Serbian strains from 2010 initially showed an EI159M mutation at this site. Over time, these strains were replaced by those containing isoleucine at the E159th position. However, starting in 2018, both the EI159 and EI159T strains began circulating simultaneously. By 2022, the EI159T became the only observed change at this position in our samples, suggesting that it may now be the dominant strain in the region. Notably, this same EI159T aa change has also been recorded in the majority of strains from Kosovo and the Vojvodina autonomous region in 2018. A similar strain shift was observed in Austria when other regional strains were examined. Strains with isoleucine at the E159 position were detected in 2008, while strains from 2014 to 2015 predominantly show the EI159T change. This pattern was also seen in sequences from Greece. Strains from 2010 to 2013 primarily exhibit the EI159 aa change, but this is later replaced by the EI159T in samples sequenced after 2020. Given the importance of the E159 site, frequent changes in its aa sequence warrant further investigation. Isoleucine is a branched, highly hydrophobic aa, similar to valine and methionine, indicating that the EI159V and EI159M changes might not significantly affect peptide stability. In contrast, threonine (EI159T) is a polar aa with a hydrophilic side chain, which may lead to differences in protein folding or stability. This is particularly important considering that these mutations are adjacent to the E154-156 glycosylation sites.

The NS1 glycoprotein plays a crucial immunomodulatory role, enabling the virus to influence the activity of the complement system. In Flaviviruses, the NS1 glycoprotein typically features a conserved glycosylation motif (N-X-S/T) at 2 positions in Dengue Virus (DFV), Japanese Encephalitis Virus (JEV), and Yellow Fever Virus (YFV) (22). However, in the case of WNV, there are three such glycosylation motifs. In our study, we identified two strains with changes in the conserved region of the NS1 gene. The SRB/WNF/8870-10/Belgrade 2022/WNV Lineage 2 (PQ053330) strain exhibited two changes: D208G and T209P within the glycosylation site. According to Whiteman et al. (22), WNV mutants with ablated glycosylation sites showed decreased virulence in *in-vivo* mouse models. The reduction in virulence was correlated with the number of glycosylation sites ablated (22). The SRB/WNF/8805-58846/Belgrade 2018/WNV Lineage 2 (PQ053328) strain exhibited a D208H aa change at the second position of the third glycosylation site. Although the glycosylation site was recognized by the NetNglyc server, the substitution of aspartic acid with histidine could disrupt the protein's secondary or tertiary structure. Aspartic acid is negatively charged, while histidine is positively charged, which might lead to protein misfolding. Previous studies have shown that any changes in glycosylation sites can affect the virus's interaction with the immune system, potentially impairing the NS1 protein's immunomodulatory role, thus resulting in attenuated WNV phenotypes (22, 23).

The NS3 protein, in conjunction with the NS2B protein, is involved in cleaving junctions between non-structural proteins that comprise the WNV polyprotein (24). Along with NS5, NS3 is essential for viral replication, and the creation of the viral replication complex (24). The NS3 protein spans all three domains and contains the RNA helicase and RNA triphosphatase activities at its C-terminal end. In a study by Brault et al. (25), a proline residue at the 249th position was identified as a virulence factor in a strain isolated from American crows, which exhibited high mortality rates. The proline residue at the 249th position was identified in all strains sequenced in our study. The same residue was found in previously sequenced strains from Serbia, except for 4 strains: West Nile virus strain Novi Sad 2010 (KC496016), West Nile virus strain Novi Sad/12 (KC496073), and 2 from 2018, WNV/SRB/Curug-5871/2018 (MW751837) and WNV/SRB/Subotica-6530-10/2018 (MW751846), where an NS3 P149H aa change was recorded. The virulent 249 proline residue was found in all strains from Greece and some strains from Hungary, while strains from central and Western Europe had the NS3 P249H aa change, which is in accordance with previously published studies (9).

A limitation of this study is the exclusion of environmental factors like climate change, which can affect mosquito populations and the spread of West Nile Virus. Future studies should include these factors for a more complete understanding of transmission dynamics. Additionally, implementing strong public health measures, such as mosquito control and public awareness programs, is crucial. Enhancing collaboration and data sharing across European countries would further improve the understanding of WNV patterns and support coordinated efforts to address outbreaks.

## 5 Conclusion

The study highlights that the Western Balkan and Central European countries are significant mixing grounds for WNV strains from both Western-Central Europe (Clade E) and Southern Europe (Clade D). The spread of these strains, including to Belgium and Poland, underscores the complexity of WNV dissemination in Europe. This is the first in-depth molecular analysis of the circulating WNV strains in Serbia. The study provides insights into the evolution of WNV in Serbia and Europe, noting the importance of continuous monitoring and phylogenetic analysis to track the emergence and spread of virulent strains. The frequent changes observed in critical sites like E159, the NS1 and the NS3 protein warrant further investigation to understand their impact on the virus's pathogenicity and epidemiology.

## Data availability statement

The datasets presented in this study can be found in GenBank NCBI. The accession numbers: PQ053322: https://www.ncbi.nlm.nih.gov/nuccore/PQ053322, PQ053323: https://www.ncbi.nlm.nih.gov/nuccore/PQ053323, PQ053324: https://www.ncbi.nlm.nih.gov/nuccore/PQ053324, PQ053325: https://www.ncbi.nlm.nih.gov/nuccore/PQ053325, PQ053326: https://www.ncbi.nlm.nih.gov/nuccore/PQ053326, PQ053327: https://www.ncbi.nlm.nih.gov/nuccore/PQ053327, PQ053328: https://www.ncbi.nlm.nih.gov/nuccore/PQ053328, PQ053329: https://www.ncbi.nlm.nih.gov/

nuccore/PQ053329, PQ053330: https://www.ncbi.nlm.nih.gov/nuccore/PQ053330, and PQ053331: https://www.ncbi.nlm.nih.gov/nuccore/PQ053331.

## Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because samples from birds and mosquitoes were collected as a part of the national monitoring for WNV.

## Author contributions

SŠ: Data curation, Methodology, Software, Writing – original draft. ŠG: Data curation, Methodology, Software, Writing – review & editing. LV: Conceptualization, Writing – review & editing. DG: Funding acquisition, Project administration, Supervision, Writing – review & editing.

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## 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.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1494746/full#supplementary-material

SUPPLEMENTARY IMAGE 1
A complete phylogenetic tree showing the genetic relationships between Serbian strains (PQ053322-PQ053331) and strains from the NCBI. Utilizing MEGA X software, the study employed the Maximum Likelihood Method alongside the Tamura Nei model for its investigation. To increase the accuracy 1000 bootstrap replicates were included, a Gamma distribution to account for varying rates across sites (+G) across five rate categories, and the assumption that certain sites remain unchanged over time (+I). Branches not supported in at least 70% of the bootstrap replicates were condensed. The software's "Find best DNA/Protein Models" functionality was utilized to identify the most suitable model for analysis.Clades and sublineages were labelled with the following colours with HTML colour notations in brackets: Clade A light green (#91cf4f), Clade B dark green (#00b04f), sublineage E1 blue (#94b2d6), sublineage E2 light blue (#91ccdb), sublineage D1 red (#ff0000), sublineage D2 peach (#fabf8f), sublineage D3 orange (#ffbf00).

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# Microbial ecology of sandflies—the correlation between nutrition, *Phlebotomus papatasi* sandfly development and microbiome

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The role and the impact of the microbial component on the biology, ecology, and development of sandflies is largely unknown. We evaluated the impact of larval nutrition on laboratory-reared sandflies in correlation to the abundance of food, light starvation, and food with/without live microbiome, by monitoring the survival and development of immature stages, and the longevity of adult sandflies. Within this study we examined 360 larvae, 116 pupae, and 120 adult flies of *Phlebotomus papatasi* for the microbial gut content. The data showed that the presence of a live and diverse microbiome plays a role in the development and survival of larvae. The mortality rate of the larvae was higher, and larval development was longer for sandflies maintained on microbiome-depleted medium, in comparison to the larvae fed with medium containing alive and complex microbiome. Actively feeding larvae reduce microbial abundance and diversity of the medium. The microbial content of the larval gut depends on the composition of the rearing medium, indicating a potential attraction to certain bacteria. The microbial content of the pupa gut was severely diminished, with overall survival of two bacterial species in adult insects - *Ochrobactrum intermedium* (found in 95% of dissected adults) and *Bacillus subtilis* (16%). Further microbial studies may aid in developing biological control methods for sandfly larval or adult stages.

KEYWORDS

**sandfly, *Phlebotomus*, bacteria, microbiome, nutrition, development**

## 1 Introduction

Phlebotomine sandflies (Diptera, Psychodidae) are hematophagous insects of great medical and veterinary importance on a global scale (1). Sandflies are able to transmit protozoan, bacterial, and viral pathogens (2), among which protozoan parasites from the genus *Leishmania* (Kinetoplastida, Trypanosomatidae) — the main causative agents of leishmaniasis, are of particular importance to veterinarians and physicians around the world. Leishmaniasis is a severely underreported disease, counting 1–2 million reported cases annually, with serious implications that incidence numbers are significantly higher (3, 4). Despite the increasing number of cases and continuous disease spread, there are no approved human vaccines, while currently used therapeutic approaches and control strategies show unsatisfactory results (5–8).

Major health authorities around the world persistently emphasize the urgent need for the development of new leishmaniasis control methods. In order to develop effective leishmaniasis control measures, among others, it is necessary to understand sandfly vectorial potential, biology, and ecology (9). A comprehensive understanding of sandfly biology requires that sandflies be studied in an ecological context, with the microbiome as an important component of the system. In the past, the microbial component and its impacts on sandfly life, fitness, fecundity, vectorial potential, etc. were greatly overlooked. The recent resurgence of interest

in sandfly microbial community investigations was prompted by abundant evidence highlighting the role of microbes in the control of vector-borne diseases such as malaria, dengue, Zika, Chagas disease, etc. (10–12).

Sandflies inhabit various environments, providing them with ample opportunity to encounter and interact with diverse microbes from the environment. Their complex life cycle, coupled with the distinct lifestyle requirements and dietary preferences of immature and adult stages (e.g., larvae that feed on decomposing organic material versus adult flies that feed on the plant juices, blood, etc. (13)) supports a maintenance of a rich gut microbiome that can be inherited and acquired (14, 15). As *Leishmania* development in the sandfly is confined to the digestive tract, the parasites will inevitably encounter and interact with this diverse microbiome (16). The gut microbiome of sandflies can either hinder or facilitate the survival and development of *Leishmania* within the sandfly (17, 18). Moreover, the microbiome egested during sandfly feeding significantly contributes to host infection by inflammasome activation (19). Up to this point, only a limited number of studies focused on investigations of the microbial impact on sandfly biology and ecology showing that the microbiome play an important role in mediating the attraction of gravid sandfly females toward certain types of breeding grounds (20, 21). Even though microbial studies in sandflies are severely neglected and sparse, existing data still indicate that the microbiome play a more prominent role in sandfly biology, ecology, and vectorial potential than previously thought. Understanding the sandfly microbial ecology is crucial as it bears significant implications for the development of novel vector control strategies aimed toward leishmaniasis reduction.

Given the challenges associated with conducting sandfly studies in nature, we designed a laboratory study that evaluated the impact of microbial components, particularly microbiome-rich/depleted nutrition, on sandfly life span and gut microbiome content in relation to the inherited microbiome, thereby contributing to the overall knowledge and understanding of sandfly biology and ecology.

# 2 Method

## 2.1 Sandfly colony

Experiments were conducted on a *Phlebotomus papatasi* colony originating from Sanliurfa, Türkiye (established in 2003). Five-to-seven-days-old females were artificially blood-fed and left to deposit eggs. The eggs were collected, surface sterilized, counted, and transferred to clean pots. All pots were maintained under standard conditions (humidity: 60–70%; temperature: 26 ± 1°C; light:dark photoperiod: 14:10 h), with the variation in food availability. The pots were examined three times per week and all observations were made on three individual replicates. In total, 12 pots with 3,823 eggs were examined during this experiment (Table 1). Larval food was prepared from equal amounts of rabbit chow and faeces (22), and all sandflies were fed with the food obtained from the same preparation batch. Tested rearing pots were divided into three groups. The first test group (T1) was fed on the autoclaved larval food weighing between 0.5–1.5 g depending on the larval stage. The second test group (T2) was overfed, e.g., immature stages of sandflies were provided with high amounts of larval food, ranging from 0.8 g for young larvae to 2.3 g for older larvae. The third test group (T3) was subjected to light starvation conditions where only a limited amount of food was provided. Young larvae were provided with less than 0.4 g, while older larvae were provided with less than 1.2 g per feeding. Control (C) rearing pots were maintained according to the standard insectary protocol and the amount of food and feeding regimen were the same as for the T1 group. Adult sandflies were released from rearing pots three times per week, were provided with 40% sugar solution and were kept under the same conditions as the immature stages.

## 2.2 Monitoring of life parameters

The life cycle of sandflies is very complex and requires a transition through four larval instars and a pupa, to reach the adult stage. Larvae burrow the rearing medium during their development, which makes their precise counting particularly challenging. Hence, eggs were counted at the beginning of the experiment, and the total number of emerged flies was recorded. The mortality rate of immature stages was calculated based on the number of eggs and emerged adults, after considering the number of dissected samples. The number of dead flies was recorded three times per week and the longevity of the flies was noted.

## 2.3 Microbial examination of the sandfly gut content

Individual gut dissections were performed on 360 larvae, 116 pupae, and 120 adults. Due to variations in the speed of larval development among the groups, the timing of dissection differed for

TABLE 1 Life parameters of sandflies observed under the impact of different nutrition.

| | Control group (C) | Group fed with autoclaved food (T1) | Group that was overfed (T2) | Group that was starved (T3) |
|---|---|---|---|---|
| Initial number of eggs[a] | 982 | 920 | 949 | 972 |
| Number of emerged adults | 794 | 573 | 749 | 283 |
| Mortality | 7.9% | 28.2% | 9.6% | 67% |
| Developmental period[b] | 47 ± 3 days | 62 ± 5 days | 48 ± 3 days | 96 ± 8 days |
| Longevity of adults | 21 ± 2 days | 20 ± 2 days | 20 ± 3 days | 19 ± 2 days |

[a]The expressed values show total number of samples from three replicates.
[b]From first egg deposition to first adult emergence; Data do not show dissected individuals.

each group. Due to the minute size of the larvae, the first dissections were conducted approximately 17–18 days post-hatching for C, T1, and T2, and 25–26 days post-hatching for T3, when the larvae reached a minimum size of 1.8 mm to ensure precise gut dissection. The second dissections were conducted on day 23–24 post-hatching for C and T2, 28–29 for T1, and 40 days post-hatching for T3, with the minimal size of the larvae reaching 2.5 mm. Third larval dissections were conducted when the larvae reached a minimum size of 3 mm (day 28–29 for C and T2, 32 for T1, and 55 days post-hatching for T3). The pupa for dissections were mature (with visible wings, and other adult body characteristics). Unfed adult flies of both sexes were dissected at 48 h post-emergence.

Prior to gut dissection, all samples were surface sterilized in a series of washes (70% ethanol) and rinsed (sterile Phosphate Buffered Saline (PBS)) for 3 cycles. Dissections were performed under the binocular stereomicroscope using sterile equipment and sterile PBS as the dissection medium. The undamaged guts were individually macerated in 100 µL of sterile PBS with a sterile pestle, and 20 µL of the homogenate was immediately plated on Brain Heart Infusion (BHI) (Merck, Germany) agar, Wort Agar (WA) (BioLife Italiana, Italy), and Potato Dextrose Agar (PDA) (Merck, Germany). BHI and WA plates were incubated under aerobic conditions for 24 h at 37°C, and PDA plates were incubated at 26°C for 7 days. Colony Forming Units (CFU) of bacteria and yeast were recorded after 24 h and the total load was calculated. All colonies that displayed morphological differences were further subcultured on BHI/WA until pure cultures were obtained. All agars were prepared according to the manufacturer's instructions and all plates were checked for potential contaminants by overnight incubation at 37°C.

## 2.4 Microbial examination of the rearing medium

Sampling for the microbial analysis of the medium was conducted on the same days as the sampling for sandfly gut dissections. Per sampling in total 0.4 g of medium was collected from the C, T1, and T2 groups, and 0.2 g from T3. The medium was resuspended in sterile PBS, and serial dilutions were prepared. 20 µL of a diluted medium was plated on BHI, WA, and PDA and incubated as previously noted.

## 2.5 Microbiome identification

Most of the isolated bacteria were identified via traditional methods (23), and only a certain number of isolates that showed similar and/or ambiguous morphological features were subjected to Sanger Sequencing (Figure 1) (14). For this purpose, DNA was extracted from fresh cultures with QIAamp DNA Mini Kit (Qiagen GmbH, Germany), following the manufacturer's instructions. The extracted DNA was subjected to PCR amplification and subsequent Sanger sequencing with 27F and 1492R primers (24). The obtained sequences were edited in BioEdit (25) and blasted in the NCBI database for homologous sequence search. Considering the problematic taxonomy of bacteria, 98% sequence similarity was considered as a lower threshold at both the genus and species levels, respectively (26). Sequences were aligned using MEGA11 (27), and a maximum likelihood phylogenetic tree was constructed under the Tamura-Nei model (Figure 1). Fungal isolates were identified based on a combination of macro- and microscopic morphology (lactophenol blue staining) (28). The yeasts were identified by morphological characteristics (29).

## 2.6 Statistical analysis

Statistical analysis was performed with Rstudio software (30). The data sets were evaluated for the equality of the two variances using the F-test. One-way and/or two-way analysis of variance (ANOVA) was performed to evaluate the statistical differences between groups. The significance of the differences between group means was evaluated via Student's T-tests. A $p$-value of 0.05 was used as the threshold.

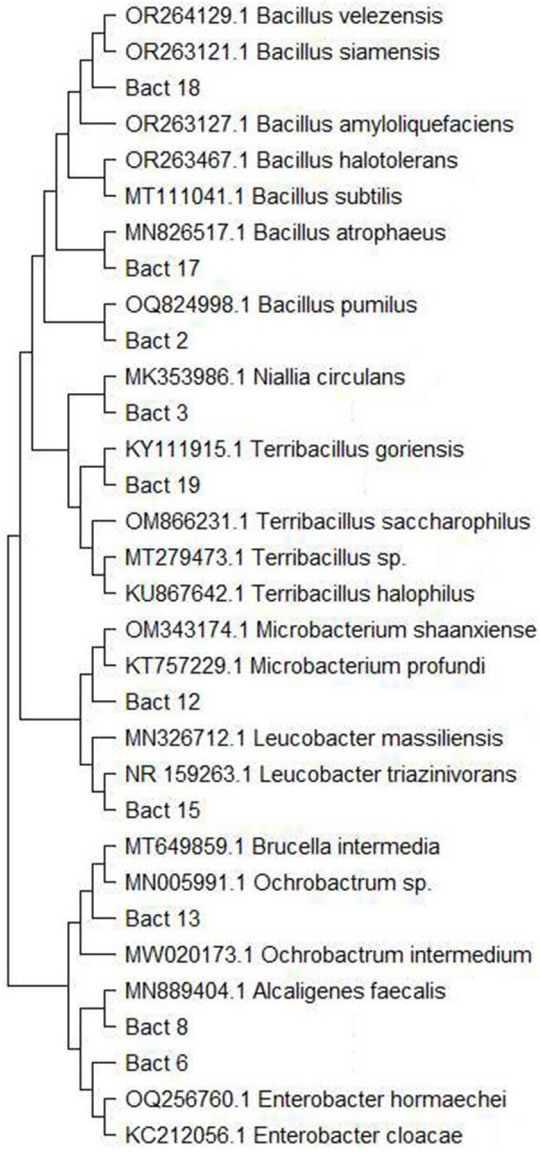


FIGURE 1
Maximum likelihood phylogenetic tree of bacteria species that were Sanger sequenced for definite species identification. The tree was constructed in MEGA 11 under Tamura-Nei parameter.

# 3 Results

## 3.1 Sandfly longevity and life parameters

The developmental parameters of the C group were normal and synchronous, falling inside the regular parameters observed during the long-term colony maintenance, which corresponded to 47 ± 3 days from the first egg deposition to the first adult's emergence (Table 1). The mortality (7.9%) and adult longevity (21 ± 2 days) rates of the C group also corresponded to the normal parameters. The T1 group that was fed with autoclaved food displayed notable differences in survival rate and developmental length when compared to the C. The mortality of the T1 group amounted to 28.2%, and the developmental time was prolonged (62 ± 5 days; *p*-value: 0.014), while adult longevity was only slightly shorter (20 ± 2 days) (Table 1). Compared with the C group, the T2 group did not display any major differences in the speed of development (48 ± 3 days) or adult longevity (20 ± 3 days), while the mortality rate was slightly higher (9.6%) (Table 1). Test group 3 displayed more irregular and drastically slower (96 ± 8 days; *p*-value: 0.0007) development, as well as strikingly increased mortality (67%), when compared to the C group (Table 1). The longevity of the adult flies in the T3 group was slightly shorter in comparison to the C group (19 ± 2 days; *p*-value: 0.006). Overall, the mortality rate of the larvae was higher, and larval developmental time was longer for sandflies maintained on autoclaved medium (54 ± 3 days), in comparison to the larvae who had plentiful available food with alive and complex microbiome present, i.e., C (38 ± 3 days; *p*-value: 0.004), and T2 (41 ± 3 days; *p*-value: 0.011). The differences in larval development were even more pronounced among sandflies that were exposed to light starvation conditions (85 ± 3 days), in comparison to the larvae from C (*p*-values: 0.0002) and T2 group (*p*-values: 0.0005). This indicates that the presence of live and diverse microbiomes, as well as a sufficient amount of food, plays a role in the optimal development and survival of larvae. No major differences were observed in relation to the duration of the pupal stage of the C, T1, and T2 groups which lasted on average for 7–9 days. Duration of the pupal stage from the T3 group was slightly prolonged (10–11 days) showing statistical significance when compared with C (*p*-value: 0.024) and T2 (*p*-value: 0.025) groups. The adult longevity among the groups was very similar, showing statistical significance only between the control and group exposed to starvation conditions (*p*-value: 0.006) (Table 1).

## 3.2 Microbial content of the rearing medium

The microbial composition of the larval food was examined at the beginning of the experiment. This microbial composition was used as a baseline with the follow-up detection of several unique records throughout the sampling period within the different examined groups (Figure 2). In the C group, *Bacillus pumilus* (≈ $0.6 \times 10^6$) (Figure 1) was recorded only at the second larval sampling (*p*-value: 0.044), and then again during pupal sampling. In the T2 group, *Microbacterium* sp. (≈ $2.4 \times 10^6$) was isolated from the second larval sampling, and *Escherichia coli* (≈ $2.8 \times 10^6$) was present during the second and third larval sampling. Even with the presence of these

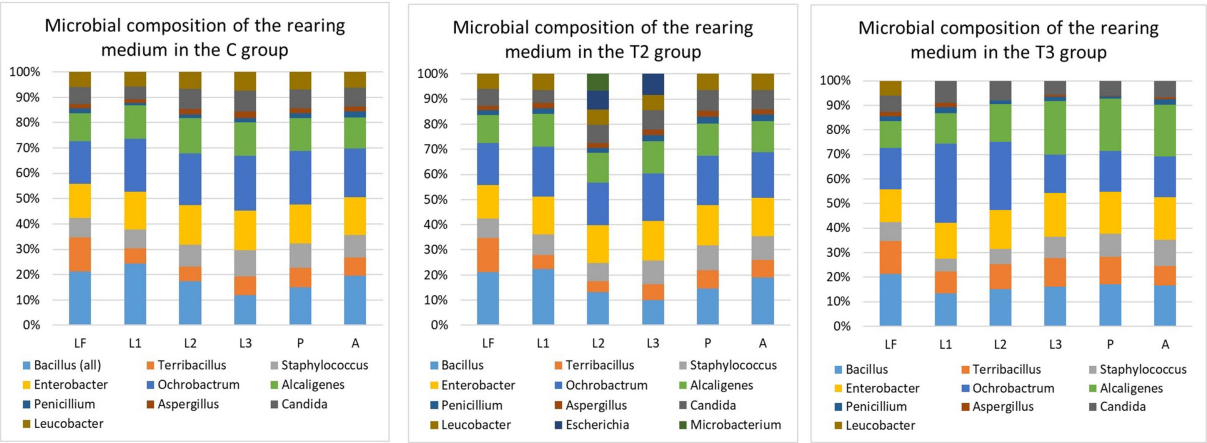


FIGURE 2
Microbial composition of rearing medium within different groups. T1, Test group 1 was fed with autoclaved medium; T2, Test group 2 was overfed; T3, Test group 3 was exposed to starvation; LF, Larval food; L1, First sampling during larval stage; L2, Second sampling during larval stage; L3, Third sampling during larval stage; P, Sampling during pupal stage; A, Sampling during adult stage. Statistically significant differences within the C group were observed when microbial content of the LF was compared with the microbial content of the rearing medium collected during L1 (*p*-value: 0.0021), L2 (*p*-value: 0.0031), L3 (*p*-value: 0.012), P (*p*-value: 0.018), and A (*p*-value: 0.022). When microbial composition of the rearing medium was compared between L1, L2, L3, P and A sampling, statistically significant differences were seen between L3 and P (*p*-value: 0.0099), L3 and A (*p*-value: 0.027). Within T2 group, statistically significant differences in the microbial content were noted between LF and sampling L1 (*p*-value: 0.015), P (*p*-value: 0.031), and A (*p*-value: 0.0436). There were no statistically significant differences in the microbial composition of the rearing medium between L1, L2, L3, P, and A sampling points. Within T3 group, when compared with microbial content of the LF, statistically significant differences were observed during L1 (*p*-value: 0.00097), L2 (*p*-value: 0.00073), L3 (*p*-value: 0.0011), P (*p*-value: 0.00093), and A (*p*-value: 0.0013). When microbial composition of the rearing medium was compared between L1, L2, L3, P and A sampling, statistically significant differences were seen between L3 and P (*p*-value: 0.033), L3 and A (*p*-value: 0.023). When microbial composition of different sampling points (L1, L2, L3, P, and A) was compared between the groups (C, T2, and T3), statistically significant differences were observed during L1 (C-T2: 0.0014; C-T3: 0.0094; T2-T3: 0.0042), L2 (C-T2: 0.019; C-T3: 0.0013; T2-T3: 0.000033), L3 (C-T3: 0.0029; T2-T3: 0.00055), P (C-T3: 0.0017; T2-T3: 0.00086), and A (C-T3: 0.0021; T2-T3: 0.0013). A *p*-value of 0.05 was used as the threshold during all calculations.

unique findings, no statistically significant differences between sampling times were observed within the T2 group (Figure 2). In general, for the C and T2 groups, the overall CFU values of bacteria were lower during the first and second sampling, whereas slightly increased numbers were observed during the third sampling. Contrary to C and T2, within the T3 group we noted the absence of these unique records, with an overall diminished microbial diversity and abundance in the medium (Figure 2). Within the T3 group, *Ochrobactrum intermedium* bacteria were present with higher CFU numbers during the first and second sampling ($\approx 4.12 \times 10^6$), while its numbers decreased in the third sampling ($\approx 2.25 \times 10^6$). The CFU numbers of *Alcaligenes faecalis* (the second predominant species within T3 group) were low during the first sampling ($\approx 2.75 \times 10^6$), and an increase in CFU was observed during the second and third sampling ($\approx 3.15 \times 10^6$). When compared with the larval food, the microbial composition of the T3 group displayed statistically significant differences during all sampling points (Figure 2). The microbial content of the rearing medium within the T1 group remained unchanged during the experiment, and plates were negative for microbial presence. The medium collected during pupation and 48 h post-emergence of adults showed slightly enriched microbial composition and abundance when compared with the medium collected during the larval period (Figure 2). The microbial increase was mainly reflected in the diversity of species from the *Bacillus* genus. These results show that the presence of actively feeding larvae changes and reduces the diversity and abundance of bacteria present in the rearing substrate (except T1).

Contrary to the relatively diverse bacterial composition, only two species of fungi and one yeast were recorded in the rearing medium. The highest abundance of fungi and yeast was detected in the rearing medium of the T2 group with the species belonging to the genera *Aspergillus, Penicillium,* and *Candida*. The slightly diminished presence of yeasts and the lowest abundance of fungi was observed in T3 group.

## 3.3 Microbial content of the sandfly guts of immature and adult stages

To better understand the composition of the sandfly gut microbiome, we examined the microbial composition of the larval food prior to the experiments and the composition of the rearing medium as larval development progressed. Variations in the microbial composition of the larval gut were observed between all examined groups. However, the microbial content within the same group did not exhibit significant variation with respect to the sampling points (first, second, or third sampling).

Overall, several species of the *Bacillus*, *Staphylococcus* and *Enterobacter* genus have been commonly found in the larvae gut within all examined groups (except T1) (Figure 3). *Ochrobactrum*

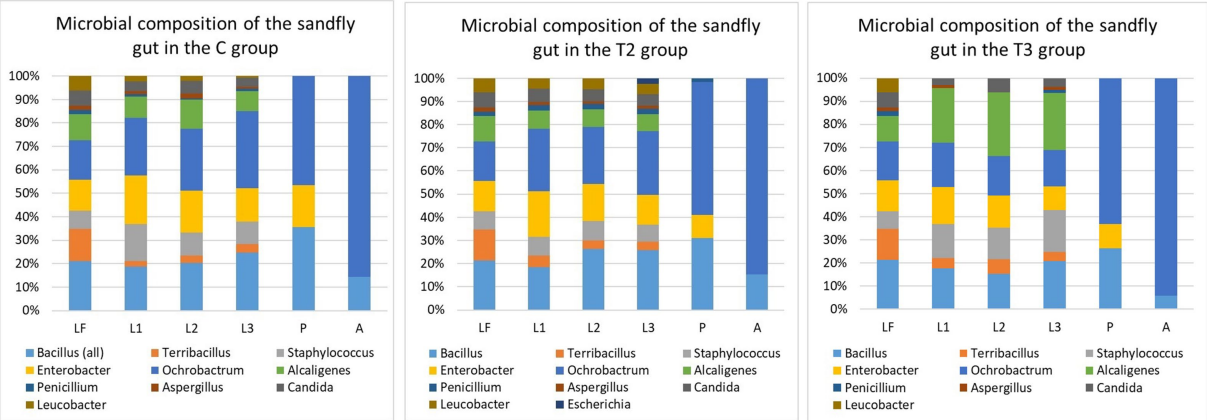


FIGURE 3
Microbial composition of the sandfly gut within different groups T1. Test group 1 was fed with autoclaved medium; T2, Test group 2 was overfed; T3, Test group 3 was exposed to starvation; LF, Larval food; L1, First sampling during larval stage; L2, Second sampling during larval stage; L3, Third sampling during larval stage; P, Sampling during pupal stage; A, Sampling during adult stage. Statistically significant differences in the microbial composition between the LF and sandfly gut were noted during all sampling points (L1, L2, L3, P, and A) within all evaluated groups (C, T2, and T3) that contained live microbiome. Statistical values within the C group: LF-L1 ($p$-value: 0.00089), LF-L2 ($p$-value: 0.00071), LF-L3 ($p$-value: 0.00075), LF-P ($p$-value: 0.00056), and LF-A ($p$-value: 0.00059). Statistical values within the T2 group: LF-L1 ($p$-value: 0.0016), LF-L2 ($p$-value: 0.0012), LF-L3 ($p$-value: 0.0023), LF-P ($p$-value: 0.0012), and LF-A ($p$-value: 0.0013). Statistical values within the T3 group: LF-L1 ($p$-value: 0.00077), LF-L2 ($p$-value: 0.0008), LF-L3 ($p$-value: 0.0008), LF-P ($p$-value: 0.00067), and LF-A ($p$-value: 0.00066). When microbial composition of different developmental forms within the group was compared, statistically significant differences were noted between L3 and P among all three groups (C group: $p$-value: 0.014; T2 group: $p$-value: 0.0048; T3 group: $p$-value: 0.022). When gut microbial content of different developmental stages was compared between the groups (C, T2 and T3), significant statistical differences during the larval sampling L1 were observed between C and T3 ($p$-value: 0.013), and T2 and T3 ($p$-value: 0.011); the larval sampling L2 between C and T3 ($p$-value: 0.012) and T2 and T3 ($p$-value: 0.013); and the larval sampling L3 between C and T3 ($p$-value: 0.029) and T2 and T3 ($p$-value: 0.012). No statistically significant differences in gut microbiome were noted between C, T2 and T3 groups during pupa and adult stages. When microbial composition of the rearing medium (M) and sandfly gut (G) content was compared, statistically significant differences within C group were noted between ML1 and GL1 ($p$-value: 0.0042), ML2-GL2 ($p$-value: 0.00011), MP-GP ($p$-value: 0.00034), MA-GA ($p$-value: 0.00039). Microbial comparison of M and G within T2 group showed differences between ML1 and GL1 ($p$-value: 0.0026), ML2-GL2 ($p$-value: 0.00036), ML3-GL3 ($p$-value: 0.043), MP-GP ($p$-value: 0.00055), MA-GA ($p$-value: 0.00085). Statistically significant differences in microbial composition between M and G of the T3 group were observed between ML1 and GL1 ($p$-value: 0.013), ML2-GL2 ($p$-value: 0.0077), ML3-GL3 ($p$-value: 0.0025), MP-GP ($p$-value: 0.0022), MA-GA ($p$-value: 0.0025). A $p$-value of 0.05 was used as the threshold during all calculations.

*intermedium* ($\approx 1.6 \times 10^3$) and *Bacillus subtilis* ($\approx 0.9 \times 10^3$) were found to be predominant in the sandfly larvae guts of the C and T2 groups, while *Alcaligenes faecalis* ($\approx 0.7 \times 10^3$) was the most predominant in the guts of the sandflies within the T3 group. *Bacillus pumilus* ($\approx 4 \times 10^2$) (Figure 1) that was isolated during the second sampling of the rearing medium from the C group, was recorded in the larval guts during the second and third dissections. Interestingly, *Microbacterium* sp. found in the T2 group during the second sampling of the medium was never recorded in the gut of larvae; while *Escherichia coli* ($\approx 2.2 \times 10^2$) recorded during the second and third sampling of the medium, was recorded in larvae gut only during the third dissection in low number (Figure 3). *Penicillium* was found in larval guts with several isolates from the C group, and predominantly T2, and with only one isolate from T3; while *Candida* was frequently found within the larval gut of the samples from the C and T2 groups, reduced in T3, and absent from T1.

The microbial content of the pupa gut was severely diminished in comparison to the larvae, and only three bacteria (*Ochrobactrum*, *Bacillus,* and *Enterobacter*), and a single isolate of *Penicillium* were detected. With regard to adult flies, only two bacteria demonstrated the potential to survive metamorphosis from the pupa - *Ochrobactrum intermedium* and *Bacillus subtilis*. *Ochrobactrum* ($\approx 10^2$) was found in 95% of all dissected adults, while *Bacillus* was found in a notably lower percentage (16%) and with very low numbers (< 20 colonies per gut).

## 4 Discussion

Sandflies have a worldwide distribution, and they occupy a high variety of habitats (13). However, the effects of the microbiome from breeding sites on the biology and ecology of sandflies, as well as the significance of microbes in larval development remains poorly understood. It has been demonstrated that the microbiome plays a significant role in *Leishmania* sp. development in the sandfly gut (17, 18), and that microbes can even affect immune-related gene expression and interaction with *Leishmania* sp. (31); but it is unknown how inherited and/or acquired microbiome impact sandfly life parameters and subsequently their vectorial potential. Although studies on the overall impact of microbiome on sandflies are very scarce, and only a limited number of publications is available, other vector borne insects such as mosquitoes have been explored in greater detail. As seen from the mosquito studies, microbial communities are known to influence mosquito life by modifying essential metabolic and behavioral processes that affect reproduction, development, immunity, digestion, egg survival, and the ability to transmit pathogens (32–38). Given the significant impact of microbes on vital aspects of mosquito biology, this study investigated how microbiome-rich/depleted nutrition, as a key factor in sandfly life, influences their survival, development and longevity with special emphasis on the microbial component. The study showed that even under controlled laboratory conditions the microbiome of both the sandfly gut and rearing medium varied among the examined groups (Figures 2, 3). In general, the presence of actively feeding larvae seems to change and reduce the microbial diversity of the rearing substrate (Figure 2). Microbial recovery was noted in all examined groups (except T1) during the third sampling within a larval stage, as well as during the sampling with predominance of pupa, and 48 h after adults started to emerge. The microbial increase could be attributed to the lower feeding rate, as most of the larvae corresponded to the late 4$^{th}$ instar in addition to the visible presence of multiple pupae and emerging adults. Alternative explanation for bacterial decline and subsequent recovery could be related to larval excretions, which can contain toxic or unfavorable substances that adversely affected the microbiome. Although microbial recovery (increased CFU numbers) was noted among all recorded species of bacteria, diversity increase was noted only among *Bacillus* genera (detection of *Bacillus pumilus* (Figure 1)), which may imply that actively feeding larvae were more attracted to *Bacillus* species. Studies conducted on adult sandflies showed that gravid females are attracted toward certain bacteria (21, 39, 40), but it was not evaluated whether larvae are guided by the same/similar attraction. It is still unclear if specific bacteria, such as species from the *Bacillus* genus, were more attractive to the larvae, or if their consumption was determined by chance. It would be recommended that further studies examine if sandfly larvae are attracted by specific microbes, as this knowledge could aid in developing biological control strategies at the larval stage.

The presence/absence of a live microbiome plays a role in sandfly development, and it can have both positive and negative effect. From C group can be observed that optimal amount of food, containing live and diverse microbiomes plays a crucial role in sandfly development and survival. Conversely, the absence of a live microbiome as seen in the T1 group, can adversely affect the sandflies by prolonging the development of immature stages and increasing their mortality. Similar observations pertaining to the impact of the microbiome on the survival and development of the immature stages were noted in mosquitoes. Reports document that a diverse microbiome from the habitat is necessary for growth and molting, and that mosquito larvae exhibit higher mortality and/or delayed development all up to the pupal stage when the abundance of microbes in the habitat was reduced (41–44).

Moreover, as seen from T2 group, too many microbes can negatively affect immature stages of sandflies. It is suggested that microbial, especially fungal overgrowth can lead to higher mortality of larvae, as larvae may entangle in fungal hyphae (22). In this study, overgrowth was a direct consequence of overfeeding, as substantial quantities of uneaten food were exposed to hot and humid conditions within the rearing pots, which are perfect for microbial growth. Although fungal overgrowth was cleaned regularly, it is possible that hyphae presence affected young larvae leading to their death, therefore resulting in higher mortality within the T2 group.

While the presence of microbiome and its composition play a role in sandfly development, another deciding factor is the amount of available food. The T3 group that was exposed to the light starvation conditions displayed a notably higher mortality rate and prolonged development. Even though approximately 96% of the eggs hatched, by the end of the 4$^{th}$ instar only a fraction of the larvae was alive. The mortality of larvae seemed to be highest within the first 2 weeks after hatching, and larval number continued to decline throughout the active phases of feeding. It is suspected that the presence of a live microbiome contributed to the overall survival of the larvae, and that the mortality rate would be even higher if the T3 group was fed with microbiome-depleted food. Although specific body measurements were not taken, it is authors' observation that all larvae that had enough food available (C, T1 and T2), regardless of the fact that microbial flora was viable or not, displayed marginally larger body size in comparison to T3 group that was exposed to light starvation conditions. The larvae

from the T3 group seemed slender and smaller, demonstrated decreased activity, and their digestive tracts were nearly devoid of content. Larvae, as the actively feeding stage of immature development are more affected by nutrition status. Hence, the prolonged duration of the immature development was mostly reflected in the larval instars, with similar findings being reported in mosquitoes (45). Even though the changes in the duration of the pupa and adult longevity were rather minor, they were most likely caused by the developmental conditions of the larvae. Nutrition does not play a major role in the process of pupation, as pupae do not feed; but preservation of the pupal gut microbiome is important as most of the microbes are eliminated during the histolysis of the digestive tract tissue during metamorphosis (46) (Figure 3). Among all groups that were fed with food containing live microbiome (C, T2, and T3 groups), it was observed that females dissected 48 h post-emergence displayed the presence of alive microbiome in their guts (Figure 3). The diversity and abundance of inherited bacterial communities were rather limited, with *Ochrobactrum* being found at a higher percent (95%) compared to *Bacillus* (16%). Previous studies demonstrated that the low presence of inherited microbiomes can recover through CFU increase (14), indicating that they play a role in the adult sandfly life functions and potential *Leishmania* susceptibility.

As all adults were exposed to the same conditions and food source, no major differences in adult longevity were observed between C, T1, and T2 groups. The T3 group, in comparison with the C group displayed a slightly shorter longevity, which could point toward the role of nutrition and microbial component in the sandfly fitness. The authors' observation is that sandflies from the T3 group were marginally smaller, which may be a direct consequence of previous observations made on the larval stage, wherein smaller larvae lead to smaller pupae, resulting in smaller adults.

Given that this experiment was conducted under laboratory conditions, with the food source whose microbial composition was limited and controlled, it was still possible to isolate and identify two bacterial communities of the inherited microbiome. It is highly likely that the diversity of sandfly inherited microbiome found in nature is much greater. The investigations of the inherited microbiome and its cultivation can contribute to the development of leishmaniasis control methods, in the same manner as the utilization of mosquito-derived microbes contributed to mosquito pathogen control (47).

In conclusion, sandflies contribute to the microbial shifts of the rearing medium under laboratory conditions, and the same can be expected in nature. Concurrently, the medium composition impacts the diversity and abundance of microbiomes present in the gut of immature stages and promotes sandfly development and survival. The diversity of microbial flora in the medium impacts the composition of the inherited microbiome, which may influence sandfly susceptibility to *Leishmania*, vector capacity and even severity of disease manifestation.

## Data availability statement

The original data are available in a publicly accessible repository of the GenBank under the accession numbers: PQ784271-PQ784280.

## Author contributions

SV: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. BA: Writing – review & editing.

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## 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.

## Generative AI statement

The author(s) declare that no Gen AI was used in the creation of this manuscript.

## Publisher's note



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# Seroprevalence and risk factors of bluetongue virus infection in sheep and goats in West Gondar zone, Northwest Ethiopia

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**Introduction:** Bluetongue is a non-contagious arthropod-borne viral disease that affects ruminants. No investigations have yet been conducted to ascertain the seroprevalence and associated risk factors of bluetongue in Northwest Ethiopia. It is essential to determine the seroprevalence and correlated risk elements to formulate an effective strategy for preventing and surveillance of the disease.

**Methods:** A cross-sectional study was carried out between February 2023 and May 2023 to determine the seroprevalence and risk factors associated with Bluetongue virus (BTV) in sheep and goats in the selected districts of West Gondar zone. A multistage cluster sampling technique was employed, with zones and districts purposively selected, and kebeles within these districts chosen through simple random sampling. Villages were treated as clusters. A total of 444 blood specimens were collected from the sheep and goats and subsequently tested for BTV antibodies using a commercially available competitive enzyme-linked immunosorbent assay kit. A mixed-effects logistic regression was employed to evaluate the relationship between Bluetongue virus seropositivity and potential risk factors.

**Results:** The overall seroprevalence rate at the individual animal level was 84.5% (95% CI: 81.09–87.82). The seroprevalence in sheep and goats was 83.8% (257/308) and 86.8% (118/136), respectively. Species and age were significant risk factors for BTV seropositivity in the study area ($p < 0.05$). Adult and older sheep and goats exhibited 3.49 (95% CI: 1.90–6.41) and 25.95 (95% CI: 9.45–71.28) times higher seroprevalence with the bluetongue virus in comparison to their younger counterparts, respectively.

**Discussion:** In conclusion, the current findings showed that BTV is highly prevalent. The specific circulating BTV serotypes and the temporal pattern

of Bluetongue in the study area remain unknown, necessitating further investigation.

KEYWORDS

**bluetongue virus, goat, risk factors, seroprevalence, sheep, West Gondar**

# 1 Introduction

Bluetongue (BT) is a non-contagious viral disease transmitted by arthropods that affects both domestic and wild ruminants, particularly in tropical and subtropical regions. It represents a significant vector-borne viral infection (1–3) caused by the Bluetongue virus (BTV) of the Orbivirus genus within the Reoviridae family. Currently, 29 known serotypes of BTV exist, with ongoing discoveries of novel types (4–7). Recent serotypes such as BTV-25, BTV-26, and BTV-27 are believed to be transmitted exclusively through vector-independent pathways, potentially leading to persistent infection in goats (8–10). The impact of BTV infection includes severe direct economic consequences (11), alongside indirect financial losses due to trade restrictions and reduced animal productivity. In Ethiopia, the disease poses substantial economic challenges, directly resulting in livestock mortality, decreased milk production, weight loss, and reproductive disorders. Indirectly, trade restrictions limit export opportunities, adversely impacting livestock-dependent farmers by reducing household income and constraining economic growth (12, 13). The molecular assays have revealed the existence of two major ancestral lineages: a Western lineage (found in Africa, Europe, and the Americas) and an Eastern lineage (found in Australia and Asia) (5, 14–16).

BTV is widely recognized as an endemic disease in Africa, yet there is a scarcity of data on its prevalence across most nations on the continent (17). BTV epidemics are often associated with periods of intense rainfall. Outbreaks affecting cattle, sheep, and goats have been reported in North and East Africa, particularly in Egypt, Algeria, Tunisia, and Kenya (18, 19). In Southern Africa, incidents involving sheep and goats have been documented in Botswana, Lesotho, Madagascar, Namibia, South Africa, and Zimbabwe (20–23). However, comprehensive data on the prevalent serotypes is available only for South Africa (serotypes 1–24) and Malawi (serotypes 1, 2, 3, 5, 8, 10, 15, 20, 21, and 22) (21). Additionally, since the late 20th century, multiple outbreaks of BT have been documented across European countries, with BTV strains predominantly believed to follow a consistent transmission route from North Africa to Southern Europe (2, 3, 24–26). Moreover, in Ethiopia, research on BTV epidemiology has predominantly focused on the southwestern regions, particularly around Jimma, Bonga, Bedele by Abera et al. (13), and the Maji districts by Haile et al. (12), with only a handful of studies conducted on the subject.

Midges belonging to an expanding array of species within the *Culicoides* genus (27) typically serve as vectors for BTV among susceptible ruminants (9, 28–30). In Northwest Ethiopia, a study by Ayele et al. (31) on *Culicoides* identification documented twelve species, eight of which, *C. corsicus*, *C. kibunensis*, *C. reioxi*, *C. kiouxi*, *C. saharienines*, *C. desertorum*, *C. reithi*, and *C. festivipennis*, had not been previously recorded in Ethiopia. Moreover, the seasonality of bluetongue infection is closely linked to the dependency of *Culicoides* on climatic variations. These midges breed in moist environments such as streams, irrigation channels, muddy areas, and regions with fecal runoff around farms, with suitable habitats being prevalent in many farming settings (9, 32–34). Cattle, acting as reservoirs and amplifying hosts, exhibit high levels of viremia.

The initial identification of BTV dates back to the late 18th century in South Africa, following the importation of prized fine wool sheep from Europe (16, 17, 20, 34). Presently, bluetongue disease is found on all continents except Antarctica, with various serotypes and strains leading to diverse disease manifestations (12). Recent studies by Gulima (35), Gizaw et al. (1), Yilma and Mekonnen (36), Abera et al. (13), and Haile et al. (12) reported seroprevalence rates of 34.1% in Amhara region, 65.2% in Oromia region, Afar region, South Nation and Nationality Regional State (SNNPRS), and Somalia region, 41.17% in SNNPRS, 30.6% in Oromia region, and 39.23% in SNNPRS, respectively. The Amhara region, which boasts a sheep population of 10,391,582 and a goat population of 7,045,305, ranks second and fourth in Ethiopia, respectively. Annually, in the Amhara region, 1,541,624 sheep and 1,239,994 goats succumb to diseases, marking the second and third highest mortality rates in the country after poultry (37); however, the specific contribution of bluetongue to these figures remains unclear. Despite the prevalence of the disease in the region, no investigations have been conducted to ascertain the seroprevalence and associated risk factors of bluetongue in sheep and goats in Amhara region, Northwest Ethiopia. Therefore, conducting research on these aspects is crucial for generating valuable data to guide future studies and to aid in the development of effective disease control and prevention strategies. The primary objective of this study was to determine the seroprevalence of bluetongue virus infection in sheep and goats and to evaluate the associated risk factors in Northwest Ethiopia.

# 2 Materials and method

## 2.1 Study area

In Ethiopia, there are nine regional states: Tigray, Afar, Amhara, Oromia, Southern Nations, Nationalities, and Peoples'

**Abbreviations:** BT, Bluetongue; BTV, Bluetongue virus; cELISA, Competitive enzyme linked immunosorbent assay; NVI, National veterinary institution; SNNPR, South nation and nationalities and people regional state; Spp., Species.

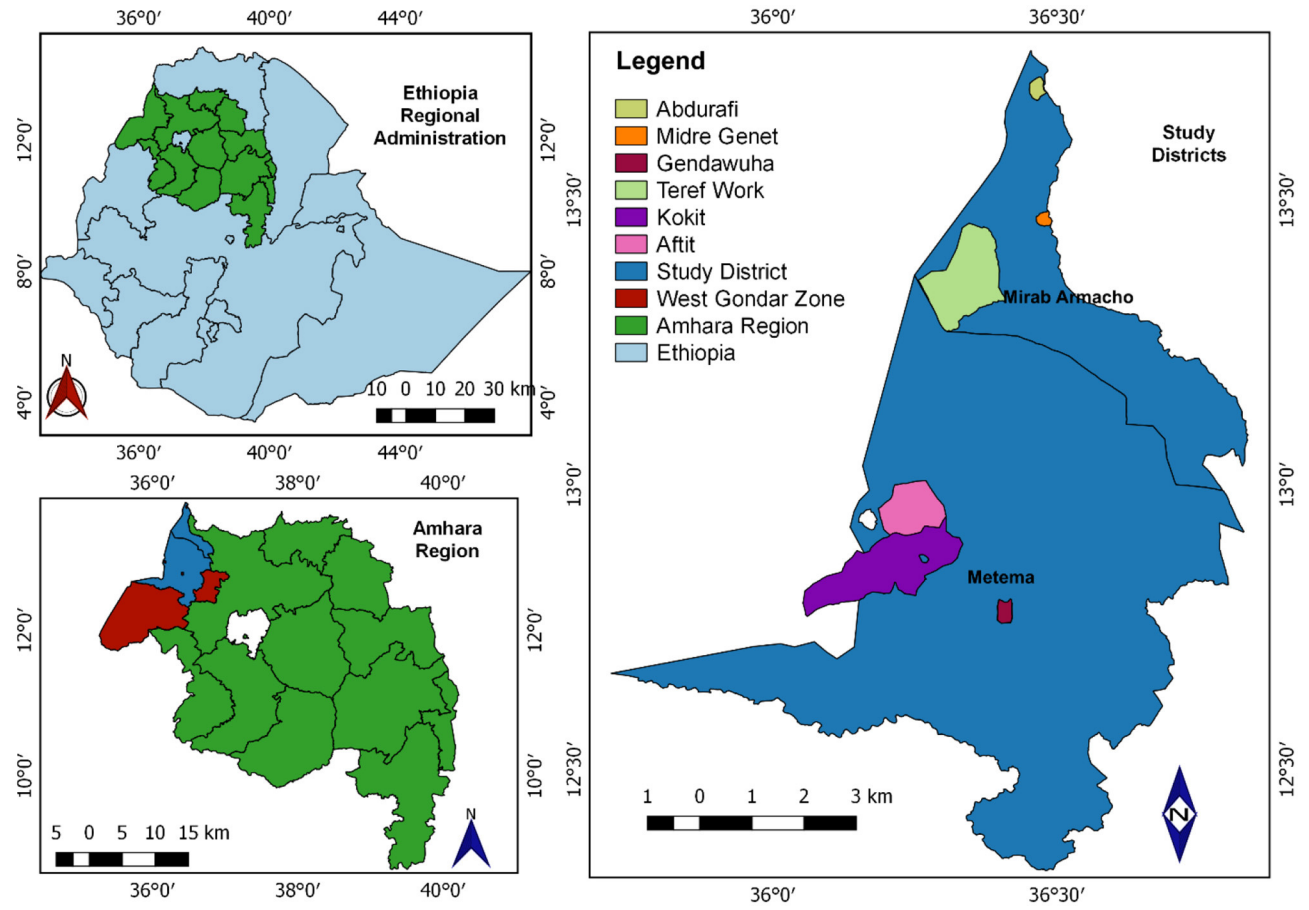


FIGURE 1
Map shows the Metenma and Mirab Armacho study districts (QGIS 3.22.6 software was used to draw).

Region (SNNPRS), Benishangul-Gumuz, Gambella, Harari, and Somali. The study was carried out in Northwest Ethiopia within the Amhara region, specifically in the West Gondar Zone. In the West Gondar Zone, the livestock populations consist of 4,677,125 cattle, 1,566,904 sheep, and 1,211,738 goats. Two districts, namely Metema and West Armacho were selected from West Gondar zone. Metema district receives annual rainfall ranging from 700 to 900 mm, and has mean annual minimum and maximum temperatures of 32 and 46°C, respectively. The altitude ranges from 500 to 700 m above sea level. Similarly, West Armacho district is positioned between latitude 13°00′00″ to 13°40′00″ North and longitude 36°20′00″ to 37°00′00″ East. The annual rainfall here varies from 600 to 1,100 mm, with mean annual minimum and maximum temperatures recorded at 30 and 45°C. The altitude in West Armacho district ranges from 500 to 700 m above sea level (Figure 1) (37).

## 2.2 Study design and period

A cross-sectional study was conducted from February 2023 to May 2023 in small ruminants.

### 2.2.1 Study populations

The study animals were apparently healthy sheep and goats of all age, sex, and species found at different agro-ecological zones in the study area. The estimated animal population in Metema district was 537,981 cattle, 120,541 sheep and 288,933 goats. Similarly, the estimated animal population in West Armacho district was 1,522,758 cattle, 1,278,321 sheep and 139,953 goats. In both districts most part is covered by forest, bush and savannah grass, extensive grazing predominates and animals are not housed.

### 2.2.2 Sample size determinations

The sample size was calculated using the method described by Tschopp et al. (38) and Dohoo et al. (39).

$$n = gc = \frac{P\,(100 - P)\,D}{SE^2}$$

Where "n": the sample size, "p": the prevalence as a percentage, "D": the design effect, "SE": the standard error, "g": the average number of individuals sampled per cluster, and "c": the number of clusters.

$$D = 1 + (g - 1)\,ICC$$

The estimate of intra-cluster correlation coefficient (ICC) for most infectious diseases does not exceed 0.2 (40). So, considering 0.2 ICC

for the cluster (village) and the possibility of collecting about 18 serum samples per village (g), D equals 4.4. Sampling 18 animals per village with an expected prevalence of 50% and a standard error of 5% gave about 24 clusters, and thus a total sample size of 444.

### 2.2.3 Sampling method

A multistage cluster sampling technique was employed to conduct the seroprevalence and risk factor study. Zones and districts were purposively selected based on the abundance of small ruminant populations and their proximity to Sudan. Specifically, two districts, West Armacho and Metema, were chosen from the West Gondar zone. Within each district, three kebeles (Kebele is the lowest level of local government and serves as a neighborhood-level administrative unit in Ethiopia) were selected using a simple random sampling technique: Gendawuha, Kokit, and Aftit kebeles from the Metema district, and Midre Genet, Abdurafi, and Teref Work kebeles from the West Armacho district. Villages within these kebeles were considered clusters, and four villages from each kebele were randomly selected. Individual animals from these villages were then randomly chosen to form the study units. The total sample collected was proportionally distributed among the selected clusters, resulting in a sample size of 444 animals, comprising 308 sheep and 136 goats. This systematic approach ensured a representative and balanced sample distribution across the study area, facilitating accurate assessments of BTV seroprevalence and associated risk factors.

## 2.3 Method of data collection

### 2.3.1 Blood sample collection

About 5 ml of blood was collected from jugular veins using plain vacutainer tube and vacutainer needle after the site was cleaned, hair removed and disinfected with 70% alcohol. The collected blood samples then stand at 45° positions until the sera were collected and transported via ice box (+4°C) to the laboratory. The sera were stored in a refrigerator at −20°C until tested, at Veterinary Microbiology Laboratory at College of Veterinary Medicine and Animal Sciences, University of Gondar. During blood sample collection, supporting data were also collected using a relevant format.

### 2.3.2 Potential risk factor

Structured data collection sheet was employed to collect information on geographical location, age, sex, breed and species of animal's sampled and flock size. Species (sheep and goats), age group was classified as [young (<1 year), adult (≤1 and ≤3 years) and older (>3 years)] as described by Yasine et al. (41) and Jemberu et al. (42), were recorded during sampling. The flock size was categorized into three groups depending on the number of small ruminants in the flock: small size ≤100), medium size (>100 and ≤200), and large size (<200) (43, 44). Information related to exposure to the putative risk factors of BTV such were included in the data collection sheet (Supplementary material 1).

## 2.4 Serological test

The competitive Enzyme-linked immunosorbent assay (cELISA) was used to discriminate BTV from another closely associated Orbivirus such as Epizootic Hemorrhagic Disease virus (EHDV). It was performed using BT antibody test kit (IDvet, 310, rue Louis Pasteur-Grabels France) following the procedures recommended by the manufacturer to the test is highly sensitive (100%) and specific (99%) serological tests (13, 45). The competition percentage (S/N%) and the cutoff were calculated and applied as recommended by the manufacturer, i.e., S/N% ≤ 70% = positive, 70% < S/N % < 80% = doubtful, S/N% ≥ 80% = negative. VP7 of BTV was targeted for antibody detection (anti-VP7) as described by Rojas et al. (46). The test was performed at National Veterinary Institute (NVI) as per the manufacturer's protocol

## 2.5 Data management and analysis

The collected data were entered into a Microsoft Excel spreadsheet. The data were then transferred and analyzed using Stata statistical software version 17. Descriptive as well as analytic statistics were used to summarize and analyze the data. Seroprevalence of BTV was computed by dividing the total number of seropositive sheep and goats by the total number of animals of each species sampled. The animal level true prevalence was calculated by adjusting the corresponding apparent seroprevalence (AP, as percentage) for 100% sensitivity (SE) and 99% specificity (SP) of c-ELISA for BTV (13) using the following formula as indicated by Dohoo et al. (39).

$$\text{True prevalence} = \frac{\text{AP} + \text{SP} - 100}{\text{SE} + \text{SP} - 100}$$

Associations between seropositivity (status of BTV infection in sheep and goats) and potential risk factors were initially examined using univariable analysis and chi-square analysis. Subsequently, a multivariable analysis was conducted utilizing a mixed-effects logistic regression model, with Kebele treated as a random effect variable. This model is employed to analyze binary outcome variables, where the log odds of the outcomes are modeled as a linear combination of both fixed and random effects. Only variables with a $p$-value < 0.5 were integrated into the multivariable model, to include the most important predictors that meaningfully contribute to the model. Moreover, it helps control confounding and enhances the practicality of decision-making. A multivariable model for the outcome variable was constructed using manual stepwise forward mixed effect logistic regression model. During the analysis confounding was checked and it was considered present if any of the remaining coefficients changed at least 25% after removing a non-significant ($p > 0.05$) variable from the model (47). Interactions were tested for all combinations of the significant main effects. Factors with a $p$-value < 0.05 in the final model were taken as a risk factor to BTV sero-prevalence.

TABLE 1 Seroprevalence of bluetongue virus in sheep and goats in the study districts.

| Variable | Category | No of animals tested | No of positive | Seroprevalence (%) | 95% CI |
|---|---|---|---|---|---|
| District | Metema | 168 | 145 | 86.3 | 80.2–90.7 |
| | W/Armacho | 276 | 230 | 83.3 | 78.4–87.3 |
| Species | Sheep | 308 | 257 | 83.4 | 78.9–87.2 |
| | Goat | 136 | 118 | 86.8 | 79.9–91.5 |
| Age | Young | 96 | 61 | 63.5 | 53.5–72.6 |
| | Adult | 173 | 144 | 83.2 | 76.9–88.1 |
| | Older | 175 | 170 | 97.1 | 93.3–98.8 |
| Sex | Male | 130 | 99 | 76.2 | 68.1–82.7 |
| | Female | 314 | 276 | 87.9 | 83.8–91.2 |
| Flock size | Small | 84 | 68 | 80.9 | 71.1–88.0 |
| | Medium | 203 | 176 | 86.7 | 81.3–90.7 |
| | Large | 157 | 131 | 83.4 | 76.8–88.5 |
| Housing | No | 363 | 314 | 86.5 | 82.6–89.7 |
| | Yes | 81 | 61 | 75.3 | 64.8–83.5 |
| MWOA | No | 209 | 181 | 86.6 | 81.3–90.6 |
| | Yes | 235 | 194 | 82.6 | 77.1–86.9 |
| Total | | 444 | 375 | 84.5 | 80.8–87.5 |

MWOA, mixed with other animals.

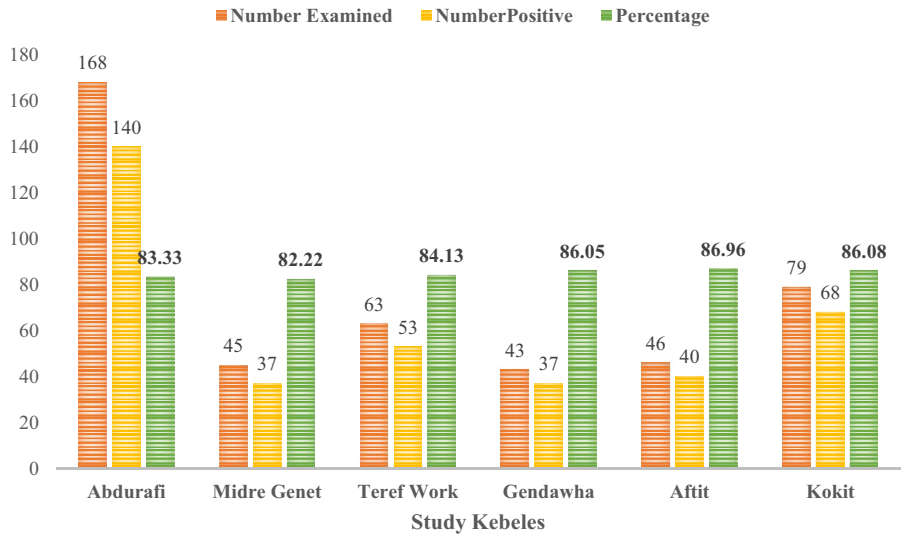


FIGURE 2
Seroprevalence of BTV in sheep and goats of different kebeles in West Gondar Zone Northwest Ethiopia.

# 3 Result

## 3.1 Seroprevalence of BTV

The overall apparent sero-prevalence was 84.5% ($n$ = 444; 95% CI: 80.8–87.5). Our study showed that higher apparent seroprevalence of BTV in sheep [83.4% ($n$ = 308; 95% CI: 78.9–87.2)] and goats [86.8% ($n$ = 136; 95% CI: 79.9–91.5)] (Table 1). In relation to Kebele, the seroprevalence of BTV antibodies was most prevalent in Aftit Kebele [86.96% ($n$ = 46; 95% CI: 73.8–94.0)] and least in Midre Genet [82.2% ($n$ = 45; 95% CI: 68.62–90.9)] (Figure 2).

## 3.2 Risk factors for bluetongue serostatus

The study commenced with the execution of univariable analysis to examine the correlation between potential risk factors and BTV infection. The findings of the chi-square analysis

TABLE 2 Chi-square analysis assessed association of potential risk factors for BTV seropositivity.

| Variable | Category | No of positives (%) | $\chi 2$ | *P*-value |
|---|---|---|---|---|
| Species | Sheep | 257 (83.4) | 0.79 | 0.373 |
| | Goats | 118 (86.8) | | |
| Age | Young | 61 (63.5) | 53.65 | 0.000 |
| | Adult | 144 (83.2) | | |
| | Older | 170 (97.1) | | |
| Sex | Male | 99 (76.2) | 9.70 | 0.002 |
| | Female | 276 (87.9) | | |
| Flock size | Small | 68 (80.9) | 1.69 | 0.430 |
| | Medium | 176 (86.7) | | |
| | Large | 131 (83.4) | | |
| Housing | No | 314 (86.5) | 6.32 | 0.012 |
| | Yes | 61 (75.3) | | |
| Altitude | >750 | 251 (82.8) | 1.91 | 0.167 |
| | <750 | 124 (87.9) | | |
| MWOA | No | 181 (86.6) | 1.40 | 0.240 |
| | Yes | 194 (82.6) | | |

have been delineated in Table 2. Subsequently, the variable was incorporated into a conclusive multivariable model and scrutinized using mixed effect logistic regression. During this evaluation, species and age emerged as statistically significant ($p < 0.05$). The final model revealed that older small ruminants were 25.95 times (95% CI: 9.45–71.28) and adult small ruminants 3.49 times (95% CI: 1.90–6.41) more likely to be seropositive with BTV compared to young small ruminants (Table 3). Moreover, there were no confounding and interactions in the final model. Only variables with a *p*-value < 0.5 were integrated into the final model.

TABLE 3 Multivariable analysis of potential risk factors for BTV seropositivity in sheep and goats using mixed effect logistic regression models with random effect variable of kebele.

| Variable | Category | AOR | 95% CI | *P*-value |
|---|---|---|---|---|
| Species | Sheep | Ref. | | |
| | Goats | 2.43 | 1.27–4.62 | 0.007 |
| Age | Young | Ref. | | |
| | Adult | 3.49 | 1.90 −6.41 | 0.001 |
| | Older | 25.95 | 9.45– 71.28 | 0.001 |

AOR, adjusted odd ratio; MWOA, mixed with other animals; Ref, reference; CI, confidence interval.

## 4 Discussion

The presence of bluetongue virus antibodies was detected in sheep and goats. The identification of antibodies against the bluetongue virus in Northwest Ethiopia signifies the endemic nature of bluetongue virus sero-prevalence among sheep and goats in the Northwest Ethiopia (18).

The seroprevalence of BTV (84.5%) identified in the current investigation aligns closely with the prevalence reported (84.6%) by Elhassan et al. (48) in Sudan. While the prevalence observed in this study is relatively high, it is lower compared to findings by Shoorijeh et al. (49) in Iran (93.5%), Najarnezhad and Rajae (50) in Iran (89.2%), Elmahi et al. (18) in Sudan (91.2%), and Gür (51) in Southern Turkey (88%). Conversely, the current findings demonstrate a higher seroprevalence of BTV in small ruminants compared to previous reports by various authors across different countries: 41.17% in small ruminants in Southern Ethiopia (36), 53.3% in sheep in India (10), 78.4% in small ruminants in Grenada (52), 56.6% in sheep in Pakistan (53), 55.3% in small ruminants in Bangladesh (54), 28.26% in sheep in Brazil (55), 54.10% in sheep in Saudi Arabia (56), and 67.7% in goats in Iran (57). The seroprevalence rates of 86.8% in goats and 83.4% in sheep documented in this study align with the findings of Mohammadi et al. (58), who reported seropositivity rates of 74.2 and 72.9% for goats and sheep, respectively, in Fars Province, Iran. However, the present findings are higher than those reported by Medrouh et al. (17) in Africa, who observed seroprevalence rates of 36.3% in sheep and 47.0% in goats, as well as by van den Brink et al. (59) in the Netherlands, who reported a rate of 7.0% in sheep. The observed disparities in seropositivity levels could be attributed to variations in sample size, study duration, geographical location, immune status, climatic conditions, husbandry practices, management strategies, and vector control interventions implemented around the different study areas. Additionally, it is well-documented that goats, even with minimal clinical signs, can harbor high levels of BTV, potentially serving as a source of infection for other susceptible animals (60, 61). BTV possesses multiple serotypes and immunity to one serotype offers little cross-protection to other serotypes (62).

The higher seropositivity rate among older animals (97.1%) aged over 3 years, compared to younger animals (63.5%) under 1 year of age, as observed in this study, aligns with the findings of Puri et al. (63), who reported a higher seropositivity rate among older animals (49.12%) over 6 months old, compared to younger animals (16.32%) under 6 months old in Nepal. Similarly, a systematic review and meta-analysis conducted by Medrouh et al. (17) in Africa, as well as a study by Ferrara et al. (64) in Italy, reported a higher seroprevalence rate of 46.2 and 46.0%, respectively, among adult animals. This difference can be attributed to several factors. Younger animals are typically sheltered indoors and receive attentive care from their owners, practices that help prevent insect and tick-borne infections. Notably, it was observed that younger animals became infected with BTV when they commenced grazing in the fields at 6 months of age (65). The variation in prevalence across age groups is likely due to the increased exposure of older animals to bluetongue virus infections over their longer lifespan, making them more susceptible to the disease.

The present investigation demonstrated a relatively higher seropositivity among females (87.9%) compared to males (76.2%). This finding aligns with previous studies, including research by Gizaw et al. (1) in Ethiopia, which reported a higher seroprevalence in females (65.54%) compared to males (49.85%). Similarly, Medrouh et al. (17) observed a greater seroprevalence in females (53.3%) than in males (28.1%) in Africa. Additionally, a study

by Puri et al. (63), in Nepal found a higher seroprevalence in females (45.6%) compared to males (26.09%). These consistent findings suggest a potential sex-based difference in susceptibility or exposure, warranting further investigation. Moreover, the discrepancy might be attributed to a potential sampling bias, as more female ruminants were included in the current study, similar to findings from a previous study by Elhassan et al. (48). The study also revealed that BTV infection rates increase with larger flock sizes: small flocks (80.9%), medium flocks (86.7%), and large flocks (83.4%). This observation aligns with the findings of Haile et al. (12), who reported seroprevalence rates of 37.42% for small flocks, 32.35% for medium flocks, and 64.91% for large flocks, as well as with the findings of Sana et al. (66), who reported rates of 36.95% for small flocks, 40% for medium flocks, and 43.59% for large flocks. However, the current results contrast with the findings of Munmun et al. (65), who documented prevalence rates of 58.33% in small flocks, 32.79% in medium flocks, and 38.46% in large flocks. This discrepancy may be due to differences in environmental conditions, vector populations, agro-ecological factors, and management practices that influence interactions with the vector of the bluetongue virus in different regions.

In the current study, older and adult small ruminants were found to be 25.95 times (95% CI: 9.45–71.28) and 3.49 times (95% CI: 1.90–6.41) more likely to be infected with BTV compared to younger small ruminants. This finding is consistent with previous reports indicating that adult small ruminants had higher odds of infection: 2.97 times (95% CI: 1.88–4.69) in Ethiopia (13), 1.76 times (95% CI: 1.09–2.85) in Ethiopia (36), 4.30 times (95% CI: 1.94–9.57) in Sudan (67), 1.48 times (95% CI: 1.12–1.9) in Italy (64), 2.41 times (95% CI: 2.13–2.74) and 2.43 times (95% CI: 2.15–2.75) in Iran (68), and 1.73 times (95% CI: 1.73–1.42) and 4.68 times (95% CI: 3.79–5.78) in Iran (69). The discrepancies in the odds of BTV occurrence observed across different studies may be attributed to variations in management practices and environmental factors. The higher seroprevalence observed in older animals is likely attributable to their prolonged exposure to the BTV over time. Additionally, differences in immune response between age groups may play a role, with older animals potentially developing higher levels of antibodies due to repeated or prolonged exposure to the virus. This phenomenon may be attributed to protective measures such as indoor housing and careful management by owners, which help shield young animals from *Culicoides* midge bites and other infections. In addition to these, a study by Ayele et al. (31) on *Culicoides* vector identification and spatial distribution demonstrated that the study area (Northwest Ethiopia) had a high abundance of *Culicoides* and provided a more favorable environment for the vector compared to findings from similar studies in Southern Ethiopia by Fetene et al. (32). Previous studies have documented that young animals become infected with BTV when introduced to grazing fields at 6 months of age (36). However, conflicting results were reported by Sana et al. (66) in Tunisia and Munmun et al. (65) in Bangladesh, which do not align with the current findings.

Moreover, a species that was not statistically significant in the univariable model became significant in the final multivariable model. This suggests that the effect of the species was initially masked by other variables in the univariable analysis. However, after adjusting for these variables in the multivariable model, the contribution of the previously non-significant species became more apparent. In this study, goats were found to be 2.43 times (95% CI: 1.27–4.62) more susceptible to infection compared to sheep, which is consistent with findings from Islam et al. (54) in Bangladesh (AOR = 4.69, 95% CI: 2.49–8.82) and Manavian et al. (69) in Iran (AOR = 0.43, 95% CI: 0.37–0.50). However, the results reported by Bakhshesh et al. (68) in Iran (AOR = 1.03, 95% CI: 0.97–1.10) and Yilma and Mekonen (36) in Ethiopia (AOR = 1.17, 95% CI: 0.68–2.01) did not align with the findings of this study. This variability could be due to differences in environmental conditions, management practices, or other unidentified factors influencing the susceptibility of these species to BTV.

This study faced several limitations that should be considered when interpreting the findings. First, the inability to identify the specific serotypes circulating in the study area posed a significant challenge. This limitation was primarily due to resource constraints, including inadequate laboratory facilities and insufficient funding. Identifying specific serotypes is essential for understanding disease epidemiology, designing effective control measures, and assessing vaccine suitability. Second, the overrepresentation of goats in the sample, although they are a key livestock species in the region, may limited the generalizability of the findings to other species, such as cattle and sheep. This underscores the importance of future studies employing more representative sampling strategies to provide a comprehensive epidemiological perspective. Similarly, herd size data were collected as categorical variables, and intra-flock seroprevalence was not reported; therefore, we acknowledge these as limitations. This underscores the need for future studies with an intra-flock level seroprevalence report.

## 5 Conclusion and recommendation

The current investigation disclosed a notable seroprevalence of 84.5% and the endemicity of BTV among sheep and goats within the designated area under study. Age and species were identified in this study as potential risk determinants for Bluetongue Virus infection in sheep and goats. A higher seroprevalence of BT was observed in goats, particularly in adult and older small ruminants, in comparison to sheep and younger small ruminants, respectively. Consequently, upcoming investigations concerning this disease should prioritize the identification of prevalent serotypes and circulating vector species.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The animal studies were approved by Institutional Review Board of the College of Veterinary Medicine and Animal Sciences of the University of Gondar, Ethiopia. The studies were

conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

AB: Data curation, Formal analysis, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. WM: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. AM: Funding acquisition, Investigation, Project administration, Resources, Writing – review & editing. MBir: Funding acquisition, Investigation, Project administration, Resources, Visualization, Writing – review & editing. SI: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Validation, Visualization, Writing – review & editing. BD: Funding acquisition, Investigation, Project administration, Resources, Visualization, Writing – review & editing. AK: Funding acquisition, Investigation, Project administration, Resources, Validation, Visualization, Writing – review & editing. AG: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing. MK: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing. GD: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Validation, Visualization, Writing – review & editing. MF: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing. HT: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing. TT: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing. LT: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing. HA: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing. ZT: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Validation, Visualization, Writing – review & editing. HD: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MBit: Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – review & editing.

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## 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.

## Generative AI statement

The author(s) declare that no Gen AI was used in the creation of this manuscript.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1565624/full#supplementary-material

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# Detection of spotted fever group rickettsiae and *Coxiella burnetii* in long-tailed ground squirrels (*Spermophilus undulatus*) and their ectoparasites

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Long-tailed ground squirrels (LTGRs, *Spermophilus undulatus*) are known as reservoirs of multiple arthropod-borne pathogens, such as *Yersinia pestis* and *Bartonella rochalimae*. However, data on the prevalence of spotted fever group rickettsiae (SFGR) and *Coxiella burnetii* in LTGRs and its ectoparasites are limited. In two alpine regions of Xinjiang Uygur Autonomous Region (XUAR, northwestern China), a total of 346 samples were collected from 142 LTGRs, including 142 livers and 204 pooled ectoparasites (*Citellophilus tesquorum dzetysuensis*: 120 pools of 484 fleas; *Frontopsylla elatoides elatoides*: 19 pools of 71 fleas; *Neopsylla mana*: 1 pool of 4 fleas; and *Linognathoides urocitelli*: 64 pools of 865 lice). From these samples, the DNA was extracted, followed by PCR amplification of different genetic markers. Particularly, genes encoding the outer membrane protein A and B (*ompA*, *ompB*), citrate synthase (*gltA*), and surface cell antigen 1 (*sca1*) were used to identify the SFGR. Additionly, the capsular outer membrane protein (*Com1*) gene and insertion sequence (*IS1111*) genes were used to detect *Coxiella*. *Rickettsia sibirica* subsp. *sibirica*, *Rickettsia felis*, and *C. burnetii* were detected in LTGRs, as well as in flea and louse pools. *Rickettsia raoultii* was found in LTGRs and flea pools. Furthermore, *Rickettsia slovaca* was also identified in the flea pools. This study provides molecular evidence for the occurrence of SFGR and *C. burnetii* in LTGRs and their ectoparasites. These findings suggest that *R. sibirica*, *R. slovaca*, *R. raoultii*, *R. felis* and *C. burnetii* are transmitted between LTGRs (as potential reservoirs) and their fleas and lice (as potential vectors).

KEYWORDS

***Spermophilus undulatus*, flea, louse, spotted fever group rickettsiae (SFGR), *Coxiella burnetii***

# 1 Introduction

Species of the genus *Rickettsia* belong to four distinct phylogenetic clades: spotted fever group, typhus group, ancestral group, and transitional group (1). The spotted fever group rickettsiae (SFGR) include over 30 distinct species that may cause severe infections in humans, domestic animals and wildlife (2). The majority of SFGR are tick-borne, while *R. felis* is typically transmitted by fleas. Notably, some SFGR species can be transmitted by lice and mosquitoes, albeit rarely (3–5).

*Coxiella burnetii*, recognized as the causative agent of Q fever, stands out as the most notorious member of its genus (6). As a globally distributed pathogen, it can infect a diverse range of mammals, from rodents to bats (7, 8). The main vectors of *C. burnetii* are hard ticks (7), but it is also potentially transmitted by fleas and lice (9, 10).

Long-tailed ground squirrels (LTGRs, *Spermophilus undulatus*) are medium-sized ground-dwelling rodents, inhabiting distinct alpine habitats in Central Asia, including Kazakhstan, Mongolia, the southern region of the Russian Federation and northwestern China (11). Previously, LTGRs and arthropods infesting them were shown to act as reservoirs or potential vectors for some pathogens, such as *Yersinia pestis*, *Borrelia burgdoferi* sense lato, *Anaplasma phagocytophilum*, *Trypanosoma otospermophili*, *Blechomonas luni*, tick-borne encephalitis virus and *Hepacivirus* C (12–16). However, the evidence of SFGR and *C. burnetii* in LTGRs and their ectoparasites, especially in fleas and lice, remains still unknown. The aim of this study was to screen these pathogens in liver samples of LTGRs and their associated arthropods.

# 2 Materials and methods

## 2.1 Sample collection and identification

In total, 142 LTGRs were captured in July, 2024 in Wenquan County and Jinghe County (1200–2,500 m above sea level, both adjacent to Kazakhstan), Bortala Mongolian Autonomous Prefecture, northwestern China (Supplementary Figure 1). To achieve this goal, Sherman traps (H.B. Sherman Traps, Tallahassee, FL, United States) were deployed at the entry points of occupied burrows. The survey encompassed a total of 150 traps per site, which were inspected once an hour (17).

All captured rodents were identified by experienced zoologists based on morphological characteristics, such as body length, fur color, tail length and other features (17). Subsequently, the rodent were euthanized and killed via cervical dislocation by certified personnel at the enhanced biosafety level 2 laboratory, Shihezi University (18). Each sampled rodent was then put in individual ziplock bags stored at −80°C. The liver was removed from individual LTGR and placed into each labeled tubes. The species was confirmed from four liver samples of representative rodents by sequencing the cytochrome b (*cytb*) gene (19). All procedures involving wild rodents adhered to the ethical guidelines of Animal Ethics Committee of Shihezi University (Approval No. A2022-029-01).

Arthropod ectoparasites (559 fleas, 865 lice and 1,136 ticks) were collected from individual rodents through gentle brushing of their fur, and then preserved in 70% ethanol. All sampled ticks were used for virus research by another team. For morphological identification, the fleas and lice were treated with 10% NaOH for 1–3 days and put onto slides for microscopical examination (11, 20). Meanwhile, the cytochrome c oxidase subunit II (*COII*) gene for fleas, and the 18S ribosomal RNA (*18S rRNA*) gene for lice were amplified and sequenced, in order to confirm their taxonomy (21–23). Subsequently, the ectoparasite samples were grouped into pools according to individual host from where they were collected, flea or louse species, number and sampling sites. Flea pools contained 2 to 5 individuals, while lice pools contained 8 to 15 individuals. Finally, fleas were allocated into a total of 140 pools and lice into 64 pools which were used to screen pathogens as described below.

## 2.2 Detection, sequencing and phylogenetic analysis

Each arthropod pool and liver sample were extracted with TIANamp Genomic DNA Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. Four genetic markers, including outer membrane proteins A and B (*ompA* and *ompB*), citrate synthase (*gltA*) and surface cell antigen 1 (*sca1*), were used to detect SFGR (17). In addition, the capsular outer membrane protein (*Com1*) gene and the insertion sequence (*IS1111*) gene were targeted to investigate the presence of *C. burnetii* (24, 25). The primer sequences and PCR conditions are shown in Supplementary Tables 1, 2. Negative controls consisted of double-distilled water, which consistently showed no detectable PCR product in all tests. Positive controls were DNA samples of *Rickettsia lusitaniae* from common pipistrelles and *Coxiella*-like symbiont from ticks, both preserved in our laboratory (26, 27). The PCR products were purified using the TIANgel Midi Purification Kit (TIANGEN, Beijing, China), and sequenced with Sanger and 454-pyrosequenced PCR amplicons (28, 29). The above sequencing was conducted three times to check the reproducibility. Obtained sequences were compared to reference sequences found in GenBank using BLAST.[1] Phylogenetic analysis was conducted using MEGA 7.0 software, employing the neighbor-joining method and 1,000 replicates for bootstrap support.

# 3 Results

## 3.1 Morphological and molecular identification of rodent species and associated fleas and lice

All sampled rodents were identified as long-tailed ground squirrels (*S. undulatus*) based on their morphology and a 98.86% sequence identity (1,124/1137 bp) to the *cytb* gene of this species found in Russia (OQ695583). A total of 559 fleas were collected. The average flea index was 3.94 (559/142). Subsequently, three species

Abbreviations: LTGR, Long-tailed ground squirrel; SFGR, spotted fever group rickettsiae; XUAR, Xinjiang Uygur Autonomous Region; ompA, outer membrane protein A; ompB, outer membrane protein B; gltA, citrate synthase; sca1, surface cell antigen 1; com1, capsular outer membrane protein; IS1111, insertion sequence.

1 http://www.ncbi.nlm.nih.gov/BLAST/

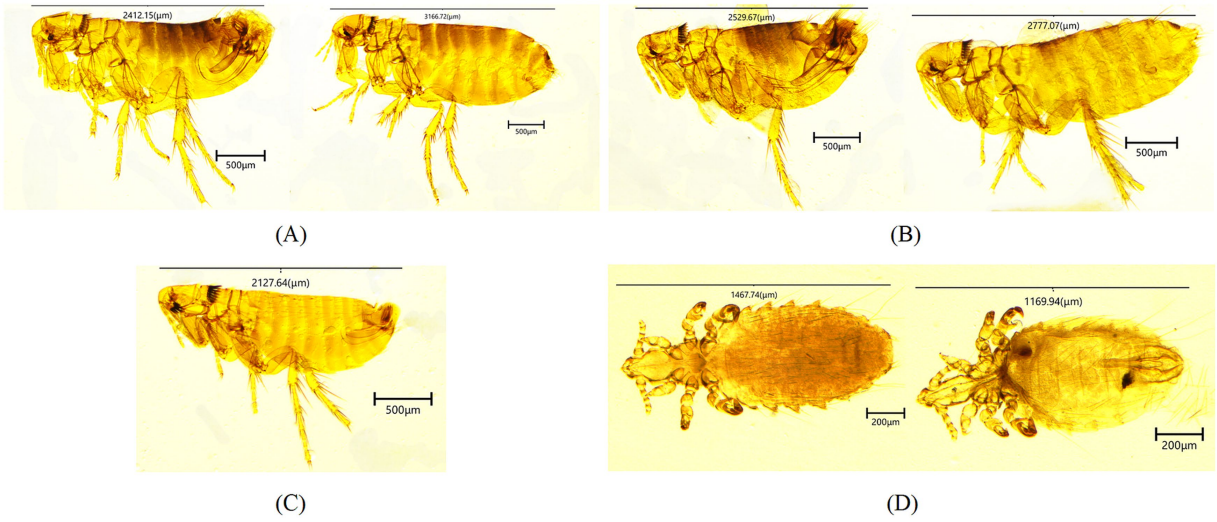


FIGURE 1
Photomicrographs of morphologically identified fleas and lice [*Citellophilus tesquorum dzetysuensis* **(A)**, *Frontopsylla elatoides elatoides* **(B)**, *Neopsylla mana* **(C)**, *Linognathoides urocitelli* **(D)**].

were identified after microscopical examination (Figure 1), including *Citellophilus tesquorum dzetysuensis* (484), *Frontopsylla elatoides elatoides* (71) and *Neopsylla mana* (4). The former flea species was morphologically indientified by genal comb absent, reduced frontal bristles (1 in males, absent in females), pronotal ctenidium with 18–22 vertical spines, labial palps extending to or beyond forecoxa. The middle was by prominent frontal tubercle, 6–7 frontal bristles, 3 ocular bristles above eye, pronotal ctenidium with 22 spines, labial palps reaching forecoxa apex. The latter was by two genal combs (outer comb with short, broad spines; inner comb with narrow, posteriorly inclined spines), pronotal ctenidium with 17–20 spines, labial palps reaching two-thirds of forecoxa. The *COII* gene sequences of these species exhibited 100, 99.86% (713/714 bp), and 100% sequence identities to those of conspecific fleas reported from China, respectively (PP475165, MF000677 and MF000670). Furthermore, 865 lice were also collected. The average louse index was 6.09 (865/142). All sampled lice in this study were identified as *Linognathoides urocitelli* by molecular detection and morphological key features, which included dorsoventrally flattened body, reduced eyes, 5-segmented antennae with sexually dimorphic spines on third segment (males), thoracic sternal plate with posterior medial lobe, and paratergal plates on abdominal segments III-VII bearing spiracular openings. The *18S rRNA* gene sequence of this species exhibited 99.81% (522/523 bp) sequence identity to the same louse species from LTGR in Mongolia (MK478719).

## 3.2 Molecular and phylogenetic analysis of spotted fever group rickettsiae (SFGR)

Eleven LTGRs (7.75%, 11/142), 12 flea pools (8.57%, 12/140) and 15 louse pools (23.44%, 15/64) tested positive for SFGR. Among the 11 SFGR-positive LTGRs, 10 liver samples were also SFGR-positive in their associated flea and louse pools. BLAST and phylogenetic analyses revealed that *R. sibirica* and *R. felis* were present in LTGRs, flea and louse pools. Additionally, *R. raoultii* was identified in LTGRs and flea pools. Moreover, *R. slovaca* was also detected in the flea pools (Table 1).

Regarding sequence comparisons based on the four genetic markers, *Rickettsia sibirica* subsp. *sibirica* showed 100% identity compared with the sequence of conspecific bacteria from a tick-bitten patient in Russia (KT006594); *R. slovaca* showed 100% identity to *R. slovaca* from *Dermacentor marginatus* ticks in Kazakhstan (MW922580); *R. raoultii* showed 99.47–99.50% identity to *R. raoultii* from striped field mouse (*Apodemus agrarius*) in China (MZ297809); and *R. felis* showed 99.76–100% identity compared with the sequence of *R. felis* from cat fleas (*Ctenocephalides felis*) in Indonesia (MT499365; Figure 2). The detailed similarities and divergences of the sequences in this study are shown in Supplementary Table 3.

## 3.3 Molecular and phylogenetic analysis of *Coxiella burnetii*

*C. burnetii* was detected in 15 LTGRs (10.56%, 15/142), 58 flea pools (41.43%, 58/140) and 20 louse pools (31.25%, 20/64). The prevalence of *C. burnetii* in both LTGRs and their ectoparasites was significantly higher than that of SFGR ($X^2 = 35.09$, $df = 2$, $p < 0.05$), suggesting a more prominent circulation of *C. burnetii* in these region. Based on BLASTn analysis, the *IS1111* sequences (PQ663250-PQ663252) in this study were closest related to a *C. burnetii* isolate from *Rhipicephalus sanguineus* ticks in Turkey (MZ073364), showing 99.54–99.69% identities (Figure 2).

## 3.4 Co-infection of *Rickettsia sibirica* and *Coxiella burnetii*

Notably, two LTGRs (1.41%, 2/142) were co-infected with *R. sibirica* and *C. burnetii*. More interestingly, their associated three

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TABLE 1 Information on specimens used in this study, including geographical location of collection site, total number, pool number, hosts, and prevalence of pathogens.

| Location | Species | Number of specimens (pools) | Host species (number) | SFGR | *Coxiella burnetii* | Co-infection |
|---|---|---|---|---|---|---|
| Jinghe County | *Citellophilus tesquorum dzetysuensis* | 357 (90) | *Spermophilus undulatus* (90) | 5/90 (5.56%) *Rickettsia sibirica* | 43/90 (47.78%) | 2/90 (2.22%) |
| | | | | 1/90 (1.11%) *Rickettsia slovaca* | | – |
| | | | | 3/90 (3.33%) *Rickettsia raoultii* | | – |
| | | | | 1/90 (1.11%) *Rickettsia felis* | | – |
| | *Frontopsylla elatoides elatoides* | 30 (9) | *Spermophilus undulatus* (9) | 1/9 (11.11%) *Rickettsia sibirica* | 7/9 (77.78%) | 1/9 (11.11%) |
| | *Neopsylla mana* | 4 (1) | *Spermophilus undulatus* (1) | – | 1/1 (100%) | – |
| | *Linognathoides urocitelli* | 785 (58) | *Spermophilus undulatus* (58) | 12/58 (20.69%) *Rickettsia sibirica* | 19/58 (32.76%) | 2/58 (3.45%) |
| | | | | 2/58 (3.45%) *Rickettsia felis* | | – |
| | *Spermophilus undulatus* | 101 (–) | – | 7/101 (6.93%) *Rickettsia sibirica* | 9/101 (8.91%) | 2/101 (1.98%) |
| | | | | 1/101 (0.99%) *Rickettsia raoultii* | | – |
| | | | | 1/101 (0.99%) *Rickettsia felis* | | – |
| Wenquan County | *Citellophilus tesquorum dzetysuensis* | 127 (30) | *Spermophilus undulatus* (30) | – | 6/30 (20%) | – |
| | *Frontopsylla elatoides elatoides* | 41 (10) | *Spermophilus undulatus* (10) | 1/10 (10%) *Rickettsia sibirica* | 1/10 (10%) | – |
| | *Linognathoides urocitelli* | 80 (6) | *Spermophilus undulatus* (6) | 1/6 (16.67%) *Rickettsia sibirica* | 1/6 (16.67%) | – |
| | *Spermophilus undulatus* | 41 (–) | – | 2/41 (4.88%) *Rickettsia sibirica* | 6/41 (14.63%) | – |

flea pools (2.14%, 3/140) and two louse pools (3.13%, 2/64) were also co-infected with *R. sibirica* and *C. burnetii*.

## 4 Discussion

In this study, 142 LTGRs and their ectoparasites, 559 fleas and 865 lice were collected in Central Asia. In these samples, four SFGR species were detected, including *R. sibirica*, *R. slovaca*, *R. raoultii*, and *R. felis*, as well as *C. burnetii*. To our best knowledge, (i) *R. sibirica*, *R. felis* and *C. burnetii* were identified here for the first time in LTGRs and their parasites (fleas and lice); (ii) in this study, first molecular evidence is also provided for *R. raoultii* in LTGRs and their ectoparasitic fleas; (iii) additionally, *R. slovaca* is newly recognized in LTGR fleas.

SFGR have worldwide distribution and affect a wide range of wild and domestic vertebrates. Although most reports focus on tick-borne transmission of SFGR, growing evidence suggests that other arthropods-including fleas (notably for *R. felis*), lice, keds, and bugs-may serve as potential vectors (2). *R. felis* is primarily transmitted by fleas, particularly by the cat flea (*C. felis*) (30). Similarly, molecular evidence was provided for *R. slovaca* in *Haematopinus suis* lice from boars in Algeria and later in *Laelaps agilis*, *Laelaps jettmari* and *Eulaelaps stabularis* mites from rodents in Slovakia (31, 32). With regard to *R. raoultii*, its occurrence was detected in fleas from yellow necked mouse (*Apodemus favicollis*) and bank vole (*Myodes glareolus*) in Germany (33). In this study, we reported novel findings of *R. sibirica* and *R. felis* in *C. tesquorum dzetysuensis*, *F. elatoides elatoides* and *L. urocitelli*, as well as *R. slovaca* and *R. raoultii* in *C. tesquorum dzetysuensis* and *F. elatoides elatoides*. In addition, SFGR were previously detected in wild boars, birds, dogs and cats, which act as reservoirs (34–37). Rodents are also notably recognized as reservoirs including yellow necked mouse (*Apodemus flavicollis*), common vole (*Microtus arvalis*) and European water vole (*Arvicola terrestris*) (38). Interestingly, we found that *R. sibirica*, *R. raoultii* and *R. felis* were present not only in the aforementioned arthropods but also in LTGRs. Notably, among 11 SFGR-positive LTGRs, 10 liver samples were also SFGR-positive in their associated flea and louse pools. This high

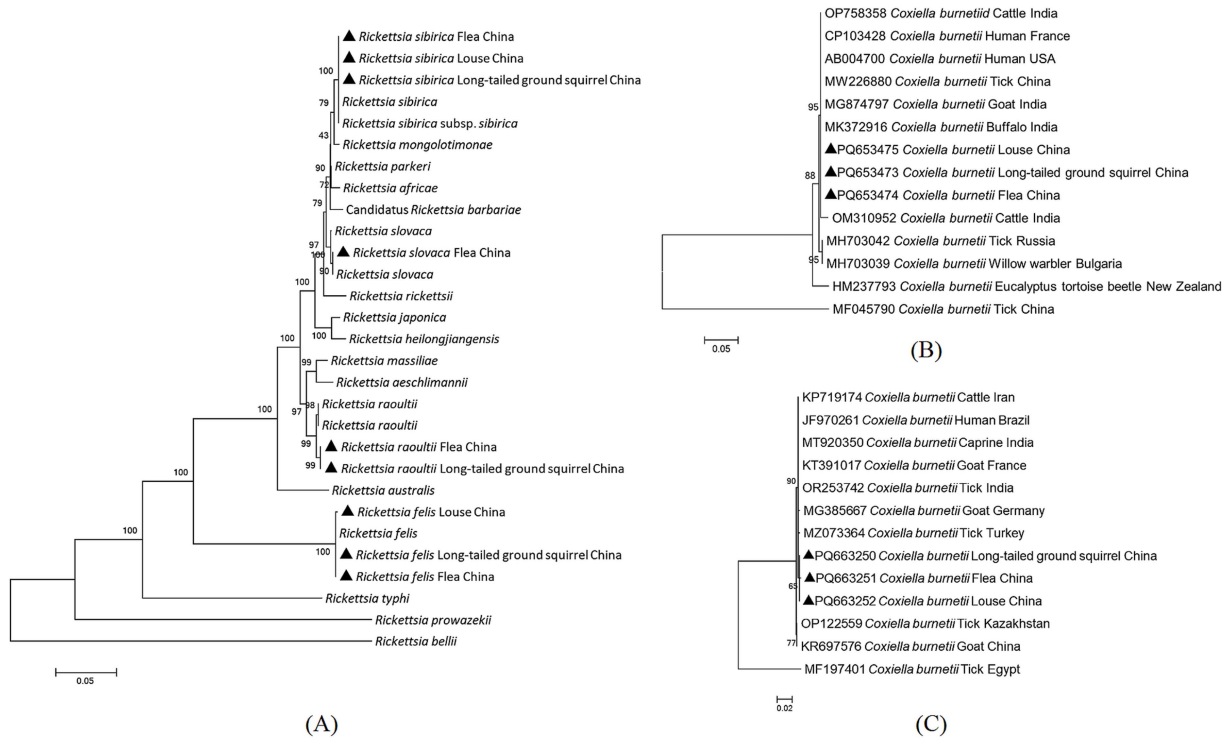


FIGURE 2
Phylogenic tree of spotted fever group rickettsiae (SFGR) and *Coxiella burnetii* from long-tailed ground squirrels and their ectoparasites [the *ompA-ompB-gltA-sca1* concatenated sequences for SFGR **(A)**, the *Com1* concatenated sequences for *C. burnetii* **(B)**, the *IS1111* concatenated sequences for *C. burnetii* **(C)**]. The new sequences provided in the present study are indicated by a black triangle (containing the accession number).

concordance suggests that ectoparasites may play a critical role in either acquiring pathogens from infected hosts or transmitting SFGR within rodent populations. Although we have detected these SFGR in LTGRs and their ectoparasites, further experiments are still needed to confirm the role of these arthropods as vectors.

*C. burnetii*, listed as category B bioterrorism agent in United States, has diverse infection routes including air-borne and venereal transmission (39). Furthermore, it is transmitted by infected arthropods, such as ticks, fleas, lice and mites (10, 40–42). Previously, *C. burnetii* was found in *Haematopinus eurysternus* lice from a cow in Egypt (10). More recently, a report revealed the presence of *C. burnetii* in fleas collected from Norway rat (*Rattus norvegicus*), black rat (*Rattus rattus*) and Cyprus red fox (*Vulpes vulpes indutus*) in Cyprus (43). In this study, we extend these findings with the novel detection of *C. burnetii* in *C. tesquorum dzetysuensis*, *F. elatoides elatoides*, *N. mana* and *L. urocitelli*. Additionally, *C. burnetii* was previously identified in deer, sheep, cattle, goats, birds and rabbits, which serve as reservoirs (7). Rodents also have an important role as reservoirs, as exemplified by the flying squirrel (*Pteromys volans*), red squirrel (*Sciurus vulgaris*), and red-backed vole (*Myodes gapperi*) (44). In our research, LTGRs have been found to harbor *C. burnetii* as well. Crucially, all *C. burnetii*-positive LTGRs in this study concurrently carried infected fleas or lice, suggesting a potential bi-directional transmission between hosts and ectoparasites.

Rodents serve as hosts for several species of blood-sucking arthropods, and infected ectoparasites may transmit pathogens to these mammals through their bites. Consequently, naïve ectoparasites themselves can become infected after biting these rodent reservoirs, leading to new opportunities for pathogens to spread by horizontal transmission (Supplementary Figure 2). Examples of such pathogens include *R. slovaca*, *R. raoultii*, *R. felis* and *R. monacensis* (33, 45). Therefore, the concurrent detection of pathogens in hosts, fleas, and lice is of significant importance, underscoring the complexity of pathogen transmission and the potential roles of these vectors in pathogen spillover and host shift.

## 5 Conclusion

In this study, we present the first molecular evidence of SFGR and *C. burnetii* in LTGRs and their ectoparasites. Our findings suggested that *R. sibirica*, *R. slovaca*, *R. raoultii*, *R. felis* and *C. burnetii* are transmitted between LTGRs (as potential reservoirs) and fleas and lice (as potential vectors) infesting them.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, Rodent species cytb: PQ653469; flea species COII: PQ653470-PQ653472; louse species 18S rRNA: PQ640277; SFGR ompA: PQ677906-PQ677914; SFGR ompB: PQ677915-PQ677923; SFGR gltA: PQ677924-PQ677932; SFGR sca1: PQ677933-PQ677941; Coxiella burnetii Com1: PQ653473-PQ653475; Coxiella burnetii IS1111: PQ663250-PQ663252.

## Ethics statement

This study was approved by the Animal Ethics Committee of Shihezi University (approval no. A2022-029-01). The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

XH: Conceptualization, Investigation, Software, Writing – original draft. ZL: Conceptualization, Investigation, Writing – original draft. ZJ: Conceptualization, Investigation, Writing – original draft. SZ: Conceptualization, Investigation, Writing – original draft. SH: Conceptualization, Writing – review & editing. MY: Investigation, Resources, Software, Writing – original draft. GL: Conceptualization, Investigation, Resources, Software, Writing – original draft, Writing – review & editing. YW: Conceptualization, Investigation, Resources, Software, Writing – original draft, Writing – review & editing.

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## 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.

## Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1553152/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The locations for capturing rodents and associated ectoparasitic arthropods in the current study.

SUPPLEMENTARY FIGURE 2
Circulation of pathogens between ectoparasites and rodents (LTGRs): **(A)** Ectoparasites (e.g., fleas and lice) acquire pathogens by biting infected rodents (LTGRs); **(B)** Pathogen-carrying ectoparasites bite uninfected rodents (LTGRs); **(C)** Uninfected rodents (LTGRs) become infected by bites from pathogen-carrying ectoparasites.

SUPPLEMENTARY TABLE 1
Target genes and sequences of primers used in this study for identifying Long-tailed ground squirrels (LTGRs), fleas, lice, spotted fever group rickettsiae (SFGR) and *Coxiella burnetii*.

SUPPLEMENTARY TABLE 2
Cycling conditions of PCR assays.

SUPPLEMENTARY TABLE 3
The BLAST analysis of Long-tailed ground squirrels (LTGRs), fleas, lice, spotted fever group rickettsiae (SFGR) and *Coxiella burnetii*.

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# Prevalence of Bluetongue and the distribution of *Culicoides* species in northern and southern regions of Kazakhstan in 2023–2024

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**Introduction:** Bluetongue virus (BTV) is a significant vector-borne pathogen affecting ruminants, leading to substantial economic losses, and adversely impacting livestock production worldwide. Recently, Bluetongue (BT) has emerged as a growing concern for European and Asian countries, including Kazakhstan. This study examines the prevalence and distribution of BTV in Kazakhstan during 2023-2024, providing up-to-date information on its occurrence in livestock and *Culicoides* species. The findings aim to contribute to better understanding and management of BT in the region.

**Methods:** A total of 972 whole blood and 972 serum samples were collected from cattle, sheep, and goats in the southern and northern regions of Kazakhstan, alongside 11,859 *Culicoides* midges in the autumn of 2023 and Spring of 2024. The serum samples were tested for BT virus (BTV)-specific antibodies using ELISA, while the whole blood and *Culicoides* specimens were analyzed for BTV RNA by Real-time RT-PCR (rRT-PCR). Morphological and molecular identification of *Culicoides* species was also conducted.

**Result:** The overall seroprevalence of BTV in Southern Kazakhstan increased across all animal species in 2024 compared to 2023, with goats showing the most notable rise (from 3.8% to 29.5%). In the northern regions, seroprevalence remained zero in 2023 but reached 10.0% in cattle by 2024. rRT-PCR results confirmed active virus circulation, with rRT-PCR-positive samples significantly higher in 2024, especially among goats (from 4.2% in 2023 to 62.0% in 2024) and cattle (from 9.2% to 34.4%). Based on morphology, nine species of *Culicoides* midges were identified, including *C. obsoletus* a known BTV vector in European countries. Four of them were genetically confirmed, and BTV RNA was detected in all four species (*C. miutissimus, C. sphagnumensis, C. newsteadi,* and *C. pectipennis*), suggesting their potential vectorial role in BTV transmission.

**Discussion:** This study provides new insights into the epidemiology of BT in Kazakhstan and serves as a valuable resource for veterinary professionals. The findings emphasize the need for continued surveillance and vector control strategies to mitigate the spread of BTV in the region.

KEYWORDS

**bluetongue, Kazakhstan, *Culicoides*, serology, RNA detection**

## Introduction

*Culicoides* biting midges are the smallest blood-sucking two-winged insects (1–4 mm) in the order Diptera. They belong to the family Ceratopogonidae and have mottled wings with dark patterns that can be used to identify the species (1).

*Culicoides* are important vectors for arboviruses in both animals and humans. The *Culicoides* midges transmit bluetongue (BT), an infectious, non-contagious, vector-borne hemorrhagic viral disease of wild and domestic ruminants. BT is characterized by fever, inflammatory-necrotic lesions in the oral cavity, tongue, the digestive tract, and the epithelium of the coronary band, along with degenerative changes in skeletal and cardiac muscles, subintimal hemorrhage in the pulmonary artery, oedema of the lungs and pericardial, pleural, and abdominal effusions (2, 3). The causative agent, BT virus (BTV) is a double-stranded RNA virus that belongs to the genus *Orbivirus* in the family *Reoviridae*. The genome of BTV is composed of 10 segments of dsRNA. This segmented structure allows the virus to undergo reassortment, leading to the emergence of variants with novel biological characteristics (4). Currently, 29 serotypes of BTV are known to exist (5).

The distribution of BTV has traditionally been observed within the geographical belt between latitudes 40° N and 35° S (6), corresponding to the habitat of *Culicoides* vectors (7). The mild, warm climates of Africa, southern Europe, and Southeast Asia favor the year-round activity of *Culicoides*, facilitating the overwintering of the BTV. Over the past 20 years, the distribution of BTV in Europe has changed significantly. Global climate change has influenced the emergence of different serotypes in geographic areas above 50° and identification of new *Culicoides* vector species (8–10). Currently, the disease has been reported on all continents, with a noticeable trend of expanding to more northern regions (8, 9, 11).

The unprecedented spread of BTV in Europe (6 serotypes across 12 EU countries since 1998), is believed to be driven by the global warming. Reports of BTV detection in countries neighboring Kazakhstan (12–14), has prompted a renewed focus on the epidemiology of BT, and the *Culicoides* vectors in the region.

Historically Kazakhstan was designated as a BT free country. However, serological studies using samples collected during 1996–1998 showed 23.2% BT seroprevalence in livestock (15, 16). Recently Zhigailov et al., reported the detection of BTV antibodies in livestock and BTV genetic material in sheep and *Culicoides* midges in the southeastern region of Kazakhstan. Through molecular genetic analysis and partial sequencing, they determined that the BTV belonged to the "western" topotype of the BTV-9 strain (17). In another study, using mathematical modeling showed that the transmission of BTV in Kazakhstan is not possible between October and March. The winter in Kazakhstan prevents the transmission of BT during this time. Assuming there are endemic BTV competent *Culicoides* midges in Kazakhstan, the modeling predicted risk of BT appearing in the south of Kazakhstan in April and spread north reaching maximum levels in northern Kazakhstan in July, decline in September and disappear by October (18).

Currently, systematic studies of the range and density of the primary vectors of BTV infection in Kazakhstan are either not conducted or are limited to serological monitoring studies. In this study we report, based on the samples collected during 2023–2024, seroprevalence of BT in livestock, the *Culicoides* species associated and their potential role as BTV vectors in Northern and Southern region of Kazakhstan.

## Materials and methods

### Collection and transport of animal biological samples

A total of 972 whole blood and serum samples were randomly collected from clinically healthy sheep, goats, and cattle from the southern regions of Kazakhstan, namely Zhetysu, Almaty, Zhambyl, and Turkestan and the northern regions of Kazakhstan, namely Kostanay, Pavlodar, and North Kazakhstan during August–September 2023 and May–June 2024 (Figure 1). The sample sizes and volumes were determined according to OIE guidelines (19), with modifications based on prevalence levels (Supplementary Table 1) (20).

### Mapping sampling sites using ArcGIS pro

The GPS coordinates of sampling locations were imported and mapped using the WGS 84 projection (EPSG: 4326), and ArcGIS Pro (21) was used to compile and generate the maps. Briefly, a layer of sampling points was created and overlaid on satellite imagery (22). The results were exported as a map with a legend and geographic grid for inclusion in the publication.

### *Culicoides* collection method

*Culicoides* were captured utilizing specialized CDC light traps containing ultraviolet lamp (Light trap, LI-MR-512, BioQuip, USA). The trapping was conducted in the evening at sunset, under calm and windless conditions, in wet, marshy regions adjacent to pastures and livestock facilities. The collected specimens were then transferred to 70% ethanol solution and stored at −20°C for subsequent sorting and morphological identification.

### ELISA

BTV antibodies were detected using the ID Screen® BT Competition kit (Ref: BTC-5P, ID-Vet, France), a competitive ELISA with 96.5% sensitivity and 99.3% specificity, designed to identify anti-VP7 antibodies in serum or plasma from cattle, sheep, and goats (23). Testing and result interpretation followed the manufacturer's instructions.

Optical density (OD) was measured at 450 nm using a Chromate® -4300 reader (Awareness Technology, USA). Samples were classified as positive if their OD was <40% of the mean negative control value (S/N) and negative if OD was ≥40% of the mean negative control value (S/N).

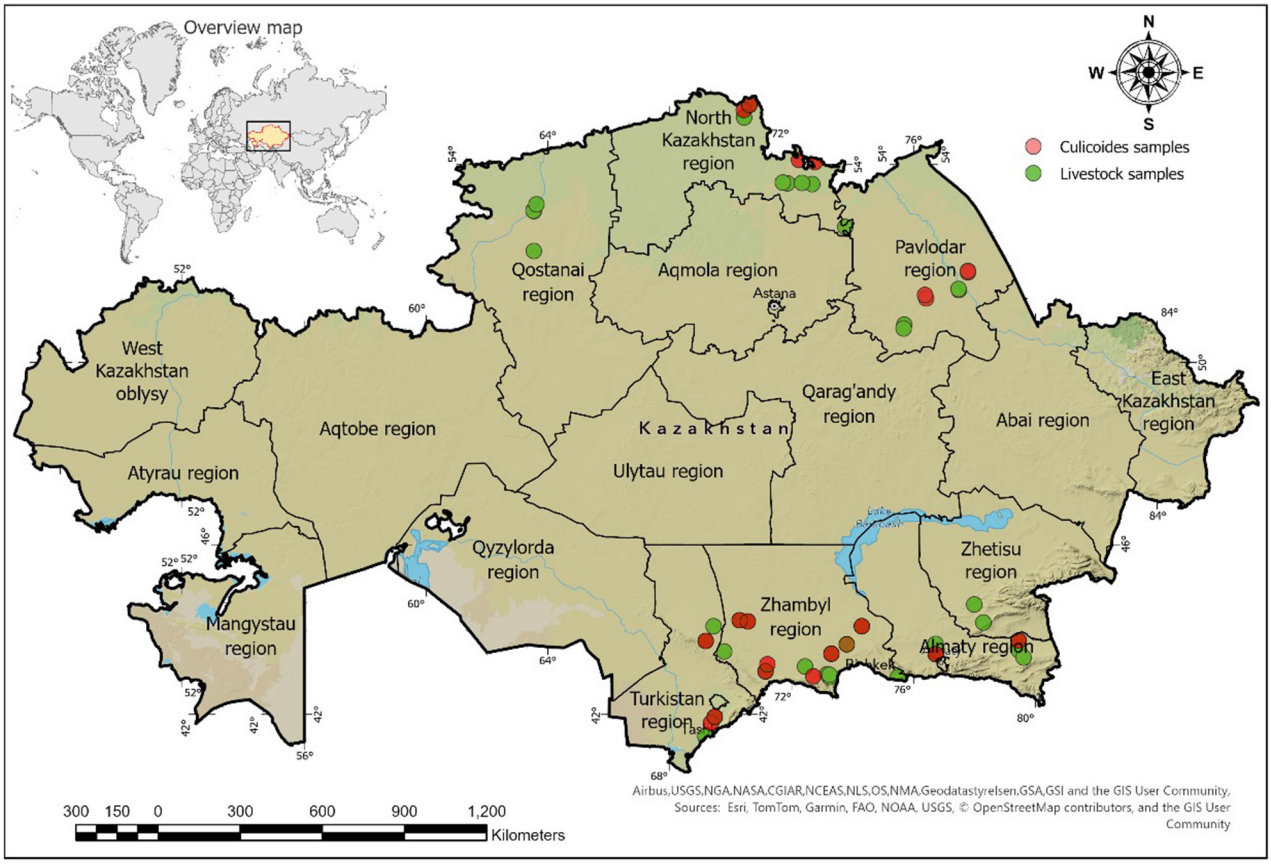


FIGURE 1
Map of Kazakhstan indicating sampling locations for livestock and *Culicoides* spp.

## RNA extraction

Viral RNA was extracted from whole blood using Qiagen RNeasy Mini Kit (Cat number, Qiagen, Hilden, Germany), in accordance with the manufacturer's guidelines. Briefly, 560 µl of AVL buffer and 5.6 µl of carrier RNA were added to 140 µl of the whole blood sample, followed by thorough mixing at room temperature for 10 min. Next, 560 µl of 96% ethanol was added, and mixed thoroughly. The resulting solution was then transferred to RNA binding columns and centrifuged at 8,000 rpm for 30 s. The columns were then washed with 700 µl of AW1 buffer each, followed by 500 µl of AW2 buffer involving by centrifugation at 8,000 rpm for 30 s. To remove any residual buffer, the columns were centrifuged at 12,000 rpm for additional 1 min, and viral RNA was eluted by adding 40 µl of AVE buffer, followed by centrifugation at 8,000 rpm for 1 min. The purity and concentration of the isolated RNA were assessed spectrophotometrically at 260/280 nm, with samples yielding an A260/A280 ratio between 1.8 and 2.0 considered pure and suitable for subsequent rRT-PCR use.

## rRT-PCR setup

ID Gene$^{TM}$ Bluetongue Duplex rRT-PCR (Cat number: IDBTV-50, Innovative Diagnostics, Grabels, France) was conducted using a Rotor-Gene Q thermal cycler (Qiagen), and the results were analyzed using Rotor-Gene Q software version 1.8.187.5. rRT-PCR setup was performed in accordance with the ID Gene BT Duplex kit instructions and the following thermal cycling conditions were followed: reverse transcription at 45°C for 10 min, initial denaturation at 95°C for 10 min followed by 40 amplification cycles (denaturation at 95°C for 15 s, annealing and extension at 60°C for 30 s). Detection of the fluorescent signal occurred during the annealing phase in the "Green" channel. Upon completion of the reaction, the amplification curve was analyzed, and a final report was generated. The threshold line was established at a maximum level of 0.05. The reliability of the obtained data was verified through appropriate values of negative and positive controls.

## Morphological and genetic identification of midges

Morphological identification of midges was performed based on wing patterns and size (24), under a stereomicroscope (Optika®, Model SZO-T, S/N 651480-482, Ponteranica, Italy). Identification keys (25, 26) and the interactive specialized software *Culicoides* Xper2 Version 2.0 (www.iikculicoides.net) were utilized for precise species determination.

For molecular characterization of the *Culicoides* species, DNA from individual midge specimen was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Ref: 69506, Hilden, Germany). Briefly, each midge specimen was added to ATL buffer, supplemented with Proteinase K, and mixed with 200 µl of Buffer AL. The mixture was vortexed for 15 s and incubated at 56°C for 10 min to ensure complete lysis. Subsequently, 200 µl of 96%−100% ethanol was added, and the mixture was vortexed for another 15 s. The lysate was transferred to a DNeasy Mini Spin Column and centrifuged at 6,000 × g (8,000 rpm) for 1 min. The column was then washed with 500 µl of Buffer AW1, centrifuged at 6,000 × g for 1 min, followed by a second wash with 500 µl of Buffer AW2, and centrifuged at 20,000 × g (14,000 rpm) for 3 min. DNA was eluted with 200 µl of nuclease-free water, incubated for 1 min, and centrifuged at 6,000 × g for 1 min. The eluted DNA was stored at −20°C until further analysis. The 28S rDNA is a molecular marker that can be used to differentiate closely related *Culicoides* species. The primers (Forward 28S rDNA 28S_C'1 5′-ACCCGCTGAATTTAAGCAT-3′, and Reverse 28S_D2 5′-TCCGTGTTTCAAGACGGG-3′) were used to amplify 28S rRNA gene from each specimen (27–29). The cycling conditions used were; initial denaturation at 98°C for 30 s, succeeded by 35 cycles comprising denaturation at 98°C for 10 s, annealing at 55–58°C for 90 s, and extension at 72°C for 45 s. This process concluded with a final extension at 72°C for 5 min. The PCR products, ∼700 bp in length, were subsequently assessed using gel electrophoresis on a 1.5% agarose gel and subjected to Sanger sequencing.

## Sanger sequencing

Sanger sequencing method, which employs terminating dideoxynucleotides on a 3,500 × l Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Japan. Serial number: 35395-010) was used to sequence the 28S rDNA amplicons. This process utilizes the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific), following the manufacturer's protocols. Capillary electrophoresis is facilitated using POP-7 (Thermo Fisher Scientific) as the polymer. DNA termination products are produced through cyclic sequencing. To purify the DNA from unbound dyes, gel filtration is carried out using Centri-Sep columns or with CleanSeq Reagent, in accordance with the kit instructions. The sequencing operations on the sequencer conform to the guidelines provided with the instrument. The resulting chromatograms were analyzed using the Sequencher software package (Gene Codes Corporation, Ann Arbor, MI), and the sequences were analyzed against the GenBank (NCBI) database with MEGA11 software to identify midge species based on percent identity (30).

## Statistical analysis

The statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). In a univariable analysis, the chi-square test was performed to examine the relationships between risk factors and BTV seroprevalence. The chi-square test was used to assess the strength of association between independent variables, effectively evaluating differences in BTV prevalence across different regions and animal species in our study.

Using multivariable logistic regression, potential risk factors with $p$-values $< 0.05$ were further evaluated. Logistic regression analysis was employed to assess the relationship between the dependent variable (presence of BTV infection) and independent variables (e.g., age, sex, geographic location). This method allowed for the simultaneous consideration of multiple variables. For the outcome variable, a multivariable model was constructed using logistic regression analysis. Confidence intervals (95% CI) and odds ratios (OR) were calculated.

# Results

## Prevalence of BTV in animals

Thus far, animals showing BT clinical signs have never been reported in the northern regions. However, in the southern regions, sheep with clinical manifestations of BT have been reported. In this study, all the samples (a total of 972 samples of whole blood and sera) were collected from clinically healthy animals in the southern and northern Kazakhstan in the autumn of 2023 and Spring of 2024. This included 642 samples from sheep, 87 from goats, and 243 from cattle. All sera samples were evaluated by ELISA for BTV-specific antibodies (Table 1). The overall serology results revealed a higher number of positive samples in the southern regions compared to the northern regions ($p < 0.0001$) (Supplementary Table 2). When the data was analyzed based on animal species, the overall seroprevalence was 4.2% (95% CI: 2.9–6.0) among sheep, 21.8% (95% CI: 14.4–31.6) among goats, and 14.4% (95% CI: 10.5–19.4) among cattle (Supplementary Table 3). When compared the year of collection, seroprevalence in the southern regions for 2023 Autumn was 5.4% (95% CI: 2.9–9.9) among sheep, 4.2% (95% CI: 0.7–20.2) among goats, and 10.5% (95% CI: 5.4–19.4) among cattle. In 2024 Spring, the seroprevalence was 6.8% (95% CI: 4.3–10.5) for sheep, 36.0% (95% CI: 24.1–49.9) for goats, and 22.9% (95% CI: 15.6–32.3) for cattle (Supplementary Table 4).

In northern regions, overall seroprevalence was zero in samples collected in Autumn 2023. In 2024, the overall seroprevalence was 0.6% (95% CI: 0.1–3.2) for sheep and 10.0% (95% CI: 4.3–21.4) for cattle, with no seroprevalence in goats (Supplementary Table 4). Detailed serological prevalence by oblasts is presented in Table 1.

rRT-PCR testing revealed that the number of positive samples for bluetongue virus (BTV) RNA was significantly higher in southern regions compared to northern regions ($p < 0.0001$). Additionally, the overall number of rRT-PCR-positive samples was higher in 2024 than in 2023 (Supplementary Table 2). BTV RNA was detected in the different animal species as follows: sheep-−8.9% (95% CI: 6.9–11.3; $p = 0.57$), goats-−36.8% (95% CI: 27.4–47.3), and cattle-−16.5% (95% CI: 12.3–21.6). Notably, the number of rRT-PCR-positive samples exceeded those identified as positive by ELISA (Supplementary Table 3).

In the southern regions in 2023, the proportions of rRT-PCR-positive results were: sheep-−7.2% (95% CI: 4.2–12.1), goats-−4.2% (95% CI: 0.7–20.2), and cattle-−9.2% (95% CI: 4.5–17.8).

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TABLE 1 Detection of seropositive and rRT-PCR-positive animal species in the southern and northern regions of Kazakhstan for the years 2023–2024.

| Animal species | Year and season | Region | Oblast | Animals tested | Seropositive samples (*n*, %) | 95% Confidence intervals (CI) | rRT-PCR-Positive samples (*n*, %) | 95% Confidence intervals (CI) |
|---|---|---|---|---|---|---|---|---|
| Sheep | 2023, Autumn | Southern regions | Zhetysu | 40 | 1 (2.5) | 0.4–12.8 | 5 (12.5) | 5.5–26.1 |
| | | | Almaty | 30 | 0 (0.0) | 0.0–11.3 | 0 (0.0) | 0.0–11.3 |
| | | | Zhambyl | 50 | 3 (6.0) | 2.1–16.2 | 2 (4.0) | 1.1–13.5 |
| | | | Turkestan | 47 | 5 (10.6) | 4.6–22.6 | 5 (10.6) | 4.6–22.6 |
| | 2024, Spring | | Zhetysu | 60 | 0 (0.0) | 0.0–6.0 | 6 (10.0) | 4.7–20.1 |
| | | | Almaty | 85 | 0 (0.0) | 0.0–4.3 | 12 (14.1) | 8.3–23.1 |
| | | | Zhambyl | 78 | 10 (12.8) | 7.1–22.0 | 15 (19.2) | 12.0–29.3 |
| | | | Turkestan | 42 | 8 (19.0) | 9.9–33.3 | 12 (28.6) | 17.1–43.6 |
| | 2023, Autumn | Northern regions | Qostanay | 39 | 0 (0.0) | 0.0–8.9 | 0 (0.0) | 0.0–8.9 |
| | 2024, Spring | | Pavlodar | 89 | 1 (1.1) | 0.2–6.1 | 0 (0.0) | 0.0–4.1 |
| | | | North Kazakhstan | 82 | 0 (0.0) | 0.0–4.5 | 0 (0.0) | 0.0–4.5 |
| Goat | 2023, Autumn | Southern regions | Zhetysu | 3 | 0 (0.0) | 0.0–56.1 | 0 (0.0) | 0.0–56.1 |
| | | | Almaty | 2 | 0 (0.0) | 0.0–65.7 | 0 (0.0) | 0.0–65.7 |
| | | | Zhambyl | 10 | 0 (0.0) | 0.0–27.7 | 0 (0.0) | 0.0–27.7 |
| | | | Turkestan | 9 | 1 (11.0) | 1.9–43.5 | 1 (11.0) | 1.9–43.5 |
| | 2024, Spring | | Zhetysu | 3 | 0 (0.0) | 0.0–56.1 | 3 (100.0) | 43.9–100 |
| | | | Almaty | 15 | 0 (0.0) | 0.0–20.3 | 6 (40.0) | 19.8–64.2 |
| | | | Zhambyl | 16 | 14 (87.5) | 63.9–96.5 | 15 (93.8) | 71.7–98.9 |
| | | | Turkestan | 16 | 4 (25.0) | 10.1–49.5 | 7 (43.8) | 23.1–66.8 |
| | 2023, Autumn | Northern regions | Qostanay | 2 | 0 (0.0) | 0.0–65.7 | 0 (0.0) | 0.0–65.7 |
| | 2024, Spring | | Pavlodar | 6 | 0 (0.0) | 0.0–39.0 | 0 (0.0) | 0.0–39.0 |
| | | | North Kazakhstan | 5 | 0 (0.0) | 0.0–43.4 | 0 (0.0) | 0.0–43.4 |
| Cattle | 2023, Autumn | Southern regions | Zhetysu | 0 | 0 (0.0) | 0 | 0 (0.0) | 0 |
| | | | Almaty | 16 | 1 (6.3) | 1.1–28.3 | 1 (6.3) | 1.1–28.3 |
| | | | Zhambyl | 33 | 2 (6.0) | 1.7–19.6 | 1 (3.0) | 0.5–15.3 |
| | | | Turkestan | 27 | 5 (18.5) | 8.2–36.7 | 5 (18.5) | 8.2–36.7 |
| | 2024, Spring | | Zhetysu | 6 | 1 (16.7) | 3.0–56.3 | 4 (66.7) | 30.0–90.3 |
| | | | Almaty | 18 | 10 (55.5) | 33.7–75.4 | 8 (44.4) | 24.6–66.3 |
| | | | Zhambyl | 56 | 11 (19.6) | 11.3–31.8 | 15 (26.8) | 17.0–39.6 |
| | | | Turkestan | 16 | 0 (0.0) | 0.0–19.4 | 6 (37.5) | 18.5–61.4 |
| | 2023, Autumn | Northern regions | Qostanay | 21 | 0 (0.0) | 0.0–15.5 | 0 (0.0) | 0.0–15.5 |
| | 2024, Spring | | Pavlodar | 25 | 5 (20.0) | 8.9–39.1 | 0 (0.0) | 0.0–13.3 |
| | | | North Kazakhstan | 25 | 0 (0.0) | 0.0–13.3 | 0 (0.0) | 0.0–13.3 |

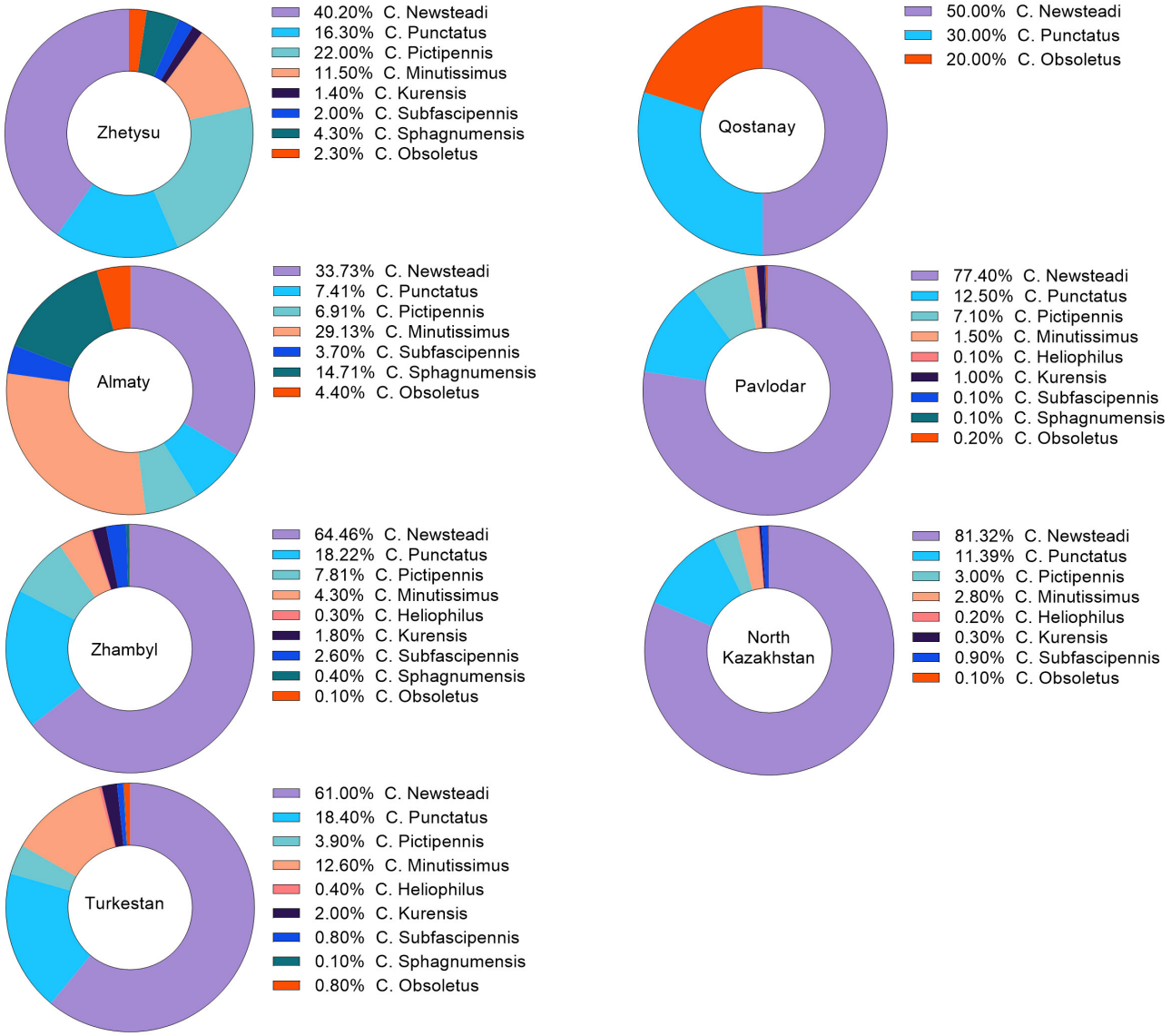


FIGURE 2
Percentage distribution of *Culicoides* species occurrence by region. The percentage distribution of *Culicoides* species occurrence was calculated based on the captured species. This percentage may vary according to the differing weather conditions in each region.

In 2024, the percentage of rRT-PCR-positive samples among sheep increased to 17.0% (95% CI: 12.9–22.0), among goats to 62.0% (95% CI: 48.1–74.1), and among cattle to 34.4% (95% CI: 25.6–44.3; Supplementary Table 4). Conversely, in the northern regions, no rRT-PCR-positive animals for BTV were detected in either 2023 or 2024 (Supplementary Table 4). The distribution of BTV RNA detection across oblasts is presented in Table 1.

## Distribution of *Culicoides* spp. in the southern and northern regions of Kazakhstan

A total of 11,859 *Culicoides* spp. were collected in the southern and northern regions of Kazakhstan. Specifically, 209 specimens (1.8%) were from the Zhetysu, 564 (4.8%) from the Almaty oblast, 2,201 (18.6%) from the Turkestan, 6,350 (53.5%) from the Zhambyl, 1,287 (10.9%) from the North Kazakhstan oblast, and 1,248 (10.5%) from the Pavlodar oblast. Detailed quantitative and percentage metrics of captured *Culicoides* in each oblast are presented in Figure 2 and Table 2. The observed variations in the capture rates of *Culicoides* across different oblasts are attributed to changes in weather conditions.

## Morphological and molecular identification of *Culicoides* spp.

According to the morphological identification based on wing patterns, the following nine species of *Culicoides* were

TABLE 2 Quantitative metrics of *Culicoides* capture in the southern and northern regions of Kazakhstan for 2024.

| Oblast (Administrative division) | Rural district, Village (Administrative division) | Species and number of midges, pcs | | | | | | | | | Total number |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Newsteadi | Punctatus | Pictipennis | Minutissimus | Heliophilus | Kurensis | Subfascipennis | Sphagnumensis | Obsoletus | |
| Zhetysy | Koksu | 13 | 11 | 19 | 9 | – | 1 | 2 | 4 | 3 | 62 |
| | Kerbulaq | 71 | 23 | 27 | 15 | – | 2 | 2 | 5 | 2 | 147 |
| **Total** | | 84 | 34 | 46 | 24 | – | 3 | 4 | 9 | 5 | 209 (1.8%) |
| Almaty | Tasqarasu, Tasqarasu village | 78 | 24 | 18 | 92 | – | – | 12 | 11 | 8 | 243 |
| | Mezhdurechensky, Aqsy village | 112 | 18 | 21 | 72 | – | – | 9 | 72 | 17 | 321 |
| **Total** | | 190 | 42 | 39 | 164 | | | 21 | 83 | 25 | 564 (4.8%) |
| Turkestan | Sholaqkorgan, Sholaqkorgan village | 476 | 208 | 15 | 117 | 4 | 23 | 11 | 2 | 4 | 860 |
| | Saryagash, Saryagash city | 552 | 162 | 42 | 98 | 2 | 14 | 2 | 1 | 11 | 884 |
| | Qyzylzhar, Zhaskeldi village | 315 | 35 | 28 | 63 | 3 | 7 | 4 | – | 2 | 457 |
| **Total** | | 1,343 | 405 | 85 | 278 | 9 | 44 | 17 | 3 | 17 | 2,201 |
| Zhambyl | Qarakemer, Qarakemer village | 415 | 15 | 108 | 24 | 5 | 38 | 118 | 4 | 1 | 728 (18.6%) |
| | Qarasu, Ashybulaq village | 852 | 42 | 46 | 52 | 2 | 11 | 21 | 2 | 1 | 1,029 |
| | Saudakent, Baiqadam village | 542 | 172 | 43 | 18 | – | 2 | 10 | 12 | 3 | 802 |
| | Zhaiylma, Zhaiylma village | 792 | 381 | 82 | 92 | 7 | 44 | 13 | 4 | – | 1,415 |
| | Merke, Merke village | 688 | 212 | 35 | 21 | 3 | 11 | 2 | 1 | 2 | 975 |
| | Tolebi, Tolebi village | 746 | 317 | 169 | 46 | – | 7 | 4 | 1 | – | 1,290 |
| | Zhanaqogam, Koktobe village | 13 | – | – | – | – | – | – | – | – | 13 |
| | Asa, Asa village | 43 | 18 | 11 | 22 | – | 2 | – | – | 2 | 98 |
| **Total** | | 4,091 | 1,157 | 579 | 275 | 17 | 115 | 168 | 24 | 9 | 6,350 (53.5%) |
| North Kazakhstan | Avangard, Roshchino village | 245 | 62 | – | – | – | 2 | – | – | 1 | 310 |
| | Bulaevo city | 372 | 43 | 18 | 8 | 2 | 2 | – | – | – | 445 |
| | Talshyk, Talshyk village | 112 | – | 12 | 21 | – | – | 8 | – | – | 153 |
| | Telzhan, Telzhan village | 318 | 42 | 9 | 7 | – | – | 3 | – | – | 379 |
| **Total** | | 1,047 | 147 | 39 | 36 | – | 4 | 11 | | 1 | 1,287 (10.9%) |
| Pavlodar | Kentubek, Kentubek village | 215 | 71 | 57 | – | – | 3 | 1 | – | 2 | 349 |
| | Koktobe, Koktobe village | 304 | – | 32 | 12 | 1 | 7 | – | 1 | – | 357 |
| | Toraygyr, Toraygyr village | 182 | 53 | – | – | – | 3 | – | – | – | 238 |
| | Aksan, Aksan village | 265 | 32 | – | 7 | – | – | – | – | – | 304 |
| **Total** | | 966 | 156 | 89 | 19 | 1 | 13 | 1 | 1 | 2 | 1,248 (10.5%) |
| **Total** | | 7,531 | 1,941 | 877 | 796 | 29 | 179 | 222 | 120 | 59 | 11,859 |





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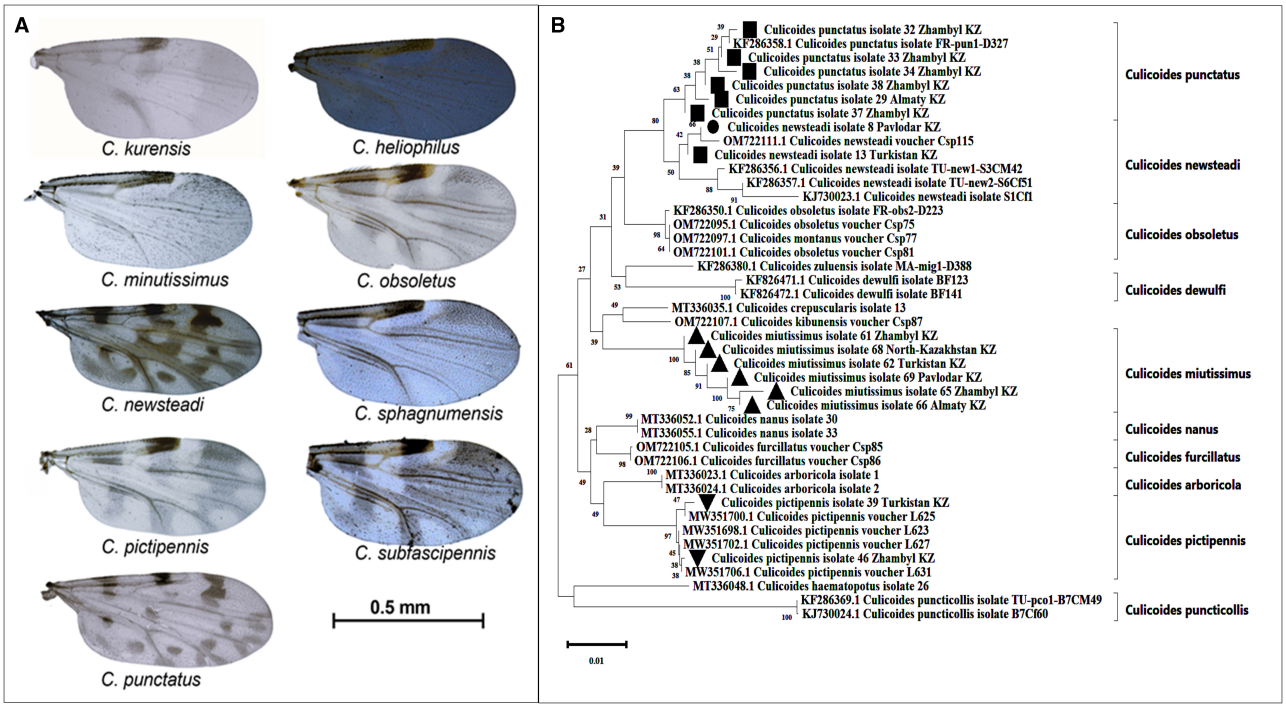


FIGURE 3
Morphological and Genetic Identification of *Culicoides* spp. **(A)**—Wings of *Culicoides* species captured in Kazakhstan. Photographs of the wings were taken using a camera mounted on a stereomicroscope (Ken-A-Vesion, USA) and processed with specialized software, PowerPoint. Scale bar: 0.5 mm. **(B)**—Phylogenetic tree of *Culicoides* species captured in the southern and northern regions of Kazakhstan. Evolutionary relationships of the taxa were analyzed using the Neighbor-Joining method (30). The optimal phylogenetic tree displays the relationships among 43 nucleotide sequences. The reliability of taxa clustering was assessed using a bootstrap test (1,000 iterations), with corresponding percentages indicated next to the branches (31). The branch lengths on the tree are proportional to evolutionary distances, which were calculated using the Tamura-Nei method (32) and expressed as the number of nucleotide substitutions per site. Compositional bias differences among sequences were taken into account in evolutionary comparisons (32).

identified (Figure 3A): *C. Newsteadi, C. Punctatus, C. Pictipennis, C. Minutissimus, C. Heliophilus, C. Kurensis, C. Subfascipennis, C. Sphagnumensis, C. Obsoletus.* Among them, the most dominant species were *C. Newsteadi, C. Punctatus, C. Pictipennis, C. Minutissimus* (Figure 2).

During the molecular identification, it was confirmed that the species *C. newsteadi, C. punctatus, C. pictipennis*, and *C. minutissimus* aligned with their morphological identification (Figure 3B). Five other species (*C. heliophilus, C. kurensis, C. subfascipennis, C. sphagnumensis*, *and C. obsoletus*) could not be confirmed molecularly due to insufficient quality and or quantity of data obtained.

## Prevalence of BTV in *Culicoides* species

Morphological and genetic identification of *Culicoides* species facilitated the creation of 79 pooled samples categorized by species and collection sites. The number of pooled samples collected in each region is presented in Table 3.

During the study, no BTV RNA was detected in *Culicoides* specimens collected from the North Kazakhstan and Pavlodar regions. In the Almaty region, RNA was identified in *C. miutissimus* (Ct 34.5–34.6) and *C. sphagnumennis* (Ct 33.4). In contrast, in the Turkestan region, RNA was detected only in *C. miutissimus* (Ct 31.1–33.5).

In the Zhambyl region, BTV RNA was identified in the following midge species: *C. newsteadi* (Ct 33.1–35.6), *C. pectipennis* (Ct 31.2–35.1), *C. miutissimus* (Ct 32.3–35.6), and *C. sphagnumennis* (Ct 35.4–35.6; see Table 3).

# Discussion

This study provides critical data on the latest seroprevalence of BT and identification of potential BTV vectors in Kazakhstan. In this study, 972 blood and serum samples were collected from sheep, goat and cattle from southern and northern Kazakhstan in Autumn 2023 and Spring 2024. The testing results indicated that seroprevalence and virus distribution were higher in the southern regions compared to the northern regions. It is important to note that sample collection was based on an estimated seroprevalence of ~5%. According to previous studies (15–18), the seroprevalence in the southern regions of Kazakhstan was at least 20%. However, the results of this study confirmed the proposed hypothesis, demonstrating a seroprevalence rate of 5% or higher. The overall seroprevalence of BTV across all animal species was as follows: 4.2% among sheep, 21.8% among goats, and 14.4% among cattle. It is important to note that, despite the trend of

TABLE 3 Detection of BTV RNA in *Culicoides* species.

| Oblast | Species | Number of examined pools[#] | Number of rRT-PCR-positive pools | Ct value range[*] | Potential host-feeders[**] |
|---|---|---|---|---|---|
| North Kazakhstan | *C. Newsteadi* | 4 | 0 | 38.0–39.4 | Cattle, Sheep |
| | *C. Punctatus* | 3 | 0 | 39.4–41.0 | Goat, Cattle |
| | *C. Pectipennis* | 3 | 0 | 38.9–39.7 | Goat |
| | *C. Miutissimus* | 3 | 0 | 39.7–40.1 | Cattle |
| Pavlodar | *C. Newsteadi* | 4 | 0 | 39.7–41.5 | Sheep, Goat, Cattle |
| | *C. Punctatus* | 3 | 0 | 37.1–43.2 | |
| | *C. Pectipennis* | 2 | 0 | 36.9–41.1 | Cattle, Sheep |
| | *C. Miutissimus* | 2 | 0 | 39.1–39.3 | |
| Almaty | *C. Newsteadi* | 2 | 0 | 37.8–39.4 | Cattle, Sheep |
| | *C. Punctatus* | 2 | 0 | 39.1–39.5 | |
| | *C. Pectipennis* | 1 | 0 | 39.7–41.5 | |
| | *C. Miutissimus* | 2 | 2 | 34.5–34.6 | Sheep |
| | *C. Sphagnumennis* | 2 | 1 | 33.4 | |
| Turkestan | *C. Newsteadi* | 3 | 0 | 37.8–39.4 | Goat, Sheep |
| | *C. Punctatus* | 3 | 0 | 39.1–39.5 | Sheep, Goat, Cattle |
| | *C. Pectipennis* | 3 | 0 | 37.8–39.4 | |
| | *C. Miutissimus* | 3 | 2 | 31.1–33.5 | |
| | *C. Kurensis* | 2 | 0 | 38.9–42.4 | |
| Zhambyl | *C. Newstedi* | 7 | 2 | 33.1–35.6 | Goat, Sheep |
| | *C. Punctatus* | 7 | 0 | 37.8–40.1 | Cattle, Sheep |
| | *C. Pectipennis* | 7 | 3 | 31.2–35.1 | Cattle, Sheep |
| | *C. Miutissimus* | 6 | 4 | 32.3–35.6 | Sheep, Goat, Cattle |
| | *C. Sphagnumennis* | 3 | 2 | 35.4–35.6 | Goat, Sheep |
| | *C. Kurensis* | 2 | 0 | 39.0–41.1 | Sheep |
| **Total** | 79 | 16 | – | – | |

(#) Each pool contained between 5 and 50 midges, depending on the total number of captured specimens. (*)—Values up to 35.9 were considered positive, values from 36.0 to 39.9 were considered suspicious, and values of 40.0 and above were considered negative. (**)—The midges were captured from the premises where these animals were housed.

higher seroprevalence in goats and cattle, no statistically significant differences were observed among the animal species ($p > 0.05$). Compared to previous years, the seroprevalence in the southern regions increased across all animal species in 2024 compared to 2023. This increase was particularly notable among goats (from 3.8% in 2023 to 29.5% in 2024) and cattle (from 7.8% to 18.5%). In the northern regions, seroprevalence was zero for all animal species in 2023. However, in 2024, seroprevalence of 10.0% was observed in cattle, indicating the onset of virus circulation in these regions.

To confirm the serological data obtained, rRT-PCR testing was conducted. The rRT-PCR results corroborated the findings from ELISA, demonstrating a significantly higher number of positive BTV RNA samples in the southern regions compared to the northern regions ($p < 0.0001$), highlighting active virus circulation in these areas. It is noteworthy that the number of rRT-PCR-positive samples was considerably higher in 2024 than in 2023. For instance, the proportion of rRT-PCR-positive samples in sheep increased from 7.2% in 2023 to 17.0% in 2024, while in cattle, the increase was from 9.2% to 34.4%. A particularly sharp rise in rRT-PCR-positive samples was observed among goats, where the percentage rose from 4.2% in 2023 to 62.0% in 2024. This growth may be associated with more intense virus circulation among this species or changes in risk factors. Additionally, in some cases, the number of PCR-positive samples exceeded the number of ELISA seropositive samples. This could indicate recent infections, where animals had not yet developed antibodies, or reflect the higher sensitivity of rRT-PCR in detecting active infections.

In this study, sequencing and virus isolation from BTV rRT-PCR-positive samples were not performed due to financial and resource limitations. However, given the importance, detailed characterization of circulating strains will be focused in future studies. The information on the genetic diversity of BTV in Kazakhstan would help to trace the origin and spread of BTV within different ecological and climatic zones within the country.

The presence and the abundance of *Culicoides* midges, influence iBTV transmission, and understanding their regional

diversity is essential for assessing the risk of BTV spread and designing effective control measures.

An ~1,400 species of *Culicoides* midges have been identified globally, with 130 species reported in the former USSR. Among these, *C. obsoletus, C. dewulfi, C. scoticus, C. pulicaris, C. stigma, C. nubeculosus, C. chiopterus*, and *C. punctatus* are widely distributed in Russia, with their range extending up to 72° latitude (26). In Kazakhstan, members of *Culicoides* subgenera *Avaritia, Culicoides, Hofmania, Monoculicoides, Oaecata, Wirthomyia, Silvaticulicoides* (*Beltranmyia* have been reported (26, 33)). According to the literature, the most abundant species in southern Kazakhstan are *C. punctatus, C. newsteadi*, and *C. obsoletus* (34, 35). In central Kazakhstan, *C. obsoletus, C. dewulfi*, and *C. montanus* dominate (36); in northern Kazakhstan, *C. obsoletus, C. punctatus,* and *C. chiopterus* are prevalent (33, 36); while in eastern Kazakhstan, *C. grisescens* and *C. obsoletus* are the most common (33, 36).

According to the literature, *C. imicola* is the primary vector of BTV in Africa, the Middle East, and southern Europe, *C. sonorensis* in North America, and *C. brevitarsis* in Australia. It is worth noting that *C. imicola* is absent from the listed species in Kazakhstan; however, *C. obsoletus* a known BTV vector in European countries, is present in Kazakhstan. In Europe, the main BTV vectors are midges from the *obsoletus* and *pulicaris* complexes (6). *C. pulicaris* was not detected in any region during this study.

Adult females *Culicoides* require blood during their gonotrophic cycle. Therefore, they feed on warm-blooded animals every 3–4 days, staying close to the ground. Different species exhibit distinct host preferences: some are anthropophilic, feeding on humans, others are zoophilic, preferring livestock, or ornithophilic, feeding on birds. Their activity patterns also vary, with some being active predominantly at twilight and nighttime, while others feed during the day, either in open spaces or livestock enclosures. Based on the samples collected in this study, host preferences of the *Culicoides* were; Sheep-−42%, Cattle-−34%, and Goats-−24%.

In this study, *nine Culicoides* spp. were morphologically identified and the identity of four of the species were confirmed by sequencing 28S rDNA. The 28S marker, is commonly employed in examining the evolutionary relationships among insects. Nevertheless, the effectiveness of the primer employed for amplifying this marker was observed to be diminished in relation to some species. This issue may be attributable to the genetic traits of the taxa influencing the primer binding process, the presence of polymorphisms in the amplification regions specific to these species, or the degradation or low quality of the DNA in the initial samples.

For further research, it is planned to revise the approach to primer selection, including the use of alternative sequences that are more specific to the species in question, or to modify the PCR conditions to improve amplification efficiency.

Out of the nine *Culicoides* species four (*C. Miutissimus, C. Newstedi, C. Pectipennis and C. Sphagnumennis*) were identified as potential BTV vectors in Kazakhstan. In southern regions of Kazakhstan, BTV RNA was primarily detected in *C. miutissimus* and *C. sphagnumennis*. These findings align with previous studies indicating active virus circulation in these warm and humid regions (34, 35). These species play a critical role in the epidemiological situation in the southern regions, as the environmental conditions there are favorable for their reproduction and activity (33).

In northern Kazakhstan, specifically in regions such as North Kazakhstan and Pavlodar, viral RNA was not detected in any *Culicoides* spp., likely due to less favorable climatic conditions for the vectors in these areas (18). Climatic factors, such as cold winters and relatively dry summer months, limit their activity and ability to transmit the virus.

As for *C. newstedi* and *C. pectipennis*, they exhibited a high level of BTV infection in the Zhambyl region, strongly suggesting their potential role as vectors (34). Previously, these species have also been reported in Russia and Central Asia as important BTV carriers (1).

Additionally, the literature indicates that *C. imicola* and *C. obsoletus*, though not widely present in Kazakhstan, are primary vectors of BTV in Europe and Africa (6). These species possess significant epidemiological potential in the global spread of the virus, and if they were to be detected in Kazakhstan, their role in the epidemiological situation could become crucial.

Notably, despite the detection of seropositive animals for BT in the northern regions of Kazakhstan, no PCR-positive animals or vectors were found among susceptible livestock (sheep, goats, and cattle). The presence of seropositive animals in could possibly be explained through animal movement of seropositive animals from the southern regions to the northern regions, or lower level of BT infections. In contrast, the BT situation in southern regions was the presence of both seropositive and PCR-positive animals, as well as PCR-positive midges, were identified. In all southern regions, the RNA of BTV was predominantly detected in *C. miutissimus* among PCR-positive *Culicoides* species. However, the prevalence of *C. miutissimus* in the southern regions varied significantly, ranging from 4.3% to 29.1% (see Figure 2).

These findings have significant and multifaceted implications for the epidemiology of the diseases they transmit (37). Analyzing the obtained results, we aimed to assess the current epidemiological situation of BT in the northern and southern regions of Kazakhstan. However, this study has highlighted the need for a more comprehensive analysis taking into account the climatic heterogeneity of the country. For a more accurate assessment of the risk of virus transmission, it is necessary to consider multiple factors, including climate zones, temperature, the presence and density of susceptible animals, age, sex, vector populations, insect biological activity, prevailing wind directions, and other epidemiologically significant parameters (18, 33, 38).

According to previous mathematical modeling studies (18), it has been scientifically established that the spread of Bluetongue virus is most likely in the southern and southeastern regions of Kazakhstan. The results obtained in our study confirm this prediction, demonstrating a high level of seroprevalence in these areas. This pattern may be associated with favorable climatic conditions that facilitate virus circulation and vector activity. A study conducted in Southern Italy (Campania region) demonstrated that moderately mild winters and high seasonal *Culicoides* activity contribute to sustained virus circulation (39). Similarly, a meta-analysis of BTV seroprevalence in

Africa (40) emphasized that the year-round warm climate and high *Culicoides* population density ensure continuous virus transmission among various animal species, including cattle, sheep, goats, and camels. However, unlike these regions, BTV circulation in Kazakhstan is restricted exclusively to the warm season, necessitating the development of region-specific control and prevention measures.

Furthermore, the transboundary spread of BTV remains a significant risk factor. However, data on the epidemiological situation in neighboring countries bordering our study regions, such as Russia, Kyrgyzstan, and Uzbekistan, are extremely limited. Therefore, future research should incorporate an assessment of the epizootic situation in border areas and the influence of climatic factors on virus circulation.

Nevertheless, the data obtained not only confirm the current distribution of vectors but also allow for the prediction of BTV dynamics in the northern and southern regions of Kazakhstan. This, in turn, may contribute to the improvement of preventive and anti-epizootic measures in the future.

## Conclusion

The results of this study highlight the importance of systematic monitoring of *Culicoides* spp. in Kazakhstan. The identification of species such as *C. miutissimus*, *C. sphagnumennis*, *C. newstedi*, and *C. pectipennis* as potential BTV vectors calls for further investigation to develop effective measures to prevent the spread of BTV in the region. The abundance of *Culicoides* species can vary during the vector season. This study was conducted in the Spring and the Autumn. Therefore, in future studies samples collected in summer months might provide information on additional species of *Culicoides* that could serve as BTV vectors. To confirm the vector competence of *Culicoides* species, it is essential to demonstrate not only their ability to acquire the virus from a host but also their capacity to retain and transmit it to a new, uninfected susceptible host. Therefore, detecting the virus in the body of a blood-feeding insect alone is insufficient evidence of its vector competence. Hence, a more precise evaluation requires separate analysis of the insect's head, salivary glands, and body. Additionally, it is crucial to consider climatic and ecological factors that influence vector activity and their ability to transmit the virus. These factors must be integrated into future studies to provide a comprehensive understanding of vector behavior and disease transmission dynamics.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

## Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because According to the Law of the Republic of Kazakhstan No. 97-VII ZRK "On Responsible Treatment of Animals" dated December 30, 2021, Ethical Committee approval is not required when sampling animals for the purpose of infectious disease monitoring.

## Author contributions

KZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. DM: Formal analysis, Methodology, Visualization, Writing – original draft. NS: Formal analysis, Methodology, Writing – original draft. MM: Formal analysis, Methodology, Writing – original draft. SK: Formal analysis, Methodology, Writing – original draft. MA: Formal analysis, Methodology, Writing – original draft. MK: Methodology, Writing – original draft. ST: Methodology, Writing – original draft. MA: Formal analysis, Methodology, Writing – original draft. AM: Formal analysis, Methodology, Writing – original draft. NR: Formal analysis, Methodology, Writing – original draft. KS: Data curation, Methodology, Writing – original draft. SB: Methodology, Validation, Visualization, Writing – review & editing, Conceptualization, Data curation, Formal analysis. AA: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – review & editing. AK: Investigation, Resources, Supervision, Writing – original draft.

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## 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 potential conflicts of interest.

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The author(s) declare that Gen AI was used in the creation of this manuscript. Artificial intelligence was utilized during

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1559636/full#supplementary-material

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# Understanding climate-sensitive tick development and diapause with a structured population model

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**Introduction:** Tick-borne diseases have become a growing public health concern globally. As climate change reshapes the environment, tick populations are expected to expand into previously unsuitable areas, further increasing human exposure to ticks and the pathogens they transmit. Understanding the environmental factors that sustain tick populations is crucial for enhancing prevention and control measures.

**Methods:** This study presents a multi-process structured population model that simulates nymph activity, development, and diapause in response to temperature and photoperiod. By integrating laboratory data and meteorological variables, the model captures the role of photoperiod in regulating diapause and the influence of temperature on development rates.

**Results:** With this model, we propose a mechanism to better understand how short- and long-day conditions synchronize nymph development, highlighting the importance of repeated sensing of external conditions for maintaining behavioral strategies to optimize fitness under changing environmental conditions. The model successfully replicates nymph development observed in laboratory conditions and extends to field applications, predicting seasonal activity under variable weather conditions.

**Discussion:** By providing a mechanistic understanding of tick phenology, our model establishes a foundation for assessing the impacts of climate on tick populations. The insights gained can inform public health tools and strategies, contributing to the mitigation of tick-borne disease risks in a changing environment.

KEYWORDS

***Ixodes scapularis*, nymph, lyme borreliosis, mathematical modeling, approximate Bayesian computation, inverse modeling**

## 1 Introduction

Lyme borreliosis is caused by spirochete bacteria of the *Borrelia burgdorferi* sensu lato complex (1). Ticks play a critical role in the transmission of lyme disease by acquiring the pathogen through feeding on infected reservoir hosts, such as mammals, birds, or reptiles, and transmitting it during subsequent feedings on other hosts, including humans.

Different tick species act as vectors in distinct geographical regions: *Ixodes scapularis* in eastern North America, *Ix. pacificus* in the western United States, *Ix. ricinus* in Europe, and *Ix. persulcatus* in Asia (2). Climate change is expected to significantly affect the

epidemiology of Lyme disease by modifying the environmental conditions necessary for tick survival and reproduction (3–6). Increasing temperatures may enhance habitat suitability by improving survival and accelerating development (7). Meanwhile, changes in land use and host distributions could facilitate range expansions into previously inhospitable areas, thus increasing the potential for human exposure (6, 8). Understanding the life cycle and environmental dependency of ticks is crucial for predicting their potential spread and risk of disease transmission, particularly in the context of climate change.

Hard ticks, including the family of Ixodidae, exhibit a complex life cycle characterized by distinct development stages—egg, larva, nymph, and adult—with larvae, nymphs, and adults taking a single blood meal, resulting in adults laying a large number of eggs (9). Each development stage is influenced by environmental factors such as temperature and humidity (10). Laboratory studies indicate that increasing temperatures accelerate development rates across all life stages; however, this relationship is not linear and is often confounded by diapause—periods of arrested development/activity triggered by environmental cues such as day length and temperature (11). Diapause can be categorized into behavioral diapause, where ticks delay host-seeking activity despite favorable conditions, and developmental diapause, where ticks halt their growth until conditions improve (12, 13). Belozerov and Naumov determined the role of photoperiod in diapause regulation by subjecting non-fed and engorged nymphs to alternating short- and long-day conditions (12). Their research revealed the existence of a complex photoperiodic control mechanism, indicating that prior exposure to short-day photoperiods plays a significant role in synchronizing developmental processes.

Various studies have utilized mechanistic models to explore how abiotic factors influence tick life cycle and population dynamics. For instance, Ogden et al. (14) developed a process-based model that incorporates temperature-dependent development rates for different life stages of *Ix. scapularis*, revealing how cumulative degree days can predict developmental success and survival rates under varying climatic conditions. By using dynamic life tables influenced by a comprehensive set of environmental drivers, Mount et al. (15) and Gaff et al. (16) studied the impact of host densities, habitat types, and weather conditions on *Ix. scapularis* populations and Lyme disease transmission.

Despite the success of these studies, no mathematical model has specifically addressed the mechanisms underlying diapause regulation reported by Belozerov and Naumov (12). Here, we introduce a generic model of climate-sensitive nymphal development and diapause in hard ticks, developed using the dynamically structured population modeling framework of Erguler et al. (17). This framework allows for a realistic representation of physiological processes by incorporating multiple time-dependent reaction rates acting on distinct life stages. By incorporating laboratory-derived data on *Ix. ricinus* and *Ix. scapularis* nymphal development under varying environmental conditions, we explore the interplay between photoperiod, temperature fluctuations, and diapause mechanisms. We expect that our findings will contribute to a more comprehensive understanding of how climate may influence tick phenology, highlight critical areas for future research, and inform strategies for managing tick-borne diseases.

TABLE 1 Environmental factors driving development dynamics as considered in this context.

| Name | Code | Description |
|---|---|---|
| $\phi$ | photo | Hours of day light |
| $\gamma$ | blood | Host availability index |
| T | temp | Mean air temperature (°C) |

## 2 Methods

We employed the dynamically structured population modeling framework (sPop) proposed by Erguler et al. (17) to account for the additive effects of temperature variation and the complex, multi-faceted decision-making processes underlying tick diapause. We defined the model using the PopJSON format (v.1.2.11), a JSON-based representation available at https://github.com/kerguler/PopJSON, and simulated using the Population software package (v.0.1.7), available at https://github.com/kerguler/Population. The environmental factors incorporated are listed in Table 1, and the model parameters, along with their ranges, are provided in Table 2.

### 2.1 Nymph diapause model

We considered three consecutive stages of tick development—larva, nymph, and adult—and focused on the nymph stage, regarding the larva and adult stages as transient compartments flanking the nymph stage. Following development, we transferred nymphs to the adult stage, serving as an indicator of development completion.

We further organized nymphs into six states—resting, questing, engorged, developing, diapausing, and molting—based on activity changes in response to environmental stimuli. The resting and questing stages apply to unfed nymphs, representing either behavioral diapause, where host-seeking and attachment are temporarily suppressed, or active host-seeking behavior (18). In contrast, the developing and diapausing states represent engorged nymphs undergoing active development or developmental/morphogenetic diapause. In this model, the diapausing state also accounts for post-diapause quiescence, with a mechanism allowing for altered photosensitivity (18).

Although several external factors affect tick development and survival, in this foundational model, we focused on the effect of day length (photoperiod) and temperature based on the experimental findings of Ogden et al. (11), Belozerov et al. (12, 19), and Campbell et al. (20). Future work will extend the model to represent the complete life cycle, refine the mechanisms of control, and incorporate additional key drivers, such as saturation deficit and land cover.

#### 2.1.1 Molting as active or resting nymphs

We initiate the model with larvae molting into the nymph stage, producing either resting or questing nymphs. The ratio of resting nymphs relative to the total strongly depends on photoperiod,

TABLE 2 Model parameters and their acceptable ranges.

| Name | Code | Type | Prior (min.) | Prior (max.) | Description |
|---|---|---|---|---|---|
| $\alpha_{CPP}$ | par_CPP | parameter | 12 | 24 | Hours of day light |
| $d_{qSD}$ | par_check_rest | parameter | 1 | 30 | Light sensitivity in questing/resting nymphs (days) |
| $d_{pSD}$ | par_check_diap | parameter | 1 | 120 | Light sensitivity in diapausing/quiescent nymphs (days) |
| $\lambda_g$ | par_engorged_mean | parameter | 1 | 14 | The shortest engorgement time (days) |
| $\lambda_m$ | par_molt | parameter | 1 | 60 | The molting period (days) |
| $\mu_r$ | par_death | parameter | 0 | 1 | Resting and developing state mortality |
| $\mu_q$ | par_quest_death | parameter | 0 | 1 | Questing and engorged state mortality |
| $\mu_d$ | par_diap_death | parameter | 0 | 1 | Diapausing and quiescent state mortality |
| $p_{LD}$ | par_frac_fast_LD | parameter | 0 | 1 | Fraction of unrested nymphs initiating synchronized development |
| $q_{LD}$ | par_frac_diap_LD | parameter | 0 | 1 | Minimum diapausing fraction under long-day conditions |
| g | par_development_mean | parameter | 0.01 | 100 | Scale factor for mean synchronized development time |
| $g_{LD}$ | par_development_mean_LD | parameter | 0.01 | 100 | Scale factor for mean unsynchronized development time |
| $T_1$ | par_development_mean_T1 | parameter | -10 | 40 | Lower functional temperature (°C) |
| $T_2$ | par_development_mean_T2 | parameter | -10 | 40 | Functional temperature range (°C) |
| $c$ | par_development_mean_loga | parameter | -20 | 0 | Briere equation scale factor (log-scale) |

as reported by Belozerov et al. (19). Based on the observed patterns, we modeled the resting ratio, $q_{SD}$, using the following sigmoidal function:

$$q_{SD}(t) = \frac{1}{1 + e^{2\phi(t-1) - 2\alpha_{CPP}}}, \quad (1)$$

where $\alpha_{CPP}$ is the critical photoperiod (CPP, day-length in hours) and $\phi(t)$ is the photoperiod at time $t$.

We assumed that nymphs regularly sense photoperiod and adjust their behavior accordingly (either stay as they are or switch to the questing or resting state). To maintain a given fraction of resting to questing nymphs, the decision is made at the same time and frequency, $d_{qSD}$, in both states.

### 2.1.2 Questing and blood-feeding

Questing nymphs attach to a host and become engorged in about 3–4 days under ideal conditions (21). We assumed that host availability, $\gamma(t)$, is a fraction between 0 (no blood source) and 1 (complete availability and accessibility), and modeled engorgement duration as a function of $\gamma(t)$ using an Erlang distribution (as frequently used in sPop). Accordingly, we assumed that the mean engorgement duration, $f_g$, is inversely proportional to $\gamma(t)$,

$$f_g(b,t) = \begin{cases} \lambda_g/\gamma(t-1) & \text{if } \gamma(t-1) > 0, \\ \infty & \text{if } \gamma(t-1) = 0, \end{cases} \quad (2)$$

where $\lambda_g$ represents the shortest engorgement time under ideal conditions.

Although sPop accommodates a range of standard deviations, setting the value too low, thus the transition too steep, can become computationally demanding. To balance model complexity and computational efficiency, we defined standard deviations as proportional to the means, assigning appropriate proportionality constants based on relevant experimental observations. For engorgement duration, we set the standard deviation, $\sigma_g = 0.2 f_g$, to ensure timely completion of engorgement when $f_g$ is short (21).

### 2.1.3 Interplay of multiple physiological processes

To implement the switch between the resting and questing stages and to keep track of the degree of engorgement and memory of exposure to short-day conditions, we exploited the multi-process feature of the sPop framework. We assumed that each state is linked to a set of processes, each handling a specific task in a defined order. Some of these processes are associated with indicator values, such as the memory of prior exposure to short-day conditions, which further subdivide individuals within the same state into distinct subgroups.

We assumed that the primary objective is survival, governed by a time-invariant daily rate of mortality. Resting and developing states, questing and engorged states, and the diapausing state have daily mortalities of $\mu_r$, $\mu_q$, and $\mu_d$, respectively. When survival is assured, three additional processes determine the fate of a pre-engorgement nymph: photoperiod sensitivity, engorgement indicator, and resting state memory.

### 2.1.4 Accumulating progress across states

Resting and questing nymphs evaluate day length to switch between states at intervals of $d_{qSD}$ (Equation 1). The frequency of these evaluations corresponds to the photoperiod sensitivity (photosensitivity) process.

Questing nymphs proceed to engorgement when a suitable host is available ($\gamma(t) > 0$). During engorgement, nymphs accumulate blood, increasing the value of an engorgement indicator. This

indicator is transferred to the resting state as a memory of engorgement, allowing the process to resume where it left off in case of an interruption, such as premature switch to the resting state caused by detachment and exposure to short-day conditions. Engorgement is allowed to resume from the same point when activity switches back to the questing state and a host is available.

Pre-engorgement nymphs also possess a memory process that categorizes individuals based on whether they have rested. This information is carried into subsequent states as a logical indicator to guide post-engorgement development dynamics. We hypothesized that, once fully engorged, nymphs transition to a temporary state (labeled "engorged") to sense photoperiod at intervals of $d_{qSD}$ days and choose one of four pathways: (i) short-day diapause with a memory of short-day encounter, (ii) long-day diapause without a memory of short-day encounter, (iii) long-day development with a memory of short-day encounter, or (iv) development while retaining their memory of prior short-day encounter.

Essentially, sensing short-day conditions after engorgement forces nymphs into a long-term state of dormancy, referred to as diapause. Under long-day conditions, or if short-day conditions are not sensed, nymphs exhibit a mixed response: some enter diapause (the option of long-day diapause without a memory of short-day encounter), some develop rapidly and synchronously (the option of long-day development with a memory of short-day encounter), and the rest develop at a rate defined by their history of sensing (and remembering) short-day conditions.

### 2.1.5 Engorged nymph development

A certain fraction of engorged nymphs, designated by $q_{SD}$, will sense short-day conditions and enter diapause with a positive short-day encounter memory. The fate of the remaining nymphs depends on prior encounter of the resting state (12).

A fraction of nymphs, denoted by $q_{LD}$, that have neither rested before nor sensed short-day conditions post-engorgement will enter diapause without retaining a memory of prior resting. We assumed that $q_{LD}$ is asymptotically constant, $q_{LD} \sim q_{LD}$, during sufficiently long days. As the photoperiod approaches or falls below $\alpha_{CPP}$, $q_{LD}$ increases, following a mechanism similar to that governing resting/questing decision-making (Equation 1):

$$q_{LD}(t) = q_{LD} + \frac{1 - q_{LD}}{1 + e^{2\phi(t-1) - 2\alpha_{CPP}}}. \quad (3)$$

Of the remaining unrested nymphs, a fraction $p_{LD}$ will initiate development as though they had rested. The rest of the unrested nymphs, along with all the rested nymphs destined to begin development, will initiate development synchronously or asynchronously based on their prior encounter of the resting state.

### 2.1.6 Synchronization of development

One of our key assumptions is that blood feeding triggers a range of physiological responses, resulting in significant variation in development rates. Some nymphs reach the molting state much faster than others, while some take months to complete development. Based on observations by Belozerov et al. (12), we hypothesized that encountering the resting state, i.e., sensing short-day conditions at least once during the nymph stage, synchronizes developmental responses and reduces variability in development time.

We assumed that synchronized development takes an average of $\lambda$ days, with a standard deviation of $\sigma$, modeled using an Erlang distribution. In the absence of resting, the response is a mixture of fast and slow development, resulting in a development time of $\lambda_{LD} \pm \sigma_{LD}$ days. Based on the observed dynamics in Belozerov et al. (12), we set $\sigma = 0.1\lambda$ and $\sigma_{LD} = 0.3\lambda_{LD}$.

### 2.1.7 Temperature-driven development rate

In addition, we assumed that temperature regulates development responses (11, 20) and modeled $\lambda$ using the Briere equation, $B(T)$ (22):

$$\begin{aligned} \lambda &= g/\rho \text{ and } \lambda_{LD} = g_{LD}/\rho, \\ \rho &= \begin{cases} B(T) & \text{if } T(t-1) > T_1 \text{ and } T(t-1) \le T_1 + T_2, \\ 0 & \text{otherwise,} \end{cases} \\ B(T) &= e^c \left[273.15 + T(t-1)\right] \left[T(t-1) - T_1\right] \sqrt{T_1 + T_2 - T(t-1)}, \end{aligned} \quad (4)$$

where $g$, $g_{LD}$, and $c$ are scale parameters, $T_1$ and $T_2$ define the functional temperature range, and $T(t)$ is the average air temperature at time $t$. For practical purposes, we adapted the Briere equation to use Kelvin units and constrained development times between 0 and 365 days (excluding inactive periods).

### 2.1.8 The dynamics of development and quiescence

During active development, nymphs sense environmental conditions—primarily photoperiod—which may trigger the initiation of diapause (19). Based on the observations of Belozerov et al. (12), we incorporated photoperiod as the primary driver of this decision. We assumed that once the decision is made, nymphs either enter long-term dormancy for $d_{pSD}$ days or remain active for the same duration. Similar to the resting or questing decision, we hypothesized that the decision for active and inactive nymphs must be synchronized to achieve the desired fraction of diapausing nymphs relative to the total number of engorged nymphs.

Although there are significant physiological differences between diapause and quiescence (23), we considered light sensitivity as the key factor in distinguishing the two states. Following this first decision of diapause, we assumed heightened light sensitivity, with nymphs sensing photoperiod at intervals of $d_{qSD}$ to switch between active development (represented by the developing state) and responsive quiescence (represented by the diapausing state). We defined an additional memory process for both the developing and diapausing states to indicate the initially low and subsequently high photosensitivity.

During quiescence, a dormant nymph can become active and proceed with development, while an active nymph can become dormant. The trigger depends on complex factors, including photoperiod, temperature, and host availability (23); however, we considered photoperiod as the primary driver in this context. Accordingly, a fraction of nymphs ($q_{SD}$) sense short-day conditions and either remain in or switch to the diapausing state with a positive resting memory. Of the remaining nymphs, those with

a positive resting memory switch to active development, while the others either maintain dormancy or continue development with a negative resting memory. When sufficient progress is made, nymphs proceed to molting, and adults emerge after $\lambda_m$ days.

## 2.2 Parameter inference

To calibrate the model for the closely related species *Ix. ricinus* and *Ix. scapularis*, we used the experimental observations of Campbell et al. (20), Yeh et al. (21), Ogden et al. (11), and Belozerov et al. (12). We determined appropriate values for the model parameters, $\theta$, listed in Table 2, by identifying an optimum parameter configuration through least squares non-linear curve fitting and—to explore parameter uncertainty—sampling a set of alternative configurations around the optimum using approximate Bayesian computation [ABC, (24)]. We refer to these partial posterior samples as the posterior mode, $\Theta$ (17, 25).

To apply ABC, we replaced the likelihood function, with a simulation-based distance function, which we refer to as the score function, $f(\mathrm{d}, y(\theta))$ (26). The score function quantifies the similarity between observations, d, and the model output, $y(\theta)$. The posterior probability, $\Pr(\theta|\mathrm{d})$, is approximated when the score function is used with a sufficiently low threshold:

$$\Pr(\theta|f(\mathrm{d}, y(\theta)) < \epsilon) \to \Pr(\theta|\mathrm{d}) \quad \text{as} \quad \epsilon \to 0. \tag{5}$$

We applied hierarchical Bayesian inference (27) by separating the three parameters related to temperature, $\theta_\alpha \in \{\mathrm{T}_1, \mathrm{T}_2, c\}$, from the remaining parameters, $\theta_\beta$, where $\theta \in \{\theta_\alpha, \theta_\beta\}$. Similarly, we treated the experimental observations from Campbell et al. (20) and Ogden et al. (11) as a distinct dataset, labeled $\mathrm{d}_\alpha$, separate from the photoperiod study of Belozerov et al. (12), labeled $\mathrm{d}_\beta$, where $\mathrm{d} \in \{\mathrm{d}_\alpha, \mathrm{d}_\beta\}$. Consequently, the posterior probability of all parameters is the joint probability of $\theta_\beta$ given $\theta_\alpha$ and $\mathrm{d}_\beta$ and $\theta_\alpha$ given $\mathrm{d}_\alpha$:

$$\Pr(\theta|\mathrm{d}) = \Pr(\theta_\beta, \theta_\alpha|\mathrm{d}_\beta, \mathrm{d}_\alpha) = \Pr(\theta_\beta|\theta_\alpha, \mathrm{d}_\beta)\Pr(\theta_\alpha|\mathrm{d}_\alpha). \tag{6}$$

Following this approach, we first sampled $\theta_\alpha \sim \Pr(\theta_\alpha|\mathrm{d}_\alpha)$, then $\theta_\beta \sim \Pr(\theta_\beta|\theta_\alpha, \mathrm{d}_\beta)$, and in both cases, applied the principles of ABC.

### 2.2.1 Inference for temperature dependence

We assumed a uniform prior for $\theta_\alpha$ to arrive at $\Pr(\theta_\alpha|\mathrm{d}_\alpha) \sim \Pr(\mathrm{d}_\alpha|\theta_\alpha)$, where $\Pr(\mathrm{d}_\alpha|\theta_\alpha)$ is the likelihood function for temperature dependence. By replacing $\Pr(\mathrm{d}_\alpha|\theta_\alpha)$ with a normalized least squares error function,

$$\mathcal{S}_\alpha(\theta) = \sum_T \frac{1}{2}\left(\frac{z_T - y_z(\theta|T)}{\sigma_T}\right)^2, \tag{7}$$

we quantified the simulation-based distance between the observed and simulated development times. In Equation 7, $z_T$ and $\sigma_T$ represent the observed mean and standard deviation of development time at temperature $T$, and $y_z(\theta|T)$ denotes the corresponding development time, simulated using $\theta$ at $T$.

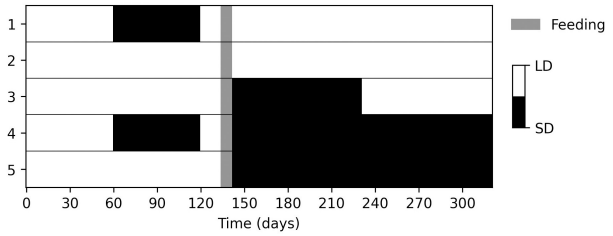


FIGURE 1
The experimental configuration applied to simulate *Ix. scapularis* nymph development under five distinct photoperiod regimens at 20°C. The setup was adapted from that described in Belozerov et al. (12).

### 2.2.2 Inference for photoperiod dependence

We assumed a uniform prior for $\theta_\beta$ to arrive at $\Pr(\theta_\beta|\theta_\alpha, \mathrm{d}_\beta) \sim \Pr(\mathrm{d}_\beta|\theta_\beta, \theta_\alpha)$, where $\Pr(\mathrm{d}_\beta|\theta_\beta, \theta_\alpha)$ is the likelihood function primarily characterizing photoperiod dependence. To replace the likelihood and quantify the development dynamics of engorged nymphs, we used a normalized least squares error function,

$$\mathcal{S}_\beta(\theta) = \sum_v \sum_t \frac{1}{2}\left(v_t - y_{v,t}(\theta|T)\right)^2, \tag{8}$$

where $v_t$ represents the observed number of developed (molting) or adult nymphs at time $t$, and $y_{v,t}(\theta|T)$ denotes the corresponding simulation output at time $t$, obtained using $\theta$ at $T = 20^\circ C$.

To determine the parameters linking photoperiod to development, we closely replicated the experimental conditions described in Belozerov et al. (12). The original study involved maintaining laboratory colonies of *Ix. scapularis* nymphs at 20°C under long-day (LD, 22L:2D) or short-day (SD, 12L:12D) regimens for two months, before feeding under LD conditions. We assumed that feeding occured with perfect fidelity, $\gamma = 1$, during the final week of a three-week LD period. Although not all nymphs in the original study fed to engorgement, we adapted the setup by allowing complete engorgement and focusing on subsequent development. After feeding, engorged nymphs were either kept in the same photoperiod or switched to the opposite regimen. Nymphs kept under LD before feeding and switched to SD after feeding were further split into two groups, one of which was later switched back to LD three months after feeding. Figure 1 provides an overview of these five experimental conditions.

# 3 Results

## 3.1 The impact of temperature on engorged nymph development

We sampled 100 parameter configurations from the posterior distribution $\Pr(\theta_\alpha|\mathrm{d}_\alpha)$ using ABC with the score function $\mathcal{S}_\alpha(\theta)$ and $\epsilon = 200$ (Equation 7). We labeled these samples as the posterior mode $\Theta_\alpha$, and displayed the values in Supplementary Figure S1. The resulting agreement between the model and the observations from Campbell et al. (20) and Ogden et al. (11) is shown in Figure 2.

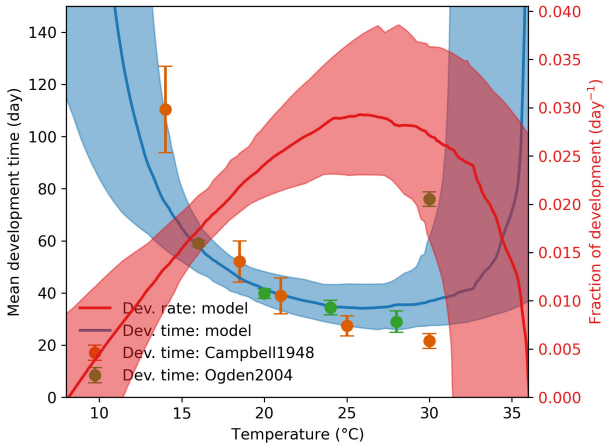


FIGURE 2
Engorged nymph development time and rate. The observed development times are shown as green and orange dots with standard deviations as vertical error bars at various temperatures. The median model prediction and the 90% range are shown as a solid blue line and a blue shade, respectively.

Using the Briere function, we identified both the lower and upper temperature thresholds for development at 10.6 ± 2.0°C and 35.3 ± 2.6°C, respectively, within a 150-day simulation period. The lower threshold aligns with the value reported by Campbell et al. (20), but the development times at high temperatures differ markedly between Campbell et al. (20) and Ogden et al. (11), with the former noting severe pathological impacts of high temperatures. Although our model permits development above 30°C, the associated uncertainty limits definitive conclusions. We note that temperature-dependent survival, unaccounted for in these simulations, will be addressed in future model enhancements.

## 3.2 The impact of photoperiod on nymph development

We applied the five experimental regimens summarized in Figure 1 and simulated the development of 100 larvae by recording the number of apolysed and ecdysed nymphs—referred to as molting nymphs and adults in our model. We sampled 100 parameter configurations from the posterior distribution $\Pr(\theta_\beta|\theta_\alpha, \mathrm{d}_\beta)$ using ABC with the score function $\mathcal{S}_\beta(\theta)$ and $\epsilon = 5,000$ (Equation 8). We labeled these samples as the posterior mode $\Theta_\beta$, and displayed the values in Supplementary Figure S2. As shown in Figure 3, we observed close agreement between our predictions and the photoperiodic control of nymph development observed by Belozerov et al. (12).

We identified the average development time at 20°C as 31.6 days for synchronized nymphs, with a range of 16.0–42.8 days, and 84.8 (47.3–126.3) days for unsynchronised nymphs. The average feeding duration was 2.9 (1.2–5.1) days, while the expected molting period was 27.4 (18.2–35.2) days.

The data did not provide sufficient information on survival rates ($\mu_r$, $\mu_q$, and $\mu_d$) or the photosensitive periods of unfed and engorged nymphs ($\mathrm{d}_{\mathrm{qSD}}$ and $\mathrm{d}_{\mathrm{pSD}}$). The inferred mortality values were below 0.004, suggesting no detectable loss over the course of the experiment. The median photosensitive period was 15.7 days for unfed and post-diapause nymphs, with a range of 4.4–29.6 days, and 46.3 (1.9–94.3) days for diapausing nymphs.

## 3.3 Nymph activity with respect to photoperiod

The median critical photoperiod in the $\Theta_\beta$ sample was 16.6 (13.2–20.4) hours. The fraction of nymphs entering diapause under continuous daylight was 0.14 (0.01–0.35), and the fraction undergoing synchronized development under these conditions was 0.19 (0.00–0.47).

We also replicated the pattern of photoperiod dependence in the development times of engorged nymphs, as described by Belozerov et al. (19) (see Figure 13.7 therein). Although the experiments in Belozerov et al. (19) were conducted with *Ix. ricinus*, a species closely related to *Ix. scapularis*, our model accurately reproduced the observed relationship between average development time and photoperiod (Figure 4).

## 3.4 Nymph activity under variable conditions

The ticks examined by Belozerov et al. (12) originated from a Middle Atlantic population in Beltsville, Maryland, USA. Using the VEClim platform [https://veclim.com, (28)], we extracted the average decadal (2010–2020) temperature and photoperiod from the ERA5 meteorological reanalysis dataset (29) for the grid cell centered at 39.0° latitude and -77.0° longitude, corresponding to the region. We simulated the fate of 100 larvae, introduced at the beginning of the year, over the course of a calendar year under the influence of these conditions.

In Figure 5, we present the number of questing nymphs and emerging adults simulated with $\Theta \in \{\Theta_\beta, \Theta_\alpha\}$. Despite considerable variability due to parameter uncertainty, sustained questing activity with intermittent peaks is evident from March to September. Our model consistently suggests that adult production peaks in mid-June and gradually declines by the end of September.

# 4 Discussion

In this study, we developed a climate-sensitive model of nymph development and diapause in hard ticks using the dynamically structured population modeling framework of Erguler et al. (17). This framework enables the realistic simulation of insect physiology by incorporating multiple processes, with time-dependent durations, while accommodating varying environmental conditions. By integrating laboratory data on nymph development, we investigated the mechanisms through which photoperiod and temperature regulate development and diapause.

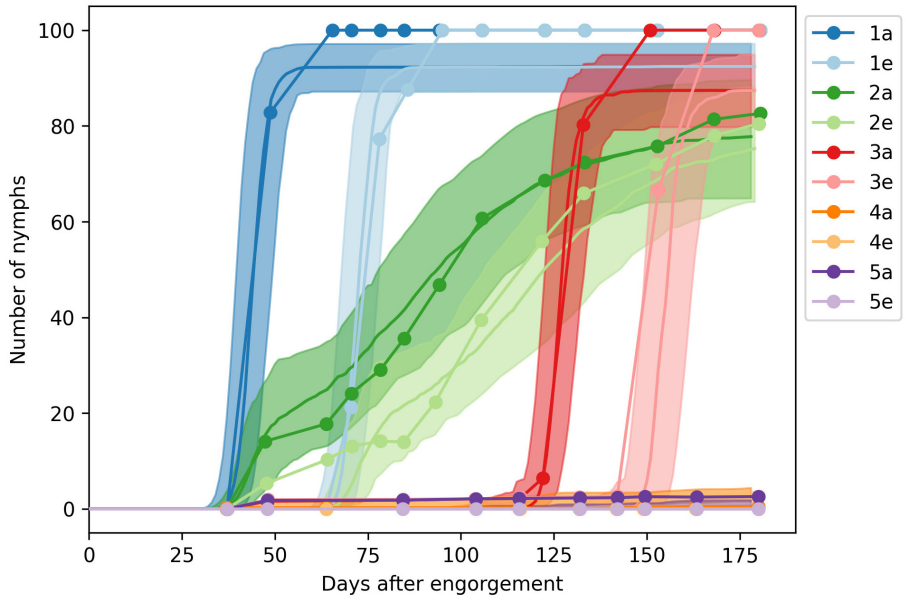


FIGURE 3
Engorged nymph development under different photoperiod regimens. The solid lines and shades show the median and 90% range of model predictions, while the dots represent observations from the laboratory experiments of Belozerov et al. (12).

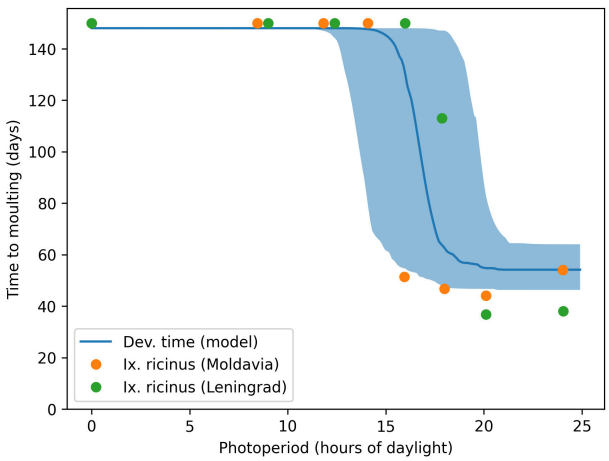


FIGURE 4
Critical photoperiod in engorged nymph development. The solid lines and shades show the median and 90% range of model predictions, while the dots represent observations from the laboratory experiments of Belozerov et al. (19).

While all life stages can transmit pathogens, nymphs are particularly important for increased risk of tick-borne disease transmission in humans due to their small size, high prevalence of infection, and peak activity coinciding with human outdoor activities in late spring and early summer (30). We presented here a foundational model that primarily tracks the nymph stage, highlighting the role of photoperiod, alongside temperature, in driving activity and development.

To investigate the driving mechanisms, we adopted an inverse modeling approach, where a plausible mechanism—formulated as a mathematical model—is evaluated for experimental support, and the insights generated are assessed. Rather than extracting parameter values from the literature, we estimated biologically plausible values from experimental observations.

Our model contributes to the extensive mathematical modeling literature (31, 32) by providing a deeper mechanistic understanding of the processes governing development and diapause control. Our analysis suggests the existence of a population-specific critical photoperiod, regular photoperiod sensing, and probabilistic switching. The model also reproduces synchronized development following prior exposure to short-day conditions. Nymphs emerging under long-day conditions that are able to feed experience slower development, increasing the likelihood of encountering short-day conditions and delaying development until the following season. After exposure to short-day conditions, these nymphs develop rapidly, and may contribute to disease transmission.

In our framework, the decision to forego diapause (or resting) must coincide with the decision to enter diapause, and they both must remain in effect for the same duration. Without this constraint, nymphs initially in diapause would receive multiple opportunities to revert, ultimately skewing the model toward non-diapause outcomes under diapause-inducing photoperiod regimens. By enforcing identical timelines for both resting and active states, the model maintains consistent proportions under steady photoperiod conditions. This analysis aligns with the evolutionary theory of bet-hedging, where the random generation of seemingly disadvantageous phenotypes—such as prolonged dormancy—enhances fitness in response to unpredictable environmental changes (33).

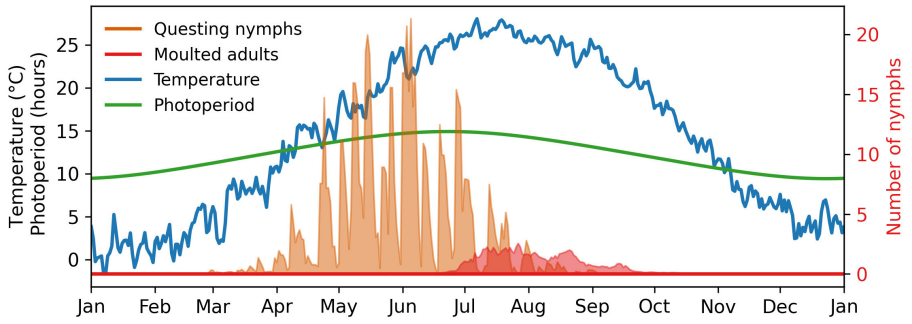


FIGURE 5
Expected nymph development under variable conditions in Beltsville, Maryland, USA. The shades show the 90% range of model predictions.

Our model incorporates significantly more detail on the potential mechanisms of photoperiodic control compared to some of the most advanced modeling approaches. Randolph and Rogers (34) and Ogden et al. (14) assumed specific calendar dates to initiate diapause, which may require revision when applying elsewhere. While Dobson et al. (35) incorporated temperature into diapause control—specifically in behavioral diapause mechanisms—they assumed that diapause begins when day length shortens at the maximal rate. In contrast, Mount et al. (15) and Gaff et al. (16) assumed a range of photoperiods during which immature ticks remain active.

Although our model replicates the dynamics observed in the Belozerov-Naumov study, this may represent just one of many plausible hypotheses. For instance, the synchronous and asynchronous development dynamics could also be reproduced by assuming an existing population structure with varying levels of development completion at the point of nymph stage entry. In this scenario, all nymphs develop at the same rate, but some have partially completed their development by the time they molt into the nymph stage. A key requirement of this hypothesis is that this population structure must also exist in unfed nymphs, as Experiments 1 and 2 are identical post-blood feeding but produce different dynamics. This would imply that some nymphs accumulate a significant portion of their development before receiving a blood meal—an unlikely scenario of autogenous reproduction.

We observed that even under asynchronous development, Experiment 2 in the Belozerov-Naumov study also produced synchronous and delayed development dynamics. Although these patterns could fall within the margin of observational error, we hypothesize that the brief initial peak in the experiment may result from nymphs developing rapidly under long-day conditions. Additionally, we propose that the small peak observed at the end of the experiment may be attributed to nymphs entering diapause without the memory of prior exposure to short-day conditions. However, these nymphs gradually exited diapause and did not contribute significantly to the final peak. Had these nymphs retained the memory of prior resting, their emergence from diapause would have resulted in an abrupt burst of development completion rather than of a small peak. Consequently, the nymphs we modeled as diapausing under long-day conditions can also be assumed dead, contributing no further to population dynamics.

Our analysis revealed that the data did not strongly inform the mortality rates of different nymphal states or distinguish between diapause and post-diapause quiescence. This foundational model will serve as the basis in future work for incorporating further laboratory observations, with a particular focus on assessing the effects of temperature. We also plan to incorporate additional key drivers, such as saturation deficit and land cover. Saturation deficit, a function of temperature and humidity, plays a critical role in questing and blood-feeding success by preventing mortality through dessication (36). Additionally, land cover can create microhabitats that shield ticks from extreme temperature and desiccation, while host abundance and dynamics may introduce variability in feeding opportunities (37). Addressing these complex interactions will be central to future model enhancements.

We applied our model in Beltsville, Maryland, USA, using decadal average temperature and photoperiod data to estimate potential temporal ranges of nymph activity. The predicted activity period aligned with observations (from May to August); however, the model predicted an earlier peak in adult activity compared to the observed peak in October and November (38). This deviation is expected, as our model reflects the total number of adults produced rather than their activity levels, which are critical for detection through standard sampling methods.

Modeling tick dynamics under field conditions requires incorporating climatic factors, host interactions, and disease transmission pathways to predict population trends and mitigate tick-borne disease risks. Our work highlights how temperature influences development and photoperiod governs diapause, providing insights into how climate change may shape tick phenology. By replicating laboratory experiments and extending predictions to field conditions, our approach demonstrates the potential of multi-process structured population modeling to capture complex physiological processes. Our foundational model offers a tool for future research, encouraging further laboratory validation and expanding to include additional environmental drivers that shape tick dynamics and disease spread. As climate change continues to alter environmental conditions, integrating these models into public health planning will be essential for anticipating shifts in disease risk.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

## Author contributions

KE: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Software, Writing – original draft, Writing – review & editing. AS: Conceptualization, Validation, Writing – review & editing. GD: Conceptualization, Validation, Writing – review & editing. LC-D: Conceptualization, Validation, Writing – review & editing.

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## 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.

## Generative AI statement

The author(s) declare that Gen AI was used in the creation of this manuscript. The ChatGPT-4o was used to assist with general language editing and improving the clarity of this manuscript. The AI was employed to refine grammar, enhance readability, and ensure consistency in phrasing, without altering the scientific content or interpretations presented.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1553557/full#supplementary-material

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# Establishment of a multiplex qPCR assay for the detection of pathogens associated with bovine respiratory disease complex

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**Introduction:** The bovine respiratory disease complex poses a significant threat to the cattle industry, necessitating a multifaceted approach to address its occurrence. The syndrome is caused by various pathogens such as bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus type 3 (BPIV3), bovine viral diarrhea virus (BVDV), bovine adenovirus type 3 (BAV3), *Mycoplasma bovis* (Mb), and infectious bovine rhinotracheitis virus (IBRV). The confluence of these pathogens causes substantial economic losses to the cattle industry. Although preventive and control measures have been implemented, containment of bovine respiratory diseases continues to present a formidable challenge, highlighting the need for innovative diagnostic and intervention strategies.

**Methods:** In this study, we designed specific primers targeting six conserved pathogen genes (*N* of BRSV, *M* of BPIV3, *5'UTR* of BVDV, *Hexon* of BAV3, *oppF* of Mb, and *gB* of IBRV). Subsequently, we established a multiplexed fluorescent real-time quantitative PCR (qPCR) assay for simultaneous detection of these pathogens.

**Results:** The developed method exhibited high specificity and sensitivity, with the lowest detection limits for plasmid DNA standards of BRSV, BPIV3, BVDV, BAV3, Mb, and IBRV being 70.1, 40.4, 15.1, 74.4, 69.6, and 4.99 copies/μL, respectively. The coefficients of variation determined by the assay established in this study were <4%, and the amplification efficiency was 93.84%–111.60%, which showed the reliability and stability of the method.

**Discussion:** The detection rates for BRSV, BPIV3, BVDV, BAV3, Mb, and IBRV were 7.59% (17/224), 11.61% (26/224), 8.04% (18/224), 22.32% (50/224), 27.23% (61/224), and 8.04% (18/224), respectively. All 224 cows were cases of natural disease. Fifty-six diseased cattle were infected with a mixture of two or more of the six pathogens at a mixed infection rate of 25% (56/224). Therefore, this study successfully developed a highly efficient, rapid, specific, and sensitive multiplex qPCR method to detect major pathogens associated with bovine respiratory diseases. This advancement is expected to significantly influence the future of the cattle industry and serve as a valuable reference for subsequent research in this field.

KEYWORDS

**bovine respiratory syncytial virus, bovine parainfluenza virus type 3, bovine viral diarrhea virus, bovine adenovirus type 3, *Mycoplasma bovis*, infectious bovine rhinotracheitis virus, multiplex real-time fluorescence quantitative PCR**

# 1 Introduction

The bovine respiratory disease complex (BRDC) is an umbrella term encompassing a spectrum of respiratory ailments in cattle arising from the interplay between various pathogens and environmental factors. The main etiological agents implicated in BRDC include viruses, bacteria, and mycoplasmas (1, 2). In this study, we focused on six key pathogens, namely bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus type 3 (BPIV3), bovine viral diarrhea virus (BVDV), bovine adenovirus type 3 (BAV3), *Mycoplasma bovis* (Mb), and infectious bovine rhinotracheitis virus (IBRV).

BRSV, a member of the family *Paramyxoviridae,* subfamily *Pneumovirinae,* and genus *Pneumovirus* (3), is a single-stranded negative-sense RNA virus. It possesses an enveloped capsid and an unsegmented genome of length approximately 15 kilobases (kb). BRSV was initially isolated from affected cattle in 1970 (4), and in recent years isolations of the pathogen have been reported from China and Turkey (5, 6). Turkish researchers revealed the relationship between calf age, season, and management conditions and BRSV infection rates, and found variations in the G protein of the virulent strains through gene sequence analysis, which may be associated with antigenic changes and provide a basis for regional prevention and control (7). BPIV3 belongs to the family *Paramyxoviridae,* subfamily *Paramyxovirinae,* and genus *Respirovirus,* and is an unsegmented, single-stranded, negative-sense RNA virus, The viral genome is approximately 15 kb in length. BPIV3 often exacerbates the clinical condition of cattle by co-circulating with other pathogens (8, 37). BVDV, classified within the family *Flaviviridae* and genus *Pestivirus*, is a single-stranded, positive-sense RNA virus that possesses an enveloped capsid. BVDV is genetically diverse, with three recognized genotypes: BVDV-1 (Pestivirus A), BVDV-2 (Pestivirus B), and HoBi-like virus (Pestivirus H), each harboring a genome of approximately 12.3 kb (10). BVDV can cause immunosuppression in cattle, predisposing them to secondary infections by other pathogens. In utero infections are particularly concerning, as they can lead to vertical transmission of the pathogen, perpetuating the cycle of infection within the herd (11). Epidemiological studies have shown that seropositivity in animal populations tends to increase with age due to long-term colonization of the virus in the host, suggesting a significant positive correlation between the cycle of infection and the duration of exposure of individuals (12). Abnormal interleukin levels and reduced antioxidant enzyme activity in the serum of cattle naturally infected with BVDV suggest a synergistic role of the inflammatory cascade response and oxidative damage in disease progression (13). BAV3, a member of the family *Adenoviridae* and genus *Mastadenovirus,* is a non-encapsulated, linear, double-stranded DNA virus (14). It is recognized as a significant pathogen in bovine respiratory diseases, with a genome length of 34.4 kb (9). Mb, a prokaryotic pathogen, is the main etiological agent of epidemics characterized by bronchopneumonia, mastitis, and arthritis, and is a major cause of bovine respiratory disease (15, 16). Mb disrupts the mammary epithelial cell barrier and induces a persistent inflammatory response through virulence factors such as adhesion proteins (17), and its genetic diversity (18) may influence differences in pathogenicity of regionally endemic strains. IBRV, a member of the family *Herpesviridae,* subfamily *Alphaherpesvirinae,* and genus Var*icellovirus*, is an enveloped, double-stranded DNA virus with a genome length of approximately 138 kb (19).

Because the clinical symptoms of these pathogens are highly similar, traditional single detection methods such as common PCR and pathogen isolation have limitations such as low efficiency, high cost, and susceptibility to leakage, which make it difficult to meet the needs of rapid clinical diagnosis and precise prevention and control. In recent years, the development of efficient multiplex qPCR detection system has become a research hotspot for the characteristics of BRDC pathogen mixed infection. Rapid advances in molecular biology techniques have provided new tools for pathogen detection and virulence factor analysis. For example, qPCR and loop-mediated isothermal amplification (LAMP)-based methods have significantly improved the sensitivity and specificity of *Mycoplasma bovis* detection (20). Serum biomarkers such as H-FABP and NT-proBNP have been shown to be effective in assessing myocardial injury associated with bovine respiratory disease, providing new ideas for early clinical diagnosis (21). Rapid test reagents for BVDV-specific antibodies (22) enable immediate screening in the field and shorten the diagnostic window period. To cope with the diversity of BRDC pathogens, multiplex real-time PCR technology significantly improves detection efficiency by simultaneously detecting multiple pathogens in a single reaction. Studies have shown that regular monitoring of nasal swab samples from healthy calves can detect low load pathogens before the onset of clinical signs, providing a window period for intervention (23). A 2019–2023 epidemiological survey in Canada revealed that the most common viruses locally were bovine coronavirus and bovine respiratory syncytial virus, and the most common bacteria were *Pasteurella multocida* and *Mannheimia haemolytica*, as revealed by multiplex qPCR analysis (24), which provides data to support vaccine development and control strategy development. Zhang et al. developed a one-step quintuplex qPCR assay capable of detecting five important bovine respiratory pathogens (25). The qPCR technique performed better in terms of both sensitivity and specificity when compared with traditional culture or immunological methods.

In this study, we developed a qPCR assay using specific primers and probes designed for the conserved sequences of six BRDC-associated pathogens. We optimized the reaction system and amplification conditions by grouping three RNA viruses into reaction system 1 for amplification, and two DNA viruses and *Mycoplasma bovis* into reaction system 2 for amplification. This method allows for the simultaneous detection of these six main pathogens, thus simplifying the diagnostic process. Herein, we amplified six pathogens using a dual-reaction system and classified them according to the type of extracted nucleic acid (DNA or RNA). Dividing the six pathogens into two groups reduces the complexity of the reaction system and minimizes competition between primers. Most qPCR instruments support limited fluorescence channels, dual system can be compatible with more conventional instruments, lowering the hardware threshold. The dual-system qPCR achieves a balance between sensitivity, specificity, and equipment compatibility, which is especially suitable for BRDC, a scenario where multiple pathogens need to be detected simultaneously and resources are limited.

# 2 Materials and methods

## 2.1 Primer and probe design

The *N* protein of BRSV demonstrates high evolutionary conservation among viral proteins (26). Comparative phylogenetic analysis reveals

that the *M* gene of BPIV3 exhibits relatively lower resolution compared to its *HN*, *P*, and *N* genes, which may be attributed to the *M* gene's significant sequence conservation (27). In contrast, the *5'-UTR* of BVDV has been established as the preferred target for pathogen identification due to its genetic stability (28). Furthermore, molecular characterization of BAV3 identifies the *hexon* gene as encoding both the major antigenic determinant and one of the most conserved genomic regions (29). The *oppF* gene is one of the key component genes of the oligopeptide permease system and is responsible for encoding ATP-binding proteins. This basal metabolic function makes the gene highly conserved in evolution with a low mutation rate, making it suitable as a stable target for detection. Bashiruddin et al. established a PCR method for the ATP-binding protein *oppD/F* gene as a target gene (30). The *gB*, the main immunoantigen in the viral envelope, is a highly conserved protein and is commonly used as a pathogen diagnostic for IBRV (31).

A comparative analysis was conducted on 12 virulent strain sequences of BRSV, 12 of BPIV3, 20 of BVDV, 6 of BAV3, 15 of Mb, and 10 of IBRV, as recorded in the National Center for Biotechnology Information (NCBI) database. Therefore, specific primers and probes were designed targeting the *N* gene of BRSV, *M* gene of BPIV3, *5'UTR* gene of BVDV, *Hexon* gene of BAV3, *oppF* gene of Mb, and *gB* gene of IBRV (GenBank accession number: MT861050.1, OR597585.1, MN417910.1, JN381195.1, AF130119.1, and JX898220.1, respectively) (Table 1), and analyzed by Primer-BLAST (NCBI) for their specificity. Positions with fewer mutations in multiple sequences were selected for specific primer and probe design using Primer Premier 5 and Oligo 7. All primers and probes were synthesized by Shanghai Sangon Biological (Shanghai, China).

## 2.2 Sample preparation

The collected nasal swabs were thoroughly mixed with 1 mL of phosphate-buffered saline solution and were centrifuged by centrifuge

TABLE 1 Primer and probe information and qPCR reaction systems.

| Reaction system (20 μL) | Target | Gene | Sequence (5′–3′) | Size (bp) | reaction volume (10 μmol/L) | Other components |
|---|---|---|---|---|---|---|
| 1 | BRSV | *N* | TTGTCACCTGTACTACGTTGAAT | 93 | 0.7 μL (0.35 μM) | 2 × Probe Master mix: 10 μL; cDNA template:2 μL; $ddH_2O$:3.3 μL |
| | | | GGCTCTTAGCAAGGTCAAACTA | | 0.7 μL (0.35 μM) | |
| | | | FAM-CTGGTTGACAACAGTTGRTCCTTGTTG-MGB | | 0.1 μL (0.05 μM) | |
| | BPIV3 | *M* | AATAGGTGACCCACCCAAAC | 106 | 0.8 μL (0.4 μM) | |
| | | | CAGATCACTGACACTCCCATATC | | 0.8 μL (0.4 μM) | |
| | | | VIC-TTTGAGATGGAGCGGTCAAAGGACA-MGB | | 0.1 μL (0.05 μM) | |
| | BVDV | *5'UTR* | GTCTAACCGACTGCTACGAATAC | 87 | 0.7 μL (0.35 μM) | |
| | | | GTGCCATGTACAGCAGAGAT | | 0.7 μL (0.35 μM) | |
| | | | CY5-TTTAGTAGCAATACAGTGGGCCTCTGC-MGB | | 0.1 μL (0.05 μM) | |
| 2 | BAV3 | *Hexon* | ACCTACGAGTGGTCCTTTAGA | 111 | 0.8 μL (0.4 μM) | 2 × Probe Master mix: 10 μL; cDNA template: 2 μL; $ddH_2O$: 3.1 μL |
| | | | GTAGAGGTTGACGCTGGTAAT | | 0.8 μL (0.4 μM) | |
| | | | FAM-AGTGCTCTGAAGGATCATGTTTACGTCC-MGB | | 0.1 μL (0.05 μM) | |
| | Mb | *oppF* | GCTGTTGATGGTGTGTCATTT | 93 | 0.7 μL (0.35 μM) | |
| | | | TGAACGTCCAACAGTGGTT | | 0.7 μL (0.35 μM) | |
| | | | VIC-CGGTCTAATTGGTGAGTCAGGAAGTGG-MGB | | 0.1 μL (0.05 μM) | |
| | IBRV | *gB* | TGCTCGACTACAGCGAGATA | 92 | 0.8 μL (0.4 μM) | |
| | | | CATATTGCCGTCCGTCTTGA | | 0.8 μL (0.4 μM) | |
| | | | CY5-CACGAGCTCCGGTTCTACGACATTG-MGB | | 0.1 μL (0.05 μM) | |

at 13,523 g (Eppendorf Ltd., Hamburg, Germany) for 1 min at 4°C. Subsequently, nucleic acids were extracted using a Virus DNA/RNA Extraction Kit 2.0 (Nanjing Vazyme Biotech Co., Ltd., Nanjing, China) with a fully automated nucleic acid extractor (Nanjing Vazyme Biotech Co., Ltd., Nanjing, China), following the RM-401 default program. RNA extracted from nasal swabs was reverse-transcribed using HiScript III All-in-one RT SuperMix (Nanjing Vazyme Biotech Co., Ltd., Nanjing, China). DNA and cDNA were stored at −20°C for further analysis.

## 2.3 Preparation of standard plasmids

The amplification products of the *N* gene (2–325, 324 bp) of BRSV, *M* gene (1–335, 335 bp) of BPIV3, *5'UTR* gene (39–280, 242 bp) of BVDV, *Hexon* gene (1,414–1,748, 335 bp) of BAV3, *oppF* (1,913–2,562, 650 bp) gene of Mb, and *gB* gene (1–650, 650 bp) of IBRV were cloned into the pMD18-T vector [Takara Biotechnology (Dalian) Co., Ltd., Dalian, China]. The recombinant plasmids were then transformed into DH5α competent cells (Guangzhou Xinkailai Biotechnology Co., Ltd., Guangzhou, China). Single-positive colonies were selected under sterile conditions, inoculated into ampicillin-resistant medium, and cultured, and their bacterial lysates were submitted to Shanghai Sangon Biotechnology for sequencing. The correctly sequenced positive plasmids were designated as pMD18-T-BRSV, pMD18-T-BPIV3, pMD18-T-BVDV, pMD18-T-BAV3, pMD18-T-Mb, and pMD18-T-IBRV. Plasmid DNA was extracted from the transformed cells using the EndoFree Mini Plasmid Kit II (Tiangen Biochemical Technology, Beijing, China) and stored at −20°C for subsequent experiments.

## 2.4 Reaction system procedure and optimization

First, we mixed six plasmids of $10^6$ copies/μL in an ep tube and mixed them as our templates. The different primers and probes were aligned and combined for amplification with the following procedure: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s, with fluorescence signal acquisition at 60°C. Determine whether cross-reactivity occurs between different primers and probes.

In this study, we established two PCR reaction systems for the detection of six pathogens. Pathogens with RNA nucleic acids, namely BRSV, BPIV3, and BVDV, were used in PCR reaction system 1. Conversely, pathogens with DNA nucleic acids, including BAV3, Mb, and IBRV, were used in the PCR reaction system 2. The same protocol was followed for both reaction systems.

The reaction systems, with a total volume of 20 μL, comprised six pairs of primers, six probes, sterile double-distilled water, 2 μL of DNA/cDNA template, and 10 μL of the AceQ qPCR Probe Master mix (Nanjing Vazyme Biotech Co., Ltd., Nanjing, China). Amplification and analysis were performed using LightCycler 480 II real-time fluorescent qPCR (Roche Diagnostics (Shanghai) Limited, Shanghai, China).

We used $10^6$ copies/μL of standard plasmids for BRSV, BPIV3, BVDV, BAV3, Mb, and IBRV as templates. The concentrations of primers and probes were optimized using the following thermal cycling conditions: initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s, with fluorescence signal acquisition at 60°C. After determining the optimal concentrations of primers and probes, we evaluated seven annealing temperature gradients ranging from 56 to 62°C to calibrate the annealing temperature.

## 2.5 Establishing standard curves

A 10-fold serial dilution of the standard plasmid was performed, ranging from $10^8$ to $10^0$ copies/μL. Real-time fluorescent quantitative PCR amplification of the diluted standard plasmid DNAs was performed in triplicate for each dilution using the optimized reaction system and protocol. The logarithm of the initial copy number of the standard plasmid DNA is plotted on the X-axis, whereas the cycle threshold (Ct) values obtained from real-time fluorescent quantitative PCR are plotted on the Y-axis. Standard curves were generated using GraphPad Prism 8 (GraphPad Software, California, United States).

## 2.6 Sensitivity test

A 10-fold serial dilution of the standard plasmid, ranging from $10^5$ to $10^0$ copies/μL, was performed. Using an optimized reaction system and protocol, real-time fluorescent qPCR amplification was performed on diluted standard plasmid samples, with sterile double-distilled water serving as a negative control. This process allowed us to determine the minimum detectable copy number for each pathogen. Graphical representations of the data were created using GraphPad Prism 8 (GraphPad Software, California, United States). The reaction was carried out with 2 μL of upstream and downstream primers (no probe required), 10 μL of 2 × Taq PCR Star Mix (Kangrun Chengye Biotechnology (Beijing) Limited, Beijing, China) and 2 μL of plasmid as templates, supplemented with 4 μL of water to a 20 μL system and performed with the following program: 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 56°C, 15 s at 72°C; and 5 min at 72°C. Finally, a comparison of the sensitivity of qPCR and PCR was made.

## 2.7 Specificity test

In this study, we assessed the specificity of a multiplex real-time fluorescent quantitative PCR assay using optimized reaction conditions and systems with DNA/cDNA templates from BRSV, BPIV3, BVDV, BAV3, Mb, IBRV, bovine torovirus (BToV), bovine enterovirus (BEV), bovine norovirus (BNoV), bovine rotavirus (BRV), and bovine ephemeral fever virus. Sterile double-distilled water and laboratory-preserved samples of healthy cattle was used as the negative control. All templates were verified by PCR or viral isolation methods to confirm their identity. The resulting data were graphically represented and analyzed using GraphPad Prism 8 (GraphPad Software, California, United States).

## 2.8 Repeatability test

To assess the reproducibility of the assay developed in this study, we selected $10^7$, $10^6$, and $10^5$ copies/μL, and the plasmid copy number

corresponding to a Ct value of around 30 and the lowest limiting copy number for the assay. Three replicates of both intra-and inter-assay experiments were conducted using these diluted plasmids with the optimized reaction system and protocol. The average Ct values, standard deviations (SD), and coefficients of variation (CV) were calculated. The reproducibility and stability of the assay were subsequently evaluated based on the obtained CV values.

## 2.9 Sample testing

In this study, a total of 224 nasal swabs were collected from cattle with BRDC in Guangdong Province, China. The samples and healthy bovine nasal swabs and serum samples were subjected to nucleic acid extraction and reverse transcription according to the method described in Section 2.2, and then the extracted nucleic acids were used as templates for amplification according to the optimized reaction system and procedure.

## 2.10 Comparative analysis of detection performance

We selected some positive samples tested and randomly selected samples that tested negative in this study for comparison with a standardized qPCR test kit (Shenzhen Anieasy Biotechnology Co., Ltd., Shenzhen, China) for bovine respiratory disease complex as well as a patented primer and probe for BAV3 (patent no. CN200910062095.9).

# 3 Results

## 3.1 Preparation of standard plasmids

Standard plasmids were extracted and their corresponding concentrations were determined using a NanoDrop One ultramicrospectrophotometer (Thermo Fisher Scientific, Shanghai, China). The measured concentrations of BRSV, BPIV3, BVDV, BAV3, Mb and IBRV were 231, 133.5, 483, 246, 254 and 182 ng/μL. The copy numbers were calculated using the following formula:

$$copy\ number\left(copies/\mu L\right)=\frac{Concentration\left(ng/\mu L\right)\times10^{-9}\times6.022\times10^{23}}{DNA length\times660}$$

The copy numbers of standard plasmids for BRSV, BPIV3, BVDV, BAV3, Mb, and IBRV were calculated to be $7.01\times10^{10}$, $4.04\times10^{10}$, $1.51\times10^{11}$, $7.44\times10^{10}$, $6.96\times10^{10}$, and $4.99\times10^{10}$ copies/μL, respectively. Furthermore, the PCR amplification and sequencing results proved that the positive standard plasmid construction was successful (Supplementary Figures 1, 2).

## 3.2 Reaction system procedure and optimization

It has been verified that no cross-reactivity occurs between mismatched primers and probes, they exhibit no amplification (Table 2). In this study, we applied the same reaction procedure and divided it into two reaction systems for real-time fluorescence qPCR amplification according to the different extracted nucleic acids. The optimized reaction systems are listed in Table 1. The optimal annealing temperature for both reaction systems was 61°C.

## 3.3 Standard curve and sensitivity analysis

Standard curves were established based on the amplification results of real-time fluorescence qPCR on positive standard plasmids, ranging from $10^8$ copies/μL to $10^0$ copies/μL (Figure 1; Supplementary Figure 3). The method showed a good amplification efficiency, with a good linear relationship between log starting quantity (X-axis) and Ct (Y-axis), as follows: BRSV expression, Y = −3.148X + 38.60, E = 107.81%, $R^2$ = 0.994; BPIV3 expression, Y = −3.479X + 40.37, E = 93.84%, $R^2$ = 0.995; BVDV expression, Y = −3.198X + 41.66, E = 105.44%, $R^2$ = 0.993; BAV3 expression, Y = −3.336X + 39.62, E = 99.42%, $R^2$ = 0.991; Mb expression, Y = −3.292X + 40.58, E = 101.26%, $R^2$ = 0.988; and IBRV expression, Y = −3.072X + 35.63, E = 111.60%, $R^2$ = 0.991.

The standard positive plasmids of the six pathogens were used as templates for sensitivity tests of the assay established in this study (Supplementary Figure 4). The lowest detectable levels of BRSV, BPIV3, BVDV, BAV3, Mb, and IBRV were 70.1, 40.4, 15.1, 74.4, 69.6, and 4.99 copies/μL, respectively. In contrast, none of the negative controls showed amplification. The detection limits of traditional PCR for BRSV, BPIV3, BVDV, BAV3, Mb, and IBRV were $7.01\times10^3$, $4.04\times10^4$, $1.51\times10^5$, $7.44\times10^3$, $6.96\times10^5$, and $4.99\times10^4$ copies/μL,

TABLE 2 Primer-probe cross-reaction results.

| Probe components | Primer components | | | | | |
|---|---|---|---|---|---|---|
| | BRSV-Primer | BPIV3-Primer | BVDV-Primer | BAV3-Primer | Mb-Primer | IBRV-Primer |
| BRSV-Probe | + | – | – | – | – | – |
| BPIV3-Probe | – | + | – | – | – | – |
| BVDV-Probe | – | – | + | – | – | – |
| BAV3-Probe | – | – | – | + | – | – |
| Mb-Probe | – | – | – | – | + | – |
| IBRV-Probe | – | – | – | – | – | + |

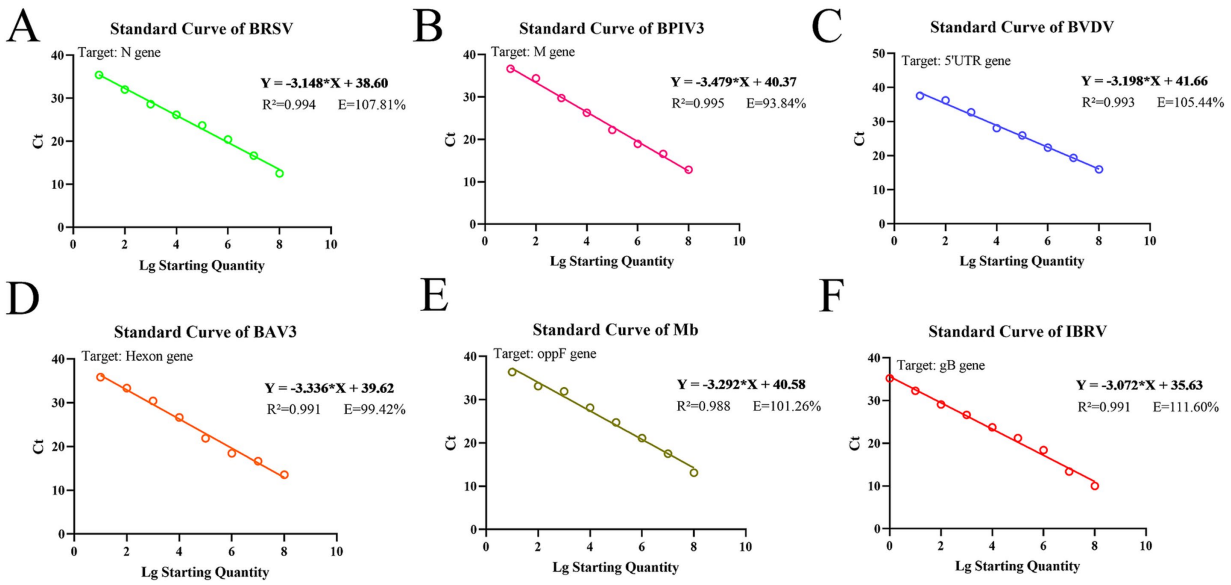


FIGURE 1
Standard curves based on the qPCR amplification of six pathogenic plasmids. **(A)** BRSV; **(B)** BPIV3; **(C)** BVDV; **(D)** BAV3; **(E)** Mb; **(F)** IBRV.

respectively (Table 3; Supplementary Figure 5). Compared to traditional PCR, the sensitivity of our detection method increased 100–10,000 times.

## 3.4 Specificity analysis

Real-time fluorescence qPCR amplification was performed by adding DNA/cDNA from different pathogens to the corresponding reaction system using the same reaction program (Figure 2). The results showed that in reaction system 1, the instrument only detected the amplification and fluorescence signals of BRSV, BPIV3, and BVDV and did not detect the non-specific amplification of other pathogens; in reaction system 2, the instrument only detected the amplification and fluorescence signals of BAV3, Mb, and IBRV, and did not detect the non-specific amplification of other pathogens. No fluorescence signal or amplification was observed in the negative control wells. This indicates that the method has good specificity.

Next, three intra-and inter-assays were performed using different concentrations of the six plasmids (Table 4). Overall, the range of CVs for BRSV, BPIV3, BVDV, BAV3, Mb, and IBRV was 0.38–2.44, 0.15–3.87, 1.22–3.08, 0.05–2.25, 0.33–2.13, and 0.67–3.24%, respectively. All CVs were <4%, indicating that this multiplex real-time fluorescence qPCR method has good repeatability and stability. The CV was calculated using the following formula:

$$coefficient\ variation = \frac{standard\ deviation(SD)}{Mean(MN)} \times 100\%$$

## 3.5 Bovine nasal swab and serum sample testing

In this study, 224 samples from BRDC-affected cattle were tested, resulting in 99 positive samples, including 17 BRSV, 26 BPIV3, 18 BVDV, 50 BAV3, 61 Mb, and 18 IBRV samples. The detection rates were 7.59% (17/224), 11.61% (26/224), 8.04% (18/224), 22.32% (50/224), 27.23% (61/224), and 8.04% (18/224) respectively. The results showed that 56 cattle had mixed infections to varying degrees, with a mixed infection rate of 25% (Figure 3).

## 3.6 Comparative analysis of detection performance

The assay was performed with the same qPCR instrument and samples, using the method established in this study, standard kits, and proprietary primers and probes for BAV3. The results showed that the assay established in this study was consistent with the positive detection rate of the kit and the patented primer and probe, and the compliance rate was 100%.

# 4 Discussion

The etiological diversity and complexity of BRDC impose a substantial economic burden on the cattle industry. This burden manifests itself primarily through decreased productivity of the affected cattle, significant economic losses attributable to mortality, and escalation of risks associated with farming practices (1, 32). Such surveillance is crucial for ensuring the long-term stable economic development of cattle farms. Therefore, the objective of this study was to develop a dual-reaction system qPCR assay that is efficient,

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TABLE 3 Comparison of the sensitivity of qPCR and PCR to positive standard plasmids.

| Plasmid concentration (copies/μL) | BRSV | | BPIV3 | | BVDV | | BAV3 | | Mb | | IBRV | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PCR | qPCR | PCR | qPCR | PCR | qPCR | PCR | qPCR | PCR | qPCR | PCR | qPCR |
| $10^8$ | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^7$ | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^6$ | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^5$ | + | + | + | + | + | + | + | + | + | + | + | + |
| $10^4$ | + | + | + | + | – | + | + | + | – | + | + | + |
| $10^3$ | + | + | – | + | – | + | + | + | – | + | – | + |
| $10^2$ | – | + | – | + | – | + | – | + | – | + | – | + |
| $10^1$ | – | + | – | + | – | + | – | + | – | + | – | + |
| $10^0$ | – | – | – | – | – | – | – | – | – | – | – | + |

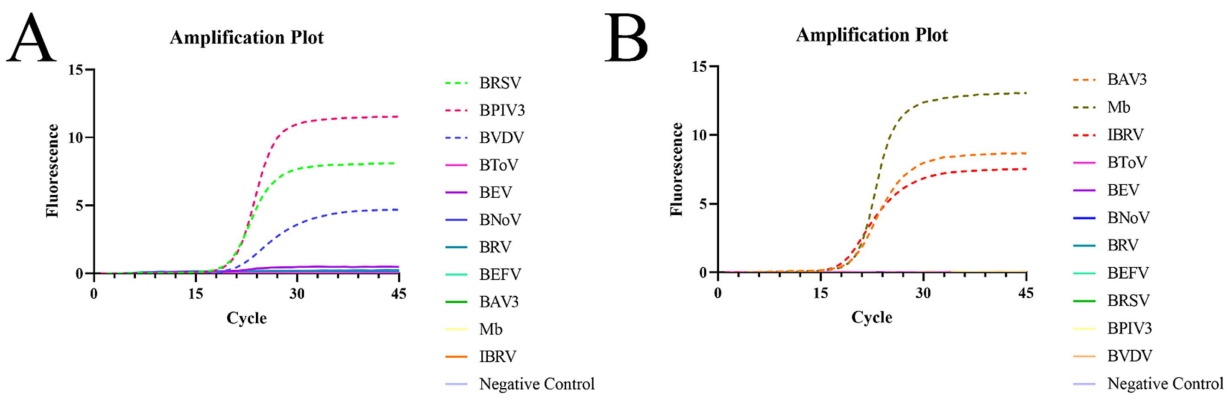


FIGURE 2
Specific amplification plots based on qPCR amplification of the six pathogenic plasmids. **(A)** Reaction system 1. **(B)** Reaction system 2.

sensitive, and robust for the detection of these key pathogens, thus augmenting preventive and control measures for bovine respiratory diseases.

The templates used in the assay developed in this study were all plasmids, and the target sequences inserted in the plasmids were identical to the viral genome sequences in the publicly available database. It has been shown that plasmid standards can be used to establish initial detection limits and linear ranges for qPCR methods. The non-use of real viral samples is a limitation of this study. Therefore, we will supplement with experiments on the detection ability of the assay when virus samples are obtained.

Recently, various assays have been used to detect pathogens associated with BRDC (25, 33–35). However, the present study introduces an innovative multiplexed qPCR assay that can be categorized into two distinct systems based on the type of nucleic acids extracted from pathogens. Specifically, the assay targets three RNA extracted from pathogens (BRSV, BPIV3, and BVDV) in reaction system 1 and three DNA extracted from pathogens (BAV3, Mb, and IBRV) in reaction system 2. This dual-system approach allows simultaneous amplification under identical reaction conditions. The assay established by Kishimoto et al. (33) was able to detect 17 pathogens with very high throughput, but lower amplification efficiencies (< 90%) for some pathogens may have an impact on the results, and the complexity of the primers and probes raises the likelihood of cross-reactivity, with higher demands on the design of primers and probes. Moreover, their assay may be adapted to higher-end equipment. The one-step qPCR assay developed by Zhang et al. (25) efficiently detects both DNA pathogens and RNA pathogens in a single tube. In order to avoid the potential interference of reverse transcriptase with DNA detection, we separated the RNA pathogen and DNA pathogen into two systems, which improved the stability of the assay and enabled the simultaneous detection of six pathogens. The limitation of this study in comparison to them is also that the separate reverse transcription step may increase the time of sample nucleic acid acquisition. Splitting the amplification into two tubes reduces the difficulty of analysis, but slightly increases the number of steps.

Since the qPCR instrument can acquire four fluorescent signals and the instrument fluorescence channels are limited, we chose to use two reaction systems to realize the detection of the six pathogens. The disadvantages are that the dual reaction system requires double reagents, consumables and instrument occupancy time, which leads to an increase in the cost of the experiment, and the need to optimize and validate the experimental conditions of the two systems separately, which increases the risk of human error. The advantage of the dual-reaction system is that it covers a wider spectrum of pathogens through the combined application, while optimizing the reaction conditions to solve the potential problem of primer cross-interference

TABLE 4 Reproducibility of the multiplex real-time fluorescence quantitative PCR method established in this study.

| Target | Plasmid concentration (copies/μL) | Intra-assay | | | Inter-assay | | |
|---|---|---|---|---|---|---|---|
| | | MN | SD | CV (%) | MN | SD | CV (%) |
| BRSV | $10^7$ | 16.58 | 0.11 | 0.66 | 16.77 | 0.41 | 2.44 |
| | $10^6$ | 20.52 | 0.09 | 0.44 | 20.49 | 0.15 | 0.73 |
| | $10^5$ | 23.67 | 0.09 | 0.38 | 23.14 | 0.42 | 1.82 |
| | $10^2$ | 31.92 | 0.08 | 0.25 | 31.73 | 0.33 | 1.04 |
| | $10^1$ | 35.80 | 0.56 | 1.56 | 35.89 | 0.46 | 1.28 |
| BPIV3 | $10^7$ | 16.14 | 0.19 | 1.18 | 16.79 | 0.65 | 3.87 |
| | $10^6$ | 19.68 | 0.03 | 0.15 | 19.45 | 0.22 | 1.13 |
| | $10^5$ | 22.85 | 0.06 | 0.26 | 23.05 | 0.26 | 1.13 |
| | $10^3$ | 30.20 | 0.11 | 0.36 | 30.51 | 0.55 | 1.80 |
| | $10^1$ | 36.36 | 0.38 | 1.05 | 36.68 | 0.67 | 1.83 |
| BVDV | $10^7$ | 18.49 | 0.29 | 1.57 | 18.50 | 0.57 | 3.08 |
| | $10^6$ | 22.08 | 0.27 | 1.22 | 22.08 | 0.36 | 1.63 |
| | $10^5$ | 25.31 | 0.31 | 1.22 | 25.56 | 0.52 | 2.03 |
| | $10^3$ | 31.45 | 0.64 | 2.03 | 31.77 | 0.71 | 2.23 |
| | $10^1$ | 37.47 | 0.82 | 2.19 | 37.84 | 0.67 | 1.77 |
| BAV3 | $10^7$ | 16.03 | 0.36 | 2.25 | 15.89 | 0.22 | 1.38 |
| | $10^6$ | 19.86 | 0.01 | 0.05 | 20.06 | 0.16 | 0.80 |
| | $10^5$ | 22.75 | 0.05 | 0.22 | 23.05 | 0.23 | 1.00 |
| | $10^2$ | 32.84 | 0.12 | 0.37 | 32.70 | 0.56 | 1.71 |
| | $10^1$ | 35.32 | 0.20 | 0.57 | 35.94 | 0.81 | 2.25 |
| Mb | $10^7$ | 16.50 | 0.29 | 1.76 | 16.41 | 0.35 | 2.13 |
| | $10^6$ | 20.32 | 0.14 | 0.69 | 20.58 | 0.12 | 0.58 |
| | $10^5$ | 24.30 | 0.15 | 0.62 | 24.50 | 0.08 | 0.33 |
| | $10^3$ | 30.35 | 0.26 | 0.86 | 30.21 | 0.32 | 1.06 |
| | $10^1$ | 36.57 | 0.25 | 0.68 | 36.86 | 0.32 | 0.87 |
| IBRV | $10^7$ | 12.86 | 0.14 | 1.09 | 13.28 | 0.43 | 3.24 |
| | $10^6$ | 17.94 | 0.17 | 0.95 | 18.06 | 0.28 | 1.55 |
| | $10^5$ | 22.43 | 0.15 | 0.67 | 22.66 | 0.22 | 0.97 |
| | $10^1$ | 31.52 | 0.38 | 1.21 | 31.76 | 0.39 | 1.23 |
| | $10^0$ | 36.15 | 0.41 | 1.13 | 36.48 | 0.38 | 1.04 |

in multiplexed assays. Splitting the six targets into two sets of assays reduces the risk of cross-reactivity between primer and probe components. Using a dual-reaction system qPCR approach, this technique reduces the detection time by at least half an hour compared to conventional methods. This avoids the inefficiencies associated with single pathogen detection, thus improving the efficiency and speed of disease diagnosis.

The proportions of samples positive for BRSV, BPIV3, BVDV, BAV3, Mb, and IBRV detected in this study were 7.59, 11.61, 8.04, 22.32, 27.23, and 8.04%, respectively. The detection of Mb in BRDC cases suggests that it may be widely colonized in cattle through continuous detoxification and vertical transmission. The high prevalence of BAV3 may be related to immunosuppression due to calf weaning stress, and its latent infection properties may exacerbate synergistic pathogenicity with other pathogens. The prevalence of other pathogens suggests that they remain important causative agents of BRDC. In particular, persistently infected individuals with BVDV may be a hidden source of transmission, and screening needs to be strengthened by a strategy that combines antigen detection with antibody surveillance. Moreover, among these positive samples, 56 cases of mixed infections were observed, which might be related to the increased risk of pathogen exposure under high-density feeding pattern, involving different permutations of pathogens, which implies the complexity of BRDC and the diversity of the associated pathogens, and confirms the difficulty of prevention and control of BRDC. In terms of pathogen combinations, Mb and BAV3 had the highest percentage of co-infection, accounting for 42.9% (24/56) of the mixed infections, suggesting a significant propensity for

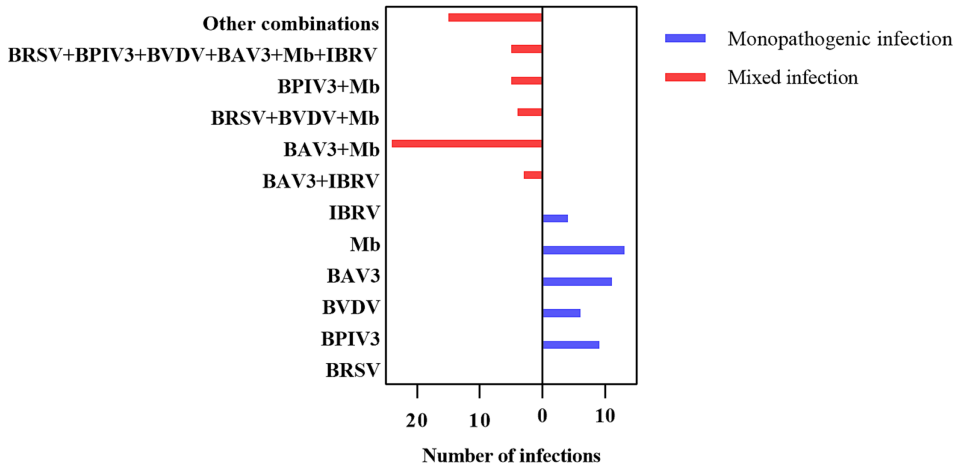


FIGURE 3
Number of single and mixed infections.

synergistic infection. Extreme mixed infections in which all six pathogens were infected (5/56) occurred in pastures with incomplete immunization and intensive cattle farming, suggesting that immunosuppression by pathogens such as BVDV and BAV3 is a central factor in multiple infections. The total detection rate of these pathogens is low, suggesting that some diseased cattle may have been infected by bacteria such as *Pasteurella multocida* and *Mannheimia haemolytica* (36). In response to these cases, medication and control of environmental humidity can reduce *Mycoplasma* infections as well as aerosol transmission of other pathogens. Severe cases can be urgently removed by quarantine and herd immunization of susceptible cattle in the herd.

In this study, based on qPCR detection of samples from Guangdong Province, the mixed infection pattern of BRDC was preliminarily revealed, but there were regional limitations. The hot and humid climate of Guangdong may contribute to the enhancement of BAV3 and Mb adaptation, and there may be differences in the pathogen combinations in other dry regions; Guangdong has a high proportion of intensive farming, and in the future, we can also compare the characteristics of mixed infections in provinces with predominantly free-range pastures, such as Inner Mongolia. This method is suitable for complex cases that require simultaneous screening for mixed *Mycoplasma bovis* and viral infections, and clinically allows for rapid screening of key targets, which can show a large advantage for medium-sized assays. In the future, experiments will be conducted with viral strains, so that the method can be closer to the clinic and more intuitively reflect the efficiency of the detection of real clinical strains.

## 5 Conclusion

In this study, we have developed a highly efficient, sensitive, and specific qPCR detection method that showed remarkable stability and capability to simultaneously identify six key bovine respiratory pathogens (BRSV, BPIV3, BVDV, BAV3, Mb, and IBRV). In particular, Mb and BAV3 showed the higher detection frequency among all the investigated pathogens. Our findings revealed 56 cases of mixed infections among the positive samples, highlighting the varying prevalence of each pathogen. Based on these test results, drug treatment and immunization programs should be rationalized to cope with the complex pathogen situation of BRDC and to reduce the economic losses caused by BRDC to farmers.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

## Ethics statement

The study was approved by the Ethics Committee for Animal Clinical Research of South China Agricultural University (protocol code 2020B108). The study was conducted in accordance with local legal and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

LL: Writing – original draft, Writing – review & editing. QJ: Writing – original draft, Software. SiL: Writing – original draft, Data curation. XL: Writing – original draft, Supervision. SS: Writing – original draft, Validation. XW: Writing – review & editing. CS: Writing – review & editing. KJ: Funding acquisition, Writing – review

& editing. ShL: Writing – review & editing, Funding acquisition, Resources.

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## 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.

## Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1594488/full#supplementary-material

SUPPLEMENTARY FIGURE 1
PCR results of standard plasmids from the six pathogens.

SUPPLEMENTARY FIGURE 2
Plasmid sequence alignment results of six pathogens.

SUPPLEMENTARY FIGURE 3
Amplification plots based on the qPCR amplification of six pathogenic plasmids.

SUPPLEMENTARY FIGURE 4
Amplification plots showing the sensitivity of the qPCR amplification of the six pathogenic plasmids.

SUPPLEMENTARY FIGURE 5
PCR sensitivity of the six pathogenic plasmids.

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# Outbreak of recombinant lumpy skin disease virus in yaks: high mortality and systemic pathogenesis in Qinghai-Tibet Plateau yak herds

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The emergence of recombinant *Lumpy Skin Disease Virus* (LSDV) strains in Asia has led to outbreaks marked by severe skin nodules, high transmissibility, and transboundary spread, resulting in significant economic losses to cattle industries in China and neighboring countries. The Qinghai-Tibet Plateau, historically a natural barrier against viral incursions, has recently experienced increasing LSDV cases in yaks (*Bos grunniens*). Current study elucidates the threat posed by recombinant LSDV strains to yaks through clinical, pathological, and molecular analyses. Field observations revealed infected yaks exhibited fever, dyspnea, cutaneous pox lesions, lymphadenopathy, and mucosal lesions. Viral DNA detection showed 100% positivity in skin samples (6/6), 53.33% (8/15) in nasal swabs, and 33.33% (5/15) in anal swabs, with an overall mortality rate of 46.67% (7/15). Necropsy identified respiratory and digestive system lesions, including tracheal congestion, pulmonary hemorrhagic plaques, and ruminal serosal hemorrhagic masses. Histopathology demonstrated dermal vasculitis, lymphocytic infiltration, and viral inclusion bodies. Immunohistochemistry localized viral antigens to hair follicle epithelia and macrophages. Phylogenetic analysis positioned the yak-derived LSDV strain (LSDV/China/GS/Yak) within the Cluster 1.2 recombinant subclade with high homology to recombinant strains circulating in East/Southeast Asia but differing from non-recombinant Indian Cluster 1.2 strains. The results emphasize increased pathogenicity of recombinant LSDV in plateau yaks and convey the critical need for region-specific control strategies.

KEYWORDS

**LSDV, recombinant strain, Qinghai-Tibet Plateau, yak, prevention and control**

# 1 Introduction

Lumpy skin disease (LSD) is a transboundary infectious disease caused by the lumpy skin disease virus (LSDV), primarily transmitted by insect vectors. It affects cattle including Water buffalo (*Bubalus bubalis*), giraffes (*Giraffa camelopardalis*), impalas (*Aepyceros melampus*), Arabian oryx (*Oryx leucoryx*), and Indian gazelles (*Gazella bennettii*), among other species (1–6). In cattle, LSD is characterized by cutaneous nodules, fever, and systemic infection, leading to reduced milk production, compromised reproductive efficacy in bulls, and economic losses in beef cattle (7, 8).

LSDV is a member of the genus *Capripoxvirus* within the subfamily chordopoxvirinae (family poxviridae), along with the sheeppox virus (SPPV) and goatpox virus (GTPV) (9, 10). It is an enveloped, double-stranded DNA virus with a 151 kb genome, sharing over 95% nucleotide homology with SPPV and GTPV, and exhibiting cross-immunogenicity among *capripoxviruses* (11). Current commercial vaccines against LSDV include homologous vaccines (attenuated Neethling field strains) and heterologous vaccines (live-attenuated GTPV and SPPV) (1). Its vaccination is very effective and provides potent protection from specific pathogens, but noteworthy is that mild "Neethling-like" signs can appear after immunization. While generally benign and harmless, there is valid reason to have reservations concerning the safety profile of live-attenuated immunization overall. It is therefore essential in this context to closely monitor the situation, followed by thorough investigation to improve immunization strategies in the future (1, 12).

Historically, LSD was endemic to sub-Saharan Africa until the 1990s (12, 13). The first African outbreaks outside the continent were reported in the Middle East in 1989, followed by sporadic cases (14–16). By 2014, LSD had spread to Balkan countries (17–19). In 2017, a vaccine-escape recombinant LSDV strain—derived from genetic recombination between Neethling-like vaccine strains and field isolates—was identified in Russia near the Kazakhstan border (20). Over the past decade, LSDV has expanded into Europe and Asia, now circulating in East, South, and Southeast Asia (21, 22). Notably, two distinct LSDV lineages co-circulate in Asia: (1) recombinant vaccine-like strains (e.g., in China, Mongolia, Thailand, and Vietnam) and (2) classical field strains (e.g., in Bangladesh, India, Nepal, and Myanmar) (18, 23–29). This dual viral pressure highlights the ongoing threat to Asian cattle populations.

Yaks (*Bos grunniens*), a cold-adapted ruminant species with a global population exceeding 20 million, are predominantly (>95%) distributed across the high-altitude (3,000–5,000 m), hypoxic regions of China's Qinghai-Tibet Plateau (30, 31). Dubbed the "ship of the plateau," yaks serve as a critical livestock resource, providing meat, hides, and dairy products for local communities (32). LSDV outbreaks have inflicted severe economic losses on global cattle industries (8). Given the harsh environmental conditions and limited veterinary infrastructure in yak habitats, the introduction of LSDV presents a catastrophic risk to yak farming on the plateau. The correspondence of heightened susceptibility among yak populations and the high transmissibility of recombinant LSDV strains requires in-depth understanding of viral pathogenesis in this novel host species. This study investigates the clinicopathological features and molecular epidemiology of LSDV infections in Qinghai-Tibet Plateau yaks, underscoring the escalating threat to high-altitude ecosystems and the urgent need for cross-border surveillance. Longitudinal research involving next-generation molecular tools, including whole-genome sequencing and phylogenetic analysis, is essential to track viral evolution and the development of new recombinant strains to implement preventive disease management and control strategies in this vulnerable ecosystem.

# 2 Materials and methods

## 2.1 Sample collection and viral DNA extraction

In 2023, samples were collected from 15 yaks (*Bos grunniens*) exhibiting cutaneous nodules or pox-like lesions at a farm in Gannan Tibetan Autonomous Prefecture, China, suspected of being infected with LSDV. Samples included blood, nasal and anal swabs (15 from each animal), and 6 skin tissues. Nasal and anal swabs were immediately placed in 800 μL phosphate-buffered saline (PBS), while blood specimens were permitted to stay at room temperature for 2 h to obtain serum and were subsequently centrifuged at 6,000 rpm for 5 min to discard coagulated red cells. Specimens were shipped to the laboratory under cold chain conditions to −20°C and were preserved at −80°C until further handling.

Approximately 1 g of skin tissue was homogenized in 1 mL PBS using an Automatic Sample Fast Grinder (Jingxin, Shanghai, China). The homogenate, along with other samples, was centrifuged at 9,000 rpm for 3 min at 4°C. Viral DNA was extracted from 400 μL of each sample using the VAMNE Magnetic Pathogen DNA/RNA Kit (Prepackaged) (Nanjing Vazyme, Nanjing, China) following the manufacturer's protocol.

## 2.2 qPCR detection of LSDV DNA

LSDV DNA was detected using quantitative polymerase chain reaction (qPCR) with primers targeting the GPCR gene (19, 33), modified as follows: Forward primer: 5′-agtcgaatataaagtaatcagtc-3′, Reverse primer: 5′-ccgcata-taatacaacttattatag-3′. Each 20 μL reaction contained: 10 μL Taq Pro Universal SYBR qPCR Master Mix (Nanjing Vazyme, Nanjing, China), 0.4 μL each of forward and reverse primers (10 μM), 2 μL template DNA, 7.2 μL distilled $H_2O$. Thermal cycling conditions: 95°C for 30 s (pre-denaturation), 40 cycles of 95°C for 5 s and 60°C for 30 S. Melt curve analysis was performed using the default settings. All reactions were performed on a LightCycler® 96 Instrument (Roche Diagnostics, Basel, Switzerland) Melt curve analysis was performed using the default settings.

## 2.3 Histopathology and immunohistochemistry analysis

Fresh nodule tissues were fixed in 10% neutral buffered formalin, washed with PBS, dehydrated through graded ethanol (70, 95, 100%), cleared in xylene, embedded in paraffin, and sectioned at 3 μm. Sections were stained with hematoxylin and eosin (H&E) for histopathological analysis. For immunohistochemistry (IHC), adjacent sections were incubated

with mouse anti-LSDV126 protein monoclonal antibody (generously provided by Prof. Yuefeng Sun, Lanzhou Veterinary Research Institute) following established protocols (34). Slides were scanned using an automated slide scanner (3DHistech, Budapest, Hungary) and visualized with SlideViewer v2.7.[1]

## 2.4 Whole-genome sequencing

The whole genome sequencing was performed on the Illumina NovaSeq 6,000 PE150 platform. Raw reads were quality-controlled using Trimmomatic v0.39 (35), and clean reads were mapped to the LSDV reference genome (China/GD01/2020; GenBank: MW355944.1) using Bowtie2 v2.5.2 (36). Contigs were assembled using MEGAHIT v1.2.9 (37) and further polished with SeqMan module in DNASTAR Lasergene v11[2] to generate complete genomes. We have submitted the genomic sequences to the GenBank database, GenBank No. MW355944.1.

## 2.5 Phylogenetic analysis

Full-genome, GPCR, and RPO30 sequences of LSDV, sheeppox virus (SPPV), and *goatpox virus* (GTPV) were retrieved from the NCBI database.[3] Sequence alignment was performed using MAFFT v7.505 (38) with the FFT-NS-1 (fast) strategy and default alignment parameters within the PhyloSuite v1.2.3 software package (39). The best substitution model was selected via ModelFinder v2.2.0 (BIC criterion) (40). Maximum likelihood trees were constructed using IQ-TREE v2.2.0 (1,000 bootstrap replicates) (41) and visualized using Chiplot[4] (42).

# 3 Results

## 3.1 Field observations

Observed clinical signs of LSDV infection of yaks were tachypnea, haemorrhagic diarrhea, fever, and skin lesions of characteristic pox-like appearance. The lesions were most prominent on hairless or lightly covered skin surfaces, i.e., nasal planum, ventral cervical region, perineum, and the inguinal region (Figures 1A–C). Initial lesions manifested as erythematous skin patches (Figure 1A), progressing to raised nodules (Figure 1B), and eventually forming scabs that shed, leaving scars (Figure 1C). Enlarged, firm superficial cervical lymph nodes (lymphadenopathy) were observed in some yaks (Figure 1D). Soft periocular swelling (Figure 1E), hoof lesions, gingival mucosal erythema, and conjunctival hyperemia were noted in individual cases (Figures 1F–H).

## 3.2 Viral detection and necropsy findings

Samples were collected from 15 yaks displaying typical LSDV symptoms. qPCR detected LSDV DNA in 53.33% (8/15) of nasal swabs, 33.33% (5/15) of anal swabs, and 100% (6/6) of skin tissues. Seven yaks with severe clinical signs died, resulting in a mortality rate of 46.67% (7/15) while the morbidity rate was recorded as 100% (15/15).

Postmortem examination revealed lesions predominantly in the respiratory and digestive systems. The trachea exhibited congestion, hemorrhage, and excessive mucus (Figure 2A). The lungs displayed patchy hemorrhagic foci protruding from the serosal surface (Figure 2B). The rumen serosa showed multifocal hemorrhagic nodules resembling pox lesions (Figure 2C). Hemorrhage was also observed in the jejunal serosa, mirroring ruminal findings (Figure 2D).

## 3.3 Histopathology and immunohistochemistry

H&E staining identified epidermal-dermal edema with disrupted rete ridge architecture (Figure 3A). Severe vasculitis in the dermis featured perivascular lymphocyte infiltration, vascular occlusion, and eosinophilic viral inclusion bodies in affected keratinocytes (Figure 3B). Mid-dermal layers showed extensive lymphocytic and erythrocyte infiltration (Figure 3C). Deep dermal regions exhibited marked arteriolar vasculitis, endothelial necrosis, and luminal obstruction (Figure 3D).

Immunohistochemistry using anti-LSDV126 antibody highlighted viral antigen (brown staining) in hair follicle epithelial cells (Figure 4A), deep dermal macrophages (Figure 4B), and perivascular lymphocytes (Figure 4C).

## 3.4 Phylogenetic analysis

Maximum likelihood phylogenetic trees constructed from full-genome sequences of LSDV, SPPV, and GTPV (NCBI database) revealed three major LSDV clades: Cluster 1.1, Cluster 1.2, and Cluster 1.2 recombinant (Figure 5). Cluster 1.1 included strains from South Africa (1954–2016) and Balkan countries. Cluster 1.2 comprised strains from Africa, the Balkans, the Middle East, Eastern Europe, and South/Central Asia (1958–2023). Cluster 1.2 recombinant subclade contained strains from East/Southeast Asia and Asian Russia (2017–2023). The yak-derived LSDV strain in this study (LSDV/China/GS/Yak, GB No. MW355944.1) clustered within the Cluster 1.2 recombinant subclade, consistent with Chinese, Thai, and Russian recombinant strains (19).

In contrast, Indian yak LSDV strains formed a distinct Cluster 1.2 non-recombinant branch (43). GPCR and RPO30 gene-based phylogenies corroborated these findings, with LSDV/China/GS/Yak grouping within the recombinant subclade (Figure 6).

# 4 Discussion

Recombinant LSDV infections in yaks have been reported in China's Sichuan Province and India's Himachal Pradesh near the

1 https://www.3dhistech.com/research/software-downloads/
2 https://www.dnastar.com/software/lasergene/
3 http://www.ncbi.nlm.nih.gov
4 https://www.chiplot.online/tvbot.html; accessed on December 1, 2024.


FIGURE 1
Clinical manifestations of LSDV infection in yaks. **(A–C)** Cutaneous pox nodules; **(D)** Enlarged superficial cervical lymph nodes; **(E)** Periorbital skin edema; **(F–H)** Pox lesions on hooves, gingival swelling, and conjunctival hyperemia.

Qinghai-Tibet Plateau (19, 43). Our study demonstrates active LSDV transmission among yaks in this region, supported by severe clinical manifestations (fever, hemorrhagic skin nodules, lymphadenopathy) and a 100% viral DNA detection rate in skin lesions. The high viral load in skin samples suggests potential environmental persistence via scab shedding critical concern given yaks' year-round outdoor grazing practices (44). Concurrent respiratory and gastrointestinal lesions (tracheal hemorrhage, pulmonary necrosis, ruminal hemorrhagic plaques) indicate systemic viral spread, likely exacerbated by high-altitude hypoxia, contributing to the observed 46.67% mortality. Of particular concern is the adaptation of the virus to cold climates (up to 3,600 m elevation in this study), raising alarms about its potential invasion into ecologically fragile core yak habitats, which host over 90% of the world's 16 million yaks (45, 46). Traditional migratory grazing and limited veterinary infrastructure may further accelerate silent transmission.

Phylogenetic analysis revealed a striking geographic dichotomy in LSDV evolution. Classical non-recombinant Cluster 1.2 strains dominate in Bangladesh (26), India (27), Nepal (28), and Myanmar (29), whereas the yak-derived strain in this study (LSDV/China/GS/Yak) clusters within the emerging recombinant Cluster 1.2 sub-lineage (Neethling vaccine-like recombinants) circulating in China, Russia, and Thailand since 2017. The dominance of this recombinant lineage across East Asia suggests independent evolutionary trajectories or differential vaccine pressures. Notably, GPCR/RPO30 gene homology with strains from Russia (47) and Mongolia (24) implies undocumented cross-border transmission via livestock movement along the Silk Road Economic Belt. The rapid replacement of ancestral strains by recombinant strains within approximately 6 years highlights the need to investigate potential adaptive advantages, such as immune evasion or changes in vector competence.

Histopathological findings underscore the heightened pathogenicity of recombinant Cluster 1.2 LSDV in yaks. Severe vasculitis with endothelial necrosis and trans-mural lymphocyte infiltration mirrors poxvirus-endothelial interactions in human smallpox, raising questions about potential zoonotic barriers (48, 49). Viral tropism for hair follicle epithelium may enhance environmental persistence via keratinocyte shedding—a key adaptation for survival in high-UV plateau environments.

While LSD morbidity typically ranges from 3 to 85% (rarely reaching 100%) with mortality below 10% (4, 8, 21), Classical Cluster 1.2 strains in India caused mild, non-fatal infections in yaks (43). In contrast, our study and prior work (19) report 46.67–53.33% mortality in yaks infected with recombinant Cluster 1.2 LSDV, highlighting heightened susceptibility to emerging recombinants. Given yaks' grazing patterns and limited veterinary resources, unchecked spread of recombinant strains across the Qinghai-Tibet Plateau could precipitate catastrophic losses.

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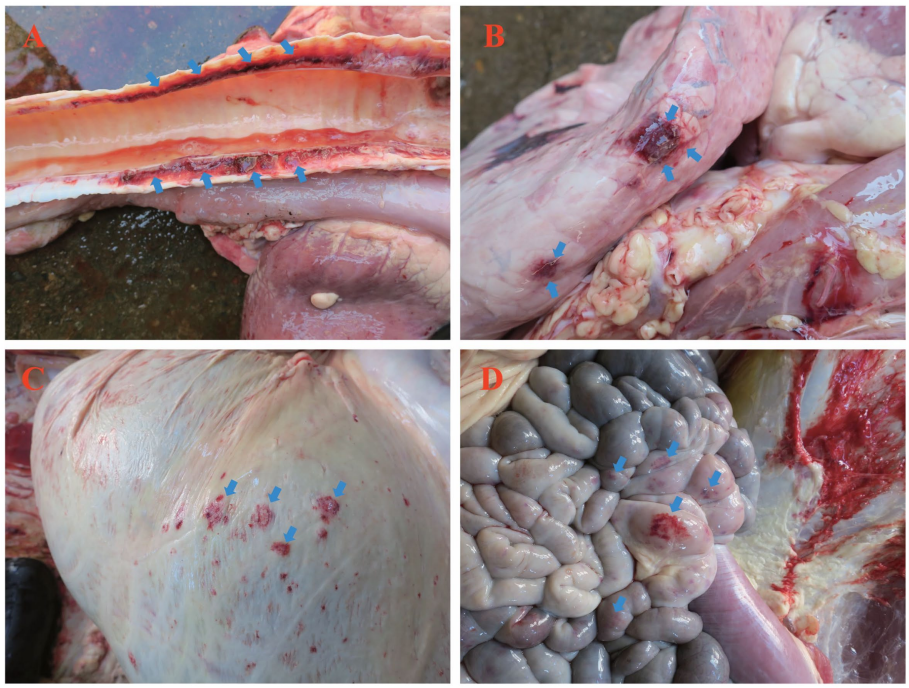


FIGURE 2
Postmortem findings in yaks, fatally infected with LSDV. **(A)** Hemorrhage in tracheal longitudinal sections; **(B)** Near-circular hemorrhagic plaques on the pulmonary pleura; **(C)** Pinpoint or patchy hemorrhage on the ruminal serosa; **(D)** Hemorrhage in the jejunal serosa. Blue arrows indicate hemorrhagic foci.

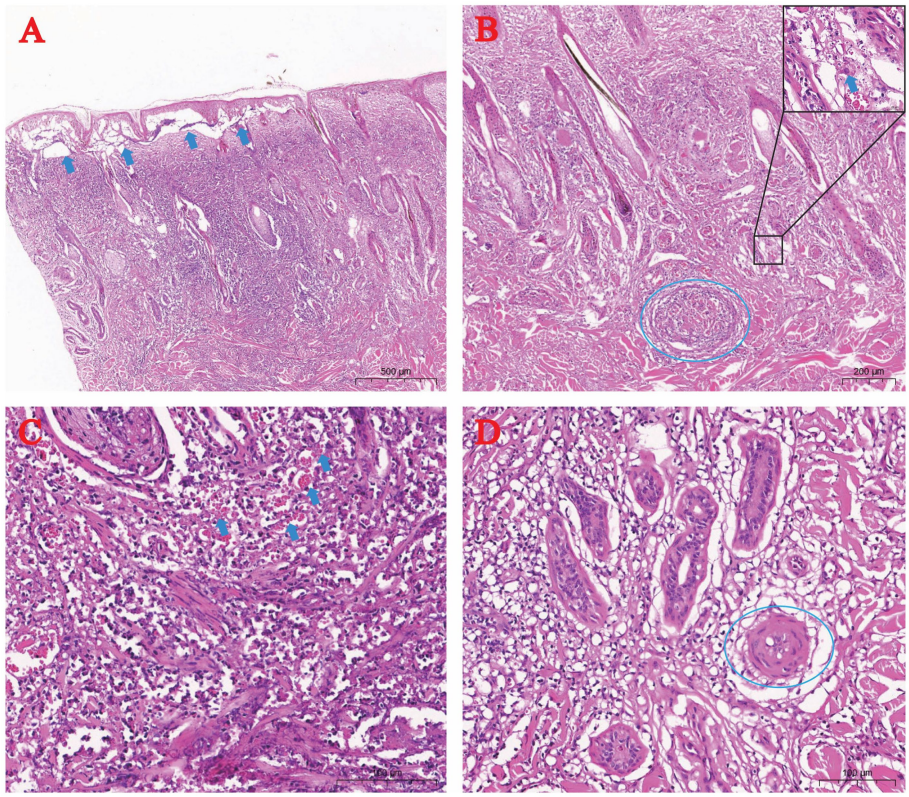


FIGURE 3
Histopathology of skin pox lesions in naturally LSDV-infected yaks (H&E staining). **(A)** Epidermal-dermal edema (arrow); **(B)** Dermal vasculitis (dashed circle) with rare cytoplasmic inclusion bodies (arrow); **(C)** Lymphocytic and erythrocytic infiltration in the dermis (arrow); **(D)** Arteriolar vasculitis in the dermis (dashed circle).

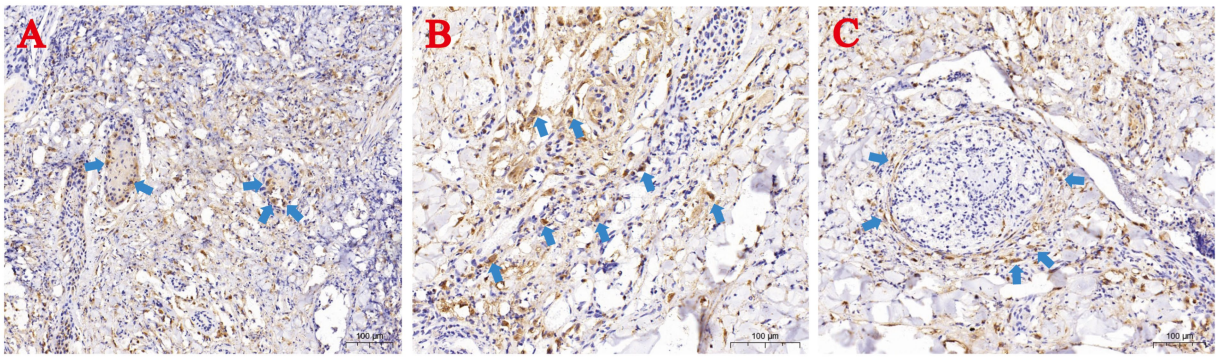


FIGURE 4
Immunohistochemical (IHC) staining of skin lesions from naturally LSDV-infected yaks. **(A)** IHC-positive hair follicle epithelial cells; **(B)** Deep dermal macrophages; **(C)** Perivascular lymphocytes. Blue arrows highlight IHC-positive cells.

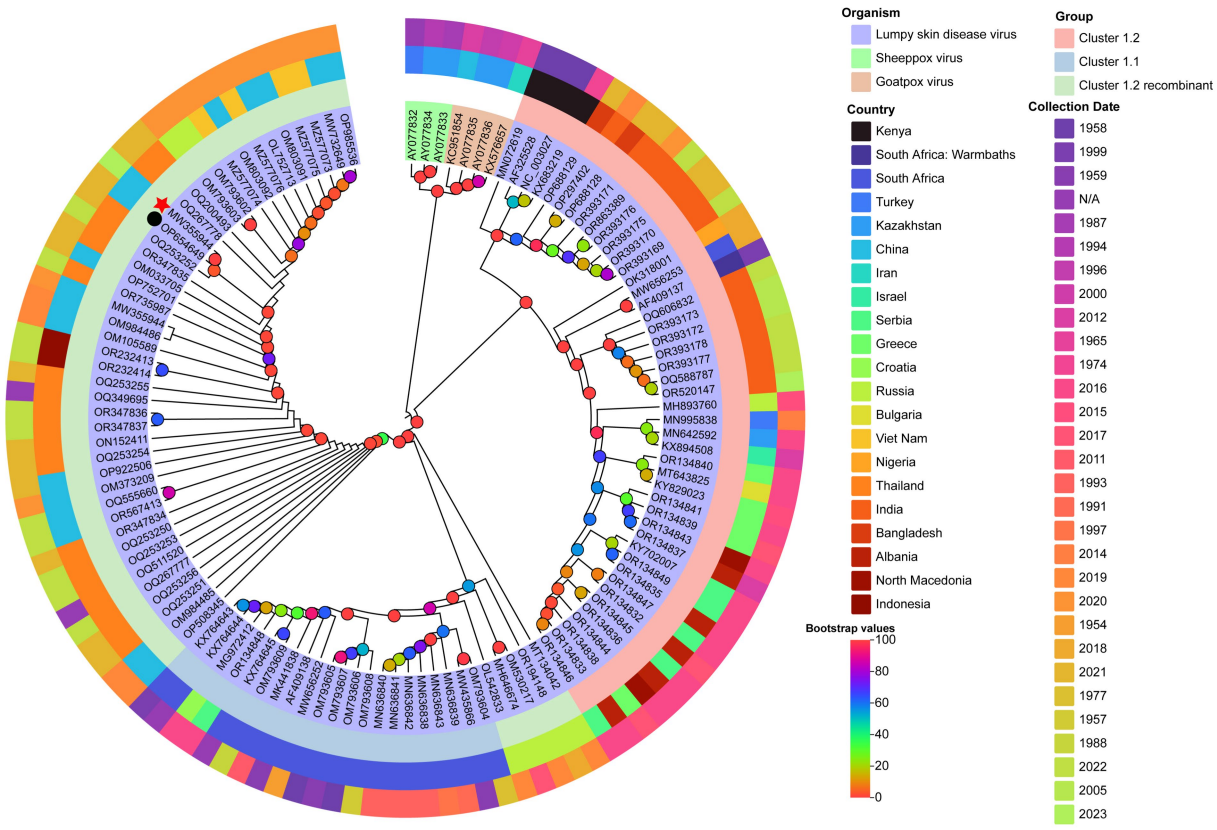


FIGURE 5
Maximum likelihood phylogenetic tree of LSDV based on full-genome sequences. The tree was constructed using PhyloSuite v1.2.3 (38) with IQ-TREE v2.2.0 (41), rooted with Sheeppox virus and Goatpox virus as outgroups. The best-fit substitution model (TVM + F + I + I + R2) was selected by BIC. Bootstrap values are color-coded at branch nodes. GenBank accession numbers, host species (color-coded), and LSDV sublineages (innermost ring) are annotated. The middle ring denotes country of origin, and the outermost ring indicates collection year. Red stars and black circles mark yak-derived LSDV genomes from this study and prior work, respectively.

## 5 Conclusion

LSDV represents an emerging threat to yaks in the Qinghai-Tibet Plateau, demonstrating exceptional virulence with a 46.67% case fatality rate. Histopathological evidence reveals dual pathogenic mechanisms: microcirculatory disruption and direct cytopathic effects. Phylogenetically, Chinese yak LSDV strains cluster within the recombinant 1.2 sublineage, showing 99.2–99.4% homology with East Asian and Russian variants. Notably, this contrasts with non-recombinant Indian yak strains (Cluster 1.1), revealing

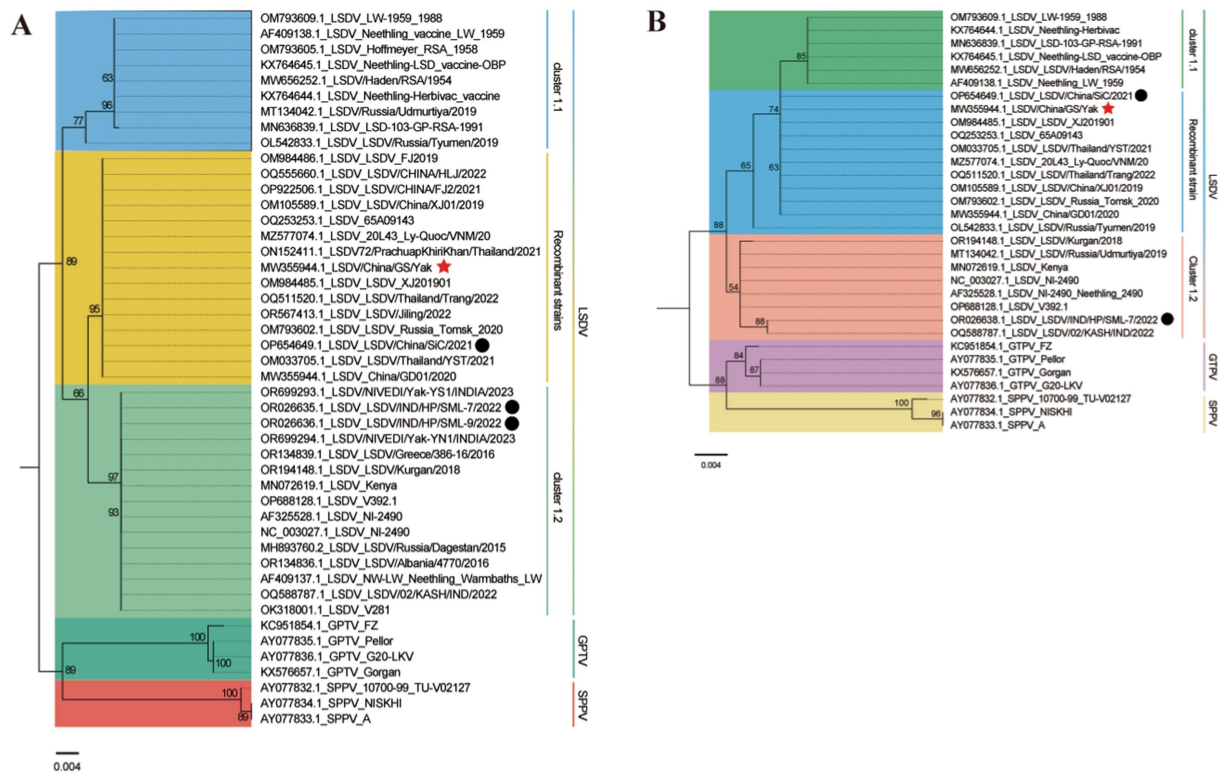


FIGURE 6
Maximum likelihood phylogenetic trees based on LSDV GPCR **(A)** and RPO30 **(B)** gene sequences. Trees were generated using PhyloSuite v1.2.3 (38) with IQ-TREE v2.2.0 (41), rooted with Sheeppox virus and Goatpox virus. Substitution models (K3Pu + F + I for GPCR; HKY + F + I for RPO30) were selected by BIC. Bootstrap values <50 are omitted. Branch labels include strain metadata, with colors denoting host species or LSDV subtypes. Red stars and black circles mark yak-derived LSDV sequences from this study and previous work.

divergent evolutionary paths between South Asian and East Asian lineages.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, MW355944.

## Ethics statement

Ethical approval not required for the studies involving animals because our study involved routine diagnostic sampling from farmed yaks with owner consent, which falls under standard veterinary practice not in experimental animal research. According to Directive 2010/63/EU, US PHS Policy, and China's Animal Epidemic Prevention Law, such sampling does not need formal ethical approval as part of disease surveillance and clinical care. Moreover, field personnel were licensed veterinary practitioners certified by the People's Republic of China, and sampling procedures strictly followed regulations. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

JH: Writing – original draft, Conceptualization, Data curation, Formal analysis, Methodology. YLi: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. XZ: Methodology, Writing – original draft. YLu: Methodology, Writing – original draft. ZZ: Methodology, Writing – original draft. MX: Methodology, Writing – original draft. SM: Methodology, Writing – original draft, Data curation. SN: Data curation, Conceptualization, Writing – review & editing. DW: Writing – original draft. TA: Writing – original draft. XL: Writing – original draft, Methodology, Resources. QM: Writing – original draft, Formal analysis, Project administration, Validation, Writing – review & editing. JL: Writing – review & editing, Writing – original draft, Funding acquisition, Resources, Supervision.

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## 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.

## Generative AI statement

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# Predicting vector distribution in Europe: at what sample size are species distribution models reliable?

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**Introduction:** Species distribution models can predict the spatial distribution of vector-borne diseases by forming associations between known vector distribution and environmental variables. In response to a changing climate and increasing rates of vector-borne diseases in Europe, model predictions for vector distribution can be used to improve surveillance. However, the field lacks standardisation with little consensus as to what sample size produces reliable models.

**Objective:** Determine the optimum sample size for models developed with the machine learning algorithm, Random Forest, and different sample ratios.

**Materials and methods:** To overcome limitations with real vector data, a simulated vector with a fully known distribution in 10 test sites across Europe was used to randomly generate different samples sizes. The test sites accounted for varying habitat suitability and the vector's relative occurrence area. 9,000 Random Forest models were developed with 24 different sample sizes (between 10–5,000) and three sample ratios with varying proportions of presence and absence data (50:50, 20:80, and 40:60, respectively). Model performance was evaluated using five metrics: percentage correctly classified, sensitivity, specificity, Cohen's Kappa, and Area Under the Curve. The metrics were grouped by sample size and ratio. The optimum sample size was determined when the 25th percentile met thresholds for excellent performance, defined as: 0.605–0.804 for Cohen's Kappa and 0.795–0.894 for the remaining metrics (to three decimal places).

**Results:** For balanced sample ratios, the optimum sample size for reliable models fell within the range of 750–1,000. Estimates increased to 1,100–1,300 for unbalanced samples with a 40:60 ratio of presence and absence data, respectively. Comparatively, unbalanced samples with a 20:80 ratio of presence and absence data did not produce reliable models with any of the sample sizes considered.

**Conclusion:** To our knowledge, this is the first study to use a simulated vector to identify the optimum sample size for Random Forest models at this resolution (≤1 km$^2$) and extent (≥10,000 km$^2$). These results may improve the reliability of model predictions, optimise field sampling, and enhance vector surveillance in response to changing climates. Further research may seek to refine these estimates and confirm transferability to real vectors.

KEYWORDS

vector-borne diseases, sample size, sample ratio, virtual species, species distribution model, machine learning, random forest, surveillance

## 1 Introduction

Emerging infectious diseases have significantly increased, with vector-borne diseases (VBDs) accounting for 28.8% of emerging infectious events globally between 1990–2000 (1). VBDs have a detrimental impact on mortality, disability-adjusted life years, and economies (2–4). The World Health Organization (WHO) estimates that 80% of the world's population are at risk of at least one VBD due to climate change, rapid urbanisation and globalisation (5). Across Europe, the incidence of endemic and (re-)emerging VBDs is changing. The warming of temperate regions has facilitated the latitudinal and altitudinal expansion of mosquitoes and ticks (6, 7). Cases of tick-borne encephalitis have increased, with six European countries considered highly endemic in 2020 (8); multiple regions reported West Nile virus for the first time in 2024 (9); and the invasive mosquito, *Aedes albopictus*, is firmly established in 14 European countries (10). Due to invasive mosquitoes, there have been 20 autochthonous dengue outbreaks, six autochthonous chikungunya outbreaks, and one autochthonous Zika outbreak in Europe since 2007 (11–13). Furthermore, it is projected that the invasive mosquitoes, *A. albopictus* and *Aedes aegypti*, will continue to expand into climatically suitable urban environments by 2050 (14).

To reduce the global incidence of VBDs by 60% from 2016 to 2030, the WHO recommends targeting the primary vectors (5). The WHO has provided a framework for effective vector control which builds on two foundational components: (1) enhanced capacity for surveillance, monitoring and evaluation and (2) increased research for vector control and innovation (5). The former was identified as a priority action for vector control in Europe (15). Field sampling monitors the distribution and abundance of vectors and is essential for surveillance, but is labour intensive and expensive (16). Species distribution models (SDMs) predict a region's suitability for a species' distribution under current or future eco-climatic conditions (17). This can optimise surveillance and reduce costs by identifying strategic locations for field sampling. As illustrated in Figure 1, correlative SDMs form associations between vector distribution and environmental variables to predict the probability of vector presence where sampling has not occurred (18). They achieve this by detecting patterns without explicitly defining biological processes, thus remaining independent of these assumptions for modelling (17). Within Europe, SDMs have successfully modelled the distribution of several arthropod vectors such as mosquitoes, sandflies and ticks (19–22).

SDMs are based on three main principles of spatial epidemiology: diseases tend to be limited geographically; the physical and biological conditions for vectors, hosts and pathogens influence the spatial heterogeneity of disease; and the current and future risks of disease are predictable if the abiotic and biotic factors can be delineated into maps (23). While it is possible to predict host and pathogen distributions, the drivers of pathogen transmission are complex, multifaceted and nonlinear (24). Furthermore, Hendrickx (25) used eco-climatic covariates to predict disease distribution in hosts and found that model accuracy decreased due to the increasing influence of other drivers, when compared to vectors. Therefore, modelling vector distributions as a proxy of VBD risk may be more appropriate. While vector presence does not necessarily infer a risk of disease, this approach aligns with the WHO and European Centre for Disease Prevention and Control (ECDC) recommendations for preparedness through vector surveillance (5, 26).

Ideally, SDMs are trained with presence and absence data since presence infers the locations which are environmentally suitable for a vector while absence infers the locations which are not (27). Vector distribution can be conceptualised as a gradient between potential distribution, which is where a species could live, and the realised distribution, which is where a species actually lives at a particular moment in time (28). Absence data is required to estimate the realised distribution, since distribution can be influenced by both abiotic and biotic factors (28). However, there is an inherent degree of uncertainty if an absence is a true absence since a species might be rare, in an inactive state, in a different habitat, or simply not captured by the trapping device (16). While absence data provides essential information, these limitations make field sampling more challenging, costly and labour-intensive to ensure their reliability (16). This has led to alternative techniques such as presence-only modelling and the generation of background samples or pseudo-absences. Background samples are generated computationally by randomly selecting points across a range of environmental conditions while pseudo-absences are manipulated to better represent a true absence (29). Several methods exist for generating pseudo-absences, such as excluding locations within a specific distance of a known presence point or comparing true absences for similar species (29). While pseudo-absences produce more accurate predictions than background samples, all three approaches have limitations: presence-only modelling fails to account for vector absence and the generation of absences makes significant assumptions which result in less accurate predictions compared to presence-absence models (29, 30). A simulated vector overcomes these limitations by generating high-quality presence and absences across different environmental gradients.

Various factors can influence model performance, including modelling algorithms, species characteristics, scale and sample size. Correlative SDMs can be developed with statistical or machine learning algorithms, but there is not a single best approach since each algorithm performs differently (31). The supervised machine learning algorithm, Random Forest (RF), can handle presence-absence data and thus model the vector's realised distributions. Species range can impact model predictions since performance generally diminishes for species with broad geographic ranges and environmental tolerances compared to those with smaller ranges and specific tolerances (32). Scale can be divided into extent and resolution. Larger extents can

Abbreviations: AUC, Area Under the (Receiver Operating Characteristic) Curve; ECDC, European Centre for Disease Prevention and Control; LST, Land surface temperature; MODIS, MODerate-resolution Imaging Spectroradiometer; NDVI, Normalised difference vegetation index; PCC, Percentage correctly classified; RF, Random forest; ROA, Relative occurrence area; SDM, Species distribution model; VBD, Vector-borne disease; WHO, World Health Organization.

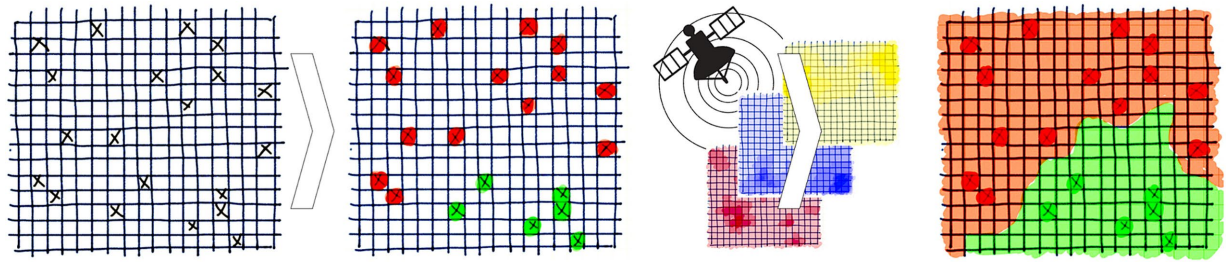

FIGURE 1
Overview of species distribution models. Random samples for vector distribution are linked to the environmental variables at each location and the model forms associations to generate a risk map based on the probability of vector presence across a region where sampling did not occur. Reprinted with permission (18).

improve model discrimination (28), but smaller extents can also enhance performance by reducing environmental variability (33). Resolutions larger than the species' niche breadth typically decrease model accuracy (34). While sample size is also known to influence model predictions, SDMs have been developed with sample sizes ranging from 10 to over 1,000 (35). There is a lack of consensus between studies which evaluate the effect of sample size, but little distinction has been made between the use of different algorithms (32, 36–41), resolutions (33, 42, 43), ensembles (44) and species (45).

As part of a wider body of work, similar studies were identified which evaluated the effect of sample size [Supplementary material (1.2)]. Comparable studies were defined as RF models which predict the distribution of terrestrial species at similar resolutions ($\leq$1 km$^2$) and extents ($\geq$10,000 km$^2$). Two studies met this criteria and quantified the effect of sample size on RF models: Liu et al. (46) predicted the habitat suitability of the snail, *Oncomelania hupensis*, which is an intermediate host for *Schistosoma* spp. and Hendrickx et al. (18) predicted the distribution of the trematode, *Dicrocoelium dendriticum,* in ruminant hosts. Both used real datasets and neither considered sample sizes for vector distributions. Two studies used virtual species to analyse the effect of sample size at a similar scale, but they did not identify an optimum sample size and reported general trends instead (29, 47).

Despite increasing interest in SDMs, the conceptual and methodological uncertainties of these models are often overlooked (27). With a lack of standardisation, an increasing amount of freely accessible species distribution data and modelling software, Jiménez-Valverde et al. (28) argue that it is essential these uncertainties are addressed through the development of a solid methodological framework. A virtual vector facilitates the systematic evaluation of different methodological designs on model performance and, with a lack of consensus for the optimum sample size, there is a need to quantify the effect of sample size on model performance. Therefore, this empirical study aims to use a virtual vector to identify the optimum sample size for reliable large extent ($\geq$10,000 km$^2$) and fine resolution ($\leq$1 km$^2$) RF models.

# 2 Materials and methods

To evaluate the effect of sample size, two confounding factors were accounted for in this study: relative occurrence area (ROA) and prevalence. Jiménez-Valverde et al. (28) argue that the effect of species range on model performance is actually a reflection of the ROA which refers to the proportion of the test site occupied by the vector. If a species occupies a small area, then there are a greater number of absences located further away from vector presence which improves model discrimination (28). Therefore, this effect is scale dependent and independent of the species' actual range size, since model performance can improve by using a larger extent which reduces the ROA (28). While the ROA is a function of extent, prevalence refers to the proportion of presence samples within the dataset and reflects the characteristics of the data (28). When compared to sample size and modelling technique, ROA and prevalence had the largest influence on model performance (44). Due to differing terminology between prevalence (28, 44), sample prevalence (38, 43) and presence prevalence (29), this study defined the proportion of presence and absence points as sample ratio. Sample size refers to the total number of presence and absence points in a sample. Both sample ratio and size refer to the dataset before partitioning, so that the results can guide the number of samples required during field sampling.

To account for ROA, the virtual vector was modelled in multiple test sites across Europe. Figure 2 illustrates a workflow whereby the effect of sample size was evaluated separately for three different sample ratios. Initially, 10 sample sizes were randomly generated. To account for variability in model performance, particularly at smaller sample sizes, sampling was replicated to generate 10 random samples, per sample size, in each test site. Model predictions were evaluated and performance grouped across the test sites. The methods were repeated twice more to refine estimates. Supplementary Figure 1 contains a flow chart detailing each step. The methodology was reported according to ODMAP, a standardised reporting protocol for SDMs (48) in Supplementary Table 1. The methods were executed in R Studio 2023.06.1 using R version 4.3.1 and the packages listed in Supplementary Table 2. QGIS 3.22.7 was used for figure generation and data quality checks.

## 2.1 Materials

For a species distribution model, datasets should describe the test site's extent, the sampling location of each presence or absence record and the environmental conditions at each point. The vector distribution and covariate datasets need to have the same resolution (49). Therefore, all datasets were projected in the geographic coordinate reference system, EPSG:4326 – WGS 84 with matching

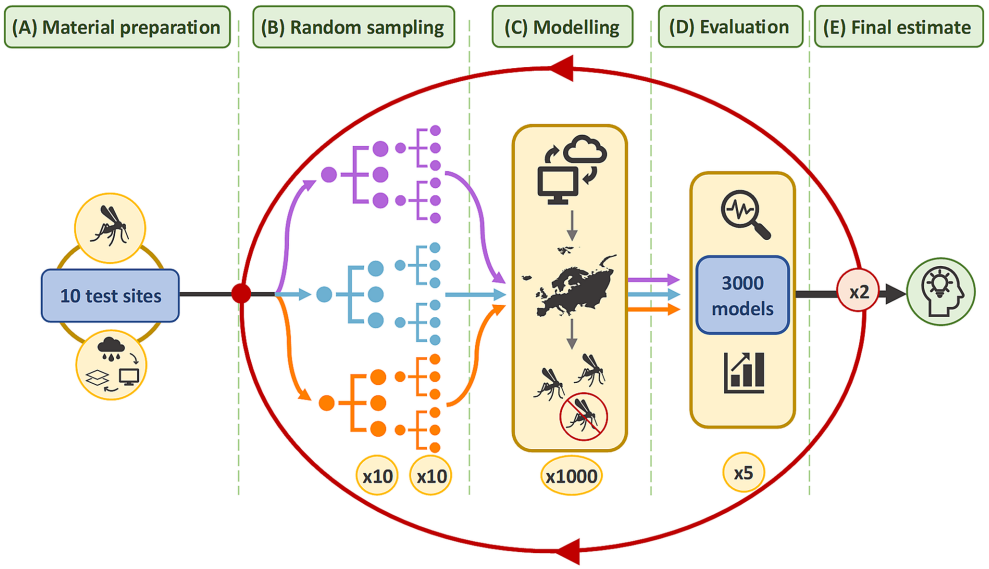


FIGURE 2
Methodology summary. In **(A)**, the virtual vector and covariate datasets were cropped to the extent of each test site. In **(B)**, 10 different sample sizes were randomly sampled from the virtual vector in each test site. Each sample size was replicated 10 times to produce 1,000 samples. This was repeated for three sample ratios. The coloured lines represent each sample ratio: purple for balanced samples with a 50:50 ratio; blue for samples with an unbalanced 20:80 ratio; and orange for samples with an unbalanced 40:60 ratio of presence and absence data, respectively. In **(C)**, 3,000 models were developed which predict the vector's distribution in each test site. In **(D)**, the performance of 3,000 models was evaluated with five metrics. The results were grouped by sample size and sample ratio to identify a narrower range of sample sizes. In **(E)**, the methods from **(B–D)** were repeated twice more within increasingly narrower ranges to refine the estimate for the optimum sample size.

spatial grids of 0.0083° × 0.0083°, which equates to approximately 1 km$^2$ at the equator (0.918 km × 0.918 km to three decimal points).

### 2.1.1 Virtual vector

SDMs should be developed with high quality, fine resolution datasets for vector presence and absence with georeferenced locations that are spatially and environmentally unbiased, but this is difficult to attain (37). With a known distribution and presence-absence data, the virtual vector enables the prediction of realised distributions, overcoming challenges associated with historical and field datasets for real vectors. The virtual vector was generated using the probability approach from the SDMvspecies package in R (50). This method is preferable to the threshold approach since it mimics vector occupancy patterns across space and time by generating species distributions across environmental gradients and takes species prevalence into consideration (37). The virtual vector was modelled to resemble a flying insect and has a fully known spatial distribution across Europe (Figure 3A). For direct comparison between the predicted and known distribution, the virtual vector was classified into binary values; a threshold of 0.5 was applied, and cells with a probability of presence greater than 0.5 were assigned a value of 1 for presence, while cells with a probability of presence equal to or less than 0.5 were assigned a value of 0 for absence (Figure 3B).

Random sampling and modelling were conducted within 10 test sites across mainland Europe (Figure 3B). Modelling is often tailored to the research objective. To estimate the optimum sample size and provide generalised guidance for field sampling and subsequent modelling, test sites with differing characteristics were selected. Each reflects varying geographic distributions of the virtual vector; thus, grouping model performance by sample size across the test sites accounts for differing habitat suitability and ROA. The test sites have an extent comparable to some European countries with an area of 11.111°$^2$, which equates to approximately 136,900 km$^2$ at the equator (370 km × 370 km). The virtual vector dataset was cropped to the extent of each test site to create 10 distinct datasets for sampling and modelling.

### 2.1.2 Covariates

Environmental variables typically represent the key aspects of a species' ecology which impact its survival in a particular environment (51). Vectors are ectothermic and while the degree of impact is species specific, temperature is one of the main environmental factors which can affect their reproduction, survival, distribution and ability to transmit pathogens (52). Environmental variables like vegetation parameters can also impact vector presence (6, 52). Remotely sensed covariates from satellites may be preferable to climatic covariates due to their high spatial and temporal resolution globally (53). Therefore, time series data for 27 covariates which describe normalised difference vegetation index (NDVI) and land surface temperature (LST) from 2001 to 2021 were obtained from MODerate-resolution Imaging Spectroradiometer (MODIS) satellite imagery (Table 1). Following methods by Scharlemann et al. (54), the covariates were temporal Fourier processed, thereby reducing data dimensionality and removing correlations to create independent covariates. The covariate dataset was then cropped to the extent of each test site.

## 2.2 Methods

### 2.2.1 Sampling

First, 10 different sample sizes (10, 30, 50, 80, 100, 250, 500, 1,000, 2,500 and 5,000) were randomly sampled from the virtual

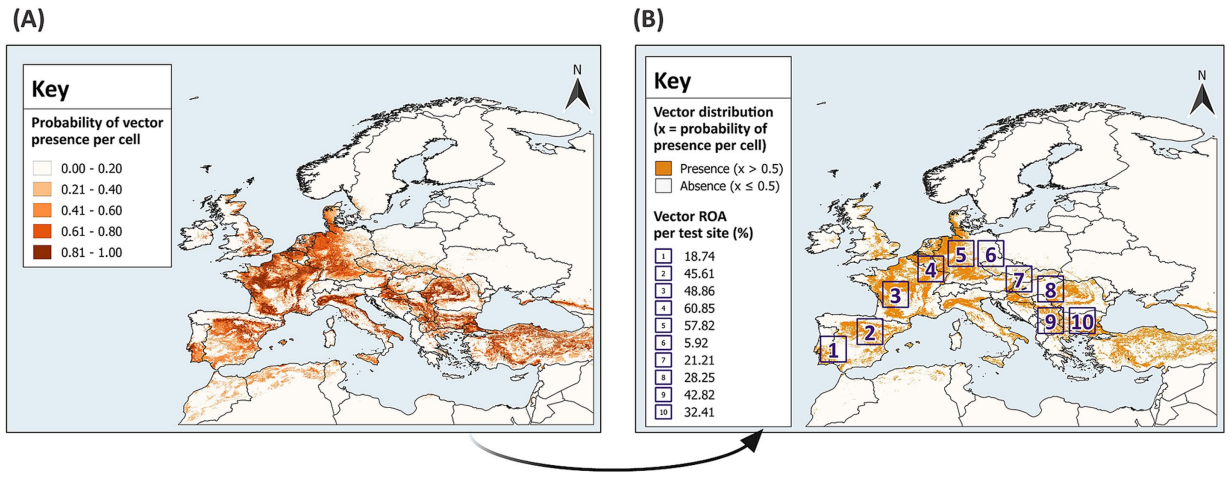


FIGURE 3
Distribution of the virtual vector across Europe. The virtual vector has a defined distribution within the extent of 13.0°W – 43.0°E, 29.0°N – 72.0°N **(A)**, which was classified into binary values for presence and absence using a threshold of 0.5 **(B)**. In **(A)**, a gradient from light to dark represents increasing probabilities of presence per raster cell, categorised into five classes using equal intervals. In **(B)**, sampling and modelling was conducted in 10 test sites across Europe. The Relative Occurrence Area (ROA) of the vector was defined as the percentage of cells with a presence value of 1, divided by the total number of cells within each test site (to two decimal points). GADM Level 0 country boundaries were utilised (79).

vector's distribution in each of the 10 test sites. Random sampling reduces the likelihood of oversampling a particular area. Geographically clustered samples can decrease predictive accuracy and may have a larger influence than sample size (18). To minimise spurious results, each sample size was replicated 10 times in each test site, generating 1,000 unique samples. Since the proportion of presence observations in a sample has an important influence on model performance (43, 44), the methods were repeated for three sample ratios with different proportions of presence and absence observations. These included a balanced 50:50 ratio and two unbalanced ratios of presence and absence points (40:60 and 20:80, respectively). Given the increased probability of sampling absences compared to presences in the field, the unbalanced sample ratios contained a greater proportion of absence points. This approach was intended to represent field conditions for vector sampling. For each of the 3,000 samples, the presence and absence points were linked to the covariates describing the environmental conditions at each location (Figure 4).

### 2.2.2 Modelling

RF has recently gained popularity due to its ease of use and ability to model non-linear relationships and complex interactions between covariates (29). RF can use classification or regression trees for binary data. However, regression RF has been shown to predict the probabilities of species distribution with greater accuracy (55). Therefore, each sample trained a regression RF model which predicted the virtual vector's distribution in its respective test site. To do so, the sample was randomly split into a test and training subset using a 30:70 ratio, respectively (Figure 5). This is a cross-validation method which uses a subset of data to train the model before comparing the predictions with the vector's known distribution from the test subset. Since RF creates robust models with the default parameters (56), the only specified parameters were the hyperparameter *mtry* (optimum) and the number of trees (500). A higher number of trees is recommended to improve the accuracy of model predictions, but this needs to be balanced against computational cost (56). Once 3,000 models formed associations between vector distribution and covariates from each training subset, the models used the covariates to make predictions for vector distribution across their respective test site. For evaluation, the predictions were dichotomised into binary values for presence (1) and absence (0) using the same threshold of 0.5. Each presence and absence point in the independent test subsets were then linked to their corresponding model predictions.

### 2.2.3 Evaluation

Model performance was assessed using five metrics: Percentage Correctly Classified (PCC), sensitivity, specificity, Cohen's Kappa and Area Under the Curve (AUC). PCC, sensitivity, specificity and Cohen's Kappa are threshold-dependent metrics, calculated from confusion matrices comparing the binary model predictions for vector presence and absence against the virtual vector's known distribution (Table 2). PCC measures the proportion of correctly predicted presence and absence observations; sensitivity measures the proportion of correctly predicted presence observations; and specificity measures the proportion of correctly predicted absence observations in the test subset (57). The values for all three metrics range from 0 to 1, with a higher value indicating better model performance. Unlike the previous metrics which solely calculate the proportion of agreement between model predictions and known observations, Cohen's Kappa also accounts for chance agreement when calculating inter-rater reliability (58, 59). The values for Cohen's Kappa range from −1 to 1, whereby 1 indicates perfect agreement between known and predicted observations while values equal to or less than 0 suggest the model's performance is no better than chance (58). Each of these metrics were cross-referenced against R functions: pcc from the PresenceAbsence package, sensitivity and specificity from the caret package, and the unweighted value for Kappa from the vcd package (60–62).

AUC, a threshold-independent metric, is derived from the receiver operating curve which analyses how different thresholds influence the classification of presence and absence by plotting

TABLE 1 Covariates used for modelling.

| Covariate | Description |
|---|---|
| dLSTa1 | Daytime Land Surface Temperature annual amplitude (K) |
| dLSTa2 | Daytime Land Surface Temperature bi-annual amplitude (K) |
| dLSTa3 | Daytime Land Surface Temperature tri-annual amplitude (K) |
| dLSTavg | Mean daytime Land Surface Temperature (K) |
| dLSTmax | Maximum daytime Land Surface Temperature (K) |
| dLSTmin | Minimum daytime Land Surface Temperature (K) |
| dLSTp1 | Daytime Land Surface Temperature phase of annual cycle (months) |
| dLSTp2 | Daytime Land Surface Temperature phase of bi-annual cycle (months) |
| dLSTp3 | Daytime Land Surface Temperature phase of tri-annual cycle (months) |
| NDVIa1 | Normalised Difference Vegetation Index annual amplitude (no units*) |
| NDVIa2 | Normalised Difference Vegetation Index bi-annual amplitude (no units*) |
| NDVIa3 | Normalised Difference Vegetation Index tri-annual amplitude (no units*) |
| NDVIavg | Mean Normalised Difference Vegetation Index (no units*) |
| NDVImax | Maximum Normalised Difference Vegetation Index (no units*) |
| NDVImin | Minimum Normalised Difference Vegetation Index (no units*) |
| NDVIp1 | Normalised Difference Vegetation Index phase of annual cycle (months) |
| NDVIp2 | Normalised Difference Vegetation Index phase of bi-annual cycle (months) |
| NDVIp3 | Normalised Difference Vegetation Index phase of tri-annual cycle (months) |
| nLSTa1 | Night-time Land Surface Temperature annual amplitude (K) |
| nLSTa2 | Night-time Land Surface Temperature bi-annual amplitude (K) |
| nLSTa3 | Night-time Land Surface Temperature tri-annual amplitude (K) |
| nLSTavg | Mean night-time Land Surface Temperature (K) |
| nLSTmax | Maximum night-time Land Surface Temperature (K) |
| nLSTmin | Minimum night-time Land Surface Temperature (K) |
| nLSTp1 | Night-time Land Surface Temperature phase of annual cycle (months) |
| nLSTp2 | Night-time Land Surface Temperature phase of bi-annual cycle (months) |
| nLSTp3 | Night-time Land Surface Temperature phase of tri-annual cycle (months) |

All files were created by transforming MODIS historical climate data, from 2001–2021, for daytime Land Surface Temperature (dLST), night-time Land Surface Temperature (nLST) and Normalised Difference Vegetation Index (NDVI) via temporal Fourier analysis. As described by Scharlemann et al. (54), the NDVI covariates denoted by the symbol * do not have units because they are dimensionless ratios and therefore, categorical. The international base unit for temperature, Kelvin, is denoted by K.

sensitivity on the y-axis against (1 – specificity) on the x-axis (63). As such, AUC provides a summary of classification accuracy with values ranging from 0–1, whereby 1 indicates perfect accuracy and 0.5 suggests the model predictions are equivalent to random chance (63). AUC was calculated using the R functions, roc and auc from the pROC package (64). All five metrics are commonly used to evaluate SDMs and, as recommended by Konowalik and Nosol (65), the use of multiple metrics overcomes the limitations associated with relying on a single metric.

### 2.2.4 Optimum sample size

For each of the five evaluation metrics, the 3,000 models were grouped by sample size and ratio across the 10 test sites and presented in boxplots. Model performance was assessed against predefined thresholds, which determine excellent model performance for each evaluation metric (Table 3). The thresholds for Cohen's Kappa and AUC were based on those presented by Landis and Koch (66) and Hosmer et al. (67), respectively. Expert advice informed the thresholds for PCC, sensitivity and specificity. The thresholds for excellent performance were defined as: 0.795–0.894 for PCC, sensitivity, specificity, and AUC; and 0.605–0.804 for Cohen's Kappa (to three decimal places). To identify the optimum sample size, the boxplots were examined to identify at what sample size the 25th percentile (first quartile of each boxplot) met these thresholds.

To refine estimates for the optimum sample size, these methods were repeated twice more within increasingly narrower ranges of sample sizes, generating 9,000 models. For simplification, all 9,000 models were then grouped by sample size and ratio and presented in heatmaps. The 25th percentile for each sample size and ratio was calculated, and the same thresholds for excellent performance applied to identify the optimum sample size. Moderate performance was also taken into consideration, defined as: 0.695–0.794 for PCC, sensitivity, specificity, and AUC; and 0.405–0.604 for Cohen's Kappa (to three decimal places).

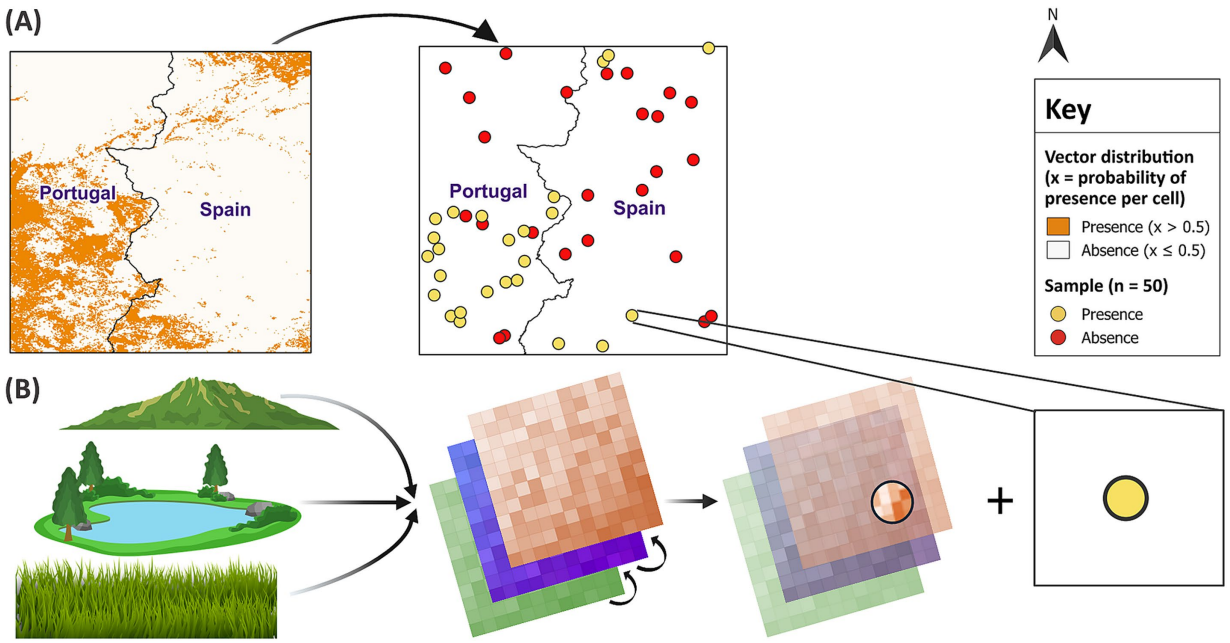


FIGURE 4
Random sampling and linkage of datasets. In **(A)** random sampling of the binary virtual vector in test site 1 generated a balanced sample size of 50. In **(B)** each presence and absence point was linked to the covariates at that location. GADM Level 0 country boundaries were utilised (79).

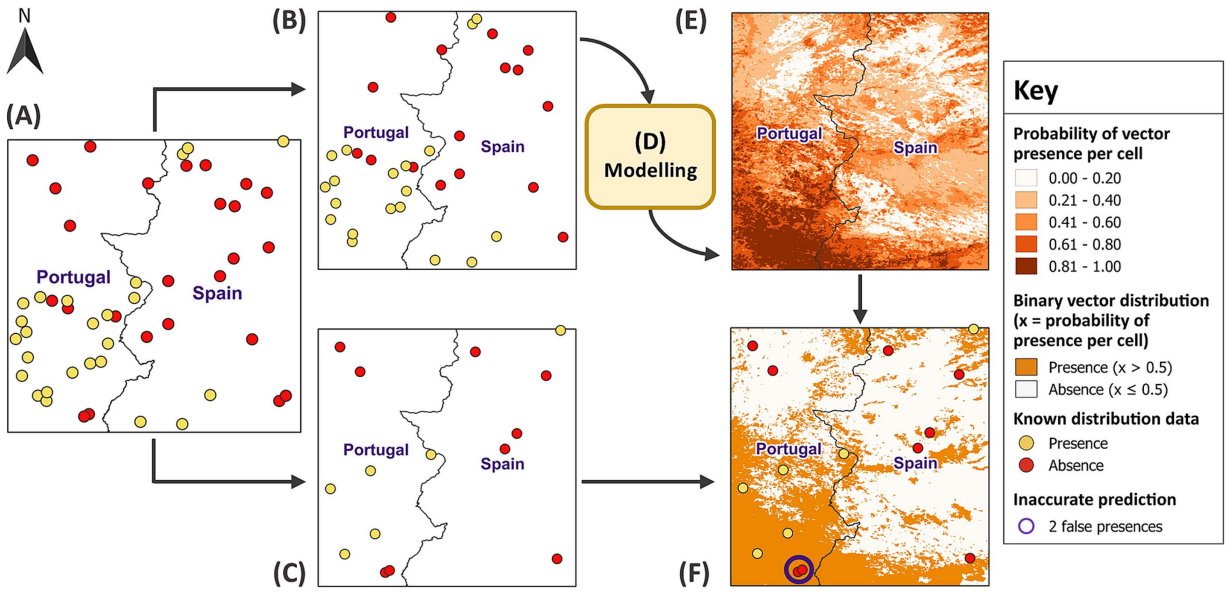


FIGURE 5
Training models and predicting vector distribution. In **(A)** a balanced sample size of 50 was randomly partitioned into **(B)** a training subset (70% of the sample) and **(C)** a test subset (30% of the sample). In **(D)** the model was developed using the training subset before the covariates for test site 1 were applied to **(E)** predict vector distribution across the remainder of the test site. The model predictions represent increasing probabilities of vector presence per raster cell, categorised into five classes using equal intervals. In **(F)**, the predictions were dichotomised into binary values for presence and absence using a threshold of 0.5. The test subset validated the model predictions, revealing two locations, circled in purple, where the model predicted presence, but the vector was actually absent. GADM Level 0 country boundaries were utilised (79).

## 3 Results

The first round of modelling evaluated the effect of sample sizes 10, 30, 50, 80, 100, 250, 500, 1,000, 2,500, and 5,000 on model performance. Boxplots for each evaluation metric were compared against the thresholds for excellent model performance, which indicated that the optimum sample size fell within the range of 250–2,500 (Supplementary Figure 2). To include a margin of error, the

TABLE 2 Calculations for four threshold-dependent evaluation metrics.

| Confusion matrix | | |
|---|---|---|
| | Actual presence | Actual absence |
| Predicted presence | A | B |
| Predicted absence | C | D |

| Evaluation metrics | |
|---|---|
| Sum | $n = (A + B + C + D)$ |
| PCC | $\frac{A+D}{n}$ |
| Sensitivity | $\frac{A}{(A+C)}$ |
| Specificity | $\frac{D}{(B+D)}$ |
| Cohen's Kappa | $Cohen's\ Kappa = \frac{P_O - P_e}{1-P_e}$, whereby: $Proportion\ of\ observed\ agreements\ (P_O) = \sum P_{ii}$, equates to: $P_O = \frac{(A+D)}{n}$ $Proportion\ of\ chance\ agreement\ (P_e) = \sum P_i + P_{+i}$, equates to: $P_e = \left(\left(\frac{A+B}{n}\right)\times\left(\frac{A+C}{n}\right)\right) + \left(\left(\frac{C+D}{n}\right)\times\left(\frac{B+D}{n}\right)\right)$ |

Each of the threshold-dependent evaluation metrics were based on a 2×2 contingency table for model performance which compared the binary model predictions to the actual presence and absence points in the test subset. The counts describe when the model (A) correctly predicts presence (true positives), (B) incorrectly predicts presence at a location where absence was actually recorded (false positives), (C) incorrectly predicts absence at a location where presence was actually recorded (false negatives), and (D) correctly predicts absence (true negatives).

TABLE 3 Thresholds for excellent model performance for each evaluation metric.

| PCC, Sensitivity and Specificity | Cohen's Kappa | AUC | Rating |
|---|---|---|---|
| N/A | < 0.00 | ≤ 0.50 | Chance |
| 0.00–0.49 | 0.00–0.20 | 0.51–0.69 | Poor |
| 0.50–0.69 | 0.21–0.40 | N/A | Fair |
| 0.70–0.79 | 0.41–0.60 | 0.70–0.79 | Moderate |
| 0.80–0.89 | 0.61–0.80 | 0.80–0.89 | Excellent |
| 0.90–1.00 | 0.81–1.00 | 0.90–1.00 | Outstanding |

range was adjusted to 150–3,000, from which 10 sample sizes were selected. The methods were repeated with the sample sizes 150, 250, 350, 500, 750, 1,000, 1,500, 2,000, 2,500, and 3,000, identifying a narrower range between 400–1,300 (Supplementary Figure 3). The results for the final round, which evaluated the effect of sample sizes 400, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, and 1,300 on model performance, are presented below.

## 3.1 Model performance

Increasing sample size improved model performance across all sample ratios and evaluation metrics (Figure 6). The sample size which first reached the thresholds for excellent model performance varied between sample ratios and metrics. For balanced ratios, the first sample size to reach the thresholds was 500, when evaluated by sensitivity (first quartile = 0.811, mean = 0.842), compared to a sample size of 1,000, when evaluated by specificity (first quartile = 0.795, mean = 0.818). The smallest sample size to reach the thresholds for unbalanced 40:60 ratios was 400, when evaluated by specificity (first quartile = 0.809, mean = 0.851), and the largest was 1,100, when evaluated by Cohen's Kappa (first quartile = 0.608, mean = 0.649). Comparatively, for unbalanced 20:80 ratios, no sample size met the thresholds for sensitivity, Cohen's Kappa or AUC, while all reached or exceeded the PCC and specificity threshold. For three out of five metrics (sensitivity, Cohen's Kappa and AUC), models developed with balanced sample ratios were more reliable than those developed with unbalanced ratios.

## 3.2 Model performance across all sample sizes

A total of 8,999 models were developed with 24 different sample sizes, some of which were evaluated multiple times (250 and 500 twice; 500 and 1,000 three times). Models were grouped by sample size and ratio and presented as heatmaps for each metric (Figure 7). A blue and orange gradient from light to dark indicates how far the first quartile lies within the thresholds for moderate and excellent performance, respectively.

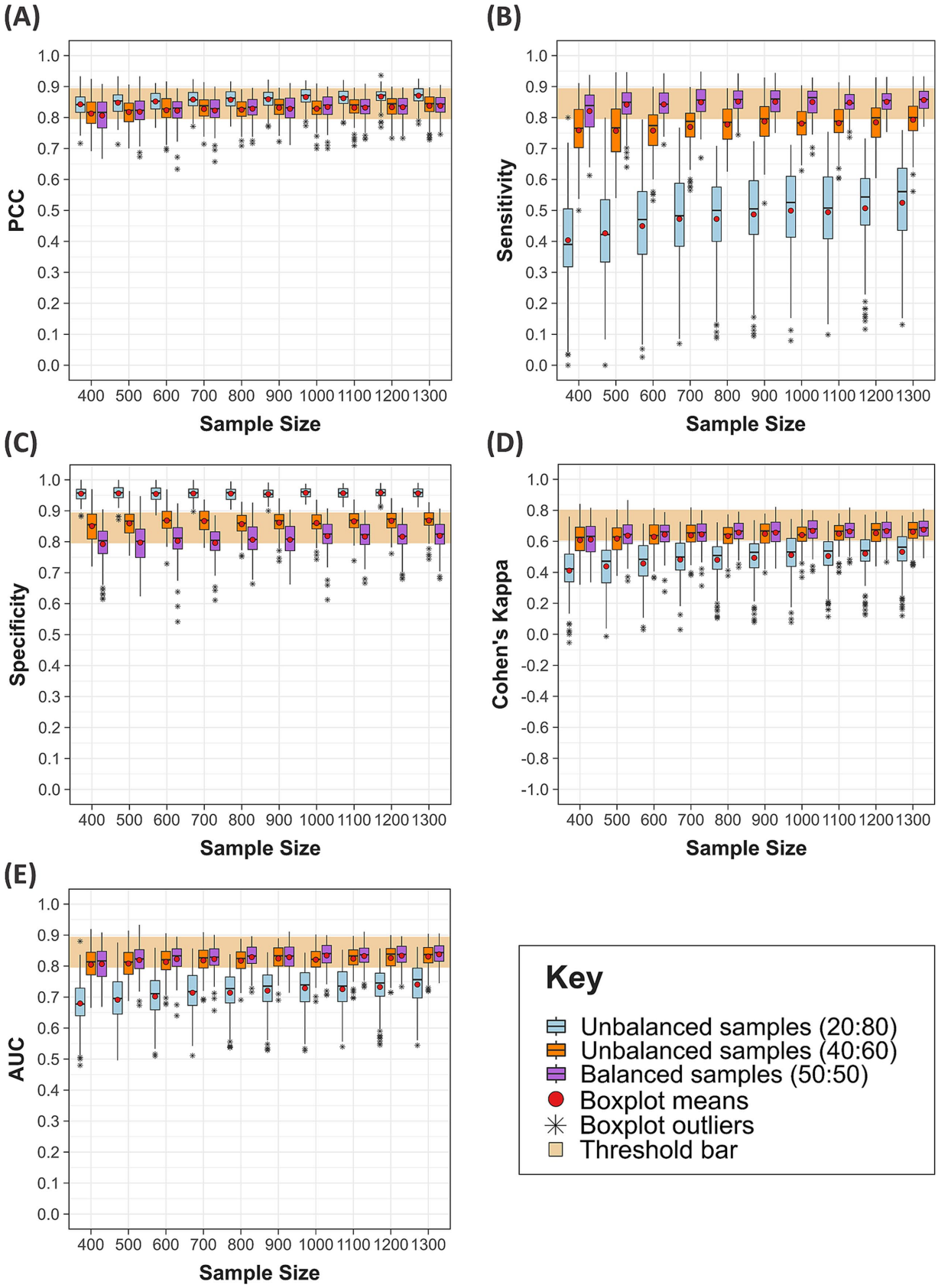


FIGURE 6
Performance of 3,000 models when evaluated by five metrics. Each boxplot represents the means, medians and quartiles of 100 models which are grouped by the 10 sample sizes (400, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, and 1,300) and three sample ratios of presence and absence data (50:50, 20:80 and 40:60, respectively). The threshold bar represents the defined thresholds for excellent models (to three decimal points): 0.795–0.894 for **(A–C,E)** which present the PCC, sensitivity, specificity and AUC metrics, respectively, and 0.605–0.804 in **(D)** which presents Cohen's Kappa.

Model performance notably deteriorated for sample sizes below 100 across all metrics, with few reaching the thresholds for moderate performance (Figure 7). One model developed with a sample size of 10 and a 20:80 ratio failed, and 93 models with the smallest sample sizes (10 and 30) had at least one incalculable metric due to a lack of presence or absence points in the test and/or training subsets. Of the 93 models with missing metrics, 60% were developed with a 20:80 ratio (56/93), 21% with a 50:50 ratio (20/93), and 19% with a 40:60 ratio (18/93). Therefore, RF models may produce unreliable predictions for vector distribution when developed with a sample size of 10 and 30, particularly with an unbalanced 20:80 ratio. Model performance for this ratio also varied substantially across the metrics at other sample sizes, indicating that model predictions with a 20:80 ratio were the least reliable.

The first sample sizes to reach each metric's thresholds varied between 400–1,100 for unbalanced 40:60 ratios and between 500–1,000 for balanced 50:50 ratios in Figure 6. However, these findings do not account for all sample sizes considered or varying model performance for the same sample sizes evaluated in multiple rounds. For balanced ratios, a sample size of 1,000 first reached the specificity threshold in the third round (first quartile = 0.795, mean = 0.818), but the same sample size did not meet the threshold in the first round (first quartile = 0.793, mean = 0.818) or the second round of methods (first quartile = 0.781, mean = 0.813). Figure 7 suggests the first sample size to reach each metric's thresholds varied between 150–5,000 for unbalanced 40:60 ratios and between 500–1,500 for balanced 50:50 ratios.

Important trends may be overlooked when solely focusing on the first sample size to reach thresholds for excellent model performance. For an unbalanced 40:60 sample ratio, the first quartile reached the PCC threshold for excellent model performance from a sample size of 600. There were minor fluctuations of 0.01 decimal places around the threshold boundary until a sample size of 1,100, after which performance consistently improved with increasing sample size (Figure 7A). For sensitivity, the first quartile only reached the threshold for excellent performance at a sample size of 5,000. Since the models were trained with a greater proportion of absence data, it may be reasonable to consider that the mean reached the threshold for excellent performance at a sample size of 1,500 (mean = 0.799) and the first quartile for a sample size of 1,100 reached the upper estimate (above 0.75) for moderate performance (Figure 7B). For specificity, the first quartile reached the threshold for excellent performance from a sample size of 150. Due to minor fluctuations at the boundary, performance was more reliably above the threshold from a sample size of 500 (Figure 7C). When evaluated by Cohen's Kappa, a sample size of 1,100 met the threshold for excellent performance, but dipped below at 1,200, suggesting models may be more reliable from 1,300 (Figure 7D). Finally, a sample size of 900 reached the AUC threshold for excellent models with fluctuations around the boundary until a sample size of 1,300 (Figure 7E).

For balanced ratios, the first quartile reached the PCC and AUC threshold for excellent performance at a sample size of 600 but remained near the boundary until a sample size of 1,000 (Figures 7A,E). For sensitivity, a sample size of 500 first reached the threshold but models were more comfortably above the threshold from a sample size of 600. However, there was a slight dip at a sample size of 900 and 1,000, albeit by 0.01 decimal places, after which performance continuously improved with increasing sample size (Figure 7B). For specificity, the first quartile reached the thresholds for excellent models from a sample size of 1,500, but performance was only 0.01 decimal places below the threshold from a sample size of 1,000 (Figure 7C). For Cohen's Kappa, the first quartile reached the threshold for excellent performance at a sample size of 750 but dipped below at a sample size of 900, suggesting models may be more reliable from a sample size of 1,000 (Figure 7D).

## 3.3 Estimates for the optimum sample size

The optimum sample size was estimated separately for the three sample ratios. To determine the optimum sample size, more emphasis was placed on sensitivity, Cohen's Kappa and AUC. While PCC is a widely used metric, it is a poor reflection of model performance since it is influenced by the ROA: if the vector occupies 5% of the test site, a 95% success rate could be achieved if the model predicted absence across the entire test site (68). Since specificity measures the percentage of absence observations correctly predicted, this should theoretically increase when models are trained on a greater proportion of absence points which explains why the 20:80 sample ratio performed best when evaluated by specificity. RF models also tend to overfit resulting in higher sensitivity and lower specificity values (44). However, it may be preferable to maximise sensitivity over specificity when the aim is to inform new vector surveys, since higher sensitivity minimises the number of true presences predicted as absences (69).

The optimum sample size was best expressed as a range since there were fluctuations around the threshold boundaries for excellent model performance between sample sizes and for the same sample size across multiple rounds. Most metrics indicated that the optimum sample size fell between the range of 750–1,000 for balanced ratios. This increased to 1,100–1,300 for an unbalanced 40:60 sample ratio of presence and absence data, respectively. Due to poor model performance, it was not possible to estimate an optimum sample size for a 20:80 ratio. Model predictions for vector distribution, developed with the lower estimate for the optimum sample size, are displayed across all 10 test sites (Figure 8). This illustrates one model's predictions with the optimum sample size, however stacked predictions should be considered due to the variation between replicate models at the same sample size (Figure 6). Additional figures which spatially present predicted vector distributions by models with different sample sizes are available in Supplementary Figures 4–9.

# 4 Discussion

To the best of our knowledge, this is the first study to use a virtual vector to identify the optimum sample size for RF models with presence-absence data at this extent (≥10,000 km$^2$) and resolution (≤1 km$^2$). This study produced three main findings; the optimum sample size for reliable SDMs fell within the range of 750–1,000 for balanced samples and 1,100–1,300 for samples with an unbalanced 40:60 ratio of presence and absence points, respectively. Secondly, model performance was poor for sample sizes below 100 and for samples with an unbalanced 20:80 ratio. Thirdly, as the proportion of presence points increased between sample ratios, model performance improved for all metrics except PCC and specificity.

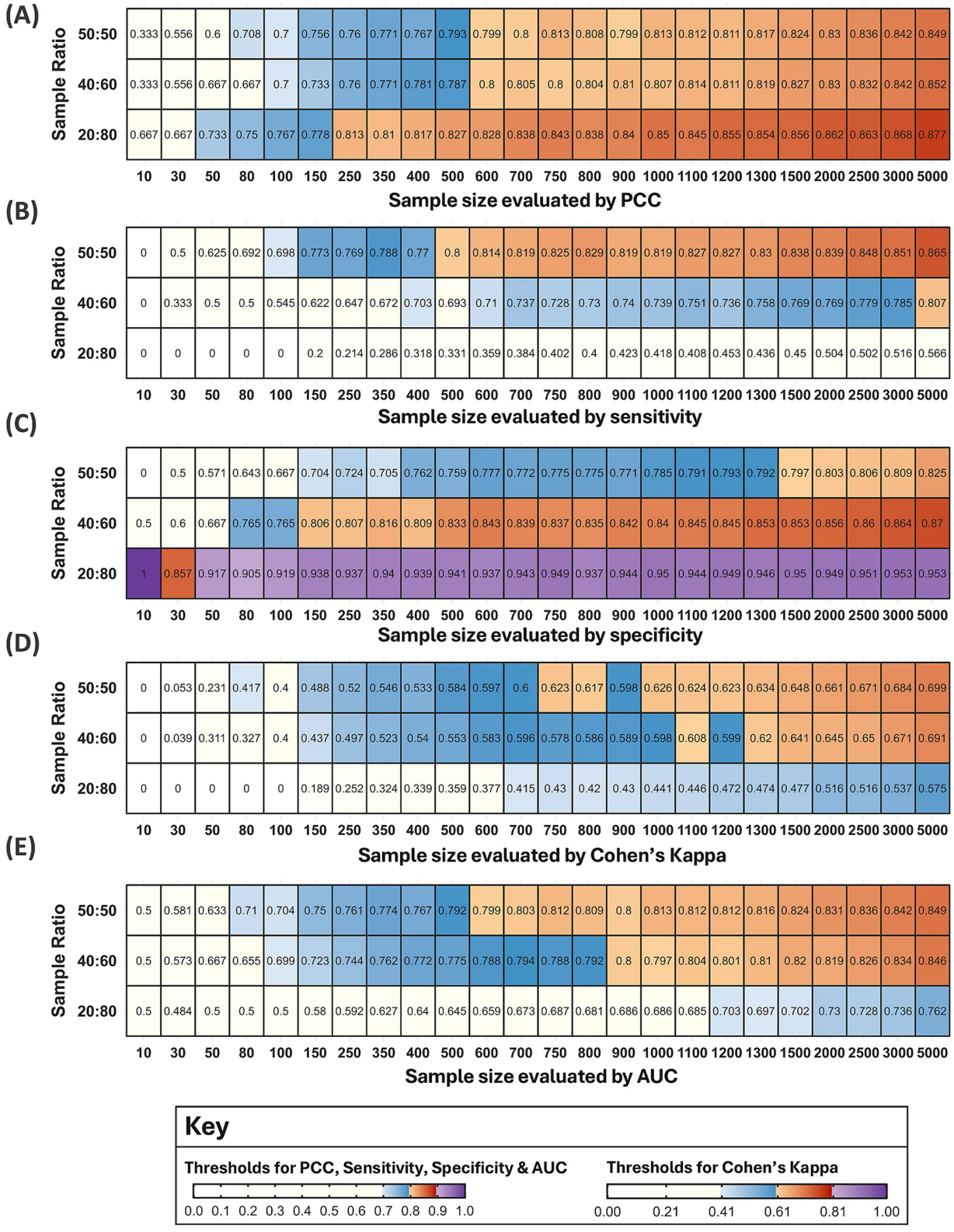


FIGURE 7
Heatmaps for five metrics evaluating the performance of 8,999 models. Each tile shows the value of the first quartile for each sample size and ratio. Note: One model (out of 9,000) failed for a sample size of 10 with a 20:80 ratio. Therefore, some tiles may represent more than 100 models if the same sample size was evaluated in multiple rounds, or fewer if a metric could not be calculated. The colour gradients from light to dark represent increasing performance within the thresholds for moderate (blue) and excellent models (orange). For **(A–C,E)** moderate performance was defined as 0.695–0.794 to three decimal points and excellent performance at 0.795–0.894 for PCC, sensitivity, specificity, and AUC, respectively. For Cohen's Kappa in **(D)** moderate performance was defined as 0.405–0.604 and excellent performance as 0.605–0.804. Performance below the moderate thresholds was coloured white and above the excellent thresholds, purple.

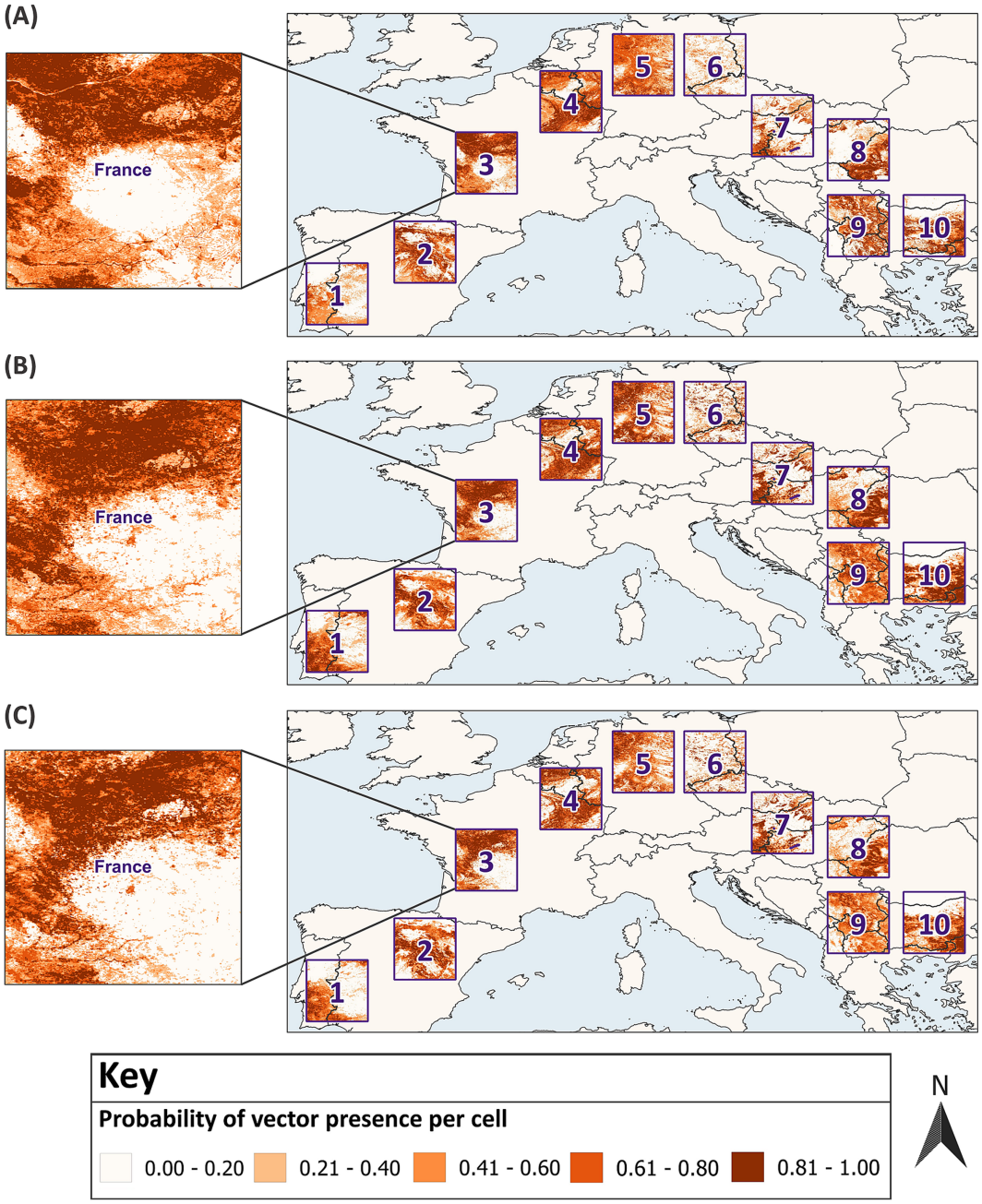


FIGURE 8
Model predictions developed with the optimum sample size compared to the virtual vector's known distribution. In **(A)** the actual distribution of the virtual vector in 10 test sites across Europe is displayed against one model's predictions which were developed with **(B)** a balanced 50:50 sample ratio and a sample size of 750 and **(C)** an unbalanced 40:60 sample ratio and a sample size of 1,100. Each map displays increasing probabilities of presence per raster cell, categorised into five classes using equal intervals. GADM Level 0 country boundaries were utilised (79).

## 4.1 Sample size for Random Forest models

An optimum sample size facilitates robust predictions for vector distributions while minimising the cost of excessive field sampling and computational processing. Many studies have demonstrated improvements in RF model performance with increasing sample size (18, 29, 33, 42–47). However, it is important to consider that these studies used different definitions for sample size when comparing results. Sample size was defined as either the number of presences in the training subset (18, 29, 33, 42, 43, 47) or the number of presences and absences in the training subset (44–46). To ensure our findings could inform field sampling, we defined sample size as the number of presences and absences in the dataset, prior to partitioning.

Our estimates for the optimum sample size were lower than two studies which quantified the effect of sample size on RF models at a similar scale (18, 46). Liu et al. (46) used field data for an intermediate host and reported an optimum sample size of 2,400 presence and absence points in the training subset, with an optimum sample ratio of 1:2 (equivalent to 33:66). Since they used an 80:20 ratio to partition

their samples into training and test subsets respectively, their estimate equates to 3,000 presence and absence points overall. Their sample ratio is closest to the 40:60 ratio considered in this study, but their estimates are nearly three times greater than our optimum sample size of 1,100–1,300. However, their models also performed better at smaller sample sizes. A sample size of 100 with their optimum ratio (first quartile = 0.894, median = 0.917) far exceeded our AUC thresholds for excellent performance (46), which differs from our findings. Since virtual vectors are a simplification of reality, estimates for the optimum sample size are expected to increase since real host and vector distributions have more complex responses to covariates (38). However, differing methodology is the most likely reason for the different estimates for the optimum sample size. Liu et al. (46) determined the optimum sample size by identifying a point at which there was a significantly small increase in AUC and sensitivity began to decrease.

On the other hand, improvements in model performance at small sample sizes likely reflect different environmental preferences. Compared to generalised species, a sample is more likely to capture the environmental conditions associated with a specialised vector occupying a smaller environmental domain, even at smaller sample sizes. This would improve predictive accuracy (32, 44). The clustered distribution of a vector due to specific environmental preferences should not to be confused with geographical clusters resulting from oversampling an area (18). Furthermore, Liu et al. (46) used a resolution of 100 m and model performance can also improve due to increased spatial accuracy, precise locations and greater delineation of habitats compared to our 1 km resolution (34, 36, 70).

Hendrickx et al. (18) used historical data for parasitic eggs in a host, as a proxy for VBD risk. They reported a minimum sample size of 1,516 (758 presence and absences each in the training subset), below which model performance rapidly deteriorated (18). The objectives to identify a minimum sample size differ from this study and thus, estimates for an optimum sample size would likely increase from 1,516, given that our findings suggested there was notable deterioration in model performance at a sample size of 100. Since their historical dataset was susceptible to geographical clustering and bias towards symptomatic cases (18), higher estimates are to be expected when predicting disease distribution in a host compared to the distribution of the virtual vector.

Considering the wider literature for RF models, our findings are more aligned with the estimates of 400–900 by Shiroyama et al. (45) and 592 by Tessarolo et al. (44). Both consider the number of presence and absences in the training subset. For balanced ratios, our estimates equate to 525–700 presence and absences in the training subset. However, both studies have differing methodologies. Tessarolo et al. (44) used historical datasets for 34 endemic terrestrial species and the estimates were reported for an ensemble of algorithms, which can perform better than a single algorithm (47, 71). Since Shiroyama et al. (45) predicted the distribution of a freshwater fish, the covariates and sampling methods are too disparate for reliable comparisons. Hanberry et al. (33) also reported an optimum sample size of 500 presences in the training subset but these estimates were obtained with a 4:1 ratio of presences and pseudo-absences (equivalent to 80:20), which was not considered in this study. Due to the scarcity of similar studies evaluating the effect of sample size on SDMs developed with RF, it was challenging to contextualise these findings with confidence due to differing model designs, data characteristics and species (Supplementary Tables 3, 4). While an extent of 10,000 $km^2$ was a somewhat arbitrary cut-off, future research could consider accounting for these methodological differences in a meta-analysis. However, this may be a challenge without detailed, transparent methods reported in standardised protocols, such as ODMAP (48).

## 4.2 Poor model performance

Our findings indicate that sample sizes below 100 produced inaccurate models. Hanberry et al. (33) reported poor performance for models trained on fewer than 200 presence points, while Shiroyama et al. (45) noted a significant decrease in AUC below 100 presence and absence points in the training subset. At these sample sizes, training subsets are unlikely to be representative of the vector's actual distribution. Broad geographic coverage and a representative range of environmental conditions in which the vector is present are key factors for accurate SDMs (44). The partition ratio may be a contributing factor since this determines the size of the training subset (which influences model accuracy) and the size of the test subset (which influences the risk of evaluation error). At partitioning, 70% of the dataset was assigned to the training subset and 30% to the test subset. This approach was taken to ensure there was sufficient data in the test subset to evaluate model performance at smaller sample sizes, reducing the risk of spurious results. This partitioning ratio has also been applied in other SDMs (72, 73). However, alternate approaches like k-fold partitioning may be more appropriate since this averages the results from several partitions and is less dependent on a single partition (68).

## 4.3 Performance by sample ratio

Between sample ratios, performance improved as the proportion of presence points increased. Other studies have reported similar findings (43), with balanced sample ratios producing the most accurate RF models (29, 47, 69). Poor model performance at small sample sizes was exacerbated for samples with a 20:80 ratio, as demonstrated by the large interquartile ranges which reflect varying performance between replicates (Supplementary Figure 2). RF can model complex, non-linear interactions between vector distribution and covariates, but becomes more susceptible to noise with less agreement between replicates at small sample sizes, particularly when the proportion of presence points decreases (29). Sample ratios with a greater proportion of absence points also performed better when evaluated by specificity, but this reflects bias towards the more prevalent class (presence or absence) on which the model was trained. Poor performance with unbalanced sample ratios is not restricted to RF, this is a well-known behaviour within machine learning, whereby models are sensitive to the majority class (presence or absence) (55).

Given the poor performance below a sample size of 100, particularly for unbalanced sample ratios, RF may not be suitable for modelling rare vectors. Maximum Entropy (MaxEnt) may be more appropriate since studies have reported reliable performance with this presence-background algorithm from sample sizes as low as three to 300 (32, 36–38, 40, 42). Conversely, several studies have also considered corrective methods for RF, such as down-sampling, to

mitigate the effects of unbalanced sample ratios and improve model performance (55, 73). It is worth noting that the spatial presentation of model predictions (Figure 8; Supplementary Figures 4–9) showed indications of model overfitting with poor discrimination towards the southeast of the test site. While complex algorithms such as RF are prone to overfitting (44, 74), further research is warranted to determine if model accuracy at these smaller sample sizes and unbalanced ratios could be first improved by optimising model design.

## 4.4 Limitations

No model will ever be 100% accurate since SDMs are sensitive to data quality, assumptions and model design. SDMs are built on the principle that a vector has a specific environmental tolerance and therefore assumes that the abiotic covariates sufficiently describe its distribution in order to make predictions (27). During quality checks, an unexpected trend was observed: test sites 2, 5 and 9 frequently produced the least accurate models per sample size, while test sites 3, 6 and 7 produced the most accurate models per sample size. There was no apparent correlation with the ROA or geographical clustering of the vector's actual or sampled distribution. To be ecologically appropriate, correlative SDMs depend on both the appropriate selection of covariates and samples which reflect the range of environmental conditions that the vector occupies (17, 44). For the former, if the selected covariates do not account for a species response to environmental gradients, model performance may deteriorate. The varied performance between test sites may be explained by different responses (abrupt versus smooth). Humidity and precipitation are two key climatic factors which can influence vector distribution (52), while land use is a key environmental variable (6, 52). These covariates were not included, which may merit further investigation to determine if their inclusion improves performance, but this was beyond the scope of this study. Our aim was to provide a general rule of thumb for the optimum sample size by grouping models across various test sites with differing habitat suitability and ROA. This approach ensures that any limitations in covariate selection were consistent across all sample sizes.

Climatic bias can reduce predictive accuracy if samples have restricted environmental variation and do not reflect the range of conditions that the vector inhabits. As such, Tessarolo et al. (44) argue that environmental coverage is more important than geographical coverage. Random sampling was among the least climatically biased designs when comparing seven sampling strategies (44). Although model performance varied little across the sampling strategie, biased sampling is expected to reduce accuracy to a greater extent for widespread, generalised species since these approaches are less likely to capture the range of environmental conditions that a vector inhabits, particularly at smaller sample sizes (44). While random sampling from a simulated vector with a fully known distribution should minimise this risk, future research should consider stratified random sampling along different environmental gradients. Random sampling minimises the risk of geographically clustered samples, but this is an idealised condition. For real vectors, not all locations are appropriate or accessible, and random sampling across large areas can be resource intensive. With a virtual vector, it is anticipated that our results would not differ significantly between stratified random sampling and random sampling. The influence of sampling strategy on model performance does constitute a separate research objective, but ensuring samples are both environmentally and geographically representative is an important consideration when applying our findings to real-world scenarios.

The optimum sample size was determined by thresholds for excellent model performance. While the five metrics are commonly used to evaluate SDMs, their thresholds can be subjective. Similar thresholds for PCC, sensitivity and specificity have been used in other studies (75, 76). However, Jiménez-Valverde et al. (28) caution that values of 0.6 can be obtained for Cohen's Kappa by under- or overpredicting by 40% for a species with an ROA of 50%. Our thresholds for Cohen's Kappa were based on those proposed by Landis and Koch (66), but McHugh (59) advocates for a more stringent criteria, suggesting that values between 0.60–0.79 actually represent moderate model performance, while values between 0.80–0.90 reflect strong model performance. This threshold for Cohen's Kappa would impact our estimates since no model reached values between 0.80–0.90 for any sample size or ratio (Figure 7D). An alternative approach would involve calculating quartile deviations and determining the statistical significance of the differences between sample sizes (46). This approach would have also verified whether the point of diminishing returns observed between 1,500–2,000 and the model deterioration below a sample size of 100 truly reflected statistically marginal improvements in performance and a minimum sample size, respectively.

While emphasis was placed on thresholds for excellent model performance, reliable models may be developed with smaller sample sizes which meet the thresholds for moderate performance. Ultimately, the value of a model depends on the research objective and smaller sample sizes may still provide useful information if the intention is to explore areas with limited information on vector distribution or to prioritise field sampling of rare species (36, 42). To confirm the value of models at smaller sample sizes, future research should consider comparing the spatial distribution of incorrect predictions for vector distribution at smaller sample sizes to predictions developed with the optimum sample size. If inaccurate predictions are spread equally across the test site, rather than concentrated areas, models at smaller sample sizes would still provide useful information.

## 4.5 Potential impact

Model predictions act as a static risk map which can theoretically guide field sampling to locations with the highest probability of vector presence. In line with ECDC recommendations for SDMs, estimates for an optimum sample size provide a framework for strategic sampling, optimising the use of limited resources to both validate model predictions and improve the surveillance of vectors and their diseases (26). Our results can help researchers determine how many samples are needed for reliable models. Since abiotic variables are used to predict vector distribution, these results are most applicable to vectors that have strong associations with climatic and environmental factors. This may include established populations of both native and invasive vectors of significance to human health, animal health and food security, provided there are at least 375 presence and absence records each (for balanced ratios) in a test site.

Table 4 illustrates how the optimum sample size can guide field sampling, based on the expected probability of vector presence in a test site. However, caution is advised since confidence intervals have not been calculated for Table 4 which account for uncertainty and the sensitivity of sampling methods. The transferability of these findings to real vectors also warrants further investigation. Vector datasets are often collected without

TABLE 4 Required number of field samples to obtain the optimum number of presences for a balanced sample ratio (50:50).

| Sample size | | Vector probability of presence | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Total | Presences | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 |
| 500 | 250 | 2,500 | 1,250 | 833 | 625 | 500 | 417 | 357 | 313 | 278 |
| 600 | 300 | 3,000 | 1,500 | 1,000 | 750 | 600 | 500 | 429 | 375 | 333 |
| 700 | 350 | 3,500 | 1,750 | 1,167 | 875 | 700 | 583 | 500 | 438 | 389 |
| 750 | 375 | 3,750 | 1,875 | 1,250 | 938 | 750 | 625 | 536 | 469 | 417 |
| 800 | 400 | 4,000 | 2,000 | 1,333 | 1,000 | 800 | 667 | 571 | 500 | 444 |
| 900 | 450 | 4,500 | 2,250 | 1,500 | 1,125 | 900 | 750 | 643 | 563 | 500 |
| 1,000 | 500 | 5,000 | 2,500 | 1,667 | 1,250 | 1,000 | 833 | 714 | 625 | 556 |
| 1,100 | 550 | 5,500 | 2,750 | 1,833 | 1,375 | 1,100 | 917 | 786 | 688 | 611 |
| 1,200 | 600 | 6,000 | 3,000 | 2,000 | 1,500 | 1,200 | 1,000 | 857 | 750 | 667 |
| 1,300 | 650 | 6,500 | 3,250 | 2,167 | 1,625 | 1,300 | 1,083 | 929 | 813 | 722 |

With an optimum sample size of 750–1,000 for a balanced sample ratio, 375–500 presences are required for excellent model performance (highlighted in yellow). Based on the expected probability of vector presence in the field, the number of samples which need to be collected will differ (calculated by dividing the number of required presence samples by the expected probability of vector presence).

standardised sampling protocols and reliably sampling absence data in the field is challenging (16). If less sensitive sampling methods are employed, it may be necessary to collect a greater number of field samples to achieve the optimum number of presence and absences required for modelling. Our results offer general guidance for vector sampling programmes, but additional factors such as funding, resource availability, and time constraints must also be considered. Since historical datasets may contain only presence records, and are susceptible to opportunistic sampling targeting areas with reported disease or easily accessible locations, such as roads (36, 77), our optimum sample size estimates will likely increase for real vectors. While RF is fairly robust to convenience samples, Kessler et al. (78) caution against their use since model predictions for tick distributions were not sufficiently accurate for detailed decision making.

SDMs ultimately depend on high-quality data and as outlined in the introduction, field sampling is costly and labour-intensive, particularly when collecting reliable absence data (16). While sufficient and comprehensive records on vector distribution are essential, model predictions can subsequently reduce costs by identifying strategic locations for future vector sampling programmes. As proposed by Lippi et al. (51), SDMs can be considered within a cyclical and iterative workflow. The increasing availability of vector presence and absence samples helps validate existing models, improves their predictive accuracy, and generates new risk maps for vector distribution. This, in turn, facilitates informed action by decision-makers and guides strategic sampling for new surveillance data.

## 5 Conclusion

We sought to evaluate the effect of sample size on model performance and to determine the optimum sample size for reliable Random Forest models which predict arthropod vector distribution. To the best of our knowledge, this is the first study which used a virtual vector and presence-absence data at this scale. A virtual vector overcomes limitations in data quality and confounding factors compared to field and historical datasets, facilitating evaluation with greater certainty. The optimum sample size estimates ranged from 750–1,000 for balanced samples and increased to 1,100–1,300 for samples with a 40:60 ratio of presence and absence points. Samples with a 20:80 ratio consistently produced unreliable models. Considering that the ROA and proportion of presence points in a sample have a large influence on model performance (44), accounting for these factors across 10 different test sites and three sample ratios was beneficial. Failure to consider the combined effects of factors may result in misleading conclusions. Since machine learning models vary slightly each time they are run (49), researchers should consider replicating models for stacked predictions, particularly when working with smaller sample sizes. While the optimum sample size may vary with different models, species and data characteristics, further research may first seek to refine or lower these estimates through optimised model design before determining how the optimum sample size differs for real vectors. Due to difficulties reliably sampling absence data in the field, it may be worthwhile investigating the effect of sample size on ratios with a greater proportion of presence points.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

LM: Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. GH: Conceptualization, Methodology, Resources, Supervision, Writing – review & editing. EM: Supervision, Writing – review & editing. CM: Conceptualization, Data curation, Methodology, Resources, Supervision, Writing – review & editing.

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## 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.

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The authors declare that no Gen AI was used in the creation of this manuscript.

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# Seroprevalence study for selected zoonotic vector-borne pathogens in sheep from endemic areas of Croatia

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Surveillance is crucial in controlling and preventing vector-borne zoonotic diseases (VBDs). We analyzed the seroprevalence of selected vector-borne zoonotic pathogens in sheep from endemic areas and their role as possible sentinels for VBDs. A total of 300 sheep from seven farms at three micro-locations were tested for the presence of IgG antibodies against tick-borne encephalitis virus (TBEV), West Nile virus (WNV), Usutu virus (USUV), *Borrelia burgdorferi* s.l., and Crimean-Congo hemorrhagic fever virus (CCHFV) using ELISA with confirmation of borderline/positive results by VNT. Seropositivity for at least one pathogen was observed in 18.0% (54/300) of sheep. The highest seroprevalence was confirmed for TBEV (9.7%; 29/300), followed by WNV (3.0%; 9/300) and *B. burgdorferi* s.l. (2.7%; 8/300), while USUV and inconclusive flavivirus (TBEV/WNV/USUV) infections had the same seroprevalence of 1.3% (4/300). None of the serum samples tested positive for CCHFV. Geographic micro-location was a significant risk factor for USUV ($p = 0.045$), TBEV ($p = 0.03$), and *B. burgdorferi* s.l. ($p = 0.015$) infections, but not for WNV. The farm distance from the household (TBEV $p < 0.001$, *B. burgdorferi* s.l. $p = 0.005$) and sheep breed (TBEV $p < 0.001$, *B. burgdorferi* s.l. $p < 0.001$) were found as risk factors for seropositivity to tick-borne (TBEV, *B. burgdorferi* s.l.), but not to mosquito-borne diseases (WNV, USUV). Of the other risk factors, sheep shearing was statistically significant, with unshared sheep showing a higher probability of tick-borne diseases ($p = 0.048$). Sex, age, herd size, and the presence of clinical signs were not associated with the seroprevalence. Serologic evidence of VBDs suggests their sentinel potential for mapping micro-foci of zoonotic pathogens' activity and identifying high-risk areas for public health. Further studies are needed to confirm this observation.

KEYWORDS

**tick-borne encephalitis virus, *Borrelia burgdorferi* s.l., West Nile virus, Usutu virus, Crimean-Congo hemorrhagic fever virus, sentinels**

# 1 Introduction

Zoonotic vector-borne diseases (VBDs) are becoming an increasing public health problem in many regions of the world (1). VBDs represent more than 17% of all infectious diseases, causing more than 700,000 human deaths annually, with the highest disease burden in tropical and subtropical areas. The spread of VBDs has been facilitated by different factors, including global travel and trade, unplanned urbanization, climate change, and the vectors' adaptation and spread (2).

Among vector-borne pathogens, *Borrelia burgdorferi* s.l. (Lyme disease; LD) and tick-borne encephalitis virus (TBEV) are most widely distributed in Europe (1). Other flaviviruses, such as West Nile virus (WNV) and Usutu virus (USUV), are also endemic in many European countries, causing outbreaks (WNV) or sporadic infections (USUV) in humans (3). In addition, several epidemics of Crimean-Congo hemorrhagic fever (CCHF) have occurred in EU/EEA neighboring countries since 2013, including the Balkan region, Russia, and Turkey (4).

Since zoonotic VBDs are spreading rapidly, surveillance is crucial in their prevention and control. To define priorities for developing integrated surveillance systems that accurately model and predict the human risk of VBDs, it is important to understand the practical options of connecting the surveillance data of both animals and humans (5). As clinical cases of emerging diseases in humans usually indicate a widespread of zoonotic pathogens in an area, various animal surveillance models are used as an early warning system, yielding valuable results. Animal surveillance can address the significant public health challenge of gathering information on the introduction and emergence of new pathogens in a given area, a prerequisite for an effective response to protect human health (6–8).

Different animal species have been tested as sentinels for VBDs, including wild birds (WNV and USUV) (9), horses (TBEV, WNV, and USUV) (10), poultry (WNV and USUV) (11), and dogs (TBEV and WNV) (12). Captive and free-ranging birds have been used for WNV surveillance for decades. As primary WNV reservoirs, infections in birds occurred more frequently than in humans and horses (13). In addition, chickens (*Gallus gallus domesticus*) have been routinely used for arbovirus surveillance and monitoring in different settings and on different continents. After infection, chickens do not exhibit any clinical signs but produce neutralizing antibodies (11). The use of sentinel chickens seems to be a more sensitive indicator of virus activity when compared with the detection of seroconversion in wild birds (14). While clinically apparent WNV infections in horses are rarely observed, seroprevalence studies in horses may allow the tracing of flavivirus transmission and help to estimate the risk for human infections (10). Whereas some studies provided evidence that dogs could be useful sentinels for WNV monitoring (15), others indicated that the role of dogs and horses in the early detection of human cases is debatable (16).

However, studies that use sheep as sentinels to predict human risk are limited. A study conducted in Germany has shown that seroprevalence in free-ranging animals, particularly in sheep and goats, can be a useful additional tool to identify TBEV foci in both endemic and non-endemic areas (17). Very few studies have analyzed WNV infection in sheep, with no data on USUV (18). Sheep and cattle became infected with the CCHFV in experimental inoculations but developed only mild and transient fever. The viremia duration and level are usually low, with detectable antibodies shortly after cessation of viremia (19). Although CCHF in domestic ruminants is typically subclinical, there is some evidence that they become reservoirs and can be used as sentinels for the circulation of CCHFV, especially in non-endemic areas (20).

In Croatia, *B. burgdorferi* s.l., TBEV, WNV, and USUV are the most commonly detected vector-borne zoonotic pathogens, all endemic in continental regions (21). The reported number of LD varies from 400 to 800. In addition, TBE is continuously recorded with a bimodal seasonality (April–August and October–November). The LD and TBE endemicity is highest in northwestern and eastern counties between the Sava and Drava rivers (21, 22). WNV infections in humans, horses, and poultry have been continuously reported in Croatia since 2012 (21), while USUV infections were detected sporadically in humans (2013, 2018, 2024) and birds (2018, 2022) (23–25). CCHFV has not been detected in Croatia so far.

The multidisciplinary approach enables the early detection of an increase in pathogen activity of VBDs or confirmation of the emergence of a new pathogen in a given area, as has also been observed in Croatia in recent years (6, 7). Chickens and horses were used as sentinels to detect seasonal WNV incursions in Croatia, revealing a significant correlation between the geographical distribution of high WNV seroprevalence in tested animals and human WNV infections (26). However, data on the sheep are limited.

This study aimed to analyze the seroprevalence of selected vector-borne zoonotic pathogens in sheep from endemic areas in eastern Croatia and assess the potential role of sheep as sentinels of VBDs.

# 2 Materials and methods

In this study, 300 sheep from Vukovar-Srijem County, the easternmost region of Croatia, were tested for the presence of IgG antibodies against TBEV, WNV, USUV, *B. burgdorferi* s.l., and CCHFV. Blood samples were collected from animals from seven farms at three micro-locations: four in Borovo, one in Vukovar, and two in Trpinja (Figure 1).

Sample size calculations were performed using the RiBESS+ tool developed by the European Food Safety Authority (27). The calculation incorporated the official sheep population data for Vukovar-Srijem County, the reported sensitivity of the enzyme-linked immunoassay (ELISA) used, and an assumed seroprevalence of 1%, reflecting the absence of previously documented USUV infections in sheep.

Serum samples were collected in the second half of April to obtain reliable data of the epidemiological status before the onset of peak vector activity.

Epidemiological and clinical data of the tested sheep are presented in Table 1. Clinical signs were assessed both at the time of sampling and retrospectively based on owner-reported observations over the preceding 12 months. The following categories were evaluated: neurological, respiratory, reproductive, gastrointestinal, dermatological signs, and lameness. Information regarding any additional observed clinical signs was also collected. Clinical examinations performed during sampling across all seven farms revealed no detectable clinical abnormalities. However, according to the owner of Farm 1, located in Borovo, episodes of dermatitis affecting all animals were reported during the winter (approximately 3 months prior), characterized by skin erythema and partial wool loss.

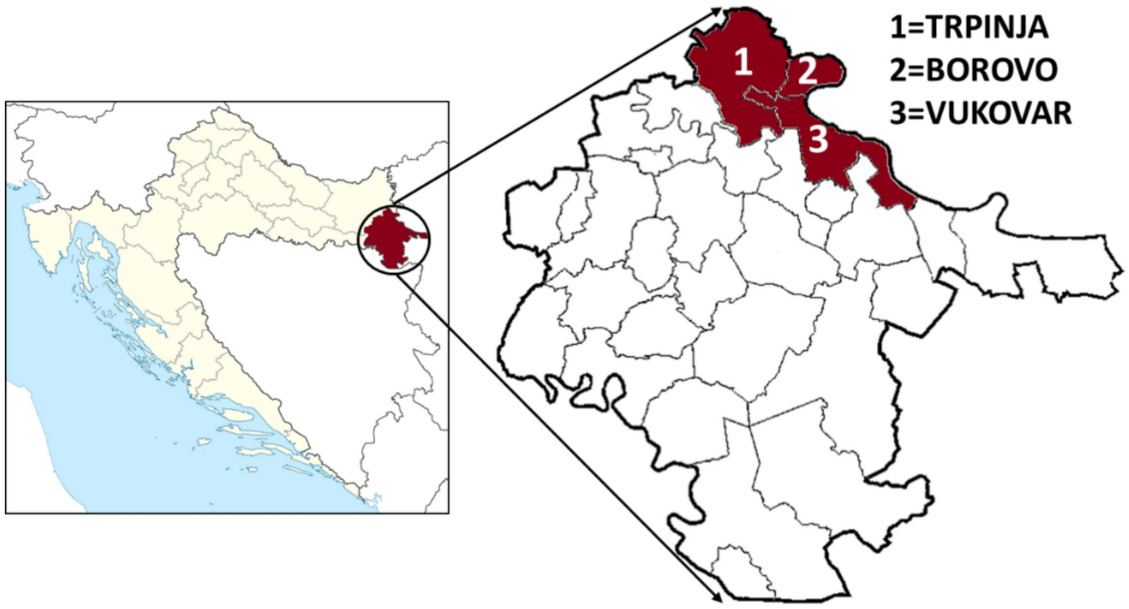


FIGURE 1
Sheep sampling area in Croatia (Vukovar-Srijem County) with three micro-locations.

TABLE 1 Epidemiological data on sheep farms from Vukovar-Srijem County included in the study.

| Characteristic | Vukovar | Trpinja | | Borovo | | | |
|---|---|---|---|---|---|---|---|
| | Farm 1 | Farm 1 | Farm 2 | Farm 1 | Farm 2 | Farm 3 | Farm 4 |
| Flock size | 100 | 286 | 50 | 50 | 10 | 150 | 70 |
| % of flock tested | 73.0 | 28.0 | 40.0 | 64.0 | 40.0 | 33.3 | 57.1 |
| Tested females/males | 73/0 | 79/1 | 20/0 | 32/1 | 4/0 | 47/3 | 38/2 |
| Breed | Romanov | Merino | Merino | Tsigai | Merino | Tsigai | Romanov |
| Average age and range (years) | 3.8 (1.1–4.8) | 4.4 (1.4–12.3) | 6.2 (2.1–9.2) | 4.1 (1.1–8.2) | 6.2 (5.6–6.6) | 4.0 (2.2–4.9) | 3.5 (2.3–5.0) |
| Observed clinical signs | No | No | No | Yes | No | No | No |
| Water body type | Stream | River | Stream | River | River | River | River |
| Distance to water (km) | 0.02 | 10.0 | 0.2 | 0.1 | 0.2 | 0.1 | 0.3 |
| Distance to households (km) | 0.5 | 0.05 | 0.05 | 2.0 | 0.05 | 1.5 | 0.02 |
| Shearing performed | No | Yes | Yes | No | No | No | No |

These lesions resolved spontaneously without the administration of any treatment.

All serum samples were screened using an ELISA for the detection of flavivirus antibodies (WNV, USUV, and TBEV) with the ID Screen® Flavivirus Competition (Innovative Diagnostics, Grabels, France). Samples with borderline or positive ELISA results were additionally tested by a virus neutralization test (VNT), as described by Ilic et al. (28), to exclude cross-reactivity and confirm infection with a specific flavivirus. A VNT titer of ≥10 was considered a positive result. In the case of positive VNT results with more than one flavivirus, the pathogen with an antibody titer at least fourfold higher than the others was considered to be the causative agent. Samples with less than fourfold titer differences using different pathogens in VNT were considered inconclusive, classified as flavivirus-positive, and excluded from further risk factor analysis for a particular disease or group of diseases.

The ID Screen® Borreliosis Double Antigen Multi-species ELISA (Innovative Diagnostics, Grabels, France) was used for the detection of antibodies against *B. burgdorferi* s.l., and the ID Screen® CCHF Double Antigen Multi-species ELISA (Innovative Diagnostics, Grabels, France) for CCHFV antibodies.

All commercial ELISA assays were performed according to the manufacturer's instructions.

Epidemiological data were systematically collected for each animal to assess possible risk factors associated with arboviral infections. The analyzed parameters included farm location, flock size, breed, sex, age, clinical signs, type of water bodies, and distance to water bodies and households. Risk factor analyses were conducted for

each VBD, with additional analyses for tick-borne infections (TBEV, CCHFV, and *B. burgdorferi* s.l.) and mosquito-borne infections (WNV and USUV) due to epidemiological differences.

Statistical analyses were performed using Statistica v.14 (TIBCO Software Inc., 2020), Medcalc Odds Ratio Calculator v.23 (MedCalc Software Ltd., 2025), and R 4.4.0 (R Core Team, Vienna, Austria, 2024). Descriptive statistics are presented as numbers and percentages. Odds ratios (OR) with 95% confidence intervals (95% CI) were used for single bivariate risk factors. Logistic regression analysis was used to calculate ORs for numerical and multivariate risk factors, while ANOVA type II was used to assess the overall influence of the whole variable. When complete separation occurred for multivariate variables (cells with 0), Firth correction was applied using the logistf package. The relation between variables was analyzed using Pearson's correlation coefficient ($r$; numerical variables) and Cramér's $V$ ($V$; nominal variables). Differences were considered significant at $p < 0.05$.

## 3 Results

Out of 300 tested sheep serum samples, 54 (18.0%) were positive for at least one vector-borne zoonotic pathogen (Table 2). The highest seroprevalence was confirmed for TBEV (9.7%). The seroprevalence of WNV was 3.0% *B. burgdorferi* s.l. 2.7%, while USUV and inconclusive flavivirus (TBEV/WNV/USUV) infections had the same seroprevalence of 1.3%. None of the serum samples tested positive for CCHFV.

TBEV seropositivity was confirmed at all micro-locations in six out of seven farms, with seroprevalence rates from 5.0 to 15.0%. WNV-positive animals were confirmed at all micro-locations on five of the seven farms, with seroprevalence ranging from 1.4 to 3.9%. The lowest seroprevalence was found for USUV, with only four positive animals confirmed on two farms at two micro-locations, with a seroprevalence of 4.1 and 1.0%, respectively (Table 2). Four samples (1.3%) were considered as flavivirus-positive due to cross-reactivity. Cross-reactivity between WNV and TBEV was confirmed in three samples and between USUV and TBEV in one sample (Table 2). Serological evidence of *B. burgdorferi* s.l. infection was confirmed in 8 animals (2.7%) at two micro-locations with seroprevalence rates of 1.0 and 5.5%, respectively.

Location as a risk factor was significant for USUV, TBEV and *B. burgdorferi* s.l. infections, but not for WNV. For mosquito-borne diseases, location had no significant influence, while it was found as a risk factor for tick-borne diseases (ANOVA $p = 0.006$) (Table 3).

When analyzing the risk between micro-locations, Borovo was selected as a reference category, as the highest number of animals was tested there. No significant association between micro-location and WNV or USUV seropositivity was found at the location Trpinja, as was the case for a group of tick-borne diseases. In contrast, on the same location, the probability of TBEV infection ($p = 0.02$) and tick-borne diseases overall ($p = 0.01$) was significantly lower and a borderline reduction in the risk of *B. burgdorferi* s.l. infection was also observed ($p = 0.07$). In contrast, in Vukovar, we observed a significant increase in the risk of USUV infections ($p = 0.03$), a decrease in *B. burgdorferi* s.l. seropositivity ($p = 0.04$) as well as lower risk of tick-borne diseases ($p = 0.03$) (Table 3).

The type of water body and the distance of the farm from it as risk factors had no significant influence on the seroprevalence of any VBDs or a group of diseases (Table 3).

The distance of the farm from the household was found as an important risk factor for seropositivity to TBEV ($p < 0.001$), *B. burgdorferi* s.l. ($p = 0.005$) and accordingly also for tick-borne diseases ($p < 0.001$), but not for WNV, USUV, and mosquito-borne infections (Table 3). To exclude the influence of location on these results because of just one farm analyzed at Vukovar micro-location and two at Trpinja, the same findings were analyzed for the four farms in Borovo micro-location, and distance to households was confirmed as a significant risk factor also on the same location at the farm level ($p = 0.01$).

TABLE 2 Seroprevalence of sheep to vector-borne zoonoses by micro-location and farm in Croatia (*N*, %).

| Location | Farm | Number of tested animals | WNV | USUV | TBEV | Flavivirus | *B. burgdorferi* s.l. | Mosquito-borne pathogens | Tick-borne pathogens | Positive to at least one pathogen |
|---|---|---|---|---|---|---|---|---|---|---|
| Vukovar | Total | 73 | 1 (1.4) | 3 (4.1) | 5 (6.9) | 0 (0.0) | 0 (0.0) | 4 (5.5) | 5 (6.8) | 9 (12.3) |
| Trpinja | Farm 1 | 80 | 3 (3.8) | 1 (1.3) | 4 (5.0) | 1 (1.3) | 1 (1.3) | 4 (5.0)* | 5 (6.3)* | 10 (12.5) |
| | Farm 2 | 20 | 0 (0.0) | 0 (0.0) | 1 (5.0) | 1 (5.0) | 0 (0.0) | 0 (0.0)* | 1 (5.0)* | 2 (10.0) |
| | Total | 100 | 3 (3.0) | 1 (1.0) | 5 (5.0) | 2 (2.0) | 1 (1.0) | 4 (4.0) | 6 (6.0) | 12 (12.0) |
| Borovo | Farm 1 | 33 | 1 (3.0) | 0 (0.0) | 6 (18.2) | 1 (3.0) | 2 (6.1) | 1 (3.0)* | 8 (24.2)* | 10 (30.3) |
| | Farm 2 | 4 | 2 (NA) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (NA) | 0 (0.0) | 2 (NA) |
| | Farm 3 | 50 | 2 (4.0) | 0 (0.0) | 11 (22.0) | 1 (2.0) | 5 (10.0) | 2 (4.0)* | 16 (32.0)* | 19 (38.0) |
| | Farm 4 | 40 | 0 (0.0) | 0 (0.0) | 2 (5.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (5.0) | 2 (5.0) |
| | Total | 127 | 5 (3.9) | 0 (0.0) | 19 (15.0) | 2 (1.6) | 7 (5.5) | 5 (3.9) | 26 (20.5) | 33 (26.0) |
| Total | | 300 | 9 (3.0) | 4 (1.3) | 29 (9.7) | 4 (1.3) | 8 (2.7) | 13 (4.3) | 37 (12.3) | 54 (18.0) |

All samples tested negative to Crimean-Congo hemorrhagic fever virus. *Numbers of positive animals without four flavivirus (TBEV/WNV/USUV)-positive animals, in which it was not possible to determine the pathogen; NA—insufficient sample size of tested animals for farm-level analysis; WNV, West Nile virus; USUV, Usutu virus; TBEV, tick-borne encephalitis virus; mosquito-borne pathogens: WNV, USUV; tick-borne pathogens: TBEV, *B. burgdorferi* s.l.

TABLE 3 Seroprevalence of vector-borne zoonoses in sheep according to geographic and environmental risk factors in Croatia (OR, 95% CI, *p*).

| Risk factor | | WNV | USUV | TBEV | *B. burgdorferi* s.l. | Mosquito-borne pathogens | Tick-borne pathogens |
|---|---|---|---|---|---|---|---|
| Location | Borovo* | $p = 0.54$ | **p = 0.045** | **p = 0.03** | **p = 0.015** | $p = 0.878$ | **p = 0.006** |
| | Trpinja | 0.76 (0.15–3.17) $p = 0.71$ | 3.86 (0.2–566.03) $p = 0.37$ | **0.3 (0.1–0.78) p = 0.02** | 0.24 (0.03–1.13) $p = 0.07$ | 1.02 (0.25–3.96) $p = 0.98$ | **0.29 (0.1–0.7) p = 0.01** |
| | Vukovar | 0.33 (0.02–2.12) $p = 0.32$ | **12.46 (1.18–1684.65) p = 0.03** | 0.41 (0.13–1.08) $p = 0.09$ | **0.11 (0.001–0.92) p = 0.04** | 1.39 (0.33–5.43) $p = 0.63$ | **0.33 (0.11–0.84) p = 0.03** |
| Water bodies | | 0.27 (0.03–2.18) $p = 0.22$ | 6.84 (0.7–66.69) $p = 0.098$ | 0.55 (0.22–1.4) $p = 0.21$ | 0.13 (0.01–2.2) $p = 0.16$ | 0.98 (0.3–3.28) $p = 0.98$ | 0.44 (0.18–1.1) $p = 0.08$ |
| Distance from water bodies (km) | | 1.034 (0.88–1.19) $p = 0.65$ | 0.99 (0.72–1.22) $p = 0.91$ | 0.91 (0.80–1.01) $p = 0.104$ | 0.91 (0.67–1.08) $p = 0.37$ | 1.02 (0.89–1.15) $p = 0.75$ | 0.92 (0.82–1.01) $p = 0.097$ |
| Distance from household (km) | | 1.07 (0.38–2.55) $p = 0.89$ | 0.58 (0.05–2.46) $p = 0.54$ | **2.50 (1.52–4.18) p < 0.001** | **4.50 (1.73–15.22) p = 0.005** | 0.90 (0.36–1.94) $p = 0.81$ | **2.74 (1.71–4.45) p < 0.001** |

*Reference category in the logistic regression; the ANOVA *p*-value represents the influence of location; WNV, West Nile virus; USUV, Usutu virus; TBEV, tick-borne encephalitis virus; mosquito-borne pathogens: WNV/USUV; tick-borne pathogens: TBEV/*B. burgdorferi* s.l. Statistically significant differences are bold.

When analyzing differences in VBDs seroprevalence at the farm level, a significant impact on seroprevalence was confirmed ($p = 0.002$), but as only animals from one farm at the Vukovar micro-location were tested, a close association between farm and location ($V = 1$) was confirmed, so we do not present the results.

The sheep breed was confirmed as an important risk factor with a significantly higher probability of infections with TBEV ($p = 0.003$), *B. burgdorferi* s.l. ($p = 0.002$) and tick-borne pathogens ($p < 0.001$) in Tsigai sheep than in Romanov sheep, which were used as the reference category. The seroprevalence rates in Merinolandschaf sheep were not significantly different (Table 4). When analyzing the correlation between the variables, sheep breed and location, the correlation was high ($V = 0.83$, 95% CI 0.79–0.86), with only Merinolandschaf present in Trpinja and only Romanov sheep in Vukovar.

Of the other risk factors analyzed, only sheep shearing was statistically significant, with unshared sheep showing a higher probability of tick-borne infections ($p = 0.048$) (Table 4).

## 4 Discussion

Infection of sheep with various zoonotic VBD pathogens has been documented in several studies, but their use as sentinel animals needs to be evaluated. In this study, we analyzed the seroprevalence of zoonotic VBDs in sheep from WNV and TBEV endemic areas in Croatia, as well as areas with sporadic evidence of USUV infection. In addition, we tested sheep for *B. burgdorferi* s.l., the most common vector-borne bacteria in Croatia, and the possible introduction of CCHFV. Sheep in all three sampling micro-locations and on all tested farms were positive for TBEV, WNV, USUV, and *B. burgdorferi* s.l. antibodies, suggesting their possible sentinel role for selected zoonotic pathogens.

The highest seroprevalence was recorded for TBEV (9.7%). It was higher than the 0.53 and 0.42% seroprevalence in Germany and Belgium, tested by VNT and plaque reduction neutralization test, respectively (29, 30). The observed difference could be attributed to the endemic occurrence and high risk of TBEV infection in this part of Croatia (22).

Very few studies analyzing WNV seroprevalence in sheep are available for European countries. In a study conducted in Turkey, 1% of sheep tested positive for WNV neutralizing antibodies (31). A seropositivity of 2.2% was observed by VNT in the Astrakhan region of Russia (32). The WNV seroprevalence of 3.0% in Croatia is similar to the seroprevalence results in sheep from Egypt, northeast Ethiopia, and Tunisia (3.5, 3.5, and 3.2%, respectively), which are also enzootic regions for WNV (33–35). With extremely high seroprevalence in horses (36), the surveillance system in this area could be compromised, making sheep a possible alternative species for WNV surveillance in regions with high virus activity.

To the best of our knowledge, this study gives the first serological evidence of USUV infections in sheep globally. The detection of USUV-seropositive sheep in Vukovar-Srijem County is not surprising, as the first serologic evidence of human USUV infection in Croatia was confirmed in 2012 in a resident of this region (37).

Athanosiu et al. (38) confirmed a *B. burgdorferi* s.l. seroprevalence of 23.58% in sheep in Greece. Similarly, a seroprevalence study in Slovakia found the seropositivity to *B. burgdorferi* s.l. of 15.8% (1999) and 17.5% (2000) (39). In the Alto Adige-South Tyrol, Italy, the seropositivity in sheep was 14.1% (1990) (40). In the present study, seropositivity was much lower (2.7%), which is consistent with the low incidence of human borreliosis in this Croatian region (21).

All sheep were tested negative for CCHFV, which was expected since this arbovirus had not yet been confirmed in Croatia. Further investigation is necessary due to the high risk of CCHF emergence, given the recent seropositive sheep confirmation in neighbouring countries (41, 42).

The antibody detection in sheep suggests their sentinel potential for VBD pathogens. For highly prevalent pathogens such as WNV, the possible advantage of sheep in endemic areas is noteworthy. In Vukovar-Srijem County, the testing area in the present study, a high IgG seroprevalence of WNV exceeding 50% was recorded in horses during 2024 (data of the Faculty of Veterinary Medicine, University of Zagreb). This challenges the continued use of horses as sentinel

TABLE 4 Seroprevalence of vector-borne zoonoses in sheep according to host- and management-related risk factors in Croatia (OR, 95% CI, *p*).

| Risk factor | WNV | USUV | TBEV | *B. burgdorferi* s.l. | Mosquito-borne pathogens | Tick-borne pathogens |
|---|---|---|---|---|---|---|
| Romanov breed* | $p = 0.17$ | $p = 0.18$ | **p < 0.001** | **p < 0.001** | $p = 0.672$ | **p < 0.001** |
| Merino breed | 5.77 (0.91–111.62) $p = 0.11$ | 0.47 (0.04–2.89) $p = 0.42$ | 0.78 (0.22–2.53) $p = 0.68$ | 3.29 (0.17–482.26) $p = 0.43$ | 1.70 (0.47–6.83) $p = 0.42$ | 0.95 (0.3–2.94) $p = 0.92$ |
| Tsigai breed | 4.31 (0.54–87.96) $p = 0.21$ | 0.19 (0.001–2.04) $p = 0.19$ | **4.02 (1.64–10.89) p = 0.003** | **22.25 (2.65–2904.02) p = 0.002** | 1.05 (0.20–4.88) $p = 0.95$ | **5.3 (2.22–14.12) p < 0.001** |
| Sex | 1.97 (0.1–37.04) $p = 0.65$ | 4.23 (0.21–85.84) $p = 0.35$ | 1.55 (0.18–13.37) $p = 0.69$ | 2.24 (0.12–42.48) $p = 0.59$ | 1.37 (0.07–25.18) $p = 0.83$ | 1.29 (0.15–11.08) $p = 0.81$ |
| Age | 1.06 0.75–1.35 $p = 0.69$ | 1.00 (0.55–1.43) $p = 0.99$ | 1.07 (0.89–1.24) $p = 0.44$ | 1.17 (0.88–1.46) $p = 0.2$ | 1.04 (0.78–1.29) $p = 0.74$ | 1.11 (0.95–1.28) $p = 0.17$ |
| Herd size | 1.00 (0.99–1.01) $p = 0.91$ | 1.00 (0.99–1.01) $p = 0.96$ | 0.99 (0.99–1.002) $p = 0.4$ | 0.99 (0.99–1.007) $p = 0.95$ | 1.00 (0.99–1.01) $p = 0.9$ | 0.99 (0.99–1.003) $p = 0.52$ |
| Shearing | 1.01 (0.25–4.13) $p = 0.99$ | 0.67 (0.07–6.53) $p = 0.73$ | 0.39 (0.14–1.06) $p = 0.06$ | 0.28 (0.03–2.3) $p = 0.23$ | 0.89 (0.27–2.98) $p = 0.85$ | **0.4 (0.16–0.99) p = 0.048** |
| Clinical signs | 1.06 (0.13–8.8) $p = 0.95$ | 0.89 (0.05–16.92) $p = 0.94$ | 2.41 (0.9–6.48) $p = 0.08$ | 2.81 (0.54–14.51) $p = 0.22$ | 0.68 (0.09–5.39) $p = 0.71$ | 2.46 (0.97–6.22) $p = 0.06$ |

*Reference category in the logistic regression; the ANOVA *p*-value represents the influence of breed; WNV, West Nile virus; USUV, Usutu virus; TBEV, tick-borne encephalitis virus; mosquito-borne pathogens: WNV/USUV; tick-borne pathogens: TBEV/*B. burgdorferi* s.l. Statistically significant differences are bold.

animals in the upcoming transmission season and underscores the need for integrating additional species into the surveillance framework. The easy access to serum samples collected for veterinary important infectious disease surveillance, such as brucellosis, and the large population of these animals offer the advantage of including sheep in the surveillance programme for VBD pathogens.

In addition to confirming the activity of zoonotic pathogens in specific areas, surveillance with sentinel animals could provide information on epidemiological risk factors in a particular location. In this study, we confirmed that location had a significant influence on the risk of infection with VBD pathogens. This was not confirmed for WNV and mosquito-borne pathogens overall, likely due to the vector population density and the high viral activity of WNV observed in this area over the last decade. The impact of location in a small geographic area, as investigated in this study, on mosquito-borne infections could be minimized compared to tick-borne diseases, given that mosquitoes travel long distances, with a mean distance of 1.33 km (43). Even though the risk of infection from less prevalent mosquito-borne pathogens, such as USUV, in this region is location-dependent in some instances. This indicates a general and highly prevalent spread of WNV throughout the study area, as well as the circulation of USUV in some micro-foci.

Location was a significant risk factor for TBEV, *B. burgdorferi* s.l., as well as tick-borne diseases overall. The observed differences between mosquito-borne and tick-borne VBDs can be explained by the significantly different movement distances of ticks compared to mosquitoes. Slovák et al. (44) confirmed that nymphs have the highest infection rates with TBEV among tick stages. At the same time, the independent movement of nymphs is very short, and they were predominantly recaptured 2–3 meters from their release points, while almost 50% of adults were found to be more than 5 meters away, with some dispersing up to 7–8 meters. These findings confirm that all tick stages exhibit minimal autonomous movement (without involvement of the host's movement), resulting in microlocalization of TBEV circulation.

From the other geographic and environmental risk factors for VBDs in this study, we found that the distance of the flock from the household is a significant risk factor for TBEV, *B. burgdorferi* s.l., and tick-borne diseases overall. This finding could be the result of the above-explained differences in the movement of the vectors, as well as confirmation that the dispersal of nymphs into pastures was minimal compared to the woodland (45).

In an analysis of host- and management-related risk factors for infection with VBD pathogens in sheep, we confirmed breed as an important risk factor. Due to the high correlation between breed and location, this finding requires further investigation. Sex, age, flock size, and clinical signs did not influence seroprevalence. An unexpected result was that the risk of tick-borne infection was statistically higher in flocks without sheep shearing practices. This could be a consequence of the easier attachment of tick vectors to a host when sheep were not sheared, but may also reflect broader farming practices. Regularly shared flocks are handled more frequently, increasing the likelihood of early tick infestation detection. Differences in exposure risk may also be influenced by other factors not addressed in our study, such as the level of flock management and the degree of system extensiveness, which is a limitation of the study. The role of shearing is, therefore, a risk factor likely multifactorial in origin and should be interpreted within the broader context of flock management and environmental exposure.

In conclusion, the results of this study suggested the potential of sheep as sentinels for mosquito-borne and tick-borne zoonotic diseases. The lower WNV seroprevalence in sheep compared to horses, the most commonly used sentinel species for WNV, suggests that even sheep may have advantages in endemic regions with high viral activity. In this study, to the best of our knowledge, we confirmed for the first time USUV infection in sheep, which expands the possibility of collecting epidemiological data using this species as sentinel animals. However, it should be noted that age was not identified as a significant risk factor for VBDs seropositivity. Due to cumulative exposure, older animals are often more likely to be seropositive, and their usefulness as sentinels for identifying recent pathogen circulation may

be questionable. Therefore, further studies are needed to evaluate the role of sheep as sentinels for mapping microfoci of zoonotic pathogens' activity and determining high-risk areas for public health.

The findings of this study are relevant within the "One Health" framework, which emphasizes the interdisciplinary collaboration between human, animal, and environmental health. The detected seropositivity to multiple zoonotic VBD pathogens (TBEV, WNV, USUV, *B. burgdorferi* s.l.) in sheep in various micro-locations supports the inclusion of livestock surveillance in integrated early-warning systems. By highlighting geographic and environmental risk factors such as farm location, breed susceptibility, and proximity to households, the study provides insight into the micro-epidemiology of zoonotic VBDs. These data can be used to improve risk mapping at the local level, allowing for more focused public health interventions, such as tick and mosquito control measures and public education campaigns in high-risk zones.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because samples tested in the study are residual samples obtained from a mandatory surveillance program on sheep infectious diseases approved by the Ministry of Agriculture, Veterinary Directorate. However, part of the research was conducted as a component of the graduation thesis of one of the co-authors, DVM. In accordance with the internal procedures of the Faculty of Veterinary Medicine, University of Zagreb, all graduate theses must receive prior ethical approval regardless of the source or nature of the samples. Therefore, ethical approval was formally requested to comply with the institutional requirements, and was granted by the Ethics Committee of the Faculty (Decision: Class: 640-01/22-02/07, Reg. No.: 251-61-01/139-22-42).

## Author contributions

LB: Conceptualization, Supervision, Writing – original draft. VSt: Methodology, Writing – review & editing. MMM: Formal analysis, Methodology, Writing – original draft. GM: Investigation, Writing – review & editing. IC: Investigation, Writing – review & editing. VSa: Funding acquisition, Writing – review & editing. VM: Investigation, Resources, Writing – review & editing. MB: Visualization, Writing – review & editing. AM: Writing – review & editing. TV-C: Conceptualization, Visualization, Writing – original draft.

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## 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.

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## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1602706/full#supplementary-material

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# Monitoring of sandflies (Diptera: Psychodidae) and pathogen screening in Slovenia with habitat suitability modeling

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Sandflies (Diptera: Psychodidae: Phlebotominae) are important vectors of pathogens, including *Leishmania* parasites and phleboviruses, but their distribution and seasonal activity in Slovenia have not been sufficiently studied. This study presents a comprehensive three-year (2020–2022) surveillance programme aimed at assessing the diversity of sandfly species, their distribution, seasonal dynamics and potential role as vectors of pathogens. A total of 1,240 sandflies were collected at 43 sampling sites across Slovenia, identifying *Phlebotomus papatasi*, *P. neglectus*, *P. perniciosus* and *P. mascittii*. The highest abundance and species diversity were observed in the Mediterranean and Karst regions. Seasonal activity peaked in July, with population fluctuations influenced by climatic conditions. Molecular analyses for *Leishmania* parasites and phleboviruses showed no positive results, indicating a low prevalence of pathogens in the sampled populations. Predictive habitat models indicate that environmental factors, particularly temperature and precipitation, play a decisive role in the spread of sandflies. While *P. mascittii* has the largest ecological range, its vector competence remains uncertain. The results provide important insights into the ecology of sandflies in Slovenia and emphasize the need for continuous surveillance in the context of climate change and emerging vector-borne disease risks.

KEYWORDS

**sandflies, monitoring, distribution, modeling, Slovenia**

## Introduction

Sandflies are arthropods (Diptera: Psychodidae: Phlebotominae) of global importance as they are important vectors of several pathogens affecting humans and animals. These insects are known vectors of several *Leishmania* species that cause visceral and cutaneous leishmaniasis (1, 2), and of *Bartonella bacilliformis*, the causative agent of bartonellosis (3).

They are also vectors of phleboviruses such as Toscana virus (TOSV), sandfly fever Sicilian virus (SFSV), sandfly fever Naples virus (SFNV), and Cyprus virus (CYPV), which cause acute febrile or even central nervous system infections in humans (4–6).

Sandflies are delicate, hairy flies with long, slender legs that occur in a wide range of habitats, from sea level to altitudes of 2,800 m and from deserts, savannas, and open forests to dense tropical rainforests. These small, nocturnal insects require sugar as a source

of energy, and only the female sandflies feed on blood as they need the nutrients to mature their eggs. The seasonal biting activity of European sandflies is mainly observed in summer (7). All sandflies breed in terrestrial habitats and are insects with relatively limited flight ability (8). In the last 10 years, several sandfly species have spread into European regions where they were not previously reported. This shift brings new public health challenges, as sandflies are vectors of *Leishmania* spp. and phleboviruses (9). Their expanding range is driven by a complex interplay of factors, including ongoing environmental and climate change, as well as increasing mobility of humans and animals. While climate change has always been a natural phenomenon, its rapid acceleration over the last century is unprecedented (10).

Six species of sandflies have been recorded in Slovenia and neighboring regions, which may influence the species composition and variability of local populations. These include *Phlebotomus* (*Transphlebotomus*) *mascittii* Grassi, 1908, *Phlebotomus* (*Larroussius*) *perniciosus* Newstead, 1911, *Phlebotomus* (*Larroussius*) *neglectus* Tonnoir, 1921, *Phlebotomus* (*Phlebotomus*) *papatasi* (Scopoli, 1786) and *Sergentomyia minuta* (Rondani, 1843).

*Phlebotomus mascittii* has been recorded in Slovenia (11) and in several neighboring countries, including Austria (12–14), Slovakia (15) and Croatia (16) as well as in parts of Germany and Belgium (17–21). *Phlebotomus perniciosus* is a common species in Italy and Croatia (22), while *P. neglectus* also occurs in neighboring Croatia (16, 23), Italy (24) and Hungary (25).

*Phlebotomus papatasi*, a predominantly endophilic and anthropophilic species, has a more southerly distribution in the region, with confirmed occurrences in Slovenia (11), Croatia (22) and southern Hungary (26).

Although *P. perfiliewi* has not yet been recorded in Slovenia, it has recently been reported in the Friuli-Venezia Giulia and Veneto regions in north-eastern Italy (27) and in Dalmatia, Croatia (22), indicating a possible expansion of the distribution area toward the Slovenian border.

Finally, *S. minuta* has been reported from Slovenia (11), Croatia (16), northern Italy (24) and Switzerland (28). However, as the females of this species mainly feed on reptiles, it is unlikely that they are involved in the transmission of pathogens to humans.

In Slovenia, research on sandflies as vectors of emerging pathogens has been relatively limited. Only a few studies have investigated their distribution, seasonal activity and possible role in the transmission of pathogens in selected areas. However, studies conducted in Slovenia and the former Yugoslavia have documented the presence of several medically important sandfly species, with the earliest records dating back to entomological surveys from the mid-20th century (29). More recent studies in Slovenia have documented the occurrence of *P. mascittii* and have highlighted illegal waste sites and peri-urban habitats as potential microfoci for sandfly proliferation (11, 30). In the broader region, human leishmaniasis has re-emerged as a public health concern, particularly in northern Italy, where retrospective analyses have identified hundreds of autochthonous cases over the past two decades (31). In Croatia, seroepidemiological surveys have demonstrated asymptomatic infections among residents in both endemic and non-endemic areas (32). Canine leishmaniasis remains widespread and of considerable veterinary relevance throughout the Balkans, with evidence of persistent transmission and a gradual northward spread in northern Italy, likely driven by environmental and climatic factors (33–37).

In addition to *Leishmania*, sandflies in Slovenia, Croatia, and Italy are competent vectors of several phleboviruses (38). Toscana virus, in particular, has emerged as an important cause of neuroinvasive disease, with multiple viral lineages detected in Croatia and endemic circulation documented across Italy (6, 39, 40). Earlier serological studies confirmed the widespread presence of sandfly fever viruses in the Adriatic region (41), and more recent data have demonstrated extensive Toscana virus exposure among humans and domestic animals (42).

Given Slovenia's diverse topography and climate, local climatic fluctuations could significantly influence sandfly populations and the pathogens they transmit. A standardized monitoring system is therefore essential to detect current trends and to predict future changes. The main objectives of this study were: (a) to document the sandfly species present in Slovenia; (b) to assess their current and potential distribution, potential range expansion, and abundance; and (c) to determine the presence or absence of viral and parasitic pathogens transmitted by sandflies. The results of this study will contribute to a clearer understanding of the epidemiological landscape of emerging pathogens in Slovenia and neighboring regions. In addition, these findings will support future research on the interactions between vectors, pathogens, and their environment, and will inform the development of improved surveillance and control strategies.

## Material and methods

### Study area

Prior to this study, data on the abundance of sandflies in Slovenia was incomplete, as these insects had only been studied in the coastal region and not in the continental part of the country until 2019. To fill this gap, a nationwide surveillance programme with systematic random sampling was carried out, covering a total of 226 sampling sites to ensure comprehensive geographical coverage (Figure 1). Sampling sites were selected to represent a variety of habitats, including suitable environments such as stone walls near human settlements, agricultural and tourist farms and animal enclosures, but also supposedly unsuitable habitats such as dense forests and areas near rivers or lakes. In this way, the presence or absence of sandflies could be detected in unexpected locations.

The site selection was based on the expectation of high species diversity. We prioritized locations that would maximize the detection of different sandfly species and their population densities, focusing on areas with historically high sandfly activity, such as the Slovenian Karst region (11, 29). In addition, we have strategically selected locations where larger numbers of sandflies have been detected in the past and which are close to regions in the neighboring countries of Italy and Croatia where leishmaniasis has occurred. This structured approach ensured a scientifically rigorous assessment of sandfly populations in Slovenia.

The study followed a longitudinal design, with repeated sampling across three sandfly seasons (2020–2022). To ensure broad spatial coverage and habitat diversity, sites were distributed

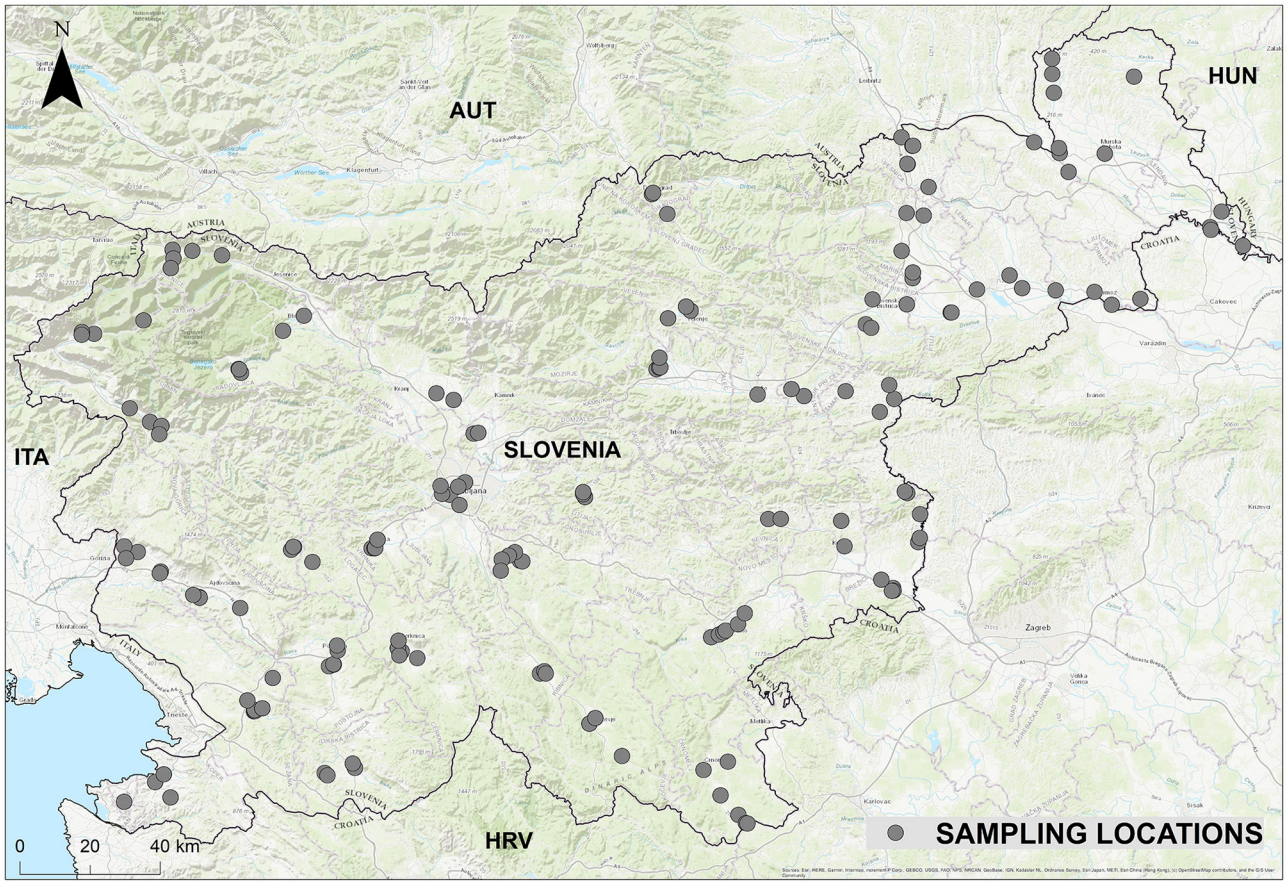


FIGURE 1
Sampling locations in Slovenia.

across different geographic regions and environmental conditions. The temporal framework allowed for long-term monitoring of seasonal population dynamics. Systematic sampling provided reliable data on species presence, seasonal activity, and population trends. Detailed information on the individual sampling locality is available in the table in the Appendix.

## Trapping methods

Fieldwork was conducted annually from May 1st to October 31st between 2020 and 2022. Each month, two CDC miniature light traps (John W. Hock Company, Florida, USA) were set at each sampling site. In addition to these traps, we also used BG-Sentinel traps (Biogents, USA) and CDC gravid traps (John W. Hock Company, Florida, USA) as part of a broader entomological surveillance programme to monitor mosquito populations at the same locations. While CDC miniature light traps were the primary method for collecting adult sandflies, we occasionally captured them in both BG-Sentinel traps and CDC gravid traps for mosquitoes.

Sandflies require a certain amount of moisture to maintain their life cycle and are most often found near hosts from which they can take a blood meal. Therefore, we mainly placed the traps near or inside buildings where domestic animals, such as chickens, rabbits and dogs were kept. We also placed the traps near dry stone walls, which are ideal resting places for sandflies in Mediterranean environments.

To better understand the spatial distribution of sandflies, we also set traps in areas typically not associated with their presence. This approach was crucial for the development of models to predict possible changes in their distribution in Slovenia. We did not attempt to sample sandfly larvae as it is difficult to find them in the wild. They usually hide in organic material outdoors or inside barns, chicken coops or rabbit hutches (2).

## Identification of sandflies based on morphology

Sandflies were identified based on morphology using identification keys (43–45). The identification of the species of adult sandflies requires the examination of internal structures, such as the arrangement of the teeth on the cibarial (pharyngeal) armature, the shape of the spermathecae in females, and the structure of the external genitalia (terminalia) in males.

As one of the aims of our study was to analyse the captured sandflies for the presence of *Leishmania* parasites and

phleboviruses, all specimens were immediately placed in a freezer upon return from the field and stored at −80°C until dissection. Dissections were performed as quickly as possible on ice plates to preserve viral RNA. The head and several posterior segments of the abdomen were carefully separated from each fly using fine entomological or insulin needles on a microscope slide and then preserved for the preparation of semi-permanent slides, which were later used for morphological identification. The transparency of the chitinous parts was achieved by heating in the Marc-André solution. The rest of the body was pooled according to location, species and sex and stored at −80°C for molecular analysis.

Before identification, the head and posterior segments of each specimen were immersed in a drop of low-viscosity CMP-9 medium (Polyscience, Cat. No. 16299). The head was mounted dorsally, the male genitalia laterally and the spermathecae anteriorly. For easier identification, the spermathecae of some females were individually prepared directly in the Marc-André solution.

## Habitat suitability modeling

To create maps of the potential distribution of sandflies, we used data on their presence at specific locations across all three sampling years. To reduce bias caused by the uneven distribution of sampling locations, we performed spatial filtering as suggested by (46), counting multiple records within a 1 km radius as a single occurrence point. The data were filtered using the "ecospat" package (47).

For the input data in the modeling process, we used 19 bioclimatic variables from the CHELSA climate database (48) (https://chelsa-climate.org/). We selected the CHELSA bioclimate variables because they have been shown to be a better choice compared to the more commonly used WorldClim variables in a previous study on mosquito modeling in Slovenia (49). Additionally, we incorporated an elevation layer from the WorldClim website (www.worldclim.org) and a CORINE land cover layer from the European Environment Agency website (https://www.eea.europa.eu/help/faq/what-is-corine-land-cover) to develop the model. The spatial resolution of the environmental variable layers was 30 arcseconds, corresponding to ~1 × 1 km. We checked predictors for collinearity to remove highly correlated and thus redundant environmental variables. To achieve this, we calculated the variance inflation factor (VIF ≥ 5) and *post-hoc* Pearson correlation coefficient between variables. VIF analysis was performed using the R package "usdm" (50), while Pearson coefficients were calculated and visualized using the R package "GGally" (51).

Due to the limited number of occurrences, we decided to create models using the MAXENT algorithm (52), which has been shown to provide better results with smaller amounts of occurrence data (53, 54).

We considered the approach proposed by (55) for modeling rare species and created several small bivariate models and their ensemble as the final model. The modeling procedure was performed using the R package "ecospat" (47). All models were evaluated based on the Boyce index. Models were based on the ensemble prediction procedure. Models with highest values of Boyce index were considered for the final model. For the visualization of final maps and for spatial procedures we used the spatial tools of ESRI ArcGIS, ver.10.7.

## Molecular detection of *Leishmania* parasites and phleboviruses

The dissected specimens were pooled together based on collection site, date, species and sex. The pooled material consisted of the thorax and abdomen sections of dissected individuals of the same species and sex. In contrast, the non-dissected specimens were pooled only by locality, date and sex and stored at −80°C until processing. As the primary aim of this study was the detection of phleboviruses, we dissected every second specimen to obtain internal tissues with potentially higher viral RNA concentrations for reliable molecular analysis. Each pool of sandflies was homogenized in 600 µL of RPMI medium using a Tissue Lyser (Retsch for Qiagen, Hilden, Germany). Nucleic acid was extracted from 200 µL of the sandfly homogenate using the BioRobot EZ1-XL Advanced (Qiagen, Germany) and the EZ1 Virus Mini Kit v2.0 (Qiagen, Germany) and eluted in 60 µL. The residual homogenate was stored for further analysis. We did not check RNA integrity after extraction, as all samples were transported on dry ice and immediately stored at −80°C, and extractions were performed within 2 weeks. However, internal control amplification in the PCR assays confirmed the presence of amplifiable RNA in all analyzed pools.

*Leishmania* DNA was detected by real-time PCR using the Primerdesign™ *Leishmania* Genesig® Advanced Kit (Genesig, Primerdesign, UK) targeting Cytochrome b (cytb) kinetoplast. Reactions were performed in a total volume of 20 µL containing 5 µL DNA, 5 µL TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems, Thermo Fisher Scientific, Grand Island, NY, USA), 1 µL *Leishmania* primer/probe mix on the StepOne (Plus) Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, USA). Cycling conditions were as follows: 50°C for 5 min, 95°C for 20 s, 50 cycles of 95°C for 3 s and 60°C for 30 s.

The sandfly pools were analyzed using Toscana virus-specific real-time RT-PCR as described by (56). Real-time RT-PCR was performed using a QuantStudio 7 system (Applied Biosystems, USA). Reactions were performed in a total volume of 20 µl and contained 5 µl of RNA, 2.5 µl of TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems, Thermo Fisher Scientific, Grand Island, NY, USA), 0.5 µmol of each primer and 0.3 µmol of probe, and water. Cycling conditions were as follows: 50°C for 5 min, 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s.

In addition, the sandfly pools were analyzed with a RT-PCR developed (57) using Phlebo forward primers 1 and 2 and Phlebo reverse primers, which allowed the amplification of a 370 bp PCR product of the S segment of viruses of the genus Phlebovirus (57). The reactions were performed in a total volume of 20 µL and contained 5 µL RNA, 10 µL PrimeScrtipt™ One Step RT-PCR Kit Buffer and 1 µL PrimeScript 1 Step Enzyme Mix (TaKaRa) and 1 µM of each primer. Cycling conditions were as follows: 50°C for

30 min, 94°C for 2 min, 55 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s.

## Results

During the study period, a total of 1,240 individuals were caught at 43 of 226 sampling sites. In 2020, we caught 552 sandflies, followed by 240 individuals in 2021 and 448 individuals in 2022. 670 individuals were identified to a species level based on morphology. The rest were pooled according to sex and location and prepared for molecular analysis to identify pathogenic microorganisms.

Based on morphological characteristics, we identified four species of sandflies, *Phlebotomus papatasi*, *P. neglectus*, *P. perniciosus* and *P. mascitii*.

Phlebotomine sandflies were found throughout the country (Figure 2), with notable differences in population structure and spatial distribution. *Phlebotomus neglectus*, *P. perniciosus* and *P. papatasi* were predominantly found in the Mediterranean and Karst regions of Slovenian Istria, while *P. mascitii* was found in all surveyed locations. *Phlebotomus mascittii*, a generally not abundant species, was found most frequently in Slovenian Istria, with significant records from the Medljan and Velike Žablje areas (Appendix 1). Data from 3 years of research show that sandfly activity peaks in July (Figure 3). As not all specimens could be identified to species level, a more precise seasonal analysis of species activity is not conclusive.

### Prediction maps

The models were created considering the following predictor variables, which showed low collinearity: elevation, Corine land cover, isothermality (bio3), maximum temperature of the warmest month (bio5), precipitation seasonality (bio15), precipitation of the wettest quarter (bio16) and precipitation of the warmest quarter (bio18) (Figure 4).

The models for *P. perniciosus* and *P. neglectus* were created using an ensemble of bivariate models that met a Boyce index threshold of 0.5 and 0.4, respectively. Despite the optimization efforts, a certain degree of model overfitting remained in the results. For *P. papatasi* and *P. mascittii*, which have a similar distribution to the first two species, the ensemble was based on bivariate models with a higher Boyce index threshold of 0.6 and 0.7, respectively.

The maps show the suitability of different regions in Slovenia for the potential distribution of sandfly species (Figure 4). The most favorable environmental conditions for all four species are found in the south-western part of the country, particularly in low-lying areas and along the coast. While three of the species are largely restricted to the sub-Mediterranean region, *P. mascittii* seems to have a larger distribution potential, extending to ecologically favorable areas in the continental part of Slovenia, especially in the warmer lowlands in the east. In contrast, the central, eastern and northern regions, which are represented by white areas on the maps, are less suitable (Figure 4). This indicates that the environmental conditions in these regions are less favorable for the establishment of sandfly populations.

### Molecular analysis

A total of 1,217 sandfly specimens were processed for molecular analysis and organized into 179 pools. Twenty-three additional samples were neither suitable for morphological identification nor for molecular testing. No pathogens were detected in any of the samples analyzed, including 551 specimens (94 pools) from 2020, 218 specimens (35 pools) from 2021 and 448 specimens (50 pools) from 2022.

## Discussion

Sandflies, important vectors of pathogens that cause diseases such as leishmaniasis and phleboviruses, are changing their distribution in Europe due to climate change, urbanization and changes in land use (9, 58). These factors create more favorable conditions for the survival and reproduction of sandflies (59). As their distribution expands, the risk of leishmaniasis outbreaks in newly colonized regions increases.

Important vector species such as *P. perniciosus* and *P. neglectus*, which were previously restricted to Mediterranean areas, have been detected further north, including in Germany and Switzerland (19, 60). *Phlebotomus mascitii*, whose vector potential is still unconfirmed, is also spreading north. Although its abundance is generally low, this species is found as far as Switzerland and Slovakia (15, 60). Its spread is probably due to milder winters and longer warm seasons, which have transformed previously unsuitable areas into viable habitats (22).

In Slovenia, sandflies are widespread throughout the country, although there are notable regional differences in population structure. *Phlebotomus neglectus*, *P. perniciosus* and *P. papatasi* are mainly found in the Mediterranean and Karst regions of Slovenian Istria, while *P. mascitii* is present in all areas studied. However, the greatest species diversity is observed in areas with a Mediterranean climate. The data from 3 years of research show that sandfly activity peaks in July (Figure 3), similar to what is observed in the neighboring Italian regions of Friuli-Venezia Giulia and Veneto (27) and in Austria (12). Our results suggest that the sandflies in this region only produce one generation per season due to the climatic conditions.

According to meteorological data from the Slovenian Environment Agency, the average annual temperature in Slovenia was 14.37°C in 2020, 13.85°C in 2021 and 14.86°C in 2022. The temperature deviation for 2021 was −0.7°C compared to the long-term average. Exceptionally high precipitation was recorded in 2020, followed by lower winter temperatures in 2020/2021 (61). These climatic conditions may have influenced the population dynamics of phlebotomine sandflies, especially in Slovenian Istria and the Karst region, where they are most abundant. Increased precipitation and lower winter temperatures probably affected the suitability of breeding sites and larval survival rates, which may have contributed to the changes in population density in 2021. A similar negative correlation between precipitation and abundance of *P. perniciosus* was observed in the Murcia region of Spain and north-eastern Italy (62, 63). As a result, the number of sandflies caught in 2021 was significantly lower than in 2020 and 2022,

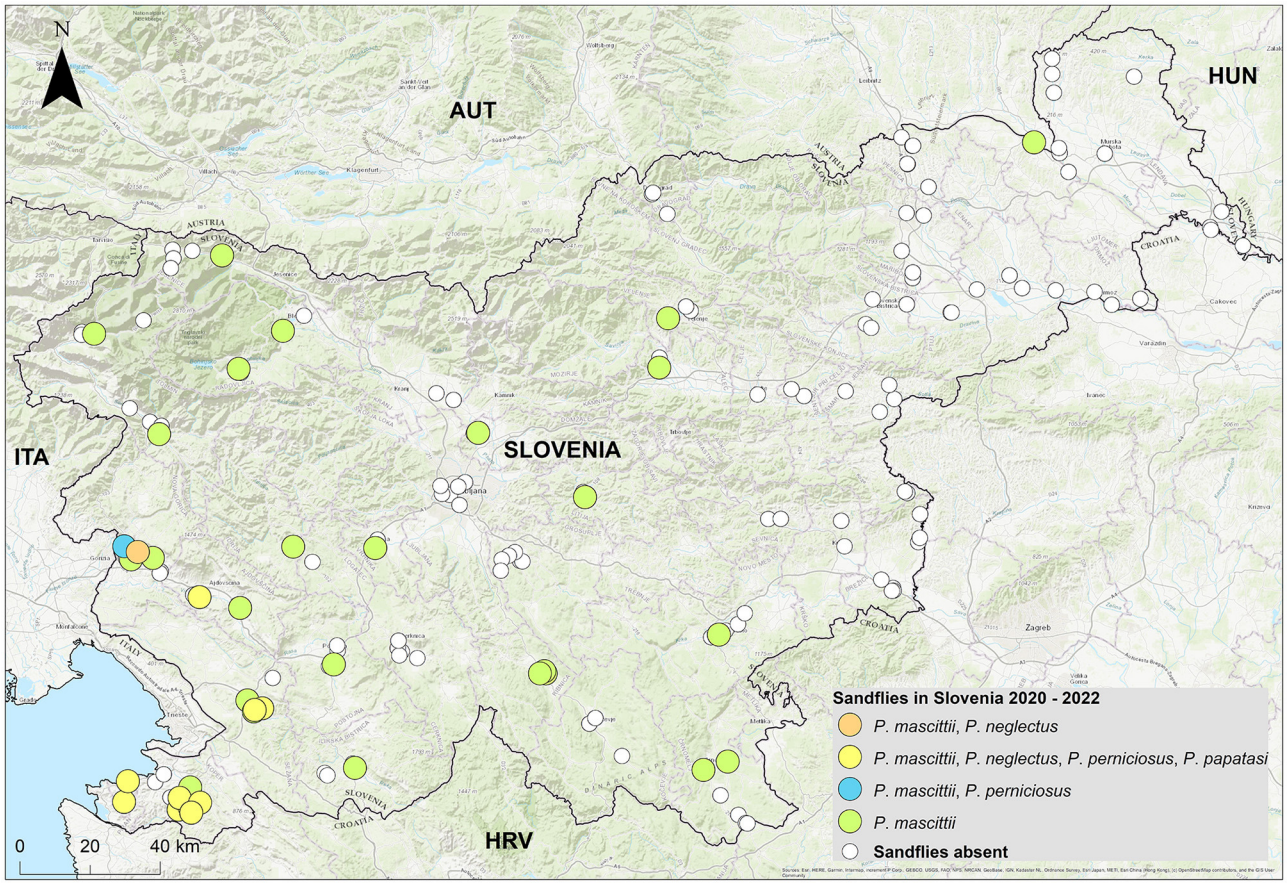


FIGURE 2
Distribution and population structure of sandflies in Slovenia.

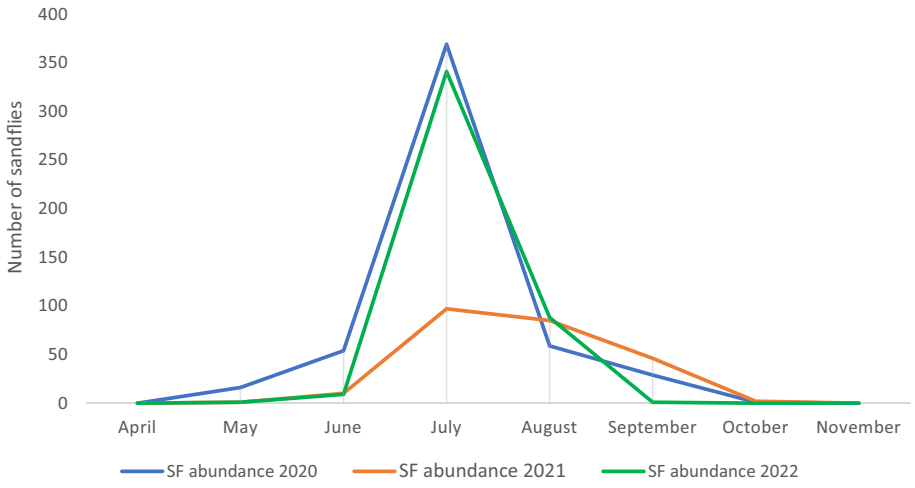


FIGURE 3
Seasonal activity of sandflies in Slovenia from 2020 to 2022.

highlighting the impact of unfavorable climatic conditions on their population dynamics.

Environment suitability models show that the studied species have different habitat preferences. *Phlebotomus papatasi*, *P. neglectus* and *P. perniciosus* show a strong preference for the south-western part of Slovenia, particularly in the coastal and Karst regions, and are very limited for other areas. In contrast, *P. mascitii* has a much broader potential range, covering almost

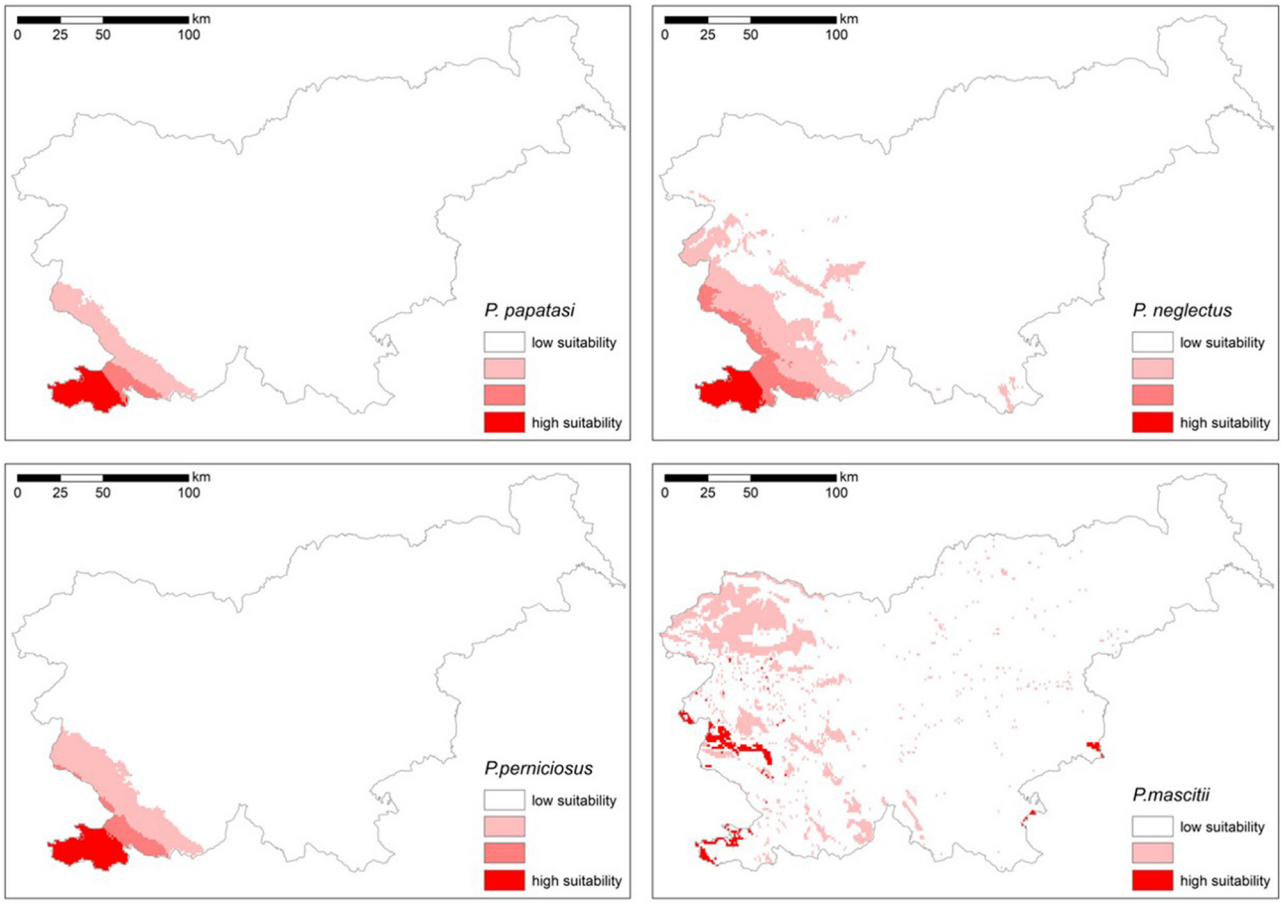


FIGURE 4
Suitability of areas in Slovenia for the settlement of *P. papatasi, P. neglectus, P. perniciosus and P. mascitii* (suitability increases from white color (inadequate) to dark red color (very suitable).

the entire country. This suggests that *P. mascitii* can thrive in a range of environmental conditions, including higher altitudes and potentially cooler or wetter regions. These distribution patterns are consistent with the environmental preferences observed in Austria and Slovakia (12, 15). Despite its wide distribution range, *P. mascitii* is not very abundant and is most frequently detected in Slovenian Istria, with significant populations found in Medljan and Velike Žablje.

The observed ecological differences between sandfly species in Slovenia may influence their role as disease vectors and their impact on public health. *P. papatasi*, *P. neglectus* and *P. perniciosus* are adapted to warm, dry regions with Mediterranean climate characteristics, while *P. mascitii* shows greater ecological flexibility. In the Mediterranean regions, *P. mascitii* is thought to be a cave-dwelling species (20), while in Central Europe it is often found in barns, chicken coops and sheds close to human dwellings and animals (12). At sites in Slovenia, this species was most frequently found in traps placed in the immediate vicinity of chicken coops or rabbit cages. *P. mascitii* is thought to be autogenous (64), although its biology remains poorly understood due to difficulties in laboratory maintenance.

Although *P. mascitii* is found near domestic animal shelters and burrows of small rodents, its low natural density is unlikely to sustain the transmission cycle of *Leishmania* infantum alone. Nevertheless, it may play a localized role in the transmission of *L. infantum*, particularly in cases of imported canine leishmaniasis. Its presence in suburban areas and animal enclosures indicates a possible epidemiological importance in regions where competent vectors are scarce.

As far as leishmaniasis in humans is concerned, no autochthonous cases have been reported to date, only imported cases from endemic Mediterranean areas (65). There is evidence that cases of canine leishmaniasis have occurred in Slovenia in the last 5 years (66, 67), similar to the northern regions of neighboring Italy and Hungary (68, 69). Unlike in Italy and Croatia, however, we were unable to detect any *Leishmania* parasites or phleboviruses in the sandfly samples we analyzed (38, 70).

The results of this three-year surveillance show the ecological differences between sandfly species in Slovenia and their potential impact on disease transmission. Understanding these differences is essential for predicting future vector dynamics and developing targeted control measures to reduce disease risks.

## Conclusion

This study provides a comprehensive assessment of the distribution of sandflies in Slovenia and shows clear regional differences in the abundance of the species and their habitat preferences. The results confirm that climatic and environmental conditions significantly influence sandfly populations, with *P. mascitii* having the largest ecological range. While the vector competence of this species remains uncertain, its presence near animal enclosures suggests a possible epidemiological relevance. The absence of *Leishmania* and phleboviruses in the samples is a reassuring result. However, because of the constant expansion of the distribution area of the known vector species, continuous monitoring is essential. Future research should focus on the potential role of *P. mascitii* in pathogen transmission and the impact of climate change on vector dynamics. Effective surveillance and proactive control measures will be crucial to mitigate the risk of emerging sandfly-borne diseases not only in Slovenia but throughout the Mediterranean region.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

## Author contributions

VI: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. PG: Formal analysis, Methodology, Software, Supervision, Validation, Writing – review & editing. SZ: Data curation, Formal analysis, Project administration, Supervision, Validation, Writing – review & editing. TK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – review & editing. TT: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. MK: Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Software, Supervision, Validation, Writing – review & editing. NK: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. UGB: Data curation, Formal analysis, Investigation, Validation, Writing – review & editing. TA-Ž: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. KA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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## 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.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1603358/full#supplementary-material

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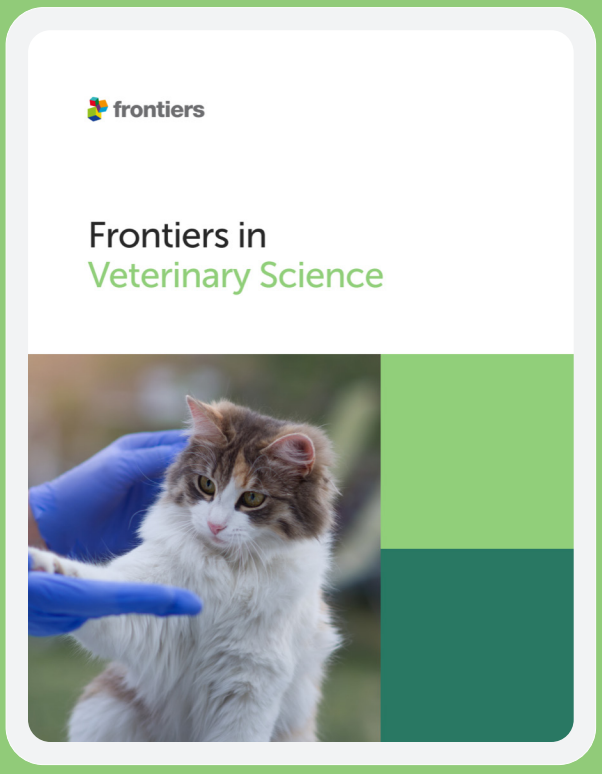


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