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Genomic and proteomic identification of *Aeromonas* isolates from diverse sources: comparative performance of WGS and MALDI-TOF MS in species-level resolution

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The accurate identification and characterization of *Aeromonas* species are essential to understand their ecological roles and potential health impacts. This study analyzed 90 *Aeromonas* isolates from various sources using whole genome sequencing (WGS) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Species identification by WGS, based on Average Nucleotide Identity (ANI $\geq 96\%$), revealed inconsistencies in 12.2% of MALDI-TOF MS results, for species not represented in its database. Phylogenetic analyses using single nucleotide polymorphism (SNP) data were concordant in resolving species-level clusters and revealing intra-species diversity. This study reinforces the value of WGS and complementary genomic approaches as reliable tools for *Aeromonas* species identification, population structure analysis, and integrated One Health surveillance. The integration of genomic tools into routine diagnostics may enhance the capacity of laboratories, particularly in low and middle income countries to monitor emerging resistance and better understand the evolutionary and epidemiological dynamics of *Aeromonas* in clinical and environmental settings.

KEYWORDS

Aeromonas species, whole genome sequencing (WGS), mass spectrometry, phylogeny, One Health

1 Introduction

Species of *Aeromonas* are increasingly recognized as emerging human pathogens, associated with gastrointestinal, wound, and bloodstream infections in both immunocompromised and immunocompetent individuals (Janda and Abbott, 2010; Fernández-Bravo and Figueras, 2020). Over 95% of clinical cases are linked to four species: *A. caviae*, *A. dhakensis*, *A. veronii*, and *A. hydrophila* (Janda and Abbott, 2010; Fernández-Bravo and Figueras, 2020), although others such as *A. salmonicida* have also been implicated in human infections (Janda and Abbott, 2010). Traditionally associated with aquatic habitats and fish diseases, these bacteria are now acknowledged as relevant opportunistic pathogens with global distribution (Janda and Abbott, 2010; Fernández-Bravo and Figueras, 2020).

Aeromonas spp. are widely distributed in freshwater, estuarine environments, and soils, and are frequently isolated from food, wastewater, and animals (Koutsoumanis et al., 2014; Fernández-Bravo and Figueras, 2020). This ecological versatility enables colonization of diverse hosts, including humans, domestic animals, and fish, reinforcing their significance within the One Health framework (Lamy et al., 2022). In tropical and subtropical regions, where exposure to untreated water and aquatic resources is common, *Aeromonas* infections may be underdiagnosed yet pose a growing burden to public health. Infections are typically acquired via ingestion of contaminated water or food, or contact with contaminated environments, and can range from localized diarrhea and skin lesions to systemic infections, particularly in immunocompromised patients (Parker and Shaw, 2011).

Clinically, accurate identification of *Aeromonas* at the species level remains challenging. Traditional phenotypic methods often yield ambiguous results due to overlapping biochemical characteristics, leading to frequent misidentification, especially among *A. caviae*, *A. media*, *A. dhankensis* and *A. hydrophila* (Janda and Abbott, 2010; Fernández-Bravo and Figueras, 2020). Whole genome sequencing (WGS) combined with average nucleotide identity (ANI) analysis has become the gold standard for bacterial species identification and taxonomic resolution. ANI provides an objective and quantitative measure of genomic similarity, with a $\geq 96\%$ threshold widely accepted to delineate species boundaries in prokaryotes. Unlike phenotypic assays or protein-based approaches, WGS delivers a comprehensive and precise view of the genome, enabling unambiguous discrimination among closely related species. The robustness, reproducibility, and high resolution afforded by WGS-ANI analyses make them indispensable for reliable taxonomic classification. However, the cost, infrastructure requirements, and turnaround time limit its use in routine diagnostics. In contrast, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) offers a rapid, cost effective alternative with growing accuracy in *Aeromonas* identification. When paired with curated databases, MALDI-TOF MS enables real time species-level resolution, facilitating timely clinical decisions. Nevertheless, phenotypic overlap and insufficient representation of well-characterized isolates in commercial databases frequently result in misclassification, particularly

between *A. hydrophila* and *A. dhakensis* (Kitagawa et al., 2022; Bartie and Desbois, 2024; Truong-Ha et al., 2024). Moreover, reference databases are often biased towards human-associated strains and not regularly updated to reflect taxonomic revisions, further reducing discriminatory power. These findings underscore that, while MALDI-TOF is valuable as a first-line tool, its limitations necessitate confirmation with genome-based approaches for reliable species-level resolution within the genus.

This study aims to improve species level identification of *Aeromonas* through a combined approach using MALDI-TOF MS and WGS. By integrating phenotypic and genotypic methodologies, we seek to overcome diagnostic limitations and provide insights into the taxonomy, resistance markers, and epidemiological relevance of this under recognized group of pathogens, with implications for surveillance and public health.

2 Materials and methods

2.1 Isolates

As part of the routine monitoring and surveillance activities conducted at the Instituto Adolfo Lutz, the public health reference laboratory for the State of São Paulo, Brazil, we included all *Aeromonas* sp. isolates received over the last ten years (2014–2024). These comprised strains from human clinical specimens, as well from animals, food and waters sources. A total of 90 *Aeromonas* strains were analyzed (Table 1). All isolates are preserved in the bacterial collection at the Bacteriology Center of the Instituto Adolfo Lutz (IAL).

2.2 Identification

The classic phenotypic characteristics of *Aeromonas* spp. were assessed, including oxidase and catalase positivity, as well as the morphology of Gram-negative rods (Supplementary Table 1). The isolates exhibited facultative anaerobic growth on standard laboratory media such as heart infusion agar, MacConkey's agar, and blood agar. Additionally, they demonstrated the ability to grow in nutrient broth without NaCl and showed resistance to the vibriostatic agent O/129.

2.3 Identification by MALDI-TOF MS mass spectrometry

Species-level identification of the isolates was initially assessed by MALDI-TOF mass spectrometry (Microflex LT, Bruker Daltonics®, Germany), following the manufacturer's protocol, which involves direct colony transfer. The isolates were grown for 24 hours on Tryptic Soy Agar (TSA) (Difco, USA), and using a sterile wooden stick, a single colony from each sample was briefly touched to transfer a thin layer of microorganisms to a polished steel plate with 96 wells.

TABLE 1 Distribution of *Aeromonas* isolates based on specimen source and host category.

Specimen source	Host category	Number of isolates
Artesian well water	Environment	4
Biodigester waste	Environment	2
Blood	Human	7
Bone fragment	Human	1
Bovine uterine	Animal	7
Cerebrospinal fluid	Human	3
Drinking tap water	Environment	1
Fish abdominal cavity	Animal	1
Fish brain	Animal	4
Fish eyes	Animal	6
Fish gills	Animal	2
Fish internal organs	Animal	2
Fish liver	Animal	3
Fish reproductive system	Animal	1
Fish skin	Animal	5
Knee discharge	Human	1
Leg secretion	Human	1
Reservoir water	Environment	1
Residual limb stump	Human	1
Skin lesion	Human	1
Stool	Human	35
Urine	Human	1
Total	—	90

The layer of microorganisms applied to a steel plate was overlaid with 1 μ L of formic acid, followed by 1 μ L of the matrix solution (10 mg/mL of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid). Each isolate was applied to three wells, and three readings were conducted. The FlexControl software (Bruker Daltonics) was used to capture the protein spectra using the MTB_autoX method within a mass range of 2 to 20 kDa. External calibration of the spectrophotometer was performed using ribosomal proteins from *Escherichia coli* (BTS - Bruker Daltonics®). For bacterial identification, BioTyper 3.0 software (Bruker Daltonics®) was utilized. The generated mass spectra were analyzed with BioTyper 3.0 software connected to the system and compared to the profiles stored in the system's library. The criteria for interpreting the patterns used in this study were as follows: scores ≥ 2.0 were considered acceptable for species attribution, while scores ≥ 1.7 and < 2.0 were used exclusively for genus identification, following the manufacturer's guidelines. In cases where the first and second match species differed, the identification corresponding to the highest score was selected.

Bacterial identification was performed using MALDI-TOF mass spectrometry and analyzed via the MicrobeNet platform (Centers

for Disease Control and Prevention, Atlanta, GA, USA; <https://www.cdc.gov/microbenet>, accessed on 23 July 2025). For each isolate, the XML export generated by MicrobeNet was retrieved and parsed to extract the top-ranked taxonomic identification, confidence score, and database version. Identifications were classified according to predefined score thresholds for species- or genus-level calls.

2.4 Whole genome sequencing

The strains were sequenced using the Ion Torrent S5 platform at the Strategic Laboratory of the Instituto Adolfo Lutz. The genetic material was extracted using the commercial Wizard Genomic DNA Purification Kit (Promega, USA). The integrity, purity, and concentration of the extracted genomic DNA were assessed using agarose gel electrophoresis (1%) at 100 V for 40 minutes, spectrophotometry with a NanoDrop™ One (Thermo Fisher Scientific, USA), and fluorometry with a Qubit™ (Thermo Fisher Scientific, USA), respectively. For the construction of the DNA library, the Ion Xpress™ Plus Fragment Library Kit (Thermo Fisher Scientific, USA) was used according to the manufacturer's recommendations. The material was applied to the Ion Chip 530 Kit.

Reads were evaluated for quality and GC content using FastQC (version 0.12.1). Contamination screening was performed with Kraken2 (version 1.1.1) via the Galaxy Europe Server (<https://usegalaxy.eu/>). Genome assembly was carried out using the FASTQ files obtained from short-read sequencing with the CLC Genomics Workbench (version 9.5.3, Qiagen, Venlo, Netherlands). QC metrics (e.g., genome size, N50, N90, contig count, and GC content) were assessed using QUAST (version 5.2.0).

2.4.1 Genome analysis

Average Nucleotide Identity (ANI) was utilized as the gold standard for species identification. Following the methodology established by Richter and Rosselló-Mora (Richter and Rosselló-Mora, 2009), ANI is defined as the percentage of identity observed in the nucleotide sequences of orthologous genes shared between two genomes. The cut-off value for *Aeromonas* was determined by Colston et al (Colston et al., 2014), who suggested that values of $\geq 96\%$ indicate that the two strains belong to same species (Maia et al., 2023). ANI analysis was performed in March 2025 using fastANI (Version 1.3) implemented on the Galaxy Europe Server (<https://usegalaxy.eu/>), with default settings applied. The reference genomes used in this analysis are listed in Supplementary Table 2. The online platform PathogenWatch (<https://pathogen.watch/> accessed on 22 April 2025) was also used for species identification.

2.5 Multilocus sequence typing

The assembled genomes were submitted to PubMLST for sequence type (ST) analysis. The MLST (Multilocus Sequence Typing) scheme for *Aeromonas* was created based on data

TABLE 2 Comparison of taxonomic identifications for *Aeromonas* isolates based on ANI and MALDI-TOF.

Id	ANI identification (% Similarity with type strain)	MALDI-TOF (Bruker) Identification (scores 1 st and 2 nd matches)	Possible explanation
Aer_19	<i>A. dhakensis</i> (97.10)	<i>A. hydrophila</i> (2.17; 2.04)	Absence of <i>A. dhakensis</i> in BDAL database
Aer_33	<i>A. dhakensis</i> (97.12)	<i>A. hydrophila</i> (2.23; 2.21)	Absence of <i>A. dhakensis</i> in BDAL database
Aer_42	<i>A. dhakensis</i> (97.23)	<i>A. hydrophila</i> (2.22; 2.18)	Absence of <i>A. dhakensis</i> in BDAL database
Aer_45	<i>A. dhakensis</i> (97.14)	<i>A. hydrophila</i> (2.28; 2.20)	Absence of <i>A. dhakensis</i> in BDAL database
Aer_47	<i>A. dhakensis</i> (97.17)	<i>A. hydrophila</i> (2.26; 2.21)	Absence of <i>A. dhakensis</i> in BDAL database
Aer_58	<i>A. dhakensis</i> (97.06)	<i>A. hydrophila</i> (2.29; 2.24)	Absence of <i>A. dhakensis</i> in BDAL database
Aer_64	<i>A. dhakensis</i> (97.1)	<i>A. hydrophila</i> (2.20; 2.12)	Absence of <i>A. dhakensis</i> in BDAL database
Aer_89	<i>A. allosaccharophila</i> (96.4)	<i>A. veronii</i> (2.15; 2.10)	Absence of <i>A. allosaccharophila</i> in BDAL database
Aer_91	<i>A. rivipollensis</i> (96.17)	<i>A. veronii</i> (2.09; 2.02)	Absence of <i>A. rivipollensis</i> in BDAL database
Aer_92	<i>A. rivipollensis</i> (96.18)	<i>A. eucrenophila</i> (2.09), <i>media</i> (2.06)	Absence of <i>A. rivipollensis</i> in BDAL database
Aer_95	<i>A. rivipollensis</i> (96.15)	<i>A. media</i> (2.07), <i>molluscorum</i> (2.02)	Absence of <i>A. rivipollensis</i> in BDAL database

obtained by Martino et al (Martino et al., 2011). utilizing six genes (*gyrB*, *groL*, *gltA*, *metG*, *ppsA*, and *recA*). Novel alleles and new allele combinations identified in this study were submitted to the PubMLST database for curation and deposition.

2.6 Single nucleotide polymorphisms analysis

Single nucleotide polymorphisms (SNPs) were identified from FASTA sequences using CSI Phylogeny (<http://www.genomicepidemiology.org/services/>, accessed on 23 July 2025) under default settings. The resulting SNP-based phylogenetic tree was visualized with Microreact (<http://microreact.org> accessed on 25 July 2025).

The Whole Genome Shotgun project has been deposited in DDBJ/ENA/GenBank under BioProject accession PRJNA1295185. Novel alleles and sequence types (STs) have been deposited in the PubMLST database, and their corresponding accession numbers are listed in the [Supplementary Table S3](#).

2.7 Statistical analysis

Pairwise SNP distances were used to assess genomic diversity and population structure among *Aeromonas* isolates. Mean pairwise distances were calculated within and between species to quantify

intra- and inter-species variation. To evaluate whether genetic distances were significantly associated with species or host/source categories, a permutational multivariate analysis of variance (PERMANOVA) (Herrando-Pérez et al., 2021). PERMANOVA was conducted using 999 permutations (default setting) to test for significant differences among groups.

3 Results

MALDI-TOF MS correctly identified 87.8% of the *Aeromonas* spp. isolates. Discrepancies between MALDI-TOF MS and genomic identification based on ANI were observed in 12.2% of the cases (Table 2). Differences were also noted between species identification by BDAL (Bruker database), MicrobeNet and the ANI-based approach. For instance, isolates identified as *A. rivipollensis* by ANI were classified as *A. veronii*, *A. eucrenophila*, and *A. media* by BDAL, and as *A. punctata* in MicrobeNet corresponding to isolates Aer_91, Aer_92, and Aer_95 respectively. Similarly, isolates identified as *A. dhakensis* by ANI were assigned to *A. hydrophila* in both the BDAL database and MicrobeNet (Aer_19, Aer_33, Aer_44, Aer_45, Aer_47, Aer_58, and Aer_64). In addition, one isolate (Aer_89) identified as *A. allosaccharophila* by ANI was classified as *A. veronii* in both BDAL and MicrobeNet.

The genomic profiles of the *Aeromonas* spp. isolates analyzed in this study are presented in [Supplementary Table S1](#). The assembled genome sizes ranged from 4.26 to 5.09 Mbp, with an average size of

4.71 Mbp. The average sequencing coverage was approximately 62×, ensuring high quality genome assemblies. The total guanine and cytosine (G + C) content of the *Aeromonas* spp. genomes ranged from 58% to 62%. Within *A. hydrophila*, the G + C content varied between 60.9% and 61.7%; in *A. caviae*, it ranged from 60.8% to 62%; and in *A. veronii*, from 58% to 58.8% (Supplementary Table S3).

The isolates analyzed originated from diverse sources, including fish, cattle, humans, and environmental samples, revealing distinct species distributions. Fish isolates were predominantly identified as *A. caviae*, *A. veronii*, and *A. hydrophila*, recovered from various tissues such as gills, eyes, brain, and skin. Uterine samples from cattle primarily harbored *A. rivipollensis*, *A. caviae*, and *A. media*, indicating species diversity within this host. Human clinical isolates, obtained from stool, blood, cerebrospinal fluid, and wound samples were largely composed of *A. hydrophila* and *A. caviae*, with occurrences of *A. veronii*, *A. dhakensis*, and *A. jandaei*. Environmental isolates, derived from reservoir and well water as well as biogas waste, were dominated by *A. hydrophila*, followed by *A. dhakensis* and *A. caviae*, highlighting their role as environmental reservoirs.

ANI-based genome comparisons (n=90) showed that *A. hydrophila* was the most prevalent species (34/90; 37.8%), followed by *A. caviae* (23/90; 25.5%) and *A. veronii* (16/90; 17.8%). Less frequent taxa included *A. dhakensis* (7/90; 7.8%), *A. jandaei* (4/90; 4.4%), *A. rivipollensis* (3/90; 3.3%), *A. media* (2/90; 2.2%), and a single isolate of *A. allosaccharophila* (1/90; 1.1%). Species identification based on ANI was further supported by results from the PathogenWatch platform, which demonstrated consistent concordance.

Regarding the sources of isolation, *A. hydrophila* was recovered from animals (8/34; 23.5%), the environment (6/34; 17.6%), and humans (20/34; 58.8%). *A. caviae* occurred predominantly in humans (17/23; 73.9%), with additional isolates from animals (5/23; 21.7%) and the environment (1/23; 4.3%). *A. veronii* was mainly animal associated (11/16; 68.8%) with human isolates (5/16; 31.3%). *A. dhakensis* was mostly human derived (6/7; 85.7%), with a single environmental isolate (1/7; 14.3%). *A. jandaei* was exclusively recovered from humans (4/4; 100%), while *A. rivipollensis* (3/3; 100%) and *A. media* (2/2; 100%) were isolated only from animals. and *A. allosaccharophila* was represented by a single animal derived isolate (1/1; 100%).

MLST results are presented in the Supplementary Table S3. A large number of isolates (n=61) were found to harbor novel STs not previously reported in the database. The distribution of STs was highly diverse, both across *Aeromonas* species and among isolates from different sources, including human, animal, and environmental samples. Notably, the *ppsA* gene, one of the housekeeping loci used in the standard MLST scheme for *Aeromonas* spp., was not detected in isolate Aer_10. Whole genome sequencing and PCR assays both failed to identify the presence of this gene, confirming its absence.

The SNP distance matrix revealed a wide range of genomic diversity among the *Aeromonas* isolates, with pairwise SNP differences ranging from 18 to 26,825 across the entire dataset.

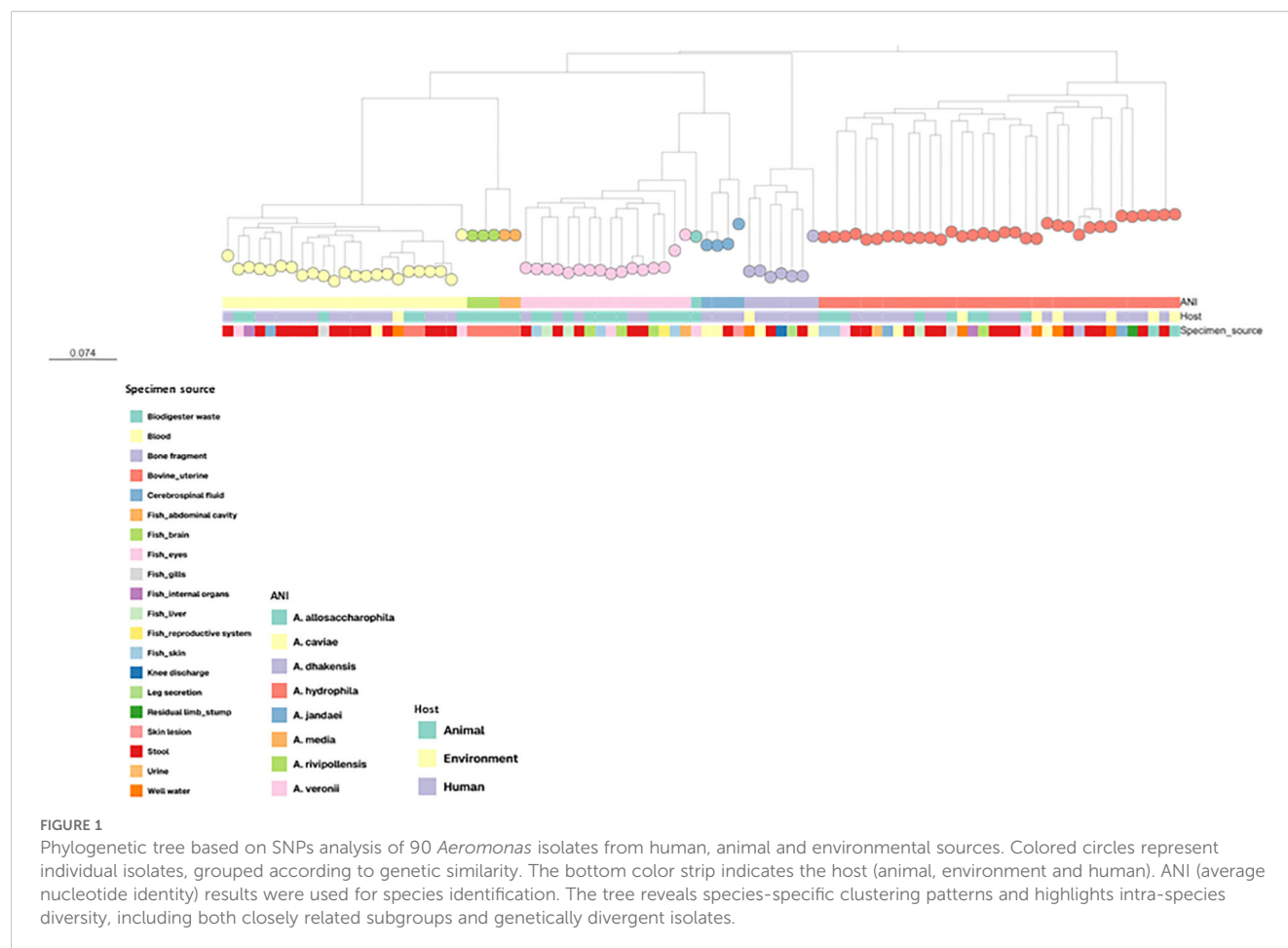
Intra-species comparisons showed variable patterns of diversity. For *A. veronii*, SNP distances ranged from 18 to 10,517 SNPs, while *A. hydrophila* and *A. caviae* exhibited the greatest intra-species genomic diversity, with SNP distances ranging from 19 to 19,447 and 22 to 7,274 respectively. In contrast, *A. dhakensis* isolates were more homogeneous, ranging from 11,597 to 12,678 SNPs. These results highlight the coexistence of closely related clonal groups and highly divergent strains, consistent with the phylogenetic analysis. Overall, SNP-based analyses provided detailed, high resolution insights into the evolutionary dynamics within the *Aeromonas* genus.

SNP-based phylogenetic analysis revealed distinct clustering patterns corresponding to *Aeromonas* species, with isolates identified as *A. caviae*, *A. hydrophila*, and *A. veronii* forming species-specific clades (Figure 1). Within these clades, *A. hydrophila* isolates tended to group more closely together, while *A. veronii* showed a more dispersed distribution across several lineages. *A. caviae* isolates displayed greater intra-species diversity, compared to the other groups. Integration of metadata indicated that *A. hydrophila* isolates included both human and environmental isolates, whereas *A. veronii* isolates of animal origin were mainly grouped in separate lineages.

Statistical analysis of the pairwise SNP distance matrix revealed a clear genomic structure among *Aeromonas* isolates. Most of the genetic variation was explained by species differences ($F = 27.54$; $p = 0.001$), confirming strong species-level clustering consistent with the phylogenetic reconstruction. A smaller but statistically significant effect was also detected for host/source categories ($F = 2.72$; $p = 0.003$), indicating that, while isolates from human, animal, and environmental origins can share closely related genotypes, their genetic composition varies slightly according to source. In summary, SNP-level diversity in *Aeromonas* reflects taxonomic differentiation, with minor contributions from ecological or host-related factors.

4 Discussion

While *Aeromonas* species are increasingly recognized as emerging pathogens with relevance in both clinical and environmental contexts, studies in Brazil integrating isolates from human, animal, and environmental sources remain scarce. Most national investigations have focused on single source isolates and have emphasized the use of MALDI-TOF MS as the primary method for species identification (Cardoso et al., 2021; Teodoro et al., 2022). In contrast, international research has progressively adopted whole genome sequencing (WGS) to improve species-level resolution and explore antimicrobial resistance, virulence, and phylogenetic relationships in *Aeromonas* (Awan et al., 2018; Klemm et al., 2024; Sagas et al., 2024). However, few global studies have simultaneously compared MALDI-TOF MS and WGS-based approaches across isolates from multiple ecological niches. This study addresses these gaps by employing a combination of MALDI-TOF MS, WGS, average nucleotide identity (ANI), multilocus sequence typing (MLST), and SNP-based phylogenetic



analysis to identify and characterize *Aeromonas* isolates from clinical, animal, and environmental sources. Our findings contribute novel insights into the genetic diversity and ecological relationships of *Aeromonas* spp., supporting integrated surveillance within a One Health framework.

The WGS-based analysis employing Average Nucleotide Identity (ANI) provided a robust and precise framework for species-level discrimination and in depth characterization of the isolates. ANI, recognized as a gold standard for bacterial taxonomy, allowed classification with high confidence (ANI $\geq 96\%$), capturing subtle genetic differences that traditional methods often miss (Martino et al., 2011; Colston et al., 2014; Kim et al., 2014; Awan et al., 2018; Cardoso et al., 2021; Teodoro et al., 2022; Maia et al., 2023; Klemm et al., 2024; Sagas et al., 2024). The identified species exhibited distinct distribution patterns across multiple sources, fish, cattle, humans, and environmental samples, reflecting their diverse ecological niches and potential epidemiological roles (Fernández-Bravo and Figueras, 2020). The concordance of ANI-based identification with the PathogenWatch platform further validates the reliability and reproducibility of this genomic approach, which is easier and faster – only upload the fasta files is enough to retrieve the identification results (PathogenWatch platform. Version 23.4.4).

Overall, MALDI-TOF MS accurately identified the majority of isolates, particularly for species well represented in the instrument's

reference database. However, discrepancies were notably observed for *A. dhakensis*, *A. rivipollensis*, and *A. allosaccharophila*, primarily because these less common species were not included in the MALDI-TOF MS reference library (Fernández-Bravo and Figueras, 2020). Several studies have further demonstrated that *A. dhakensis* remains particularly prone to misidentification by MALDI-TOF, often being classified as *A. hydrophila* due to the phenotypic and proteomic overlap between these taxa (Bartie and Desbois, 2024; Truong-Ha et al., 2024). In the study by Truong et al. (2024), MALDI-TOF achieved only 12% concordance with WGS-based ANI assignments, systematically misidentifying *A. dhakensis*, which emphasizes the critical impact of incomplete databases. This limitation highlights the challenges posed by incomplete spectral databases and reinforces the importance of continually updating and expanding these libraries to improve species coverage and diagnostic accuracy.

While traditional phenotypic methods such as MALDI-TOF MS remain valuable for rapid routine identification, they may lack sufficient resolution to reliably distinguish closely related species. In this context, WGS-based identification using ANI $\geq 96\%$ provided high resolution classification of 90 *Aeromonas* isolates and served as a robust reference for evaluating the performance of MALDI-TOF MS (Colston et al., 2014; Kim et al., 2014; Maia et al., 2023). By evaluating the same spectra in another public database (MicrobeNet, developed by the CDC and partners), the results

similarly failed to correctly identify the isolates that had been previously misidentified or not identified by the Bruker MALDI-TOF MS library. These findings highlight the limitations of current MALDI-TOF MS platforms in identifying less common *Aeromonas* species and suggest that integrating complementary methods or relying on high resolution genomic approaches remains essential for accurate species level identification.

In addition to ANI, Multilocus Sequence Typing (MLST) was employed to further characterize the *Aeromonas* isolates. The MLST analysis revealed considerable genetic heterogeneity with many isolates harboring novel sequence types (STs) not previously reported in the PubMLST database (Navarro and Martínez-Murcia, 2018; Meng et al., 2020; Lu et al., 2025). The identification of these novel STs underscores the ongoing diversification within the genus and provides valuable epidemiological markers for future surveillance and comparative studies. Their inclusion in global databases is essential to improve the resolution of molecular typing frameworks and to facilitate accurate tracking of emerging lineages. This finding underexplored diversity within *Aeromonas* genus and highlights the necessity to expanded genomic surveillance. The observed variability across isolates from human, animal, environmental sources reflects the ecological versatility of this genus and its potential for circulation between different hosts and environments (Fernández-Bravo and Figueras, 2020). Notably, one isolate (Aer_10) lacked the *ppsA* housekeeping gene, a result confirmed by both WGS and PCR, suggesting a possible gene loss or genomic rearrangement, an uncommon but significant event that may contribute to our understanding of genome plasticity in *Aeromonas*.

The overall phylogenetic structure underscores the genomic distinctiveness among *Aeromonas* species and highlights differences in intra-species diversity, with *A. caviae* showing broader dispersion compared to other species (Fernández-Bravo and Figueras, 2020; Singh et al., 2025). This wider distribution may reflect its ubiquity across environments, as previously reported, and may indicate higher genomic variability. Moreover, the close phylogenetic proximity of certain *A. hydrophila* strains from environmental and human samples is consistent with its presence in multiple ecological niches and its occurrence across different hosts. In our dataset, *A. caviae* was the most frequently isolated species from stool samples, slightly surpassing *A. hydrophila*. This observation aligns with previous reports identifying *A. caviae* as a predominant species associated with gastrointestinal infections in humans (Vila et al., 2003; Du et al., 2021; Ruiz de Alegria-Puig et al., 2023; Chong et al., 2024). Together, these findings support the use of SNP-based phylogenomics as a powerful and reliable tool for species-level resolution and for investigating evolutionary and epidemiological patterns in *Aeromonas* populations.

Our SNP-based phylogeny also revealed distinct and well-supported clades for *A. dhakensis* and *A. jandaei*, underscoring the genomic distinctiveness of these species within the genus. The clear separation between *A. dhakensis* and *A. hydrophila* corroborates recent findings that have corrected long-standing misclassifications and supports the need for re-evaluation of

public health isolates previously identified as *A. hydrophila* (Bartie and Desbois, 2024; Truong-Ha et al., 2024). Among the species analyzed, *A. rivipollensis* and *A. media* exhibited the lowest mean intra-species distances (approximately 31 and 42 SNPs, respectively), followed by *A. jandaei* (approximately 8,299 SNPs), indicating moderate genomic relatedness among the human isolates analyzed. Given the limited number of *A. jandaei* isolates analyzed and the lack of environmental or animal representatives, this finding must be viewed within the context of restricted sampling, which limits broader inferences about the population structure, ecological distribution, and potential reservoirs of the species. The statistical analysis of SNP-level variation revealed that the genomic structure of *Aeromonas* populations is mainly shaped by species-level differentiation. This strong taxonomic signal is consistent with the phylogenetic reconstruction and supports the clear separation of species within the genus, as previously observed in phylogenomic studies of *A. media*, *A. hydrophila*, and *A. veronii* complexes (Bartie and Desbois, 2024; Truong-Ha et al., 2024).

This study highlights the complementary roles of multiple molecular and phenotypic techniques in accurately identifying and characterizing *Aeromonas* species. WGS and ANI emerged as gold standard methods, offering high resolution taxonomic classification and enabling detailed exploration of phylogenetic relationships and population structure (Fernández-Bravo and Figueras, 2020; Bertran et al., 2021). Their precision was particularly valuable in distinguishing closely related species and in validating other identification tools. However, the study is not without limitations, particularly the uneven distribution of isolates across hosts and environments, as most originated from human stool samples collected through routine public health surveillance, while environmental and animal isolates were only available through specific collaborations. This sampling imbalance may limit the strength of inter-host or ecological comparisons.

This study underscores the complementary roles of molecular and phenotypic techniques in improving the identification and characterization of *Aeromonas* species. WGS and ANI provided high-resolution taxonomic classification, enabled detailed phylogenetic analysis, and served as a gold standard for validating other tools. Importantly, while WGS-based surveillance may not yet be universally accessible, especially in low and middle-income countries, combining WGS with MALDI-TOF MS and curated online platforms offers a robust framework for accurate identification. Together, these findings underscore the need for integrated diagnostic and surveillance strategies that leverage both advanced genomic approaches and accessible technologies, strengthening One Health surveillance and informing public health interventions to mitigate *Aeromonas*-associated risks widely distributed worldwide.

Data availability statement

All the sequences generated in this study were deposited in the GenBank (BioProject: PRJNA1295185).

Ethics statement

This study was submitted to and approved by the local ethics committee under CTC-IAL 05-Q/2024.

Author contributions

TV: Conceptualization, Software, Methodology, Investigation, Writing – original draft, Formal Analysis, Validation, Data curation, Visualization. GC: Investigation, Writing – review & editing, Methodology. AY: Writing – review & editing, Methodology, Data curation. AB: Investigation, Software, Formal Analysis, Writing – review & editing, Methodology. MM: Writing – review & editing, Supervision, Conceptualization, Data curation. LM: Methodology, Validation, Writing – review & editing, Formal Analysis, Data curation. AM: Formal Analysis, Validation, Writing – review & editing, Supervision. CARC: Investigation, Data curation, Formal Analysis, Writing – review & editing, Supervision, Validation. KC: Validation, Software, Writing – review & editing, Methodology, Visualization. MB: Data curation, Visualization, Software, Formal Analysis, Writing – review & editing. CS: Methodology, Conceptualization, Software, Writing – review & editing. CHC: Supervision, Visualization, Resources, Formal Analysis, Writing – review & editing, Software, Validation, Writing – original draft. MT: Supervision, Validation, Conceptualization, Funding acquisition, Writing – original draft, Resources, Data curation, Visualization, Project administration, Formal Analysis.

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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/fbri.2025.1708292/full#supplementary-material>.

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