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# Insights in emerging or re-emerging zoonoses

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# Insights in emerging or re-emerging zoonoses

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# Editorial: Insights in emerging or re-emerging zoonoses

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KEYWORDS

**public health, zoonoses, emerging, epidemiology, surveillance**

Editorial on the Research Topic
Insights in emerging or re-emerging zoonoses

The recent COVID-19 pandemic and the frequent cross-species transmission from birds to mammals of highly pathogenic avian influenza (HPAI) A(H5N1) strikingly underscore that the battle against emerging infectious diseases is far from over.

According to the World Health Organization (WHO), 60% of human pathogens originate from animals, and 75% of pathogens responsible for emerging and reemerging animal diseases have the potential to cross the animal-human interface. Thus, the emergence of zoonotic diseases is a global multifaceted problem that should be addressed through an integrated approach by the scientific community.

The main goal of this Research Topic was to provide a comprehensive collection of research studies on the epidemiology, diagnosis and pathology of infections caused by emerging or re-emerging zoonotic pathogens, including viruses, parasites, and bacteria affecting humans, domestic animals and wildlife. The 13 manuscripts of this Research Topic explore different aspects of emerging infectious diseases across the globe, including 10 original research studies, one review, and two brief research reports.

As a major outcome, around 70% of the published papers are related to viral pathogens, with a special focus on Asian countries, while the remaining focus on bacterial and parasitic diseases across the globe. Both domestic animals (companion and livestock) and wildlife have been investigated throughout the Research Topic as a potential source of infectious diseases with zoonotic potential in different socioeconomical and environmental contexts.

A strong link between poor animal welfare levels and zoonotic risks has been found by Mace and Knight in their review describing risk factors for the transmission of avian influenza (AI). They highlighted how the risks from industrial animal farming, especially mixed swine and poultry farms, are high. Reducing stress in farmed animals is crucial for their immunocompetence and to minimize disease risks. The authors advise against intensive farms and recommend lowering stocking densities to improve animal health and welfare.

Hinjoy et al., explored how poultry movement patterns in Thailand may explain AI spread over the networks once introduced using network analysis approaches. In 2021, poultry trades were recorded among 338 farmers and eight traders. The authors mapped 99 subdistricts and 181 trade links, finding feedback loops in 56 subdistricts. This network

illustrates that the poultry trade in three border provinces of Thailand was relatively localized highlighting potential AI spread pathways. Enhanced surveillance and control in high-trade areas can mitigate AI transmission, and social network analysis improves risk communication and biosecurity, enabling targeted strategies for more effective and efficient disease risk reduction across the value chain.

The evolution and possible origin of influenza viruses isolated from bats and birds is reported by Karamendin et al.. Bat H9 viruses likely originated from avian H9, with neutralizing antibodies detected in African bats. The Caspian region, a major migratory route, could facilitate viral mixing in different species. Increasing human H9N2 infections in Asia raise pandemic concerns, highlighting the need for regular monitoring of H9 virus in animal and human populations to ensure pandemic preparedness.

Different international organizations, such as WHO or EFSA, have pointed out the need to monitor other emerging and zoonotic viruses, such as SARS-CoV-2. In this Research Topic, a large-scale survey was carried out in Thailand by describing the sero-epidemiology of this virus in dogs and cats during the first and fourth waves of the Thailand COVID-19 outbreak between 2020 and 2021. The results of their study revealed seropositivity in eight out of 3,099 individuals sampled (0.26%), specifically in three cats and five dogs. Ongoing surveillance in companion animals, especially cats, is crucial due to their potential susceptibility to new variants. This could create viral reservoirs and facilitate human-animal transmission.

It is of paramount importance to maintain active surveillance of zoonotic emerging infectious diseases also at the livestock/human interface and wildlife/human interface, as highlighted by Ryoo et al. and Zhou et al., they report infection with a novel strain of foot and mouth disease virus (FMDV) in cattle and goat and Parainfluenza virus 5 (PIV5) in a 12-year-old captive male Siberian tiger from China, respectively. While Ryoo et al. provided evidenced how emergency vaccination and intensive surveillance programs reduced the spread of the new FMDV strain, Zhou et al. underlines the urgent need to control PIV5 in zoo animals to prevent interspecies transmission. PIV5 mutations in wild animals offer potential candidates for researching virus evolution and transmission mechanisms.

As a re-emerging neglected zoonosis, rabies represents one of the most significant public health concerns worldwide due to its fatal evolution. The study by Punyapornwithaya et al. aimed to predicting the incidence of canine rabies in Thailand using time series methodologies. They identified 4,678 confirmed canine rabies cases, showing seasonal patterns. The TBATS model demonstrated good predictive accuracy, forecasting an annual average of 285 cases (23 monthly) for 2023–2025. The study by Punyapornwithaya et al. offers advanced time series methodologies for infectious disease forecasting, introducing surveillance methods for rabies prevention, and control efforts.

Another comprehensive contribution on rabies has been done by Cunha et al., who reported the circulation of three Lyssavirus variants and their epidemiological importance in the north and northeast regions of Brazil, which harbor a large diversity of hosts, including bats. As highlighted by the authors, the emergence of Lyssavirus, facilitated by diverse bats and interspecies interaction, poses risks of spillover events and new viral strains. Marmosets (*Callithrix jacchus*) in Brazil serve as a unique reservoir for rabies, with implications for human infections. Control efforts should focus also on urban rabies while major challenges persist with wild animals.

Bacterial infections can also be causative agents of important zoonotic emerging or re-emerging diseases, such as brucellosis and salmonellosis. In their cross-sectional study, Ahad et al. described seroprevalence of brucellosis in camels, sheep, and goats, and their owners from Ethiopia, and analyzed risk factors collected via questionnaire. The study revealed a 5% brucellosis seroprevalence in camels, sheep, goats, and humans. Herd size and retained fetal membranes were risk factors for animal seropositivity, while human seropositivity correlated with contact with seropositive animals and assisting during calving or birthing. This study highlights again the usefulness of the "One Health" approach while planning control strategies and surveillance programs.

Even in remote islands such as the Cayman Islands (Caribbean), the epidemiological surveillance of zoonosis at the wildlife/livestock interface is of paramount importance to prevent new outbreaks in humans. Watler et al., investigated whether Salmonella could be introduced in the local poultry by import through the collection of boot swabs, paper bedding, and cecum samples from imported and feral chickens in the Cayman Islands. The results highlighted that Salmonella in the Cayman Islands mainly originates from imported day-old chicks. While feral chicken serotypes match those commonly found in humans, their low virulence and the absence of antimicrobial resistance mitigate overall health risks. Antibiotic resistance is concerning and underscores the need for stricter import controls and ongoing surveillance programs.

In particular, multi-drug resistance (MDR) has been considered a major plague globally in recent years, and both livestock and companion animals are considered sentinels to prevent this phenomenon in humans. Abad-Fau et al., reported an alarmingly high percentage (71.15%) of multidrug resistant *E. coli* from urine samples of dogs suffering from urinary infections in Spain, underlying how dogs may harbor MDR pathogenic *E. coli*, potentially facilitating antimicrobial resistance transmission to humans. Continuous surveillance of antimicrobial resistance and updating therapeutic guidelines is imperative in veterinary clinics to mitigate these concerns.

While of minor spread, *Streptococcus suis* can be an important zoonotic pathogen, especially if considered as an occupational disease in the swine sector causing a wide range of fatal diseases (among others, septicemia, endocarditis, meningitis, and pneumonia) in humans. Zong et al., investigated genes and metabolites crucial for the survival of *S. suis* 2 aiming to identify potential targets for preventing and managing *S. suis* 2 infections.

Finally, some parasitosis can be regarded as significant re-emerging neglected zoonotic diseases, such as leishmaniosis caused by *Leishmania infantum*. Domestic dogs are the most important reservoir of this parasitic protozoa, and surveillance to enable early diagnosis is key to controlling it. In this regard, Sarquis et al. contributed with novel important insights in the management of canine leishmaniosis through the validation of a new clinical diagnostic biomarker. Circulating immune complexes were tested

as potential biomarkers to diagnose canine leishmaniosis, and also to track the progression of the disease. Again, from a One Health perspective, this study will further contribute to prevent and control a major parasitic disease that can affect humans and animals.

## Author contributions

JC: Writing – original draft, Writing – review & editing. SM: Writing – original draft, Writing – review & editing. MA: Writing – original draft, Writing – review & editing. BM: Writing – original draft, Writing – review & editing.

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## Conflict of interest

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# Phylogenetic analysis of rabies surveillance samples from north and northeast Brazil

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Viruses of the *Lyssavirus* genus are classified into several genotypes (GT1 to GT7), of which only GT1 (classic rabies virus—RABV) has a cosmopolitan distribution and circulates in Brazil. GT1 is subdivided into several antigenic variants (AgV) maintained in independent cycles with a narrow host range and distinct geographic distributions, namely, AgV1 and AgV2 found in dogs, AgV3 in the vampire bats *Desmodus rotundus*, and AgV4 and AgV6 in bats non-hematophagous *Tadarida brasiliensis* and *Lasiurus cinereus*, a common variant of marmoset (*Callithrix jacchus*), and crab-eating fox (*Cerdocyon thous*). In this study, we performed phylogenetic analysis to identify at the antigenic variant level; six RABV genomes derived from the Rabies Surveillance in the north and northeast regions of Brazil. The analysis resulted in the formation of 11 monophyletic clusters, each corresponding to a particular variant, with high bootstrap support values. The samples were positioned inside the AgV3, AgV6, and *Callithrix* variant clades. This is the first report of the AgV6 variant found in northern Brazil, which provides valuable information for rabies surveillance in the country. The possibility of viral spillover has been much debated, as it deals with the risk of shifting transmission from a primary to a secondary host. However, more genomic surveillance studies should be performed, with a greater number and diversity of samples to better understand the transmission dynamics of each variant to detect changes in its geographic distribution and spillover events.

KEYWORDS

**rabies, surveillance, *Lyssavirus*, genotypes, bats, variants, northern Brazil**

# 1. Introduction

Bats (order Chiroptera) are the main reservoir hosts for most viruses of the *Lyssavirus* genus (1). Species assigned to the genus are associated with acute progressive encephalomyelitis. Among them, the best-described species belong to *Lyssavirus* rabies, which is a disease that affects mammals. Lyssaviruses have different geographic distributions in different parts of the world; however, only the *Rabies virus* (RABV) has a cosmopolitan distribution, except for Antarctica and some isolated islands (2, 3).

Together with carnivores (order Carnivora), bats maintain the circulation of RABV. Rabies is transmitted directly between susceptible individuals by bites, scratches, or infection of mucous membrane virus-containing saliva, without the participation of arthropod vectors (2, 3).

Currently, 17 species are assigned to the *Lyssavirus* genus, which is divided into phylogroups I and II, according to their antigenic and genetic characteristics. Three species (*L. lleida, L. ikoma, and L. caucasicus*) are not included in the phylogroups, although they are well defined within the genus (4). These species are distributed into seven genotypes (GT1 to GT7), and only GT1, which includes the classic RABV, is of epidemiological importance given its association with a greater number of human cases of encephalomyelitis compared with other genotypes (2, 4).

GT1 has several antigenic variants associated with different animal species and regions or countries of origin. Animal hosts play a fundamental role in the maintenance of each of the RABV variants, which exist in nature in independent cycles, such as those related to hematophagous, frugivores, insectivorous bats, and wild canids, among others (2).

Seven antigenic variants have already been isolated in Brazil as follows: AgV1 and AgV2 found in dogs; AgV3 isolated from the vampire bat *Desmodus rotundus*; and AgV4 and AgV6 isolated from non-hematophagous bats, *Tadarida brasiliensis* and Lasiurus cinereus, common marmoset variant (*Callithrix jacchus*), and crab-eating fox variant (*Cerdocyon thous*) (2–4).

It is well established that certain variants are associated with specific animal species. Therefore, the distribution of a viral population is conditioned by the regional variation in the distribution of the host species, as well as by its biological aspects. The interaction between hematophagous and non-hematophagous bats and other wild animals facilitates the sharing of antigenic variants given their adaptation to certain regionally distributed secondary wild hosts (4).

Another widely discussed factor of great epidemiological relevance is the real and imminent possibility of changing the dynamics of viral transmission from a primary host, such as non-hematophagous bats, to secondary hosts, such as dogs and cats. Therefore, the prolonged maintenance of virus circulation in a new population can lead to the expansion of its geographic distribution, which justifies the introduction of new variants (3, 4).

## 1.1. Author summary

Rabies is a neglected disease directly related to socioeconomic factors. Rabies remains a worldwide public health problem due to its fatal evolution and the high number of human cases leading to a large number of people submitted to post-exposure treatments annually. The real magnitude of this problem is still unknown since epidemiological surveillance systems provide insufficient information to accurately measure the current scenario of the disease. Here, we report the circulation of three RABV variants and their epidemiological importance in the north and northeast regions of Brazil, which harbor a large diversity of hosts, including bats.

# 2. Materials and methods

## 2.1. Sampling location

Six tissue samples collected from six animals captured in the north and northeast regions of Brazil were the source material for sequencing. Three of them came from Cabixi (n = 1, *Artibeus lituratus*) and Cacoal (*n* = 2, *Molossus molossus*) municipalities, Rondônia (RO) state. Two samples came from *A. literatus* captured in Palmas and Boa Vista, cities of Tocantins (TO) and Roraima (RR) states, respectively. From the northeast region, we obtained a sample from a non-human primate (NHP) of the genus *Callitrix sp.*, from the city of Teresina, Piauí (PI) state (Figure 1). The samples came from routine surveillance to investigate possible cases of rabies in these areas, with no need for submission and approval by the Ethics Commission on Animal Use (CEUA).

## 2.2. RNA extraction, double-stranded cdna synthesis, and genomic library preparation

Approximately 10 mg of brain tissue was added to a 2 ml microtube together with 1 ml of TRIzol™ reagent and a 5-mm diameter steel bead and macerated in a TissueLyser II equipment for 2 min at a frequency of 30 Hz. Subsequently, 200 µl of chloroform was added, and the material was transferred to a tube containing a phase separator polymer, Phasemaker™ Tubes (Thermo Fisher Scientific), and kept at room temperature for 15 min. After centrifugation at 12,000 g at 4°C for 5 min, ~560 µl of the upper precipitation phase was recovered and transferred to a new tube, and 560 µl of pure ethanol was added. Total RNA from the samples was, then, purified using the PureLink RNA Mini Kit (Invitrogen Life Technologies), following the manufacturer's protocol starting with the column purification step described in the kit. After purification, the RNA was quantified using the Qubit RNA HS Assay Kit (Thermo Fischer Scientific) in the Qubit 4.0 equipment, according to the manufacturer's recommendations.

The synthesis of the first and second strands of cDNA was performed using the SuperScript TM VILO TM Master Mix Kit (Thermo Fisher Scientific, Waltham, MA, USA) and NEBNext® Second Strand Synthesis Module (New England Biolabs, Ipswich, MA, USA), respectively. The cDNA purification reaction was performed with the PureLink® PCR Purification Kit (Invitrogen Life Technologies). All steps, from cDNA synthesis to purification, followed the recommendations of the manufacturers. The synthesized cDNA was quantified with the Qubit Assay DNA HS Kit in the Qubit 4.0 equipment.

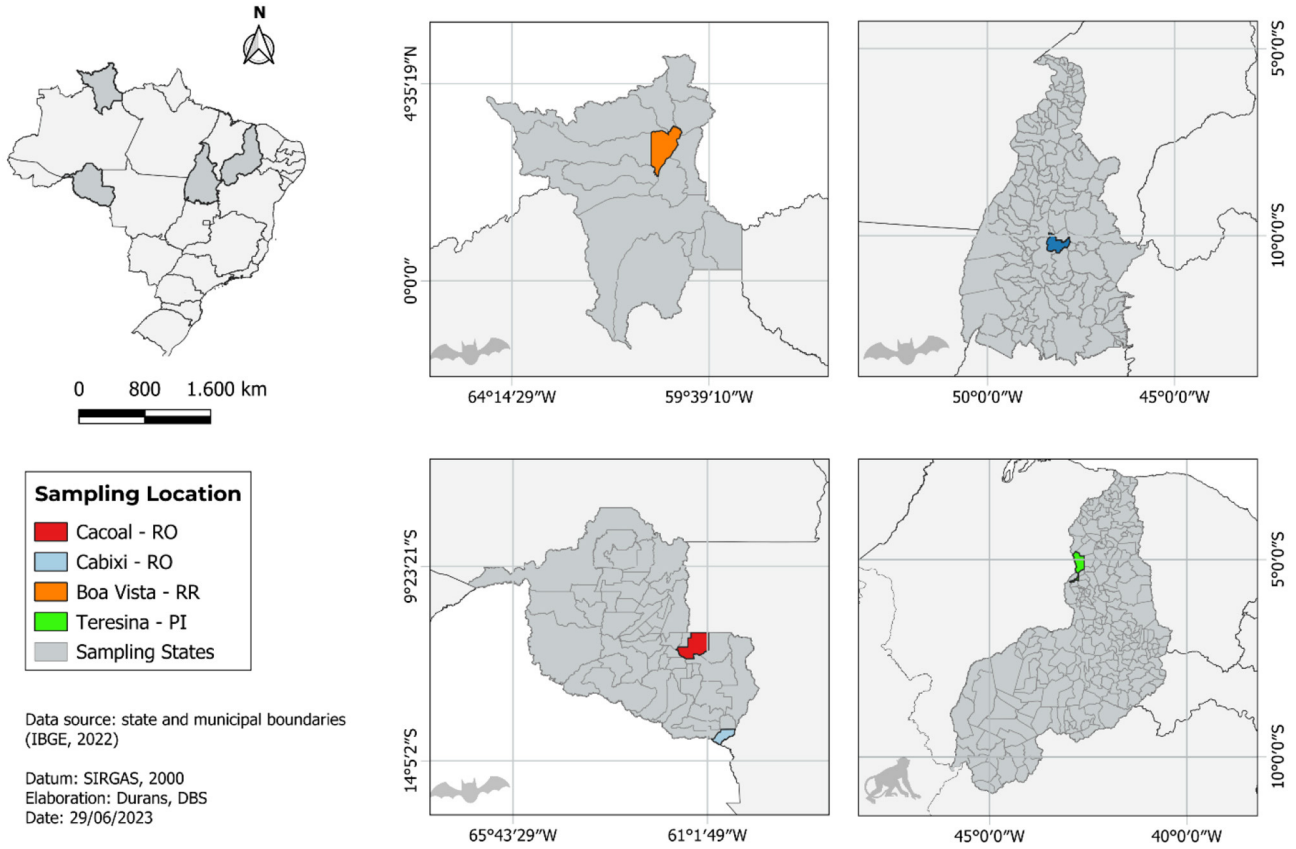


FIGURE 1
Map showing all locations of sample collection that have been analyzed in this study.

To prepare the genomic library, the Nextera XT DNA Kit (Illumina) guidelines were followed. Subsequently, the library was sequenced on the NextSeq 500 platform (Illumina) using the NextSeq 500/550 High Output Kit v2.5 (Illumina) (300 Cycles) and employing the paired-end methodology (2 x 150 bp), as recommended by the manufacturer.

## 2.3. Data analysis

Initially, the quality of the generated reads was evaluated using FastQC (5). Subsequently, DIAMOND software (6) was used to align the reads against the non-redundant (nr) protein database, taking into account the e-value (10–6) and amino acid identity. The output files generated by DIAMOND were converted into tabular output files, and these files were visualized in Krona v. 2.8 (7).

SortMeRNA v.2.1 was used to remove reads corresponding to ribosomal RNA (rRNA) (8). Next, Trim Galore v.0.4.5 (9) was used to remove short reads (<75 nt), adapters, and reads containing more than 10 undetermined bases and generate a FastQC file for the processed data.

The files generated in the data treatment step were assembled by the de novo method using SPAdes (10) (kmers: 21, 33, 55, and 77) and IDBA-UD (11) (kmers: 20, 40, 60, 80, and 100). The grouped contigs were aligned with DIAMOND under the same parameters described above.

## 2.4. Phylogenetic inference

A set of 44 nucleotide sequences corresponding to the nucleoprotein segment of RABV were analyzed. Of these, six were target sequences, five from the states in the north region (four from Rondônia state and one from Tocantins state) and one from the northeast region (Piauí state). Additionally, five of the target sequences are from bat samples and one from a non-human primate, from the years 2018, 2019, and 2021.

Using Geneious v.11.0 software (12), the nucleotide sequences were aligned using the MAFFT algorithm (13). Then, to evaluate the metrics of nucleotide distance between the sequences in the dataset, MEGA X software (14) was used, considering the *Maximum Likelihood Composition* substitution model. The final products were organized in a matrix of distances, which were later used for the analysis of intra/intergroup distances. The graphical representation was performed using R software (available at https://www.r-project.org/) together with the *ggplot2* libraries (available at https://ggplot2.tidyverse.org/), *reshape2* (https://cran.r-project.org/web/packages/reshape2/), and *pheatmap* (https://cran.r-project.org/web/packages/pheatmap/). Statistical means

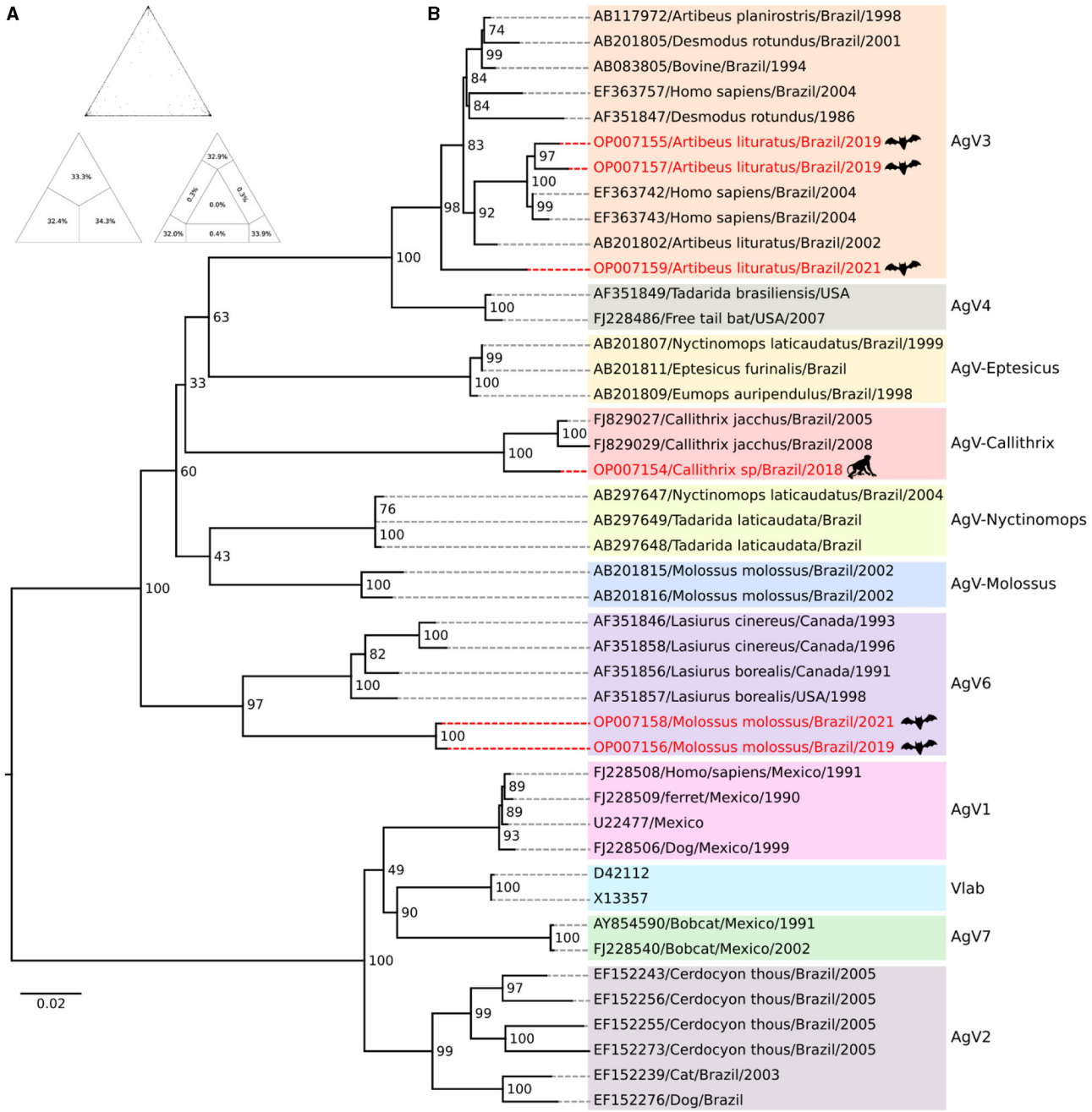


FIGURE 2
Phylogenetic inference of *Rabies virus*. **(A)** The value triangle shows a percentage representation of the analysis verifying the quality of the phylogenetic signal in the available sequence set which is shown in the value triangle. **(B)** The phylogeny is reconstructed using the *Maximum Likelihood* method. Bootstrap values are determined from 1,000 replicates and are indicated on each node. The highlighted red lines indicate the target taxa for the evolutionary positioning analysis.

demonstrating the significance of the relationship between the assessed taxa were generated by applying Student's *t*-test (considering $p < 0.05$), with a 95% confidence interval and a sample error margin of 5%.

To evaluate the evolutionary selection pressure acting on the studied sequences, the ratios between the proportions of non-synonymous (*dN*) and synonymous (*dS*) substitutions (*dN/dS*) among the analyzed sequences were obtained using CodeML software (belonging to the PAML package) (15, 16). Additionally, to verify the existence of sites under diversifying selection pressure (positive) throughout the evaluated region, the online tool Datamonkey (17) was used, employing its analysis modalities BUSTED (Branch-site Unrestricted Statistical Test for Episodic Diversification) (18) and MEME (Mixed Effects Model of Evolution) (19).

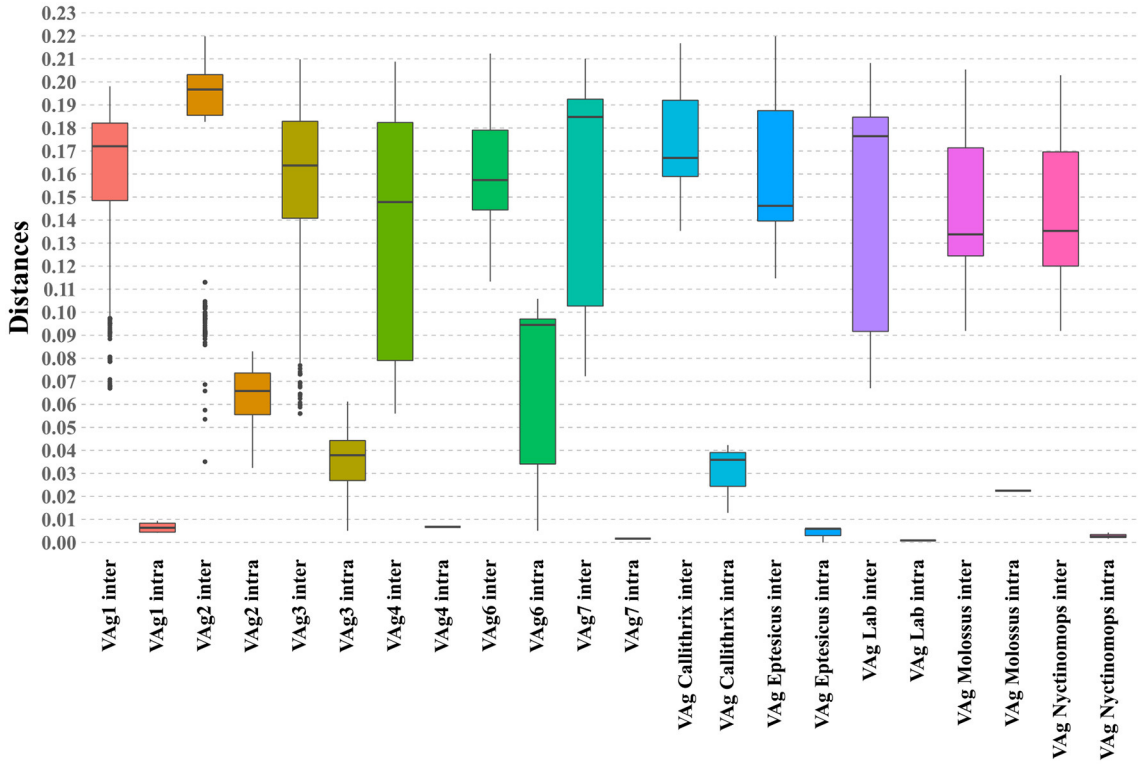


FIGURE 3
Boxplot graph illustrating the intragroup and intergroup distances of the phylogenetic grouping obtained, including the evaluated sequences. The analyzed clusters are indicated on the X-axis, and the Y-axis displays the observed percentages of nucleotide distance.

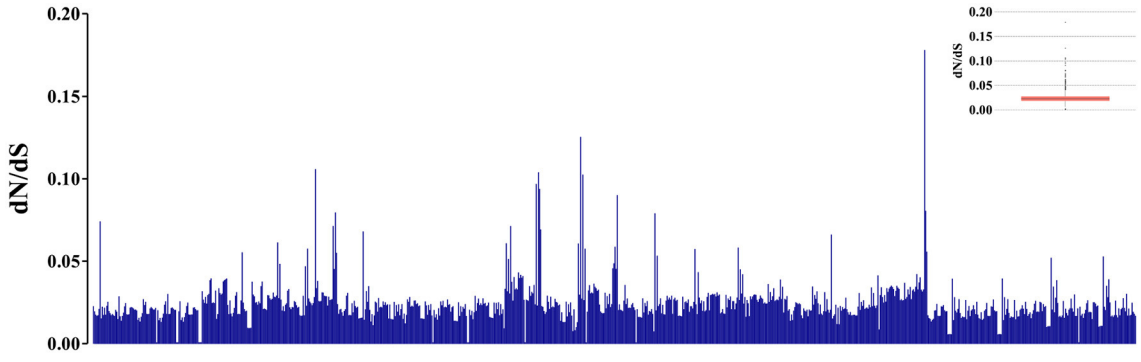


FIGURE 4
Graphic representation of 946 pairs formed by the analyzed sequences, illustrating the estimation of evolutionary pressure values using the *dN/dS* ratios. The Y-axis displays the indicative values of evolutionary pressure based on the *dN/dS* ratio. The internal boxplot graph indicates a prevalence of the *dN/dS* ratios between 0 and 0.05 for the evaluated pairs.

Using the aligned sequence file, the phylogeny was reconstructed using the *Maximum Likelihood* method with IQ-TREE v.1.6.12 software (20). The software automatically defined the best replacement model based on the Akaike Information Criterion (AIC) (GTR+F+I+G4) and generated bootstrap values (BPP) based on 1,000 repetitions. An additional analysis was performed using the software to evaluate the phylogenetic signal of the sequence set based on the defined AIC model. Finally, the topology was visualized using Figtree software v.1.4.4 (21), with definition of the midpoint anchorage of the typology obtained by the midpoint method (22) and edited using Inkscape (available at: https://inkscape.org/pt-br/).

## 3. Results

Based on the sequence quartet evaluation methodology, the phylogenetic signal analysis resulted in a positive signal, with 98.8% of trees generated exhibiting high reliability and 1% of trees generated with medium reliability (Figure 2A). The presence of

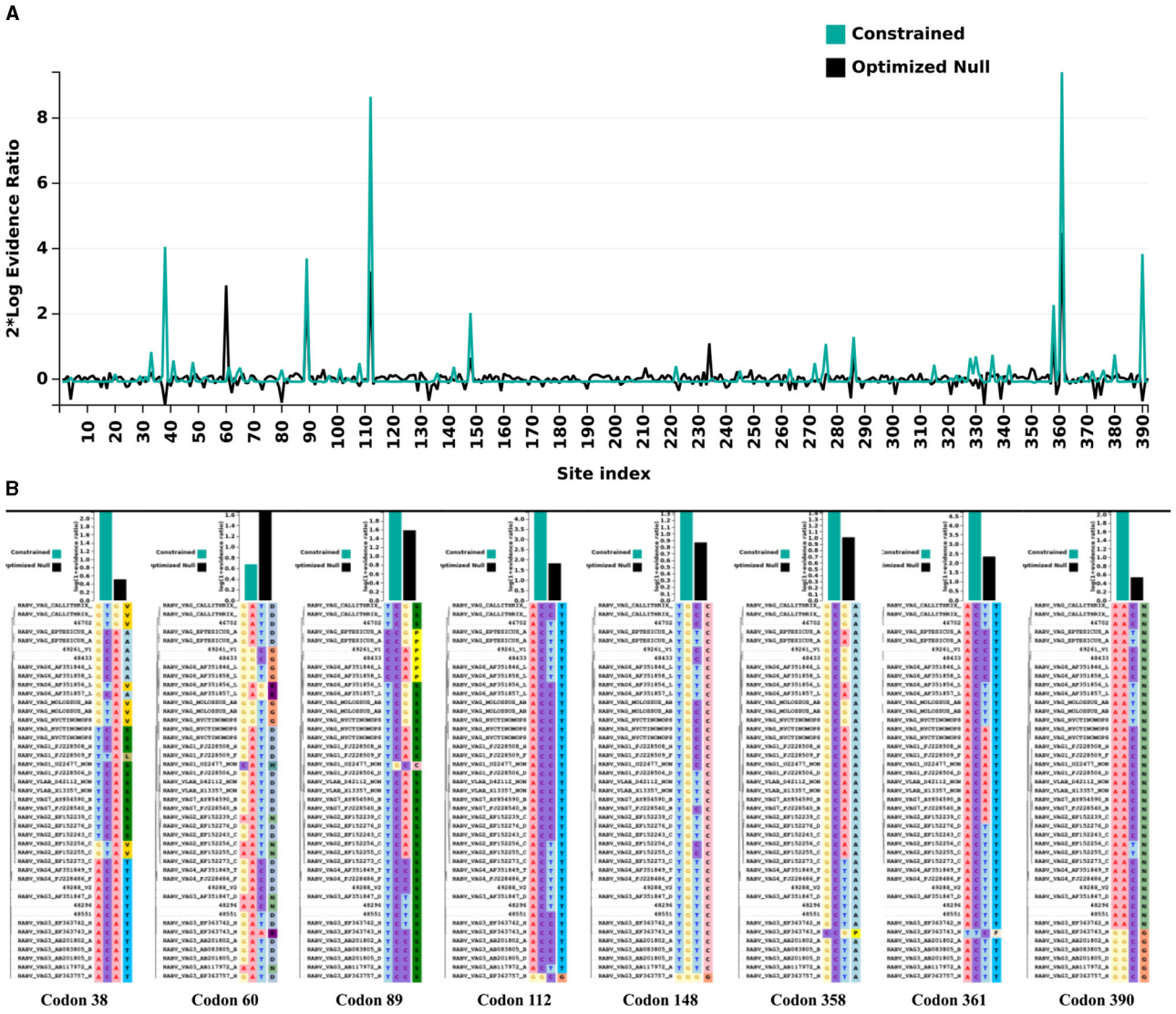


FIGURE 5
Evolutionary pressure analysis by *Datamonkey*. **(A)** Graphic representation illustrates the positions of sites along the evaluated gene region that experienced positive selection pressure effects. The numerical markings of the site positions are displayed on the X-axis, while the Y-axis represents the thresholds of evidence for positive selection pressure as defined by the tool used. **(B)** Sites (codons) along the evaluated region that exhibited diversifying (positive) selection effects, based on the defined parameters of the employed tool.

a negative phylogenetic signal, which is unsuitable for phylogeny reconstruction, is considered when the sum of unresolved and partially resolved regions exceeds 30%. Upon reconstructing the phylogeny, it was observed that 11 monophyletic groups were formed, demonstrating strong internal support. The target taxa were distributed among clusters that included sequences of VAg3 clade (taxa OP007155, OP007157, and OP007159) (BPP = 98), Variant *Callithrix* clade (taxon OP007154) (BPP = 100), and Variant 6 clade (taxa OP007158 and OP007156) (BPP = 98) (Figure 2B).

When evaluating the average nucleotide distances based on metrics using the *Maximum Likelihood Composition* model, an average distance of 0.14 was observed between the assessed taxa. Furthermore, in the evaluation of these distance metrics, significant differences ($p < 0.05$) were observed within the intra/intergroup comparative analysis when comparing the means obtained for the taxonomic grouping (Figure 3). These findings support the previous results regarding the formation of wellestablished taxonomic grouping among the evaluated variants as represented in the phylogenetic reconstruction analysis.

When analyzing the evolutionary pressure acting on the evaluated sequences, based on the non-synonymous to synonymous substitution ratios (*dN/dS*), it was observed that the studied gene region is undergoing global negative selection (purifying selection). This is indicated by the *dN/dS* ratios, which are consistently below 1, suggesting high levels of structural conservation. The range of the ratios is $0 \pm 0.17$ (Figure 4). Additionally, when examining the presence of sites under positive selection pressure in the set of evaluated sequences using the BUSTED tool, it is observed that the studied gene region, which

evolves globally under negative selection pressure according to the previous analysis, still exhibits at least eight sites (Figures 5A, B) experiencing positive selection effects. The results obtained using the MEME tool are consistent with those from BUSTED, revealing an overall average *dN/dS* ratio of 0.0394 and detecting diversifying selection in at least one site within the evaluated region.

## 4. Discussion

In recent years, the reports of emerging *Lyssavirus* have become frequent, and these occurrences are facilitated by the great diversity of bats, mainly non-hematophagous ones, and their interaction between different bat species with other animals. This behavior favors spillover events facilitating the adaptation of viruses to new hosts, which contributes to the emergence of new viral strains with pathogenic potential for other animals and humans (2, 3).

RABV circulation was verified in the northeast region of Brazil in marmosets (*Callithrix jacchus*), corresponding to a unique antigenic group, unrelated to samples found in bats or terrestrial mammals. This reinforces the hypothesis that marmosets are a natural reservoir for the virus, since its reading profile is incompatible with any monoclonal antibody available for circulating variants in the USA, maintaining a unique pattern.

Regional genetic variations can also be observed in the northern region. A retrospective study characterized RABV samples from different mammal species, including humans, from different regions in the Pará state, isolated between the years 2000 and 2005, and suggested the circulation of at least three distinct genetic lineages of a variant associated with vampire bats, one of which is maintained in the Marajó region. This reinforces the hypothesis that the region, where the first major outbreak of human rabies transmitted by vampire bats occurred, constitutes a unique niche for the maintenance of the cycle related to these animals (23, 24).

The epidemiological profile of rabies has changed in recent years, and wild animals, including the various species of bats, are considered efficient transmitters of the disease to other animals and humans. Since 2015, human rabies occasioned by the canine variants, AgV1 and AgV2, has not been recorded, and the presence of non-hematophagous bats in urban areas, although protected by law due to their important ecological role, become a threat to the population, as the number of RABV positive species grows each year in Brazil (2, 25).

In the present study, five samples from non-hematophagous bats and one from an NHP from Rondônia, Roraima, and Tocantins in the north region and Piauí state, northeast region, were analyzed. Three sequences from non-hematophagous bats *Artibeus lituratus*, from Tocantins (OP007155), Rondônia (OP007157), and Roraima (OP007159) states, were grouped into the AgV3, clade AgV, although this variant is more frequently associated with *Desmodus rotundus* vampire bats.

The AgV3 variant is strongly established and dispersed in the north and northeast regions of the country (4). The presence of this variant in *A. lituratus* may be related to close interactions between hematophagous and non-hematophagous bats, which can, to a certain extent, live in proximity and share the same shelter. In such a manner, disputes over territory may facilitate the transmission of RABV variants between different species of bats (26).

In contrast to the sequences from Rondônia and Tocantins, the sequence from Roraima, although undoubtedly clustered with the AgV3 group, was the most divergent, positioned in the basal branch in the clade. This suggests that the genetic variability within the AgV3 lineage circulating in these regions is higher than previously known. However, further analyses with a larger number of sequences must be carried out to confirm this hypothesis.

The only sequence from an NHP sample (OP007154), from the Piauí state, was positioned in a wellestablished way in the *Callithrix sp.* variant clade, together with samples from other locations in the northeast region of the country. It is noteworthy that since the end of the 1980s, RABV has been isolated from NHPs, mainly from the white-tufted marmoset (*Callithrix jacchus*), in at least four states of that region (Rio Grande do Norte, Ceará, Piauí, and Pernambuco). In two of these states (Ceará and Piauí), human cases of rabies transmitted by marmosets were reported (27).

Marmosets became well adapted to the region and a more common source of infection for humans. Data from laboratory-confirmed cases in 20 years suggest the occurrence of regional transmission and a gradual increase in the geographical distribution, supporting the emergence of marmosets as a new reservoir for RABV. In addition, tourism, wildlife traffic, and the culture of keeping these animals as pets, especially in coastal regions, are the main risk factors for the increase in human cases of rabies (27).

As a result of multiple efforts, Brazil achieved satisfactory levels of control for urban rabies maintained by dogs and cats. However, rabies in wild animals is still a major challenge for public and private services focused on human and animal health. The scenario is patent in the north and northeast regions of Brazil, where several cases of rabies have been recorded in wild animal species (27, 28).

A great diversity of variants is found in non-hematophagous bats, which points to the existence of multiple independent transmission cycles, involving different species of bats. Isolates from several genera/species of bat, including the *Molossidae* family, have already been genetically characterized in studies from different regions of the country, which provided an important basis for the analyses of this study (2, 3, 23, 24, 29–32).

Surprisingly, the RABV sequences, OP007156 and OP007158, from the insectivorous bats, *Molossus molossus*, collected in 2019 and 2021, in the Cacoal municipality, Rondônia state (Supplementary Figure 1), were grouped into the AV6 clade, with sequences from *Lasiurus cinereus*. Several studies demonstrate a restricted distribution of AgV6 to the south and southeast regions of Brazil (31). This is the first description of the variant in the north region and detected in a species not characteristic of its lineage.

Bats from the *Molossus* genus are among those most frequently found infected with RABV, probably due to the ecological and behavioral characteristics of the genus, increasing their susceptibility to become infected by specific variants from other bats, as observed in other studies (31, 33, 34).

Similar events tend to become even more frequent, given the great diversity of non-hematophagous bats, as well as the real and constant possibility of transmission between different bat species. These animals have an incredible ability for true flight, which makes them travel great distances, thus facilitating the sharing of territories and favoring the transmission and dissemination of RABV among them.

Ultimately, more genomic surveillance studies, particularly AgV, must be conducted with a larger number and diversity of samples, which will allow a more comprehensive understanding of the correlation between the genetic and ecological characteristics of the RABV variants. This would also enlighten the relationships between the divergent sequences within the established clades, thus strengthening the results of this study. This study highlights the heterogeneity and richness of the genomic information when it comes to viral diseases in the Amazon region, with the remarkable role of rabies in this context.

## 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 below: https://www.ncbi.nlm.nih.gov/genbank/, OP007154; https://www.ncbi.nlm.nih.gov/genbank/, OP007155; https://www.ncbi.nlm.nih.gov/genbank/, OP007156; https://www.ncbi.nlm.nih.gov/genbank/, OP007157; https://www.ncbi.nlm.nih.gov/genbank/, OP007158; https://www.ncbi.nlm.nih.gov/genbank/, OP007159.

## Author contributions

TCu: Methodology, Writing—review and editing. FS: Writing—review and editing, Methodology, Formal analysis. SS: Methodology, Writing—review and editing, Formal analysis. AR: Methodology, Writing—review and editing. FP: Writing—review and editing, Methodology, Formal analysis. LC: Writing—review and editing, Formal analysis. AN: Writing—review and editing, Investigation. IO: Investigation, Writing—review and editing. MB: Investigation, Writing—review and editing. RO: Investigation, Writing—review and editing. DD: Writing—review and editing, Formal analysis, Methodology. TP: Writing—review and editing. TCo: Conceptualization, Project administration, Supervision, 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.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1257558/full#supplementary-material

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# Transcriptome and metabolome profiling to elucidate the mechanism underlying the poor growth of *Streptococcus suis* serotype 2 after orphan response regulator CovR deletion

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The deletion of orphan response regulator CovR reduces the growth rate of *Streptococcus suis* serotype 2 (*S. suis* 2). In this study, metabolome and transcriptome profiling were performed to study the mechanisms underlying the poor growth of *S. suis* 2 caused by the deletion of orphan response regulator CovR. By comparing *S. suis* 2 (Δ*covR*) and *S. suis* 2 (SC19), 146 differentially accumulated metabolites (upregulated: 83 and downregulated: 63) and 141 differentially expressed genes (upregulated: 86 and downregulated: 55) were identified. Metabolome and functional annotation analysis revealed that the growth of Δ*covR* was inhibited by the imbalance aminoacyl tRNA biosynthesis (the low contents of L-lysine, L-aspartic acid, L-glutamine, and L-glutamic acid, and the high content of L-methionine). These results provide a new insight into the underlying poor growth of *S. suis* 2 caused by the deletion of orphan response regulator CovR. Metabolites and candidate genes regulated by the orphan response regulator CovR and involved in the growth of *S. suis* 2 were reported in this study.

KEYWORDS

***Streptococcus suis* type 2, orphan response regulator CovR, Δ*covR*, metabolome profiling, transcriptome sequencing, poor growth, aminoacyl tRNA**

## Introduction

*Streptococcus suis* (*S. suis*) is one of the main pathogenic bacteria on swine farms around the world (1). *S. suis* is also an emerging zoonotic pathogen that can cause a wide range of diseases (e.g., septicemia, endocarditis, arthritis, meningitis, and pneumonia) (2, 3). *S. suis* mainly causes death in 5- to 10-week-old post-weaned piglets, resulting in significant economic losses to the pig farming industry (4). According to the differences in capsular polysaccharide (CPS) antigenicity, 35 serotypes of *S. suis* strains were found and defined (5). Among these 35 serotypes, the *S. suis* 2 strain not only has the highest isolation rate but also poses a serious threat to the development of pig farming and human health (6).

Two-component systems (TCSs) play a crucial role in bacterial infection and response to changes in the external living environment, which have been identified in the vast majority of bacteria (7). The vast majority of TCSs in bacteria consist of a histidine kinase (HK)

that receives stimulus signals and a cognate response regulator (RR) that transfers stimulus signals. Once HK receives external stimulus signals, a histidine residue located on the HK will be phosphorylated, and then the phosphoryl group is transferred to the aspartic acid residue on the RR. Subsequently, the phosphorylated RR will interact with the promoter areas of the target genes associated with the response to the environmental stimuli (8). TCSs can determine the survival and pathogenicity of bacteria by regulating various cellular processes, including competition, metabolism, antibiotic resistance, biofilm formation, pathogenicity, and stress response. Therefore, TCSs are viewed as attractive candidates for the development of new strategies for preventing bacterial infections (7, 9).

The CovR/S system consists of a sensor kinase gene (*covS*) and a RR gene (*covR*), which directly or indirectly regulate 15% of genes connected with bacterial pathogenesis in *Streptococcus* spp. (10–12). Due to the impact of CovR/S on a variety of critical virulence factors, it is considered the most essential TCS system for group A *Streptococcus* (GAS, *Streptococcus pyogenes*) (13). The CovR that can regulate the virulence of bacteria has been reported in many studies. In *Streptococcus agalactia*, for example, the Δ*covR* strain has stronger hemolytic activity, adherence ability to epithelial cells, and higher lethality in the neonate rat sepsis model (14). Previous studies reported that CovR can bind to the promoter areas of the majority of virulence factor genes in GAS (13). These reports suggested that CovR can negatively regulate bacterial virulence. In bacteria, protein tyrosine kinases are linked to pathogenicity and exopolysaccharide synthesis (15). In *Edwardsiella piscicida*, adenylosuccinate synthase has been reported to have an inhibition effect on the host's nuclear factor-kappa B (NF-κB) signaling pathway, which is a common and important immune signaling pathway (16). Hemolysin is the key virulence component of *S. suis* 2, which creates holes in the target cell membrane and activates the inflammasome NLRP3 in order to release cytoplasmic $K^+$ efflux and cause Streptococcal toxic shock-like syndrome (17). Initiation factors IF1, IF2, and IF3 allow Shine-Dalgarno (SD) sequence-lead mRNAs initiate transcription (18). Ser/Thr kinase (STK) controls a number of cellular activities, including stress response, biofilm formation, membrane production, sporulation, metabolism, pathogenicity, and growth processes in bacteria. Recently, STK has been reported to be related to the virulence of *S. suis* (19). However, it is still unclear how the CovR/S system regulates genes and metabolites to promote the survivability of *S. suis* 2.

In recent years, transcriptomics and metabolomics have been extensively utilized to explain the survival and pathogenic mechanism of pathogenic bacteria. To reveal the mechanism by which the orphan RR factor CovR regulates the survivability of *S. suis* 2, liquid chromatography-tandem mass spectrometry (LC-MS) was employed to dig for the differentially expressed metabolites between *S. suis* 2 (wild type) and Δ*covR* (mutant) strains. Furthermore, the assessment of the differentially expressed genes (DEGs) was carried out using RNA-Seq and qRT-PCR. This study will not only reveal the genes and metabolites that are regulated by the orphan RR factor CovR involved in the survivability of *S. suis* 2 but also provide some potential targets for preventing and controlling *S. suis* 2 infections.

# Materials and methods

## Bacterial strains and growth condition

The *S. suis* 2 SC19 strain was donated by Prof. Chen Tan of Huazhong Agricultural University. SC19 is plated on Tryptose Soya agar (TSA) or cultured in Tryptose Soya broth (TSB) (Difco Laboratories, Detroit, MI, USA) with 10% (v/v) newborn bovine serum (Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) at 37°C. Δ*covR* mutant and complemented CΔ*covR* strains are stored in our laboratory and cultured as described above (20).

## Growth characteristics

The growth characteristics of wild-type SC19, Δ*covR* mutant, and complemented CΔ*covR* strains were determined by transferring overnight bacterial cultures into fresh preheated TSB supplemented with 10% newborn bovine serum at a ratio of 1:1000. The bacteria were grown at 37°C, accompanied by shaking at 180 rpm for 12 h, and $OD_{600}$ was determined by an Automatic Microbial Growth Curve Analyzer (MGC-200, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) for every hour. One-way ANOVA test was used to verify the significance of growth characteristics of SC19, Δ*covR*, and CΔ*covR* for every hour.

## RNA extraction, library construction, and sequencing

The total RNA was extracted using the TRIzol reagent (Takara, Dalian, China) from SC19 ($n = 3$) and Δ*covR* ($n = 3$) as described by Rio et al. (21), and the genomic DNA in the total RNA was removed by using DNase I (Takara). A total of 2 μg of RNA was used to build the RNA-seq transcriptome library by using the TruSeq™ RNA sample preparation Kit (Illumina, San Diego, CA). Briefly, the Ribo-Zero Magnetic Kit (epicenter) was used to exhaust ribosomal RNA (rRNA), and then the fragmented mRNA was broken into short fragments (200 bp) by using a fragmentation buffer. The mRNA fragment was performed by RT-PCR using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, CA) with random hexamer primers (Illumina). The dTTP was replaced with dUTP when the second strand was synthesized. Then, after end-repair and phosphorylation, the synthesized cDNA was treated with "A" base addition according to the protocol of Illumina's introduction. The UNG enzyme was used to recognize and degrade those second-strand cDNA with dUTP. The library was constructed using 200-bp cDNA target fragments of 2% low-range ultra-agarose. Subsequently, these fragments were amplified by performing 15 PCR cycles with Phusion DNA polymerase and quantified using TBS380. Finally, the paired-end RNA-seq sequencing library was constructed using Illumina Novaseq (2 × 150 bp read length).

## Transcriptome data analysis

The raw data collected from Illumina Hiseq sequencing were converted into raw data using base calling, and the results were saved in FASTQ file format. For mapping assembly sequences and distinct genes to reference sequences (GenBank: CP020863.1), Bowtie with default parameters (http://bowtiebio.sourceforge.net/index.shtml) was employed. FPKM (fragments per kilobase million) was used to quantify the quantity of genes and transcripts (http://deWeylab.biostat.wisc.edu/rsem/, https://ccb.jhu.edu/software/stringtie/). The transcriptome data were analyzed by the cloud platform of Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). Additionally, the DEGs between Δ*covR* and *S. suis* 2 strains were detected using the DESeq2 software. To control the probability of error with multiple test corrections (Benjamini/Hochberg, BH), the *p*-values were adjusted. DEGs were identified as their fold change ≥1.2 or ≤0.8 and adjusted *p*-values <0.05. The GO analysis for DEGs was performed using the Goatools software, while the KEGG analysis was performed using R script.

## Extraction of metabolites from bacteria and quality control measurement

Overnight, SC19 ($n = 4$) and Δ*covR* ($n = 4$) cultures were diluted at a 1:1000 ratio into TSB supplemented with 10% newborn bovine serum, and the mixture was then incubated to the logarithm phase. A total of 100 ml of the bacterium was transferred to a clean tube and centrifuged at 12,000*g* at 4°C for 10 min, and then the supernatant was removed. The metabolites were extracted from the bacterium (SC19 and ΔcovR) by a 400-μl isovolumetric methanol and acetonitrile solution and then sonicated by an ultrasonic cleaner (SBL-10TD, Ningbo Xinzhi Biotechnology Co., Ltd.) at 40 kHz for 30 min at 5°C. After being placed at −20°C for 30 min, the mixture was centrifuged at 13,000*g* at 4°C for 15 min. The supernatant was carefully transferred to a new microtube and evaporated to dry under a gentle stream of nitrogen. Then, the samples were resuspended with 100 μl of loading isovolumetric acetonitrile and water solution by short sonication using an ultrasonic cleaner (Ningbo Scientz Biotechnology Co., Ltd.) in a 5°C water bath. The extracted metabolites were centrifuged at 13,000*g* for 15 min at 4°C and then UHPLC-MS/MS analysis was performed. The same volumes of all samples were mixed to be used as a pooled quality control.

## UHPLC-MS/MS analysis

The LC-MS System (UHPLC-Q Exactive HF-X System, Thermo Fisher Scientific, Shanghai, China) was used to analyze the extracted metabolites. First, the sample was injected into a Waters HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm), which was followed by mass spectrometry detection. The mobile phases consisted of two solvents: solvent A, which consisted of 95% 0.1% formic acid and 5% acetonitrile, and solvent B, which consisted of 47.5% 0.1% formic acid in acetonitrile with 47.5% isopropanol and 5% water. The solvent gradient employed the following conditions: First, the gradient changed from 0 B to 24.5% B at a flow rate of 0.4 ml/min for 3.5 min; then, the gradient changed from 24.5 B to 65% B for 1.5 min; the gradient changed from 65 B to 100% B for 0.5 min; the gradient remained at 100% B for 1.9 min with the flow rate being increased from 0.4 to 0.6 ml/min; the gradient changed from 100 B to 51.5% B for 0.2 min; the gradient changed from 51.5 B to 0% B for 0.2 min at a flow rate of 0.6 to 0.5 ml/min; the gradient remained at 0% B for 0.2 min at a flow rate of 0.5 to 0.4 ml/min; the system equilibration was performed as the gradient remained at 0% B for 1 min at a flow rate of 0.4 ml/min. The sample injection volume was 2 μl at a flow rate of 0.4 ml/min. The column temperature was maintained at 40°C throughout the analysis, and the samples were stored at 4°C. The mass spectrometric data were collected using continuous scanning in either positive or negative ion modes. Data acquisition was performed in the data-dependent acquisition mode. The full MS resolution was set at 60,000, and the MS/MS resolution was set at 7,500. The heater temperature and capillary temperature were set at 425°C and 325°C, respectively. The sheath gas flow rate was set at 50 arb and the aux gas was set at 13 arb. The ion-spray voltage floating was −3,500 V in negative mode and 3,500 V in positive mode. Normalized collision energy for MS/MS was set at 20-40-60 V rolling. The detection range for mass spectrometry was from 70 to 1,050 m/z.

## Metabolite data and differential metabolites analysis

A three-dimensional data matrix that included sample information, metabolite name, and mass spectral response intensity in CSV format was obtained by analyzing metabolite data with Progenesis QI (Waters Corporation, Milford, USA). Internal standard peaks, as well as any known false positive peaks that include noise, column bleed, and derivatized reagent peaks, were removed from the data matrix, which was then de-redundant and peak pooled. The metabolic mass spectra features were recognized by accurate mass, MS/MS fragment spectra, and isotope ratio differences were searched in reliable biochemical databases such as the Human Metabolome Database (HMDB) (http://www.hmdb.ca/) and the METLIN database (https://metlin.scripps.edu/).

After searching in the database, these data were uploaded to the Majorbio cloud platform (https://cloud.majorbio.com) for analysis. In each set of samples, at least 80% of the metabolic features detected were retained. The metabolic levels in specific samples that fell below the lower limit of quantitation were imputed with minimum metabolite values, and each metabolic feature was normalized by sum. The response intensity of the sample mass spectrum peaks was normalized using the sum normalization method to obtain the normalized data matrix. Those QC samples with variables that had a relative standard deviation >30% were removed, and a logarithmic transformation (log10) was applied to the final data matrix for subsequent analysis.

Next, principal component analysis (PCA) and orthogonal least squares partial discriminant analysis (OPLS-DA) were conducted on the preprocessed data array using the R package (Version 1.6.2). The stability of the model was evaluated through

seven cycles of cross-validation. To identify significantly different metabolites, VIP values obtained from the OPLS-DA model and Student's *t*-test were used, with VIP >1 and $p < 0.05$ indicating significance. Metabolic pathway annotation was carried out using the KEGG data (https://www.kegg.jp/kegg/pathway.html) to determine the pathways with which the differential metabolites were associated. Pathway enrichment analysis was performed using the Python package scipy.stats, and Fisher's exact test was used to identify the most relevant biological pathways to the experimental treatments. The raw data were finally uploaded to MetaboLights, where they were given the unique identifier MTBLS8495 (www.ebi.ac.uk/metabolights/MTBLS8495).

## Quantitative real-time polymerase chain reaction

In this study, the accuracy of RNA-seq data was validated by performing qRT-PCR on the selected DEGs. Among them, B9H01_RS09035 is glutamine ABC transporter substrate-binding protein; B9H01_RS06585 is glutamine ABC transporter permease; B9H01_RS06580 is glutamine ABC transporter permease; B9H01_RS05040 is peptide ABC transporter ATP-binding protein; B9H01_RS05035 is amino acid ABC transporter permease; B9H01_RS09790 is ABC transporter ATP-binding protein; and B9H01_RS01490, B9H01_RS06570, and B9H01_RS09040 are ABC transporters. To begin, RNA extraction was followed as described in the preceding part of the text, and 1 μg of RNA was reserved for transcription using a PrimeScript® RT reagent Kit and gDNA Eraser (Takara, Dalian, China). Next, a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to perform the PCR reaction using Taq Pro Universal SYBR qPCR Master Mix (Q712, Vazyme, Nanjing, China). The 16S RNA gene served as the control gene, and all mRNA transcription levels were reported as $2^{-\Delta\Delta Ct}$. The primers in Table 1 were designed according to the genomic sequence of SC19 and used for qRT-PCR.

## Statistical analyses

The data in this study were analyzed using SPSS (Version 17.0, SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8. Unpaired *t*-tests between two groups or one-way ANOVA among multiple groups were used to obtain *p*-values ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; NS, not significant).

TABLE 1 Primers used in this study.

| Gene | Forward (5′-3′) | Reverse (5′-3′) |
|---|---|---|
| B9H01_RS09035 | GGGGCTAACAAACAGACT | AAGACACCGATGAGGAGA |
| B9H01_RS06585 | CCGAGATTATCCGTGGTG | GCCTCAAAGTAGCGTCCC |
| B9H01_RS06580 | TGCGTCGGACTCTATCAC | TGGAAGAATGGTCGGAAT |
| B9H01_RS05040 | CCTTCCGTTCAAATGTCG | CGTTGCTTCTGACCACCT |
| B9H01_RS05035 | CGTTATCGCTACCACAAA | ACAGCACCAGCAGAAAGG |
| B9H01_RS01490 | TTGGCAGGACATCTATTA | AGGAAACGAAACATTACTC |
| B9H01_RS09790 | AAATCGGGCAGGCTTACT | CCTTACGCATACGGTTGG |
| B9H01_RS06570 | TTTTGGGAATCAATAAGGC | CGGATAGCATAGCAGGGT |
| B9H01_RS09040 | CTCAATCATCGGTTCATC | CACGGTAAGTCGTCAAAT |
| 16S rRNA | ACTTGAGTGCAGAAGGGGAGAG | GCGTCAGTTACAGACCAGAGAGC |

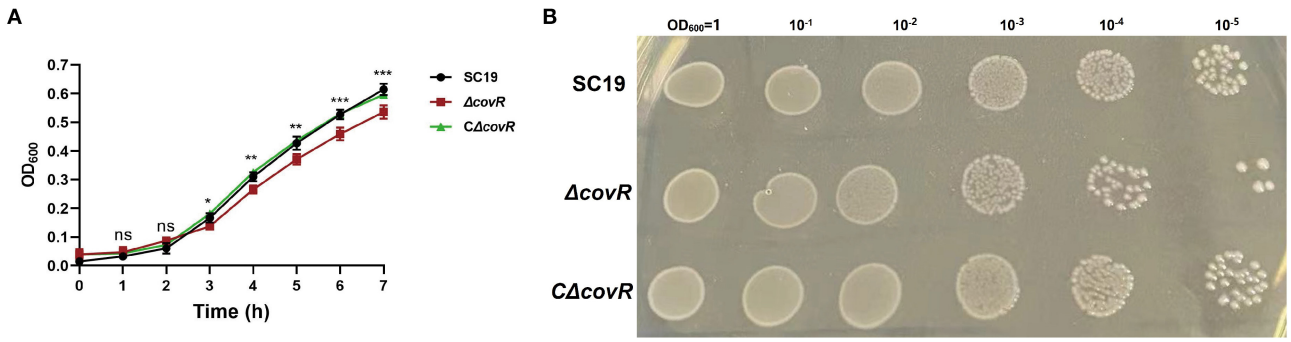


FIGURE 1
The growth characteristics of SC19, Δ*covR*, and complemented CΔ*covR* strains. **(A)** The growth curves of SC19, Δ*covR*, and complemented CΔ*covR* strains. **(B)** The clones of SC19, Δ*covR*, and complemented CΔ*covR* strains. Compare SC19 and Δ*covR*; ns, no significance; $^{*}p < 0.5$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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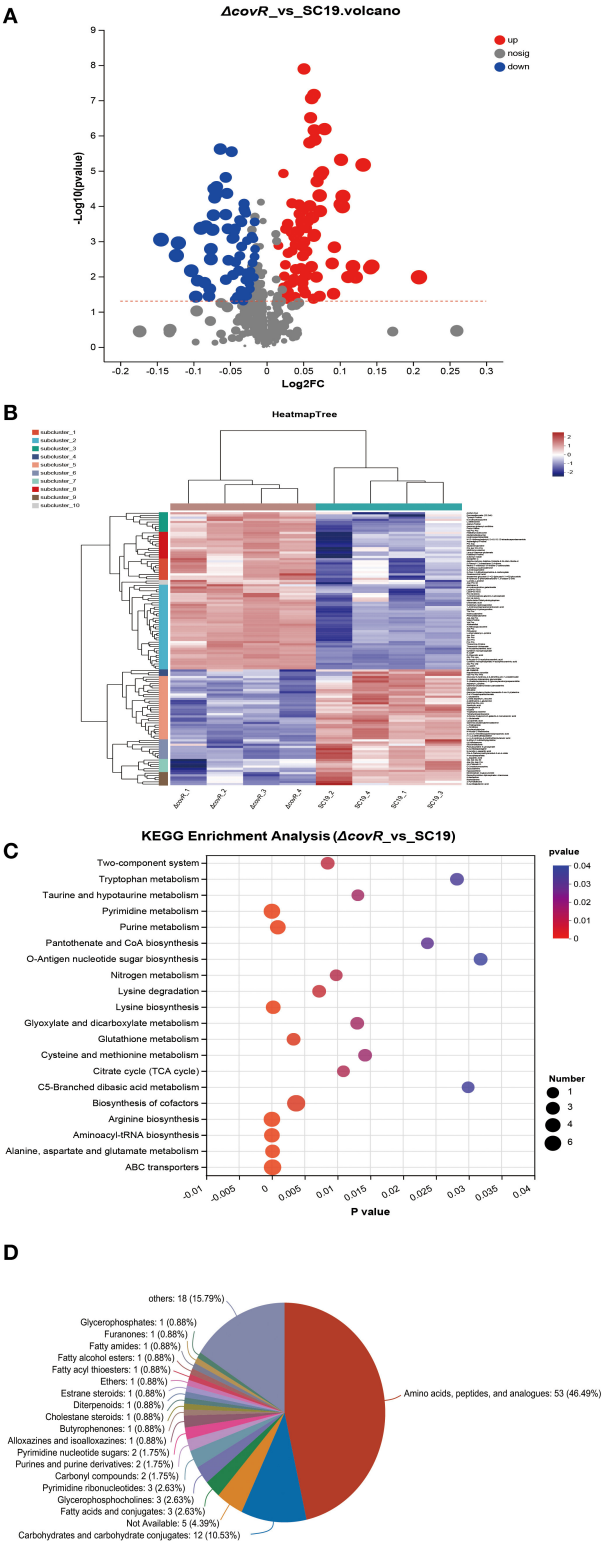


FIGURE 2
Differentially accumulated metabolites between SC19 and mutant Δ*covR* strains. **(A)** Volcano plots displayed DAMs that showed significant change (upregulated and downregulated) or no significant change between SC19 and mutant Δ*covR* strains. **(B)** The heat map of mutant Δ*covR* and *two* SC19 strains. **(C)** KEGG enrichment analysis of DAMs. **(D)** Pie chart depicting the categories of DAMs.

# Results

## The effects of covR deletion on the growth characteristics of SC19, Δ*covR*, and cΔ*covR* strains

As shown in Figure 1, the growth of Δ*covR* was significantly slower compared to that of SC19 and the complemented CΔ*covR* strains after 3 h.

## Metabolome profiling

For the purpose of determining the poor growth of mutant Δ*covR*, metabolome profiling via untargeted LC-MS was performed on SC19 and mutant Δ*covR*. The metabolites identified from LC-MS were presented in Supplementary Table S1. There was a total of 514 metabolites identified, which were classified into 11 categories. Supplementary Figure S1 provides the PCA of SC19, Δ*covR*, and QC samples, which present significant separation. The coloration of each sample was analyzed using metabolite concentration data and shown in Supplementary Figure S1, which suggests that different samples were distinguished significantly. This means that the metabolome data were highly reliable. These results also suggested that SC19 and Δ*covR* samples had substantially different metabolite profiles.

## DAMs between SC19 and mutant Δ*covR* strains

The uniquely mapped read ratio of all samples was higher than 96%, which means the reference sequence was reliable. To acquire Differentially accumulated metabolites (DAMs) between SC19 and mutant Δ*covR* strains, parameters such as *p*-values $<0.05$, $|\text{Log2 Fold Change}| > 1$, and variable importance in the projection (VIP) $>1$ were used to analyze all metabolite data. In total, 146 DAMs (83 were upregulated and 63 were downregulated) were acquired (Figures 2A, B, Supplementary Table S2). The results of the Kyoto Encyclopedia of Genes and Genomes (KEGG, Figure 2C) analysis showed that arginine biosynthesis (map00220), pyrimidine metabolism (map00240), aminoacyl-tRNA biosynthesis (map00970), alanine, aspartate and glutamate metabolism (map00250), ABC transporters (map02010), and lysine biosynthesis (map00300) were significantly enriched. Further analysis results showed that DMAs in these enrichment pathways were likely involved in aminoacyl-tRNA biosynthesis, energy metabolism, and amino acid biosynthesis and metabolism (Table 2). In addition, the 146 DMAs could be categorized into $>10$ classes (Figure 2D). These analysis results suggest that DAMs involved in aminoacyl-tRNA biosynthesis, energy metabolism, and amino acid biosynthesis and metabolism may play a key role in slowing down the growth rate of the mutant Δ*covR* strain.

TABLE 2 The list of 15 DAMs identified between *S. suis* 2 SC19 and mutant Δ*covR* strains.

| | Metabolite | Formula | Peak area | | VIP | FC (Δ*covR*/SC19) | *p*-value | Type |
|---|---|---|---|---|---|---|---|---|
| | | | Δ*covR* | SC19 | | | | |
| Aminoacyl-tRNA biosynthesis (peptides) | L-Aspartic acid | C4H7NO4 | 4.666 ± 0.048 | 5.072 ± 0.130 | 2.566141 | 0.919771 | 0.001118 | Down |
| | L-Methionine | C5H11NO2S | 3.878 ± 0.026 | 3.637 ± 0.082 | 1.965388 | 1.066263 | 0.001472 | Up |
| | L-Lysine | C6H14N2O2 | 4.474 ± 0.048 | 4.493 ± 0.042 | 0.271064 | 0.99555 | 0.5672 | Down |
| | L-Glutamate | C5H9NO4 | 5.949 ± 0.015 | 6.032 ± 0.021 | 1.171411 | 0.986077 | 0.000725 | Down |
| | L-Glutamine | C5H10N2O3 | 4.344 ± 0.024 | 4.456 ± 0.037 | 1.297029 | 0.974871 | 0.002603 | Down |
| Amino acid biosynthesis and metabolism | 1-Aminocyclopropanecarboxylic acid | C4H7NO2 | 6.041 ± 0.009 | 5.933 ± 0.029 | 1.344261 | 1.0182 | 0.000447 | Up |
| | Citrulline | C6H13N3O3 | 6.063 ± 0.015 | 6.132 ± 0.009 | 1.071567 | 0.988748 | 0.000282 | Down |
| | L-Ornithine | C5H12N2O2 | 4.701 ± 0.008 | 4.912 ± 0.022 | 1.904153 | 0.957053 | 2.41E-06 | Down |
| | N-acetylaspartate | C6H9NO5 | 4.426 ± 0.057 | 4.891 ± 0.141 | 2.755653 | 0.904742 | 0.000897 | Down |
| | Allysine | C6H11NO3 | 5.775 ± 0.009 | 5.580 ± 0.043 | 1.81366 | 1.03494 | 0.000127 | Up |
| | N-Acetyl-L-aspartic acid | C6H9NO5 | 5.640 ± 0.048 | 6.002 ± 0.091 | 2.444165 | 0.939687 | 0.000427 | Down |
| | N-Acetylglutamic acid | C7H11NO5 | 4.726 ± 0.068 | 5.054 ± 0.236 | 2.083366 | 0.935101 | 0.0373 | Down |
| Energy | Oxalosuccinic acid | C6H6O7 | 4.227 ± 0.049 | 4.139 ± 0.034 | 1.082973 | 1.021256 | 0.02676 | Up |
| | Acetyl-CoA | C23H38N7O17P3S | 4.885 ± 0.059 | 4.585 ± 0.205 | 1.97304 | 1.065416 | 0.03078 | Up |
| | D-4'-Phosphopantothenate | C9H18NO8P | 5.545 ± 0.091 | 5.847 ± 0.088 | 2.132044 | 0.948521 | 0.003222 | Down |





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## Transcriptome profiles between SC19 and mutant Δ*covR* strains

The DEGs between SC19 and mutant Δ*covR* strains were analyzed. There were 145,666,522 (SC19, 72,699,732; mutant Δ*covR*, 72,966,790) total reads generated by RNA-seq. A total of 141,860,342 (SC19, 70,781,282; mutant Δ*covR*, 71,079,060) clean reads were obtained by eliminating the low-quality reads. A total of 141,860,342 (SC19, 69,990,276; mutant Δ*covR*, 70,298,114) mapped reads were collected by aligning the high-quality reads to the reference genome (two SC19 chromosomes, complete genome, GenBank: CP020863.1). The correlation analysis showed significantly different relationships between Δ*covR* and SC19 strains (Figure 3A).

## DEGs between 2 SC19 and mutant Δ*covR* strains

To explore the candidate genes responsible for poor growth of Δ*covR* strains, DEGs (|Log2 Fold Change| >1.2 or <0.8) were identified between SC19 and mutant Δ*covR* strains. A total of 141 DEGs (86 were upregulated and 55 were downregulated) were identified. The GO annotation analysis result showed that the 141 DEGs were classified into three categories (Figure 3B), among which the integral component of membrane (36, 25.5%) was the largest group; the second largest group was ATP binding (26, 18.4%); only a few were related to metal ion binding (3, 2.1%) and iron-sulfur cluster assembly (3, 2.1%). As shown in Figure 3C, the enrichment pathways in KEGG terms were ABC transporters, glycerolipid metabolism, propanoate metabolism, carbon fixation pathways in prokaryotes, valine, leucine, and isoleucine degradation, and glycosaminoglycan degradation (Supplementary Table S3).

## The correlation analysis of DAMs and DEGs

To validate the accuracy of the identified DEGs by RNA-seq, nine DEGs were validated by qRT-PCR (Figure 4). Consistent with the RNA-Seq results (Supplementary Table S3), qRT-PCR results showed that the expression levels of the identified genes in the mutant Δ*covR* strain were significantly increased compared with those in the SC19 strain. Further research showed that all the gathered data from metabolome and transcriptome profiles had high reliability according to the correlation analysis (Figure 5). We could conclude that variations in metabolite accumulation between SC19 and mutant Δ*covR* strains were closely regulated by different gene expressions.

# Discussion

Prior studies have reported the importance of the TCS CovR in *S. suis* 2 virulence and growth characteristics (14, 22–25). However, the mechanism by which CovR deletion affects the growth of *S. suis* 2 is not yet clear.

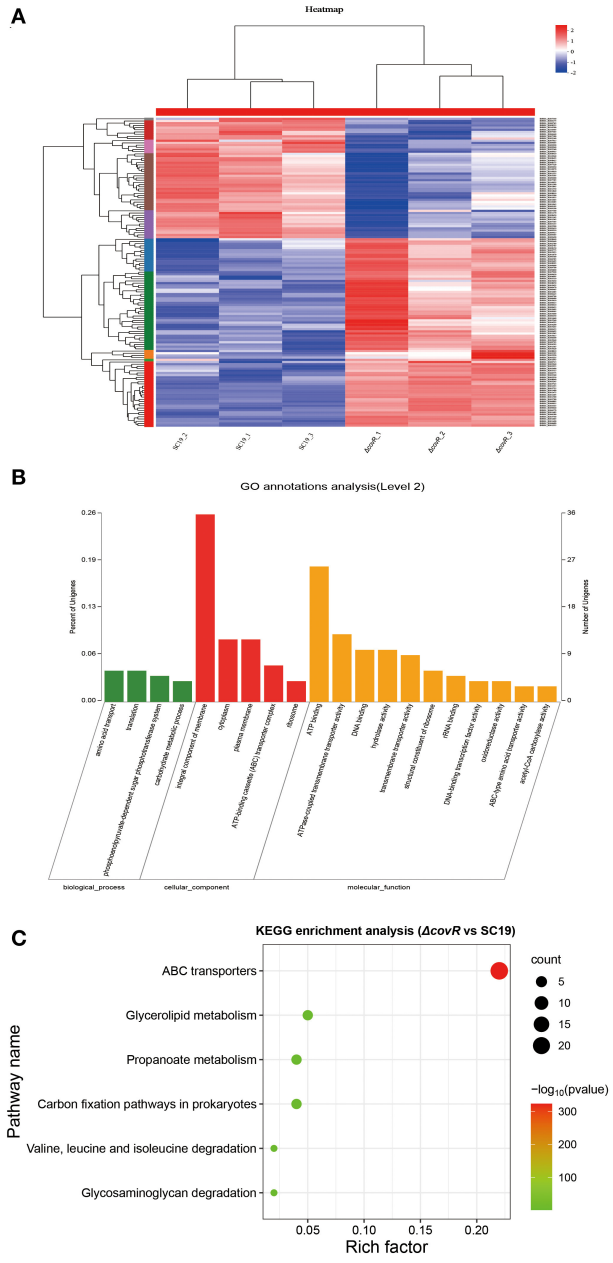


FIGURE 3
DEGs between mutant Δ*covR* and SC19 strains. **(A)** The heat map of mutant Δ*covR* and SC19 strains. **(B)** Go annotation analysis. **(C)** The KEGG enrichment pathways of 141 DEGs.

## *CovR* is essential for the growth of *S. suis* 2

First, we constructed the *covR* deletion mutant strain. We discovered that the mutant *covR* grew significantly more slowly than the parental strain SC19 on the growth curve (Figure 1A) and it also growth slow on the TSA plate (Figure 1B). This phenomenon was also presented by Pan et al. (22). To further understand the mechanism of poor growth of the Δ*covR* mutant, transcriptome and metabolome profiling were performed.

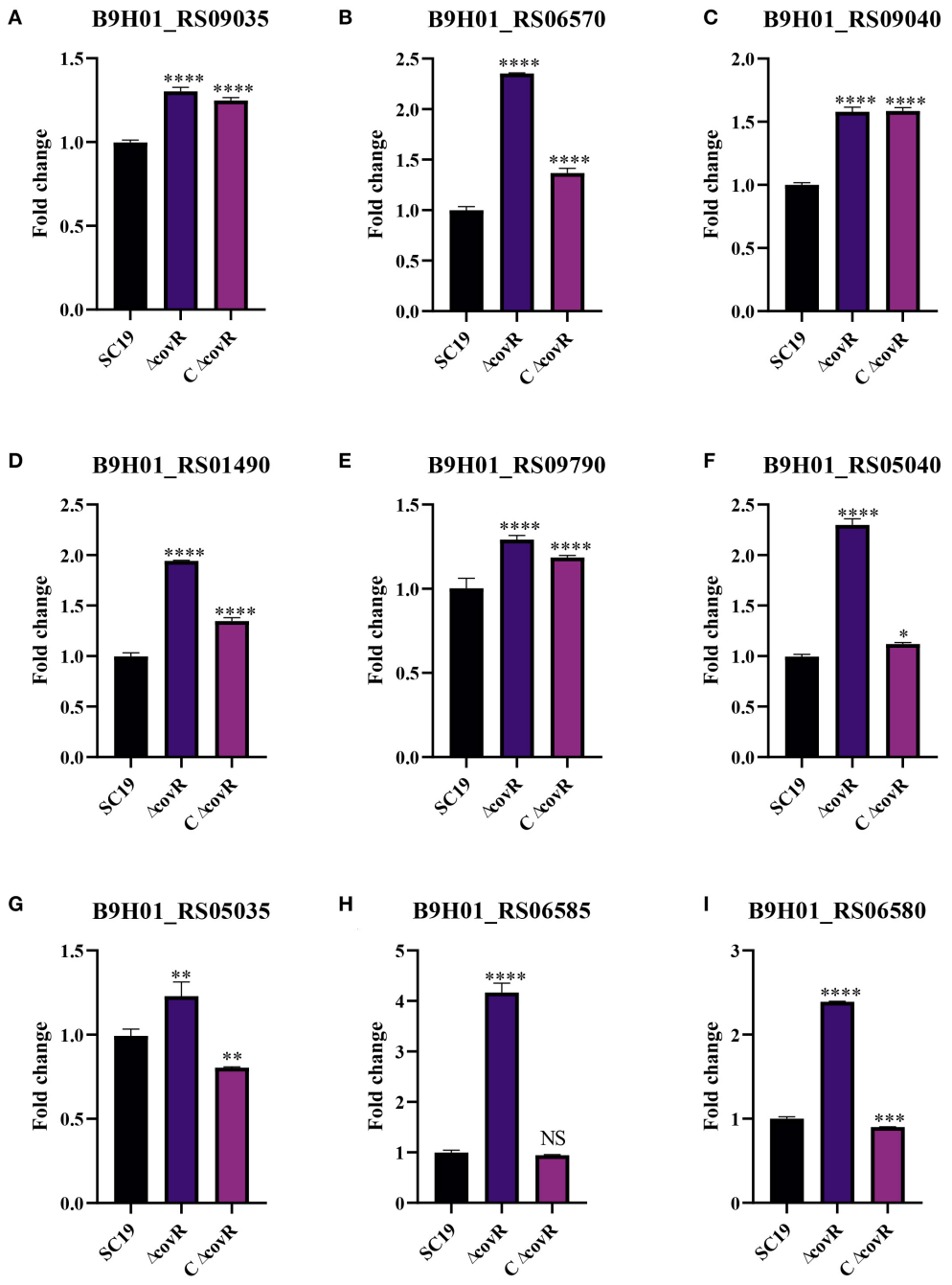


FIGURE 4
The qRT-PCR validation of genes related to the growth of *S. suis* 2. **(A)** Gene expression levels of B9H01_RS09035. **(B)** Gene expression levels of B9H01_RS06570. **(C)** Gene expression levels of B9H01_RS09040. **(D)** Gene expression levels of B9H01_RS01490. **(E)** Gene expression levels of B9H01_RS09790. **(F)** Gene expression levels of B9H01_RS05040. **(G)** Gene expression levels of B9H01_RS05035. **(H)** Gene expression levels of B9H01_RS06585. **(I)** Gene expression levels of B9H01_RS06580. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

## The poor growth of Δ*covR* was related to energy metabolism

Energy metabolism, including the tricarboxylic acid cycle (TCA), pentose phosphate pathway, and glycolysis pathway, is one of the most important metabolic pathways in the growth of microorganisms. The TCA is the main pathway by which many substrates, including glycogen, fatty acids, and proteins, enter central carbon metabolism via acetyl-coenzyme A (acetyl-CoA) (26). It is also the main pathway that provides energy for the biosynthesis of complex macromolecules such as glycogen, amino acids, and proteins (27). As we all know, acetyl-CoA and oxalosuccinic acid are the intermediate substances in the TCA, and phosphopantothenate is considered a precursor to the synthesis of coenzyme A (28).

In our study, the metabolism profile showed that oxalosuccinic acid and acetyl-CoA were up-accumulated and D-4'-Phosphopantothenate was down-accumulated. However,

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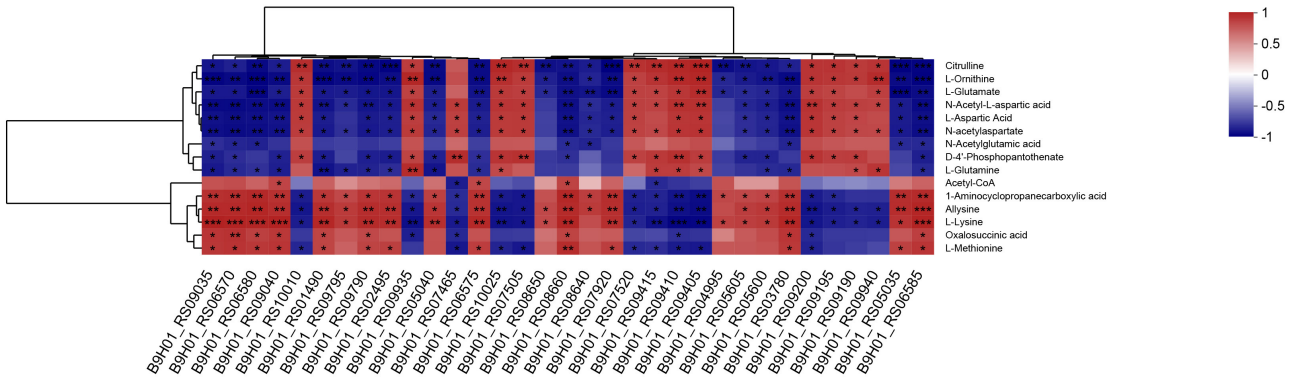


FIGURE 5
Correlation analysis of DEGs and DAMs in the mutant Δ*covR* and SC19 strains. The levels of correlation of each sample were indicated by the color, from high (red) to low (blue), and the correlation coefficient between samples was represented by the Z-score. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

the transcriptome profile showed that there were no significant changes in gene expression levels of acetyl-CoA and oxalosuccinic acid between mutant Δ*covR* and SC19 strains. The reason for the up-accumulation of acetyl-CoA and oxalosuccinic acid may be due to less consumption rather than excessive production. The D-4'-Phosphopantothenate was down-accumulated in metabolism and down-expression in the transcriptome, which means there were fewer precursors of coenzyme A, but the level of acetyl-CoA was still up-accumulated. Less consumption is the main reason for the up-accumulation of acetyl-CoA and oxaloacetylsuccinic acid in the mutant Δ*covR* strain. Therefore, the mutant Δ*covR* consumed less acetyl-CoA compared to SC19 and caused less energy supply to the biosynthesis of complex macromolecules, which resulted in poor growth.

## The poor growth of mutant Δ*covR* was related to amino acid biosynthesis and metabolism

Amino acid synthesis and metabolism play a key role in bacterial growth. There are over 300 naturally occurring amino acids, and only 22 constitute the monomer units of proteins, which can be classified as acidic or basic and hydrophilic or hydrophobic according to their respective side chain R (29). 1-aminocyclopropanecarboxylic acid (ACC) is a cyclopropane-containing non-proteinogenic amino acid isolated from various fruits and plant tissues and is considered the immediate precursor of the plant hormone ethylene (30). Recently, ACC has been found to perform similar synthesis machinery to plants in microorganisms (31, 32). Citrulline and ornithine, as we all know, are the intermediate substances of the urea cycle and have recently been reported to be involved in the metabolism of arginine in different bacteria (33). N-acetylglutamate in prokaryotes is also the first intermediate in arginine biosynthesis (34). As we all know, the level of amino acids can influence the amount of aminoacyl-tRNA. Several reports have shown that, in *E. coli* and *S. typhimurium*, the levels of aminoacyl-tRNA synthetases change with the rate of growth in different media (35). Our study found that seven metabolites related to amino acid biosynthesis and metabolism were differently accumulated, including two up-accumulated (1-aminocyclopropanecarboxylic acid and allysine) and five down-accumulated (citrulline, L-ornithine, N-acetylaspartate, N-acetyl-L-aspartic acid, and N-acetylglutamic acid) in mutant Δ*covR* compared to *S. suis* 2 SC19. The down-accumulation of citrulline, ornithine, and N-acetylglutamate were all involved in arginine metabolism, which means that arginine may play a key role in the growth of SC19.

The *argF,* as a gene code ornithine carbamoyltransferase, was downregulated in mutant Δ*covR* compared to SC19 in the transcriptome. Ornithine carbamoyltransferase can catalyze the condensation of ornithine with carbamoyl phosphate to form the amino acid citrulline, which is subsequently delivered to the cytosol (36). In bacteria, urea and ornithine produced by the decomposition of arginine can be further utilized as nitrogen and carbon sources (37). In other words, the mutant Δ*covR* produced less arginine than *S. suis* 2 SC19, which decreased the availability of nitrogen for the biosynthesis of amino acids. Thus, the CovR deletion influenced the rate of amino acid biosynthesis, resulting in poor growth of *S. suis* 2.

## Poor growth of mutant was related to aminoacyl-tRNA biosynthesis

Aminoacyl-tRNA synthetases play a key role in the process of aminoacyl-tRNA biosynthesis, which produces aminoacyl-tRNA, AMP, and PPi by esterifying an amino acid to the 3′ end of a tRNA and hydrolyzing one molecule of ATP (38). Previous studies showed that translation elongation, which is a process of the aminoacyl-tRNA translocation of the ribosome, is an essential step in protein synthesis (39), and translation is the key to defining the growth rate (40). Therefore, the quantity of aminoacyl-tRNA will have an impact on the pace of bacterial growth.

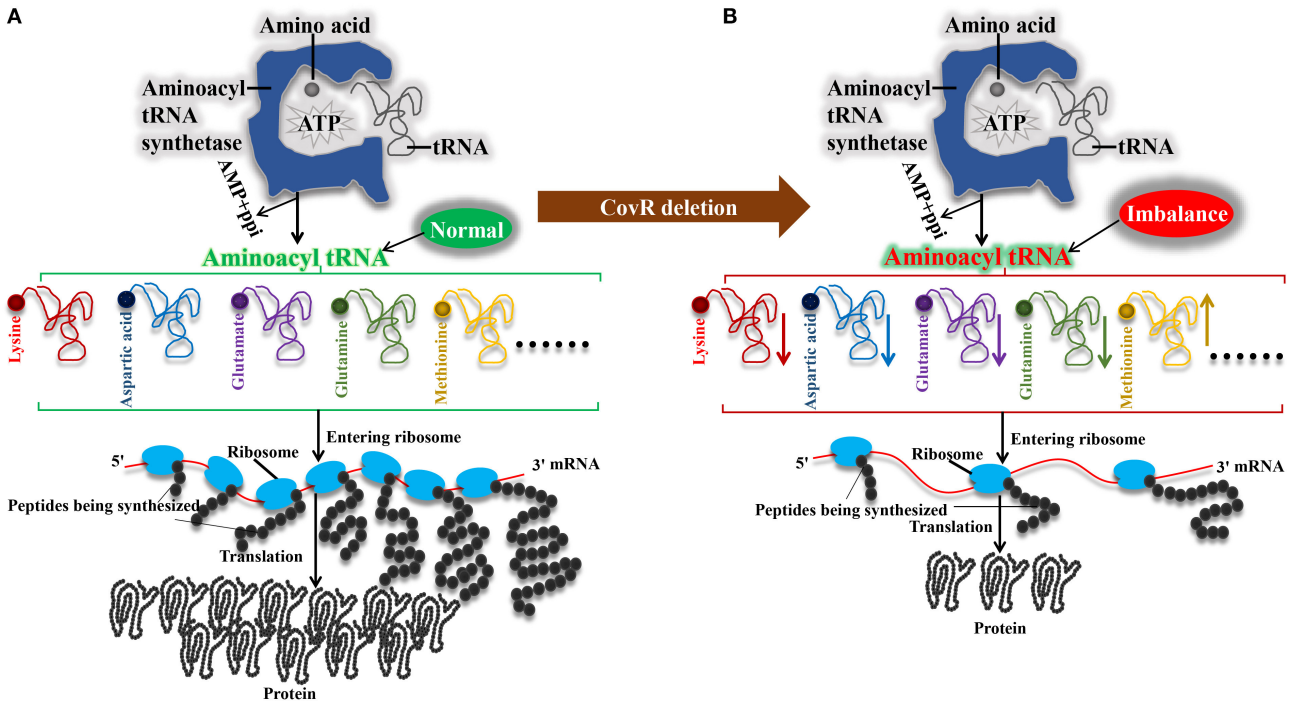


FIGURE 6
Mechanisms of the poor growth of *Streptococcus suis* serotype 2 after orphan response regulator CovR deletion. **(A)** Synthesis of aminoacyl tRNAs in SC19 in Δ*covR* strain. **(B)** Synthesis of aminoacyl tRNAs in Δ*covR* strain.

In our study, the aminoacyl-tRNA biosynthesis related to metabolism (L-methionine, L-lysine, L-aspartic Acid, L-glutamate, and L-glutamine) is down-accumulated except L-methionine in the mutant Δ*covR* strain. Meanwhile, the amino acid ABC transporter substrate-binding protein genes (B9H01_RS09940) and the amino acid ABC transporter permease gene (B9H01_RS09935) were downregulated, and the amino acid ABC transporter ATP-binding protein genes (B9H01_RS06570, B9H01_RS09040, and B9H01_RS05040) were upregulated in mutant Δ*covR* compared to SC19. The amino acid ABC transporter is a kind of active transporter for amino acid uptake (41). Therefore, we assume that mutant Δ*covR* has less amino acid ABC transporters that involve in amino acid uptake, thus expressing more ATP-binding proteins to transport more amino acids to maintain homeostasis, but still making the imbalance of aminoacyl tRNA biosynthesis, ultimately leading to slower growth.

## Conclusion

The mechanism of slow growth of Δ*covR* was proposed in Figure 6 can be explained as follows: first, the mutant Δ*covR* exhibits reduced consumption of acetyl-CoA, resulting in a decrease in ATP production for biosynthesis. This decrease in ATP production, in turn, leads to a shortage of nitrogen resources due to reduced arginine biosynthesis, which consequently slows down the rate of amino acid biosynthesis. Ultimately, the combination of decreased ATP and amino acid production results in a reduced availability of aminoacyl tRNA, leading to poor growth in mutant Δ*covR*.

## Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ebi.ac.uk/metabolights, MTBLS8495 and https://www.ncbi.nlm.nih.gov/, PRJNA879942.

## Author contributions

YZ: Data curation, Funding acquisition, Writing—original draft, Writing—review & editing. BZ: Data curation, Formal analysis, Writing—original draft, Writing—review & editing. YX: Data curation, Formal analysis, Writing—original draft, Methodology, Writing—review & editing. RL: Writing—review & editing, Data curation. HL: Data curation, Formal analysis, Writing—review & editing. PW: Methodology, Writing—review & editing. XY: 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.2023.1280161/full#supplementary-material

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# Time series analysis and forecasting of the number of canine rabies confirmed cases in Thailand based on national-level surveillance data

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**Introduction:** Rabies, a deadly zoonotic viral disease, accounts for over 50,000 fatalities globally each year. This disease predominantly plagues developing nations, with Thailand being no exception. In the current global landscape, concerted efforts are being mobilized to curb human mortalities attributed to animal-transmitted rabies. For strategic allocation and optimization of resources, sophisticated and accurate forecasting of rabies incidents is imperative. This research aims to determine temporal patterns, and seasonal fluctuations, and project the incidence of canine rabies throughout Thailand, using various time series techniques.

**Methods:** Monthly total laboratory-confirmed rabies cases data from January 2013 to December 2022 (full dataset) were split into the training dataset (January 2013 to December 2021) and the test dataset (January to December 2022). Time series models including Seasonal Autoregressive Integrated Moving Average (SARIMA), Neural Network Autoregression (NNAR), Error Trend Seasonality (ETS), the Trigonometric Exponential Smoothing State-Space Model with Box-Cox transformation, ARMA errors, Trend and Seasonal components (TBATS), and Seasonal and Trend Decomposition using Loess (STL) were used to analyze the training dataset and the full dataset. The forecast values obtained from the time series models applied to the training dataset were compared with the actual values from the test dataset to determine their predictive performance. Furthermore, the forecast projections from January 2023 to December 2025 were generated from models applied to the full dataset.

**Results:** The findings revealed a total of 4,678 confirmed canine rabies cases during the study duration, with apparent seasonality in the data. Among the models tested with the test dataset, TBATS exhibited superior predictive accuracy, closely trailed by the SARIMA model. Based on the full dataset, TBATS projections suggest an annual average of approximately 285 canine rabies cases for the years 2023 to 2025, translating to a monthly average of 23 cases (range: 18–30). In contrast, SARIMA projections averaged 277 cases annually (range: 208–214).

**Discussion:** This research offers a new perspective on disease forecasting through advanced time series methodologies. The results should be taken into consideration when planning and conducting rabies surveillance, prevention, and control activities.

KEYWORDS

**rabies, confirmed cases, time series model, forecasting, Thailand**

# 1 Introduction

Rabies is one of the most significant public health problems in several countries worldwide (1, 2). This disease is caused by the rabies virus belonging to the genus *Lyssavirus* of the family *Rhabdoviridae*. Rabies is a fatal disease in humans and is considered to be somewhat neglected (3). Based on World Health Organization (WHO) data, it is estimated that ∼59,000 people die from dog-mediated rabies each year, mostly in Asia and Africa (4). Due to the global burden of rabies, the WHO, in cooperation with the Global Alliance for Rabies Control, the Food and Agriculture Organization of the United Nations (FAO), and the World Organization for Animal Health (OIE) have set a goal to reduce human rabies deaths to zero by 2030 (5).

In Thailand, rabies is considered an important notifiable disease (6, 7). From 2010 to 2015, rabies claimed 46 lives, and annually, over 600,000 individuals undergo post-exposure prophylaxis treatment. Dogs are identified as the primary reservoirs for the rabies virus and play a crucial role in transmitting the disease to humans and other animals (8, 9). Notably, a significant proportion of confirmed rabies cases in Thailand are linked to dogs (9, 10). As a result, rabies prevention efforts predominantly focus on curbing the transmission from dogs (11, 12). Collaborative efforts across various organizations in Thailand aim to reduce rabies incidence in both humans and animals. Within the animal sector, the Department of Livestock Development (DLD) assumes a pivotal role in overseeing rabies trends and executing control strategies to diminish animal rabies cases. The surveillance of animal rabies in the country encompasses both active and passive methodologies (9, 10).

Accurate prediction of infectious disease trends is pivotal for optimizing resource allocation and strategizing future prevention and control measures. Essentially, predictions about future events are often based on historical data (13, 14). In the field of infectious disease epidemiology, forecasting the number of prospective cases or fatalities is a predominant focus, particularly when the magnitude of the susceptible population is not clearly defined (15–19). At present, several advanced time series methods are available, providing a wide range of techniques to work with various types of data with high-level predictability (14, 18).

Time series analysis is universally acknowledged as a cornerstone for forecasting across various fields, including economics (20), medicine (21, 22), veterinary science (23, 24), environmental studies (25), and agriculture (26–28). For instance, many recent studies have employed time series analysis to project COVID-19 case numbers, aiding in the formulation of disease control strategies and evaluating intervention efficacy (29–31). In the context of animal health, time series analysis has been instrumental in forecasting trends in diseases like rabies (24) and infectious disease in livestock (23, 32).

This research evaluates the efficacy of the seasonal autoregressive integrated moving average (SARIMA) in forecasting the number of confirmed canine rabies cases, representing classical time series modeling. Additionally, we explored advanced time series models (13), encompassing error trend seasonality (ETS), the trigonometric exponential smoothing state-space model with Box-Cox transformation, ARMA errors, Trend and Seasonal components (TBATS), and seasonal trend decomposition procedures based on loess (STL). The study also incorporates the neural network autoregression (NNAR), a common method aligned with machine learning approaches.

To our knowledge, prior studies have not employed advanced time series techniques to analyze and project the incidence of canine rabies cases in Thailand based on national surveillance data. This presents a significant gap in predictive knowledge, which is imperative for livestock authorities and relevant stakeholders to devise effective strategies against canine rabies. Therefore, this study aims to determine the patterns, seasonality, and to forecast the incidence of canine rabies using time series methodologies, including SARIMA, NNAR, ETS, TBATS, and STL models.

# 2 Materials and methods

## 2.1 Data and time series decomposition

### 2.1.1 Rabies cases data

In this study, we utilized data on confirmed canine rabies cases sourced from the Department of Livestock Development (DLD). This data was gathered through both active and passive rabies surveillance initiatives done by the Thai government (9). Passive surveillance primarily involved the laboratory submission of animal samples, either carcasses or heads, suspected of rabies. These samples were typically provided by veterinarians or animal owners. Meanwhile, active surveillance, done by veterinary services, focused on animals that succumbed to ambiguous symptoms. A baseline level of active sampling is also maintained, ensuring the collection of at least one sample from every subdistrict annually (9). Diagnostic procedures for all suspected animal rabies cases were conducted in nine DLD-accredited laboratories and one affiliated with the Queen Saovabha Memorial Institute. Diagnostic methods employed included the fluorescent antibody test and the mouse inoculation method (9, 10). All laboratory findings were subsequently uploaded to the centralized "ThaiRabies.net" platform (9). The dataset evaluated spans canine rabies cases recorded from January 2013 to December 2021.

#### 2.1.2 Time series decomposition and determination of seasonality in the time series data

The Ollech and Webel's combined seasonality test (WO-test) was used to ascertain the presence of seasonality in the rabies dataset. The WO-test combines the results of both the QS test and the KW-test, which are computed based on the residuals of an automated non-seasonal ARIMA. A time series is classified as having seasonality by the WO-test if the *p*-value of the QS test is <0.01 or if the *p*-value of the KW-test is <0.002 (33).

The WO-test was performed using the R statistical software, leveraging the "*seastests*" package via the "*combined_test*" function (33). Comprehensive details of the test can be found in the package's manual. Additionally, the ETS model procedure was utilized to evaluate the seasonal component present in the data. Further details about the ETS can be found in the next section.

## 2.2 Time series analysis and forecasts

### 2.2.1 Analytical and modeling procedure

This study encompassed two primary phases: (i) Identification of the most efficacious forecasting model by finalizing a model from each time series method and subsequently evaluating its performance using a validation dataset, and (ii) Utilization of the superior time series method, as determined from the previous phase, to predict future canine rabies cases based on a comprehensive dataset. Forecasts derived from other time series models, aside from the top-performing one, were also examined for comparison.

This study involved two different approaches. First, the full dataset (January 2013 to December 2022) was divided into a training dataset (January 2013 to December 2021) and a test dataset (January to December 2022). Forecasts for January 2022 to December 2022 were then generated from the training dataset and compared with the actual values within the same period in the test dataset to assess the forecasting model's performance. Second, in an effort to ensure up-to-date and accurate forecasts, it is suggested that the most current data be used for prediction (28, 34).Therefore, all time series models were applied to the full dataset to generate forecasts for the following 3 years (January 2023–2025). A schematic representation of the modeling procedure is provided in Figure 1 for clarity.

### 2.2.2 Time series models

#### 2.2.2.1 SARIMA model

The SARIMA is an extension of the autoregressive integrated moving average (ARIMA) that explicitly supports univariate time series data with a seasonal component. It adds three new hyperparameters to specify the autoregression, differencing, and moving average for the seasonal component of the series, as well as an additional parameter for the period of seasonality. The form of SARIMA is written as (35).

$$\phi_P(B)\,\Phi_p\left(B^s\right)(1-B)^d\left(1-B^s\right)^D x_t = \theta_Q(B)\,\Theta_Q\left(B^s\right)\omega_t \quad (1)$$

where $\varphi$ and $\theta$ are the parameters of the autoregressive and moving average, respectively. The terms $\Phi$ and $\Theta$ represent the parameters of the seasonal autoregressive and seasonal moving average. Additionally, SARIMA can be defined as SARIMA $(p,d,q)(P,D,Q)$. The terms $p$, $d$, and $q$ denote the order of autoregression, degree of differencing, and order of moving average, respectively. Meanwhile, $P$, $D$, and $Q$ represent the orders of the seasonal autoregression, degree of seasonal differencing, and order of seasonal moving average, respectively. The term $S$ refers to seasonal periodicity. The parameters were estimated using the maximum likelihood method. The best fit model was identified based on the minimum value of the corrected Akaike's Information Criterion (AIC) (13).

#### 2.2.2.2 NNAR model

The NNAR model can be thought of as a network of neurons or nodes displaying complex non-linear relationships and functional forms. The term NNAR $(p, k)$ is defined to indicate that there are $(p)$ lagged inputs and $(k)$ nodes in the hidden layer. With seasonal data, the NNAR model can be written as

$$\text{NNAR}\ \left(p,P,k\right)_m \quad (2)$$

where $p$ is the number of non-seasonal lagged inputs for the linear autoregressive process (AR), $P$ indicates the seasonal lags for the AR process, $k$ denotes the number of neurons in the hidden layer, and $m$ represents the seasonal period.

The forecasting process can be divided into two phases. Initially, the order of autoregression is determined. Subsequently, with the training dataset, the neural network is trained in accordance with the previously determined autoregression order. The number of input nodes or time series lags within the neural network is determined from this autoregression order.

#### 2.2.2.3 ETS model

The state-space equations can be written as follows (36).

$$y_t = w\left(x_{t-1}\right) + r\left(x_{t-1}\right)\varepsilon_t \quad (3)$$

$$x_t = f\left(x_{t-1}\right) + g\left(x_{t-1}\right)\varepsilon_t \quad (4)$$

where $w$, $f$, $r$ and $g$ are coefficients while $\varepsilon_t$ denotes the Gaussian white noise series. Equation (3) is known as the measurement equation, describing the relationship between the unobserved states $x_{t-1}$ and the observation $y_t$. Equation (4) is the transitional equation, describing the evolution of states over time. The use of identical errors in these two equations makes it an innovative state-space model (13).

The final ETS model is represented by a three-character string (Z, Z, Z), where the first, second, and third characters represent the error (A or M), type of trend (A or Ad or N), and the type of season (A or N or M), respectively. The letters A, Ad, N, and M denote additive, additive damped, none, and multiplicative (36). Additionally, the ETS forecasts are based on a weighted average of past observations, and the weight decays exponentially over time. As a result, the final observations have a greater weight than the earlier ones.

#### 2.2.2.4 TBATS model

The TBATS is an advanced adaptation of the BATS model, designed to accommodate multiple seasonal cycles. This model is

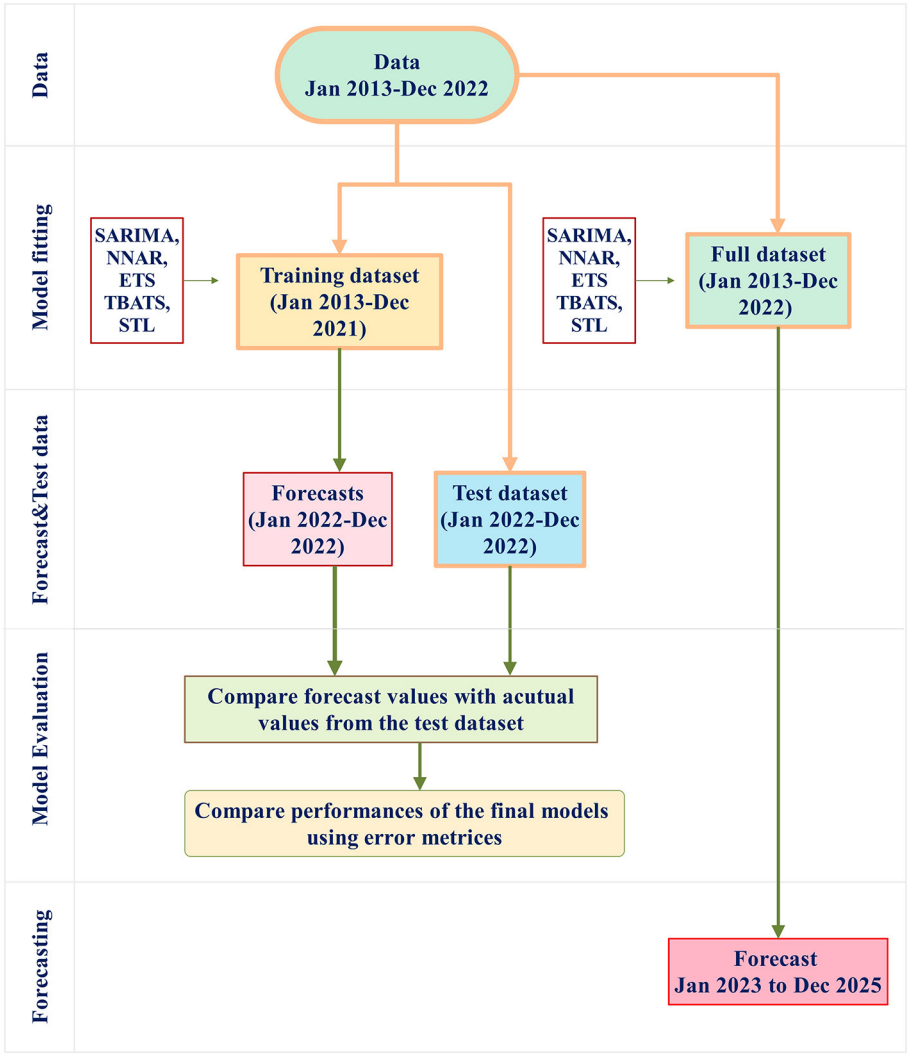


FIGURE 1
The modeling procedure involves several steps. First, the full dataset (January 2013 to December 2022) is divided into a training dataset (January 2013 to December 2021, shown as the orange box) and a test dataset (January to December 2022, shown as the blue box). The training dataset is used for model fitting using SARIMA, NNAR, ETS, TBATS, STL, and THETA methods. The final models from these methods are then evaluated for performance by applying them to the test dataset. Error metrics from these models are compared. Furthermore, the full dataset is used to build forecast models and implement the forecasting procedure to forecast the number of rabies cases in the period of January 2023 to December 2025.

supported by a trigonometric framework adept at navigating the intricacies of seasonality within a time series (19, 37).

The TBATS models is represented as $TBATS(\omega, p, q, \varphi, \{m_1, k_1\}, \{m_2, k_2\}, ..., \{m_T, k_T\})$. This formulation leverages a trigonometric representation of seasonal characteristics, drawing from the Fourier series. Within this model, the parameters p and q are associated with the ARMA process. The terms $m_{1,....}m_T$ specify the respective seasonal periods. The parameter $k$ represents the number of harmonics designated for the seasonal characteristic. Additionally, $\omega$ is indicative of the Box-Cox transformation, and $\varphi$ represents the dampening parameter value.

#### 2.2.2.5 STL model

The STL model employs a locally weighted regression method to partition a time series into its constituent trend, seasonal, and remainder components. The trend component is estimated through LOESS regression. In contrast, the seasonal component is typically assessed via SARIMA or ETS models, as detailed in (38). A notable strength of the STL model is its adaptability to shifts in the series trend. Furthermore, it exhibits robust resistance to outliers present within the series. Another salient feature of the STL model is its proficiency in managing seasonal frequencies that exceed one, as highlighted in (39).

### 2.2.3 Model performance evaluation and forecasting

In this study, we employed a range of evaluation error metrics to assess prediction performances across models developed from both the full and training datasets. These metrics included the mean absolute error (MAE), mean absolute percent error (MAPE),

mean absolute scaled error (MASE), and root mean squared error (RMSE) as detailed in (13).

To ensure the utilization of the most recent data for forecasting, we applied all the final time series models to the full dataset. This approach facilitated the modeling and forecasting of canine rabies cases for the three subsequent years post the last observation data incorporated in this study, as described in (28).

Data organization, decomposition, and segmentation of the time series were executed using the R statistical software, leveraging the "*xts*" and "*TSstudio*" packages. The "*forecast*" and "*forecastHybrid*" packages (13) provided a suite of functions instrumental in the development of the time series model and the subsequent evaluation of its predictive performance. These functions were also pivotal in forecasting the canine rabies cases for the upcoming 3 years. Specifically, the functions *auto.arima*(), *nnetar*(), *ets*(), *tbats*(), and *stl*() were employed to facilitate the SARIMA, NNAR, ETS, TBATS, and STL models, respectively. A comprehensive description of these functions can be found in the package manual (40). For graphical representation, we utilized packages such as "*ggplots*", "*plotly*", "*scales*", and "*ggsci*".

# 3 Results

## 3.1 Descriptive and time series components

A total of 4,678 reported cases of canine rabies were reported, affecting 1,908 females and 2,770 males. Among these cases, 2,405 dogs were linked to individual or public owners. The remaining cases pertained to either stray dogs or dogs without a traceable owner history. From a geographical perspective, positive rabies cases were observed in 64 out of 78 provinces. Notably, Chon Buri reported the highest number of rabies cases, totaling 463, followed by Songkhla with 347 cases, Surin with 278 cases, Roi-Et with 211 cases, and Bangkok with 203 cases. A map depicting these provinces is available in the Supplementary Figure S1.

Figure 2 presents the confirmed positive and negative canine rabies cases as documented by government laboratories. Over the study period, a total of 4,678 canine rabies cases were recorded. Between 2013 and 2017, there was a consistent rise in the number of cases, ending in a peak in 2018. Subsequently, a decline was observed from 2020 to 2021, with a resurgence in 2022.

Figure 3A delineates the annual and monthly distribution of canine rabies cases. Figure 3B depicts the monthly number of rabies cases, including the mean and standard error values (mean and error bar). On a monthly average, 39 dogs were identified as rabies-positive. The peak of canine rabies cases was recorded in March 2018, closely followed by March 2017. Notably, the period from February to April consistently registered the highest number of cases each year (Figure 3B). Yet, certain years also witnessed spikes in June, October, November, and December.

To effectively visualize the raw time series data, which comprises multiple components, data decomposition becomes imperative. Figure 4 decomposes the time series data of canine rabies cases into its trend, seasonality, and residual (or error) components. The trend analysis reveals a growth trajectory from January 2013 to December 2017, succeeded by a decline from

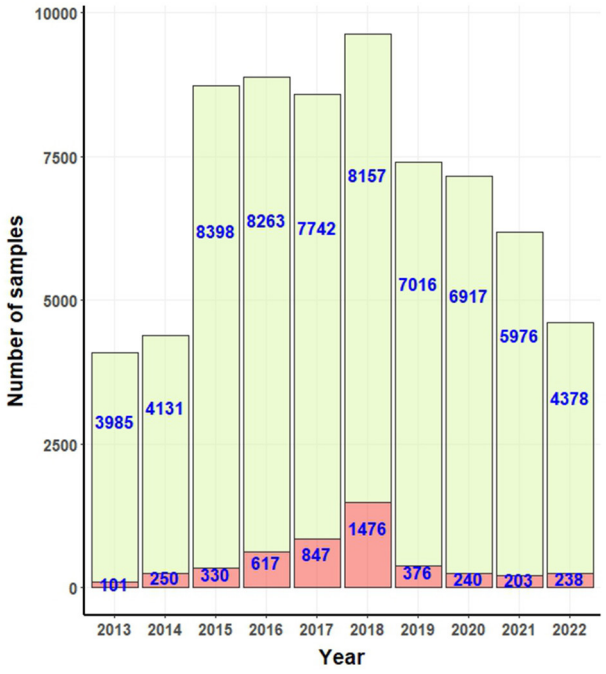


FIGURE 2
Bar graphs for number of positive (light red bar) and negative (light green bar) rabies cases confirmed by government laboratories.

August 2018 to January 2020. Post this period, the trend stabilizes. The seasonality inherent in the dataset is further confirmed by the WO-test, consistent with the ETS result. The model, with the form ETS (M, A, M), indicates the presence of a seasonal component in the data.

Figure 3B portrays the mean and standard deviations of canine rabies cases spanning 2013 to 2022. A pronounced surge in cases is evident during January and April relative to other months. It is pertinent to note that the heightened cases in January and April can be linked to the pronounced incidence of canine rabies in specific years, such as 2018.

## 3.2 Prediction performance

Figure 5 provide visual depictions of the observed vs. predicted canine rabies cases, derived from the testing and training datasets, respectively. A comparative analysis of the TBATS model against other models reveals a notable alignment of its forecasted values with the actual data. While the predictions from the SARIMA and NNAR models remain stable with slight variations, the ETS and STL models consistently project an upward trajectory. Notably, the STL model's forecasts display a mix of increasing and decreasing trends, with certain predictions deviating significantly from the actual values, as illustrated in Figure 5.

Table 1 presents the error metrices for the time series models applied to the dataset. Among these, the NNAR, THETA, and SARIMA models exhibited commendable performance on the training dataset. The NNAR model, in particular, showcased

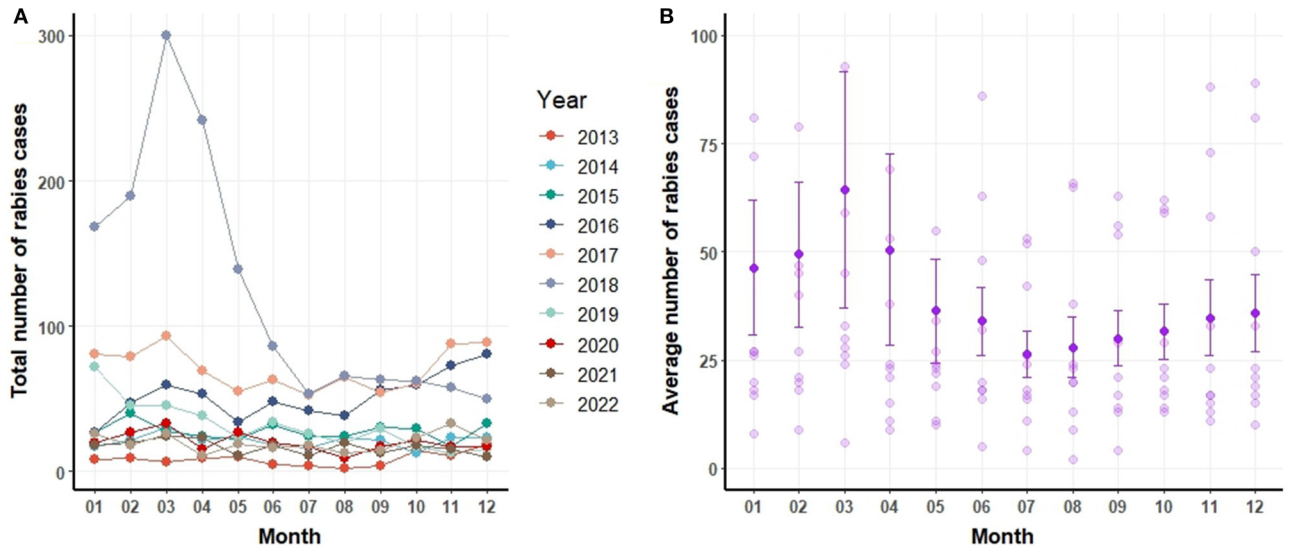


FIGURE 3
Number of canine rabies cases by year and month **(A)** and mean ± standard error of the mean for canine rabies cases by month based on data from 2013 to 2022 **(B)**.

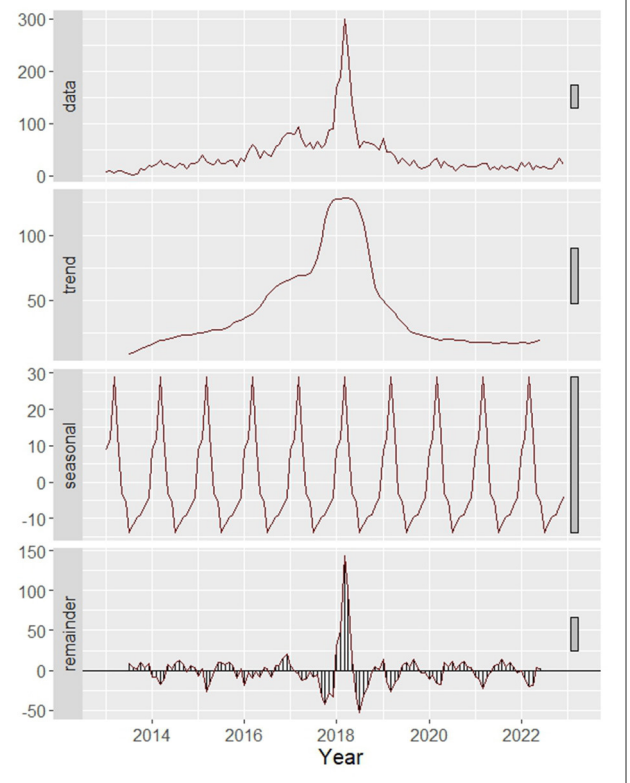


FIGURE 4
Decomposition of data on time series rabies into trend, seasonality, and remainder.

superior performance on the test data. However, it displayed signs of overfitting, as evidenced by its strong performance on the training data but suboptimal results on the test data. In the test dataset, it was observed that the error metrics values for the TBATS model were comparatively lower than those of other models. This suggests that the TBATS model exhibited superior performance in comparison to the other models.

## 3.3 Forecasting

Figure 6 illustrates the forecasted number of canine rabies cases for the years 2023 to 2025, as predicted by TBATS and SARIMA models applied to the full dataset. The forecasts from all models are further detailed in Supplementary Table S1 and Supplementary Figure S2. The aggregated predictions across all models estimate the average canine rabies cases to be 278, 288, and 300 for the years 2023, 2024, and 2025, respectively.

The TBATS model, which exhibited the highest predictive accuracy, estimates an annual average of ∼285 canine rabies cases for the period 2023 to 2025. This translates to a monthly average of 23 cases, with a range of 18–30 cases. In contrast, the SARIMA model, the subsequent top performer, projects a gradual increase in cases: 207 in 2023, 212 in 2024, and 213 in 2025 (Figure 6). The NNAR model's forecasts suggest consistent values across months, while the STL model's predictions indicate variability, with peaks in February and March and troughs in July and September each year (Supplementary Figure S2).

# 4 Discussion

For an effective rabies intervention program and efficient resource allocation, it is crucial to have a deep understanding and precise forecasting of rabies trends. The primary objective of this study was to analyze and forecast the trends of canine rabies cases

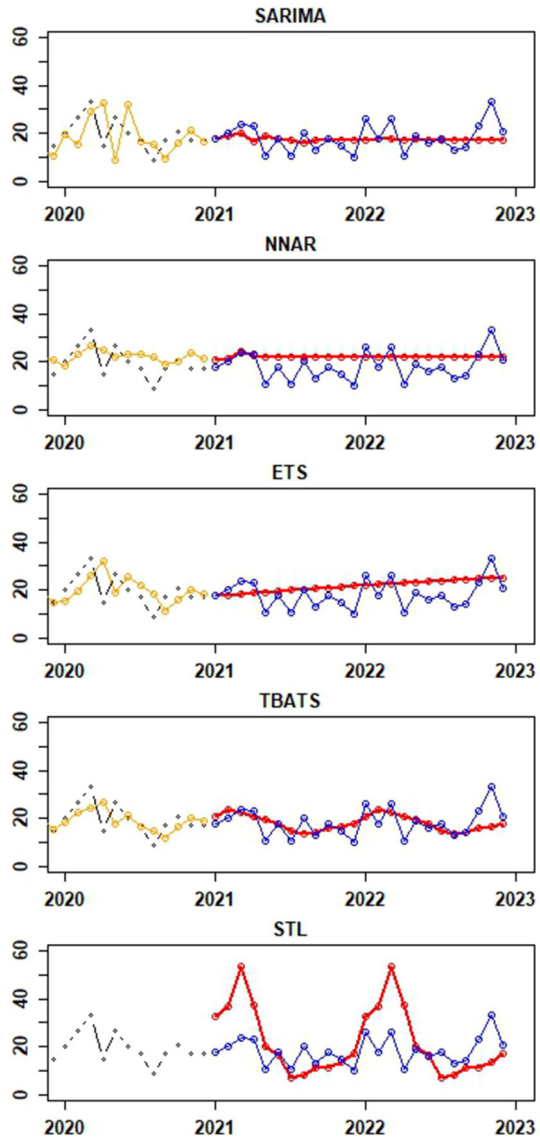


FIGURE 5
Depictions of the actual values of canine rabies cases (blue dots) from the test dataset (January to December 2022) and forecast values for the period of January to December 2022 (red dots) generated from time series forecast models applied to the training dataset (January 2013 to December 2021). The black and orange dots represent actual and fitted values from the last 24 observations of the training dataset, respectively. Notably, the fitted values from the STLM are not available for presentation.

in Thailand. Drawing on a decade's worth of national data, various time series methodologies were employed to achieve this.

Historically, there was a noticeable rise in canine rabies cases from 2013 to 2017, reaching its peak in 2018. However, a subsequent decline was observed until early 2020. This decrease is potentially attributable to the concerted efforts of the government to curtail human rabies cases. In fact, the last decade has witnessed an increase in rabies surveillance, leading to a higher number of reported canine rabies cases (41–43).

The post-2018 decline might be proof of the collaborative endeavors of both the public and private sectors. These strategies encompassed a range of measures, from enhanced surveillance and community engagement to robust public relations and the dissemination of knowledge. Approach to rabies control includes (i) setting up animal rabies monitoring, (ii) overseeing animal shelters, (iii) strengthening human rabies surveillance, (iv) assisting local communities in rabies prevention, (v) prioritizing public awareness, (vi) facilitating data sharing on rabies, (vii) concentrating on monitoring rabies cases, and (viii) disseminating knowledge and technological advancements (9).

Furthermore, the data also revealed apparent seasonal patterns in rabies occurrences, with pronounced spikes in March, June, and December. Such findings emphasize the importance of sustained rabies awareness throughout the year, rather than confining it to specific seasons.

In the aspect of prediction, the TBATS model stood out as the top performer. One of the primary reasons for its superior performance could be its proficiency in managing the complexities associated with seasonal variations and the non-linear nature of the data (19, 37, 44). This model is specifically designed to address such complexities, making it particularly suited for the task. Similarly, the SARIMA model showcased impressive results. Like the TBATS, SARIMA is equipped to handle datasets that have strong seasonal patterns, which is evident from its performance (45–47). Both these models, TBATS and SARIMA, have proven their capability in dealing with data that has a high degree of seasonal variation, making them invaluable tools in this context. On the other hand, the NNAR model, despite its merits, faced challenges when it came to managing data with intricate seasonal patterns. While it is a robust model in many scenarios, its limitations became evident in the face of complex seasonality, which affected its forecasting accuracy (13, 32).

The implications of this study are diverse. It explains how diverse forecasting models can be instrumental in shaping policy decisions. These forecasts can act as pivotal reference points, enabling authorities to set aspirational targets for the future. For instance, a tangible objective might be to ensure that the actual number of rabies cases remains below the forecasted figures. Furthermore, forecasting techniques should be integrated into existing rabies surveillance systems to assure that projections are based on the most recent data available.

However, it is imperative to acknowledge certain limitations. The data predominantly came from passive surveillance, which hinges on samples dispatched to labs either by individuals or pertinent authorities. This method might inadvertently lead to underreporting, given that some potential rabies cases might remain untested (6). However, such underreporting is a common challenge in epidemiological data (48–51). To address this issue, it is essential to intensify public awareness campaigns to encourage more extensive testing of suspected cases. Furthermore, the models deployed were somewhat myopic, focusing solely on the number of rabies cases and the corresponding months, without exploring into other potential determinants of rabies trends.

In this study, we employed time series models to analyze data available up to 2022. Should more recent data, such as from 2023 onward, become accessible, these models can be adapted accordingly. Future research should consider the development and evaluation of hybrid time series models (32, 52), as well as other time series models (23, 53), in order to determine their potential

TABLE 1 Error metrics for time series models applied to training data (January 2013 to December 2021) to forecast canine rabies cases for January to December 2022, compared with actual values present in the test dataset (January to December 2022).

| Method | Training set | | | | Testing set | | | |
|---|---|---|---|---|---|---|---|---|
| | MAE | MAPE | MASE | RMSE | MAE | MAPE | MASE | RMSE |
| SARIMA | 11.91 | 30.69 | 0.36 | 21.01 | 4.27 | 25.32 | 0.13 | 5.60 |
| NNAR | 3.86 | 14.69 | 0.12 | 5.04 | 5.78 | 40.64 | 0.17 | 7.03 |
| ETS | 12.41 | 34.97 | 0.37 | 23.08 | 14.52 | 94.74 | 0.44 | 16.95 |
| TBATS | 10.56 | 25.69 | 0.32 | 20.61 | 4.15 | 24.87 | 0.12 | 5.58 |
| STL | 12.00 | 40.25 | 0.36 | 19.46 | 10.41 | 57.46 | 0.31 | 13.51 |

SARIMA, seasonal autoregressive integrated moving average; NNAR, neural network autoregression; ETS, error trend seasonality; TBATS, trigonometric exponential smoothing state-space model with Box-Cox transformation, ARMA errors, Trend and Seasonal components; STL, seasonal and trend decomposition using LOESS.

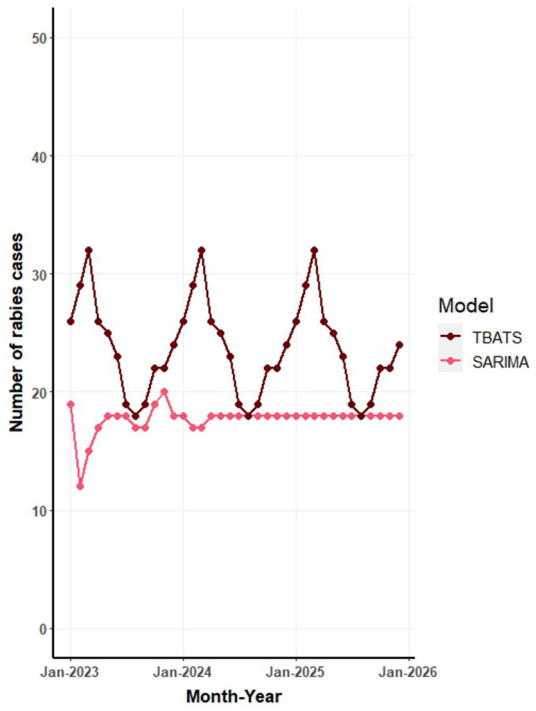


FIGURE 6
Forecast of monthly canine rabies cases in Thailand from 2023 to 2025 based on seasonal autoregressive integrated moving average (SARIMA) and trigonometric exponential smoothing state-space model with Box-Cox transformation (TBATS) models.

for enhancing the accuracy of canine rabies cases predictions. Additionally, while the primary focus of this study is on canine rabies, a comprehensive examination of the relationship between human and canine rabies cases at a national scale is recommended for subsequent investigations.

## 5 Conclusion

In a pioneering effort, this study utilized time series modeling to analyze and predict canine rabies cases based on national data. Observations revealed a decline in rabies cases from 2017 to 2019, stabilizing between 2020 and 2021. Of the models tested, the TBATS emerged as the most accurate predictor. Forecasts suggest that rabies cases will likely remain consistent in the near future, underscoring the need for intensified efforts to reduce these numbers. These predictions can guide authorities in strategic planning and resource allocation for rabies prevention and control. Ultimately, the methodologies showcased here offer a valuable tool for anticipating canine rabies trends in the years ahead.

## Data availability statement

The datasets presented in this article are not readily available because the data used in this study can be obtained from the Department of Livestock Development (DLD) in Thailand. An official letter must be sent to foreign@dld.go.th in order to request access. The DLD has granted approval for the use of this data in our research, as indicated by Approval number: 1601(01)560. Requests to access the datasets should be directed to foreign@dld.go.th.

## Author contributions

VP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing. WT: Conceptualization, Project administration, Resources, Supervision, Validation, Writing—review & editing. CJ: Data curation, Visualization, Writing—review & editing. PC: Data curation, Investigation, Resources, Validation, Visualization, Writing—review & editing. OS: Conceptualization, Data curation, Investigation, Resources, Visualization, Writing—review & editing. RS: Data curation, Writing—review & editing. OA: Conceptualization, Data curation, Formal analysis, Investigation, 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.

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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.2023.1294049/full#supplementary-material

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# Influenza risks arising from mixed intensive pig and poultry farms, with a spotlight on the United Kingdom

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Diseases passed to humans from animals (zoonoses) constitute 75% of emerging infectious diseases. Farmed animals are considered a high zoonotic risk, especially poultry and pigs as evidenced by recent outbreaks of avian and swine influenza. This review sought to collate recent knowledge of the disease risks from keeping pigs and chickens intensively and in close proximity to each other. Recent knowledge on influenza viruses compounds the public health concerns; no longer are concerns about "mixing vessel" hosts limited to pigs, but several other animal species too at a high level of probability—most notably chickens and humans. More generally, scientific literature establishing positive associations between intensive animal farming, human population growth, reduced biodiversity, and increased zoonoses risks is abundant. This includes the publication of relevant systematic reviews. The collected scientific evidence on this issue is clear: there is exceptionally strong evidence for a link between low animal welfare levels and high zoonotic risks, exacerbated by animal crowding, low genetic diversity, compromised hygiene, and high animal stress levels which compromise immune systems. Based on this evidence, further industrialized animal farms—especially poultry and pig farms or a mix thereof, and particularly in areas that already have a high concentration of farmed animals—should not generally be permitted to proceed. Instead, efforts should concentrate on supporting arable agriculture (or transitions toward this) and de-intensifying remaining animal farms, in alignment with One Health/One Welfare approaches within which animal health and welfare are integral parts of any farming operation. Among numerous other factors, this would involve reducing stocking densities down to 11 kg/m$^2$ (around five chickens/m$^2$) for meat chickens, and down to one pig/1.5 m$^2$ for pigs (assuming a 100 kg pig).

KEYWORDS

**influenza, avian, poultry, swine, pig, porcine**

## 1 Introduction

In this article we review recent scientific evidence regarding the disease risks posed to public health by intensive pig and poultry farms that are situated in close proximity to each other (hereafter, mixed pig/poultry farms). Recent examples of planning applications for such large-scale mixed swine-poultry farms exist, such as concurrent 2023 planning

applications in the Borough Council of King's Lynn and West Norfolk (UK) for the expansion of existing pig and poultry farms (1, 2). These particular applications come following the most severe avian flu outbreak in the UK since records began (3), and following post-covid increased awareness of viral disease risks for public health, including mutation risks (4). As the aforementioned planning applications concern pigs and chickens reared for meat (hereafter, meat chickens), this review will focus predominantly on pigs and meat chickens. First, evidence for the magnitude of the problem is outlined. Next, key drivers of the problem, and evidence establishing these, are reviewed. Then, methods to mitigate the risks are described. This article closes with a conclusion concerning the disease risks posed by mixed intensive swine and poultry farms, based on the evidence reviewed.

## 2 Public health risks arising from farmed animal diseases

Infectious diseases in animals can be prevalent within animal populations at varying levels of severity. Diseases may be enzootic (persistent at low levels within a population), epizootic (denoting an outbreak of a higher number of cases within a particular region), or panzootic (denoting an outbreak covering a wide region). These terms are now sometimes used interchangeably with the equivalent terms used for human diseases and populations: endemic, epidemic, and pandemic, respectively [(5), pp. 131–132]. Not only do all diseases in farmed animals compromise animal welfare, but many are also known to transmit from vertebrate animals to humans. Such a disease is termed a zoonosis, or "zoonoses" in plural form (6). The disease-causing agents can be, for instance, bacterial, viral, fungal, parasitic, or protozoic (7). Tomori and Oluwayelu (7) describe key salient examples of recent zoonoses spanning endemics, epidemics, and pandemics. Examples include MERS (Middle East respiratory syndrome) since 2012, Ebola since 1976 in Africa/the Middle East, and SARS (severe acute respiratory syndrome) since 2003 in Asia (see Table 1 for further information and examples).

Zoonotic diseases from farmed animals constitute a public health risk, which may be severe. Zoonoses form the dominant group of diseases among emerging infectious diseases (EIDs)—currently 75% of EIDs (8). Zoonoses are typically spread via contact with feces or saliva of an infected animal, either directly, or through contamination of the environment (6). While approximately three quarters of zoonoses stem from wild animals, the remaining 25% arise from domesticated species (7). Moreover, due to intensive resource requirements (e.g., land to grow feed crops for farmed animals), growing human and farmed animal populations, and growing consumption of meat, milk, and eggs, industrialized intensive animal farming (IIAF) is a leading driver of habitat destruction (9, 10). This brings the interface between humans and wild animals closer, and thereby increases the risk of disease transmission from wild animals [(11), p. 138]. Thus, IIAF confers both *direct* increased risks of zoonoses from farmed animals (arising from the vast numbers of animals kept, the ways in which they are kept, and close human-farmed animal proximity), and *indirect* increased zoonoses risks from wild animals (i.e., through intensive resource use by IIAF leading to increased habitat destruction and increased human-wild animal proximity). Antimicrobial use is also high, and this is an exacerbating factor. Seventy-three per cent of antimicrobials consumed globally are used on farmed animals; this is projected to increase by 8% by 2030 (12). These antibiotics are widely used, at low levels, for long periods—all factors known to create antibiotic resistance. Thus, IIAF plays a significant role in the increased risk of antibiotic resistance emergence. For all of these reasons, farmed animals are considered to pose a high zoonotic risk, while other domesticated animals such as cats are deemed a low zoonotic risk [(13), p. 5]. This is further discussed in section 4.

Viruses, and specifically influenza viruses, currently constitute the greatest zoonotic threats. This is because they demonstrate the greatest likelihood of being transmitted between different species, including to humans (7), with the extent to which viral transmission to humans is successful depending on the abundance of all species involved, as well as on the viral richness (the number of viral species found in a host) and the life history traits of host animals [(14), p. 646]. Hence, hereafter the focus will be exclusively on emergent zoonotic influenzas. Nevertheless, it should be noted that there are other viral disease risks aside from influenzas. Coronaviruses (e.g., COVID-19, SARS, MERS) are of the next greatest concern after influenza viruses, and they may have wild or domesticated animal hosts (7).

## 3 Influenza scrutinized: the connection between human, avian, and swine influenza

In a review of zoonotic influenza, Abdelwhab and Mettenleiter (13) outlined the four types of influenza virus that are known: A, B, C, and D. Each is a distinct viral species, differing in their genome and in the diversity of hosts infected, with type B demonstrating the narrowest host range (humans and pigs), followed by type C (humans, pigs, and seals), type D (cattle, several other ruminant species, pigs, and horses), and type A. Type A is the most widespread as it can infect a wide range of mammals and birds; hence, it is the type of most concern (13). Thus, henceforth, type A will be exclusively focused on.

Type A can be subdivided into a further 18 "H" and 11 "N" subtypes, which are effectively abbreviations of the two proteins found on the virus's surface—hemagglutinin and neuraminidase—giving viral names such as "H5N1" and "H9N2" (15). The former compound is considered to play a role chiefly in cell entry, and the latter in cell exit for the dispersal of virus progeny [(16), p. 637]. A key reason for type A's prevalence is its propensity toward *both* antigenic shift and antigenic drift. Antigenic shift describes the genetic reassortment of strains that can occur when two different strains infect the same host, potentially leading to the creation of a new type A virus strain; while antigenic drift denotes mutations that can occur over a longer period of time to enable a type A virus to adapt to (i.e., overcome) any host immunity that may have developed [(17), p. 2]. Different influenza A viruses (e.g., swine, human, avian) require specific sialic acid receptors on the host animal cell that the viral protein hemagglutinin binds to (18); these will be further delineated in the applicable subsections below.

Influenza viruses infect the respiratory or digestive tracts of their host animals (19). Influenza cases across humans, poultry, and swine can range from a low pathogenicity (asymptomatic or mild respiratory signs) to high pathogenicity, causing pneumonia, severe neurological problems, and high mortality (13, 20). Crucially, low pathogenicity (no or mild symptoms) in one species does not

TABLE 1 An exemplar list of viral zoonoses.

| Viral zoonosis | Geographical reach | Animal hosts | Key transmission routes |
|---|---|---|---|
| Swine flu | Global | Pigs | Contact with infected pigs or fomites |
| Hong Kong flu | Global | Birds | Contact with infected chickens or fomites |
| Asian flu | Global | Birds | Contact with infected chickens or fomites |
| Spanish flu | Global | Birds | Contact with airborne respiratory secretions from infected people |
| Rabies | Global | All warm-blooded animals, esp. dogs | Bite from infected animal |
| Hantavirus | Global | Rodents | Airborne excreta, saliva, bite of infected animal |
| Yellow fever | Global | Nonhuman primates | Bite of infected mosquitoes |
| Chikungunya | Global | Nonhuman primates, birds, small mammals | Bite of infected mosquitoes |
| West Nile | Global | Birds | Bite of infected mosquitoes |
| Crimean-Congo hemorrhagic fever | Global | Domesticated ruminants | Bite of infected ticks, contact with blood of viremic farmed animals or humans |
| Coronavirus (COVID-19) | Global | Unknown (probable animal origin) | Contact with infected people |
| Coronavirus (MERS) | Africa/Middle East | Camels | Contact (direct or indirect) with infected camels |
| Rift Valley fever | Africa/Middle East | Domesticated ruminants | Bite of infected mosquitoes, contact with blood of viremic farmed animals |
| Ebola | Africa/Middle East | Fruit bats, nonhuman primates, antelopes | Contact with secretions of infected animals |
| Marburg | Africa/Middle East | Bats | Contact with infected bat feces or other secretions |
| Lassa Fever | Africa/Middle East | Peri domestic rodent | Contact with rodent feces/urine, spread in healthcare facilities |
| Monkeypox | Africa/Middle East | Rodents, nonhuman primates | Contact with secretions of infected animals |
| Equine encephalitis | Americas | Wild bird, equids | Bite of infected mosquitoes |
| Colorado tick fever | Americas | Rodents | Bite of infected Rocky Mountain wood ticks |
| Zika | Americas | Nonhuman primates | Bite of infected mosquitoes, transplacental |
| Tahyna | Europe | Mammals | Bite of infected mosquitoes |
| Tick-borne encephalitis | Europe | Small rodents, mammals | Bite of infected ticks, unpasteurized milk |
| Usutu | Europe | Birds | Bite of infected mosquitoes |
| Coronavirus (SARS) | Asia | Variety of domestic and wild animals | Contact with secretions of infected animals or people |
| Japanese encephalitis | Asia | Pigs, birds | Bite of infected mosquitoes |
| Nipah | Asia | Bats, pigs | Direct or indirect contact with secretions of infected animals or people |
| Hendra | Australia | Bats, horses | Contact with secretions of infected animals |
| Kunjin | Australia | Birds | Bite of infected mosquitoes |

Adapted from Tomori and Oluwayelu (7).

necessarily indicate low pathogenicity of the same strain in humans [(13), p. 7]. It is thought that the viruses replicate primarily in the animals' intestinal or respiratory tracts and remain active in feces, saliva, or other discharges for weeks. Transmission takes place through direct contact with infected saliva, nasal secretions, or feces, or occurs via airborne particles or contaminated equipment such as vehicles and clothing (19). Transmission via the consumption of properly cooked chicken meat or pork is not currently thought possible (21, 22).

Avian and swine influenzas are of the most concern due to several factors. These include the numerous type A subtypes hosted in these animals, the vast numbers of farmed pigs and poultry, how farmed poultry and pigs are kept, the presence of both domesticated and wild birds and swine, and the close proximity of farmed pigs and poultry to humans (13, 17). Hence, the focus hereafter will be on avian, swine, and human influenzas.

## 3.1 Avian influenza

Avian influenza viruses (AIVs) include H types 1–16 and N types 1–9 (13); this constitutes all of the subtypes available bar two that are only found in bats. The hemagglutinin viral protein requires so-called α2,3-linked sialic acid cell receptors in the host to achieve cell attachment. Wild birds are the natural host for AIVs, especially mallards and gulls. Many species of natural avian influenza hosts are migratory species, which facilitates the international spread of AIVs—epitomized by the spread into

South America for the first time in 2022 (13). This is exacerbated further by the global poultry market, as wild birds transmit influenza to poultry. In poultry, all subtypes are low pathogenic types. However, if poultry catch H5 and H7, these can then morph into high pathogenic types, thus avian influenza is a notifiable disease requiring immediate reporting to authorities where suspicion exists.

The world saw its largest highly pathogenic H5N1 avian influenza outbreak in 2022, causing the culling or death of over 130 million domesticated birds globally (23) and at least 50,000 wild birds in the UK alone (24). It has now reached 81 countries and five continents after spreading into the Antarctica in October 2023 (23, 25). There is currently a high risk of avian influenza cases throughout the year in Europe, whereas until recently, it remained a seasonal epidemic in cooler months [(3, 26), p. 812].

In the UK, the bird species most negatively impacted by avian influenza since 2021 are the barnacle goose and the northern gannet (24, 27). Birds can also transmit influenza to a variety of mammals, including humans and pigs. Table 2 highlights the recorded cases of mammals that AIVs have been transmitted to. The table also highlights that H5N1 is the AIV most commonly transmitted to mammals. As well as being recorded in a range of domesticated and wild birds, between January 2022 and July 2023, H5N1 has been recorded in various non-avians globally including sea lions, minks, foxes, badgers, otters, polecats, racoons, cats, dogs, and humans (28). The potential for poultry to serve as mixing vessels for avian influenza is discussed within section 3.3.

## 3.2 Human influenza

Human influenza viruses prefer α2,6-linked sialic acid receptors for attachment to host cells, which humans have (18). The primary types are H1N1, H3N2, and H1N2 (i.e., mirroring the swine subtypes outlined in the following subsection), with the H1N2 type being a human/swine influenza mix that evolved in the 2000s (13). Humans are natural hosts for certain influenza A viruses, and these (along with some type B viruses) cause the common, mild seasonal influenzas in cooler months of the year, and there will be partial immunity in the population for pre-existing strains. However, there have been four major agreed-upon influenza pandemics affecting humans thus far (and possibly more), as introduced earlier in Table 1: H1N1 causing the Spanish flu in 1918, H2N2 causing the Asian flu in 1957, H3N2 causing the Hong Kong flu in 1968, and H1N1 from North America in 2009. Collectively, the number of casualties were in the millions. Each of these four pandemics is considered zoonotic in that the first three stemmed from avian influenza, and the last one from swine influenza [(13), p. 7; (17), p. 3].

Other zoonotic cases (not reaching pandemic level—i.e., widespread at national or international level), of AIVs in humans between 1959 and 2023, are reported in Table 3. Taking the two subtypes with the highest number of cases, H5N1 and H7N9, the case mortality rates are very high at around 53 and 39%, respectively. Most cases occurred in slaughterhouse workers via airborne transmission, and there is evidence of some viral replication occurring outside of respiratory organs, even including the brain [(13), p. 6]. Based on their review, Abdelwhab and Mettenleiter (13) estimate <3,000 clinical

TABLE 2 Confirmed AIV infections among mammals.

| | H4 | H5N1 | H6N1 | H7 | H9N2 | H10 | H11N6 | H13 |
|---|---|---|---|---|---|---|---|---|
| Humans | | X | X | X | X | X | | |
| Pigs | X | X | | X | X | X | X | |
| Seals | X | X | | X | | X | | |
| Cats | | X | | X | X | | | |
| Dogs | | X | | | X | | | |
| Minks | | X | | | X | X | | |
| Ferrets | | X | | | X | | | |
| Raccoons | | X | | | | | | |
| Foxes | | X | | | | | | |
| Zoo animals | | X | | | | | | |
| Camels | | X | | X | | | | |
| Pikas | | X | | | X | | | |
| Rodents | | X | | X | | | | |
| Skunk | | X | | | | | | |
| Badgers | | X | | | X | | | |
| Bears | | X | | | | | | |
| Meerkats | | X | | | | | | |
| Otters | | X | | | X | | | |
| Dolphins | | X | | | | | | |
| Whales | | | | | | | | X |

After Abdelwhab and Mettenleiter (13).

TABLE 3 Human infections with AIV from 1959 to 2023.

| Subtype | Year of first human identification | Year of last human identification | Cases (fatalities) | Countries |
|---|---|---|---|---|
| H3N8 | 2022 | 2022 | 2 | China |
| H5N1 | 1997 | 1997 | 18 (6) | Hong Kong |
| H5N1 | 2003 | 2023 | 868 (457) | Many |
| H5N1 | 2022 | 2023 | 5 | UK, US, Spain, Ecuador |
| H5N6 | 2014 | 2021 | 83 (33) | China |
| H5N8 | 2020 | 2020 | 7 | Russia |
| H6N1 | 2013 | 2013 | 1 | Taiwan |
| H7N2 | 2002 | 2016 | 8 | UK, US |
| H7N3 | 2004 | 2012 | 5 | Canada, Mexico, UK |
| H7N4 | 2018 | 2018 | 1 | China |
| H7N7 | 1959 | 2013 | 96 (1) | US, Australia, Netherlands, Italy, UK |
| H7N9 | 2013 | 2017 | 1,568 (616) | China, Taiwan |
| H9N2 | 1998 | 2014 | 19 | China, Bangladesh, Hong Kong |
| H9N2 | 2015 | 2022 | 85 (2) | China, Cambodia, Egypt |
| H10N3 | 2021 | 2022 | 2 | China |
| H10N7 | 2004 | 2010 | 4 | Egypt, Australia |
| H10N8 | 2013 | 2014 | 3 (2) | China |
| Total | From 1959 to 2023 | | 2,775 (1,117) | |

The four pandemics discussed in section 3.3 are estimated to have also collectively caused up to 108 million fatalities, with the Spanish Flu in 1918 being the most notable contributor to these fatalities (29). Records typically begin from 1959 as this is when today's most common and problematic subtype (H5N1) was first isolated [(30), p. 217]. After Abdelwhab and Mettenleiter (13).

(laboratory-confirmed) cases of AIV infections in humans, as per Table 3, and < 7,000 subclinical (no symptoms, just antibodies) AIV cases. There is particularly concern regarding the continued pandemic potential of H5N1 due to the recent outbreaks in different species, as discussed in section 3.1, and due to genetic variations within the H5N1 strain that have been detected (28).

In terms of swine-origin influenza in humans, Abdelwhab and Mettenleiter (13) reported approximately a 75% higher number of cases globally within the most recent decade 2010–2021 (almost 700 cases), relative to the previous five decades combined, 1959–2014 (under 400 cases). Moreover, both Kessler et al. (17) and Abdelwhab and Mettenleiter (13) link these case increases to anthropogenic factors – see section 4 for further discussion of this. There are also a few cases of human-to-human transmission of avian/swine influenza, but this is still rare at present. A major public health concern is that the next zoonotic influenza mutation could enable sustained human-to-human transmission and lead to a pandemic.

## 3.3 "Mixing vessel" host animals and swine influenza

In terms of influenzas, a "mixing vessel" is a host animal able to be infected with two or more types of influenza virus at once; such a host animal can then serve as a "fertile breeding ground" for new mixes and strains of a virus [(17), p. 11]. This potentially allows the transmission of genetic material from one strain to the other, and the emergence of a new influenza strain(s), as per the process of antigenic shift mentioned earlier in section 3 [(13), p. 3]. All four major influenza pandemics have stemmed from avian influenzas that formed new types in humans, pigs, or in another currently undetermined mixing vessel host. Further mixing can also occur via reverse zoonotic infection, whereby influenzas within humans can transmit to other animals, allowing further mixing to occur with any other influenza strains present within those animals [(31), p. 615; (17), p. 10].

Pigs are natural hosts for several influenza viruses, though of fewer types than birds; the primary types of influenza today are H1N1, H1N2, and H3N2, and cases have occurred globally. These stem from genetic mixes of human, avian, and swine influenzas, largely arising from transmissions to pigs during human and avian influenza epidemics (17). Pigs can host an unusually high number of influenza strains, many of which can infect other species (Table 2); hence pigs are particularly well-suited to serving as mixing vessels. This is based on the presence of both the so-called α2,6-linked as well as α2,3-linked sialic acid cell receptors in pigs to which the viral hemagglutinin of both human influenzas and avian influenzas can attach, respectively [(32), p. 161].

Despite pigs' infamous reputation as a mixing vessel, according to the 2023 review by Abdelwhab and Mettenleiter, there is also a high risk of numerous other animals acting as mixing vessels, as they also have both types of sialic acid cell receptors meaning they can receive both human and avian influenzas. Most worryingly, humans, chickens, and other poultry are included among the list of "high probability" species cited (Figure 1). Thus, concern for mixing vessels within the farming sector should not end with pigs, and in the case of the planning applications outlined in the introduction, there is a triple threat of mixing vessel hosts in close proximity to each other: pigs, chickens, and humans.

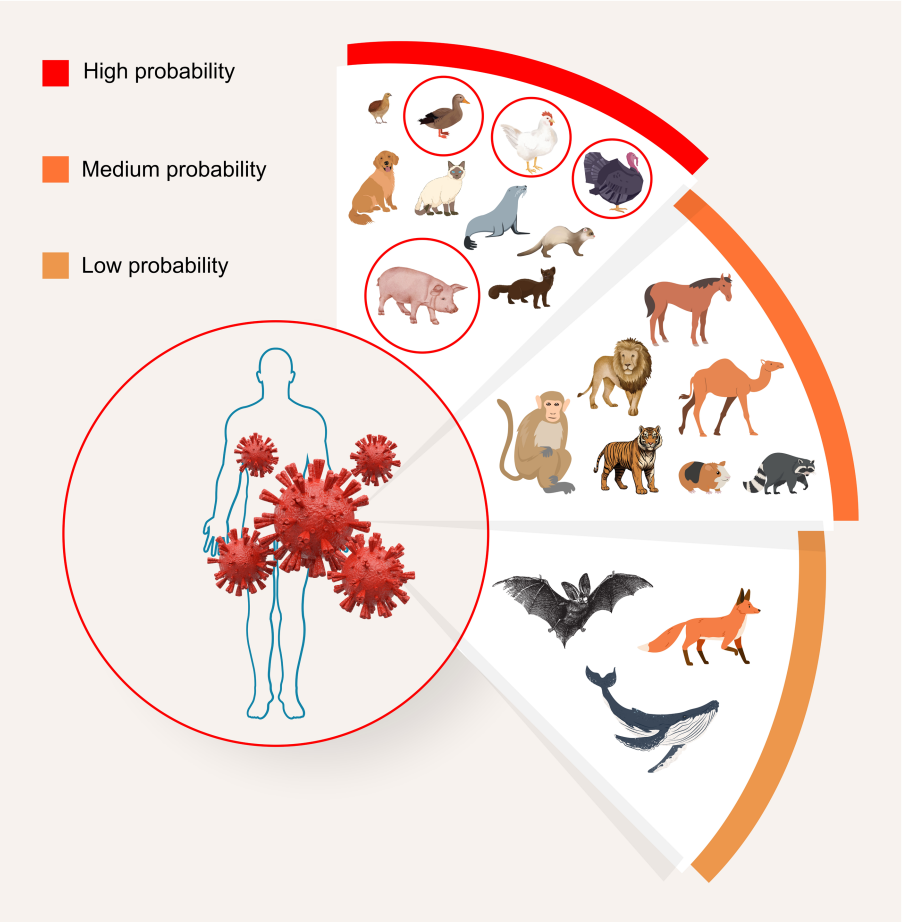


FIGURE 1
Potential for avian influenza viral mixing, and emergence of new variants, among various animal species. Potential mixing vessel hosts are grouped based on frequency of infection, human proximity, high population numbers, and presence of both avian- and human-type receptors. A diverse range of type A subtypes can be found among different animals in each group. "High probability" mixing vessel hosts include humans, pigs, minks, ferrets, seals, dogs, cats, and birds, particularly turkeys, chickens, quails, and ducks. "Medium probability" hosts are nonhuman primates, raccoons, camels, pikas, zoo animals, including tigers and lions, and horses. "Low probability" hosts include foxes, bats, and whales. After Abdelwhab and Mettenleiter (13). Image: MAD Ideas.

## 4 Causes of high zoonotic risks in farmed animals

Multiple factors increase viral transmission and infection risks for domesticated species. Through an analysis of published data, Johnson et al. (33) (p. 6) calculated that there is an average of 19.3 viruses in domesticated mammals (although 31 exist in pigs, including hepatitis E) such as Rift Valley fever from the phlebovirus genus; this is in contrast to an average of 0.3 viruses in noncaptive wild mammals, including hantaviruses and henipavirsues (for more examples of viral zoonoses see Table 1). A plethora of studies and reports indicate that the following eight factors account for the higher risk of viral infection among domesticated mammals [e.g., (8, 11, 17, 34–36)]:

1. High population numbers among farmed pigs, farmed chickens, and humans
2. How the vast majority of farmed pigs and chickens are kept
3. How the vast majority of farmed pigs and chickens are bred
4. Close proximity of farmed pigs and chickens to humans (staff)
5. The globalized and international nature of IIAF
6. The high land/resource requirements of IIAF disrupting ecosystems and increasing proximity to wild animals
7. The locations of IIAF operations
8. Misperceptions of low risks to and posed by farmed animals

Each of the preceding eight points will be addressed in turn, with examples focusing on pigs and chickens, as they are the most consequential farmed species for the purposes of this study. The eight points are also interdependent, as highlighted by Morand (34, 35) within modeling studies. These anthropogenic factors function together to create what is effectively a human-made reservoir, as described by Kessler et al. (17). This human-made reservoir increases the zoonotic risk not just in one way, but in three ways: increased risk of disease emergence, disease amplification (fast replication), and disease transmission [(11), p. 137].

First, the human population surpassed eight billion in November 2022, with a medium projected forecast of 10.4 billion by the 2080s (37). Much of this growth is occurring within developing countries.

Driven by the adoption of westernized lifestyles, greater disposable incomes, and industry lobbying, consumption of animal products is also increasing across the globe (8). Jointly, these factors greatly increase the demand for animal produce, and to produce the quantities demanded, while maximizing profits, IIAF has become the norm. This has resulted in enormous farmed animal populations. These greatly surpass the human population—over 80 billion farmed terrestrial vertebrates were slaughtered globally in the most recent available year of 2021 (38), and by 2021 there were well over 25 billion chickens alive at any one time (38). The vast majority (over 70 billion) of the animals slaughtered each year are meat chickens, as they are slaughtered at the age of 6 weeks on average, resulting in a very rapid turnover (38). Additionally, there are close to 1 billion pigs alive at any one time (39). These concurrent massive populations mean less space on Earth for wildlife, more pressures on wildlife, and closer living proximity between humans, chickens, and pigs. This increases the frequency of interaction between humans/pigs/chickens and wild animals, and increases the risks of disease transmission (see the seventh point below for further discussion of this). As an example, Figure 2 highlights the correlation between the number of farmed chickens' slaughtered over time and the number of human cases of the avian flu subtype generating the most concern (H5N1).

Second, these high farmed animal populations necessitate certain high-stress living conditions to make the rearing and slaughter of over 80 billion land animals per year feasible. These include high stocking numbers, which facilitate disease transmission (17). The latest UK data demonstrate a 7% increase since 2016 in IIAF operations, with poultry and pig farms comprising the entirety of this number [(41), pp. 59–61], meaning there were over 1,700 such farms by 2023 (i.e., housing at least 40,000 poultry, 2,000 meat pigs, or 750 breeding sows) in the UK. However, there was also a conservative estimate of 1,100 "megafarms," with the four biggest UK poultry farms stocking 1 million birds each, and the biggest three UK pig farms housing 20,000 pigs each. Lymbery (41) suggests that the increase in these megafarms occurs subtly, often being due to expansions of current farms, rather than the construction of completely new operations—i.e., just as is being proposed in the planning applications cited in the introduction of this review.

Alongside very high stocking numbers of poultry and pigs, are very high stocking densities. The legal maximum stocking density of meat chickens in the UK is 39 kg/m$^2$ or roughly 17 chickens per m$^2$ (42). High stocking densities are known to increase stress in farmed animals, which, if chronic—as is normal under such conditions, can lead to impairment of the immune system (immunocompromisation) and an impaired ability to resist disease (36). The inhibition of many highly motivated chicken and pig behaviors within such crowded conditions also contributes greatly to high stress levels, as does the lack of environmental enrichment/stimulation (36). For instance, pigs in the UK often resort to tail biting in the absence of other exploration-based and foraging enrichment (43), and sows are confined to farrowing crates for around a month surrounding birth. These crates are too small for the sow to even turn around in (44). Poor litter quality also frequently prevents dust-bathing behavior in meat chickens (43).

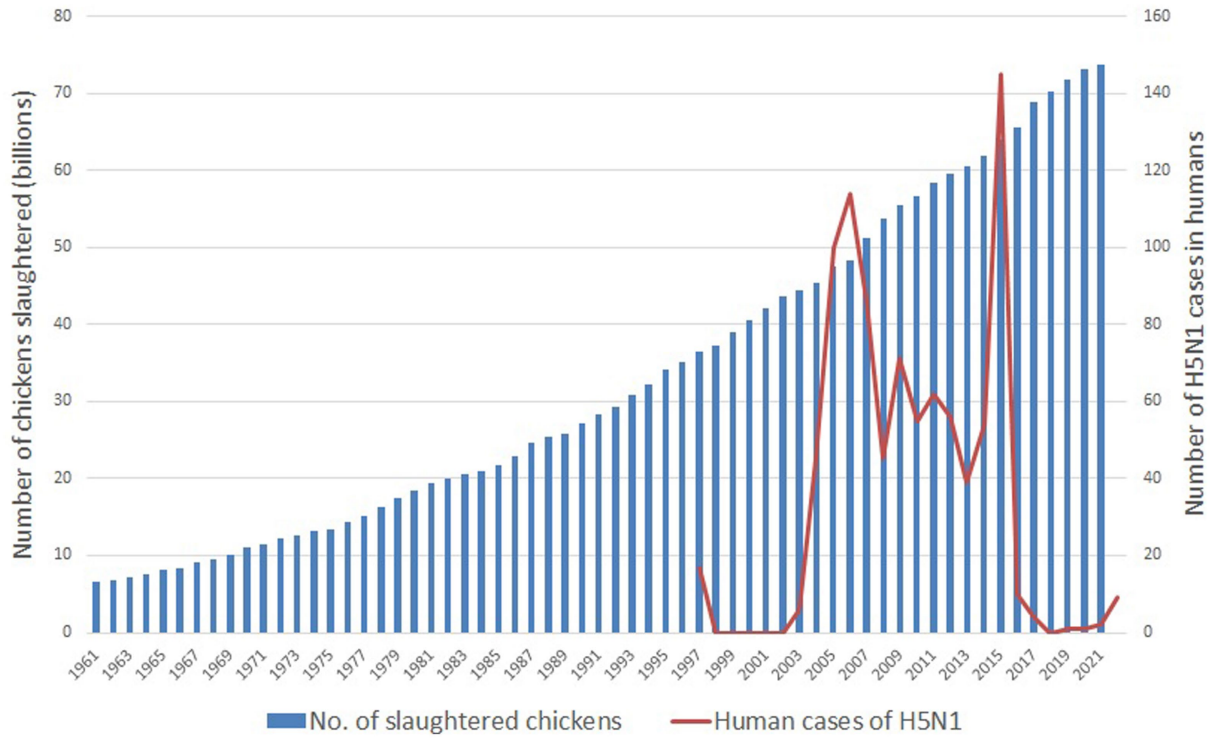


FIGURE 2
Global number of farmed chickens slaughtered and global cases of H5N1 in humans between 1961 and 2022. An H5 vaccine was introduced in 2017 [(17), p. 5]. H5N1 case data are from the CDC (40) with records beginning in 1997; number of chickens slaughtered are from the UN FAO (38). After Kessler et al. (17).

Moreover, interruptions to social bonds can occur, creating another stressor. Examples include piglets being weaned prematurely and their social group being frequently changed (45). Other significant stressors are physical restraint methods, and mutilations. For instance, despite routine tail docking of piglets being against the law in the UK, in practice, around 80% of piglets have their tails docked, and without anaesthetic (46) even though this is a painful procedure. Feed can also be restricted. Among those most affected by this are so-called "broiler breeders"—the parents of meat chickens—where feed is restricted to slow growth (which would otherwise occur very rapidly in these fast-growing breeds) and to maintain reproductive ability. Such feed restriction is an additional stressor (43). Sanitation is often an additional physiological stressor. For instance, meat chickens' barns will typically not be cleaned once during their short 6-week life (43). Suboptimal or broken ventilation systems and waste disposal can also lead to pathogen release [(17), p. 10] and further elevated stress levels, if the animals are forced to endure temperature extremes during adverse weather. The links between substantial and chronic stress in farmed animals, resultant immunocompromisation, and consequently increased disease susceptibility, cannot be overstated.

Third, IIAF has used selective breeding over many decades to create the pigs and chickens used in the industry today. The focus within such breeding has been primarily on optimizing the productivity of animals. This may impair the animals' welfare (i.e., cause stress, as problematized in the previous point above) if aspects of their breeding are likely to cause them harm. For instance, meat chickens have been genetically selected for growth rates that are now over 400% of those less than 40 years ago. This means their physiological and skeletal systems often struggle to cope with the speed at which they put on weight, leading to lameness and thus reduced mobility (36). Pigs have been genetically selected for larger litter sizes. This has led to increased sow size, intensified sow milk production, and aggressive competition for teats among piglets (45, 47). Pigs' increased growth rates have led to increased lameness and metabolic disease [(48), p. 181]. Moreover, in IIAF, the animals are all bred to be genetically very similar, which reduces the populations' resistance ("hybrid vigor") to novel threats such as pathogens [(17), p. 10].

Fourth, IIAF necessitates close proximity of staff (including farm staff, transport staff, and slaughterhouse workers), to an exceedingly high number of animals. These humans are the most at risk of being infected with a zoonosis (13). Fifth, IIAF exists within an international market involving the annual live transportation of over 2 billion farmed animals nationally and internationally with journey durations of hours to weeks. Akin to bird migration, this market has the potential to easily spread new pathogens around the world (36). However, distinct from migratory birds, these farmed animals normally experience more severe physical and emotional stress during their journeys, due to cramped conditions, foreign environments, lack of autonomy, changes to social dynamics, thwarted highly motivated behaviors, exhaustion, hunger, dehydration, fear, discomfort, and physical injuries (36).

Sixth, the considerable impact of IIAF on the climate has become more widely known, albeit scarcely acted upon. This is relevant to this study's focus as climate change means changes to ecosystems, which often increase the risk of zoonoses (7). However, less frequently discussed are the broader and additional environmental impacts of IIAF. While cattle farming is usually the worst offender from an environmental perspective, other kinds of intensive animal farming, including of chickens and pigs, still demand more resources than arable farming does and contribute to considerable environmental pollution (49, 50). The high use of resources necessitates habitat destruction, which is the leading cause of the sixth mass extinction event since fossil records began, which is currently underway globally [(51), p. 317]. The resultant profound reduction in biodiversity includes a reduction in species that are *less* likely to harbor pathogens, increases the proximity of wild animals to humans, and disrupts ecosystems—all factors leading to increased risks of zoonoses emerging (11).

Seventh, IIAF operations often congregate in clusters. Norfolk is already one of the IIAF hotspots in the UK, second only to Herefordshire and Shropshire (41), and it is within this very region that the pig and poultry farm expansions have been proposed, as outlined in the introduction. Moreover, IIAF operations are often situated in rural areas close to wildlife habitats, increasing the risk of pathogen "spillover" from wild animals into farmed animals, who then act as a "bridge" for pathogen transmission into humans [(36), p. 330]. Of course, other geographic considerations can also increase the zoonotic risk such as farm positions in close proximity to migratory paths of wild birds, congregation sites of wild birds (e.g., nesting, reproduction), slaughterhouses, and fragile ecosystems [(52), p. 6].

Finally, the EFSA (European Food Safety Authority) is beginning to challenge the perception of some farmed animal types as low risk in terms of zoonosis threat such as meat chickens or so-called breeder chickens [(53), p. 5]. Indeed, there seems abundant recent evidence to support the position that all farmed chickens are at high risk, with numerous cases of avian influenza being found in meat chicken farms and indoor farms, including when under avian influenza restrictions over Winter 2022–2023 when many captive birds were under heightened biosecurity practices such as in the UK [(54, 55); EFSA, personal communication, September 20, 2023].

## 5 Recommendations for minimizing zoonotic disease risk from farmed animals

Before focusing on recommendations *for* particular actions, it is important that we recommend *against* "silver bullet" thinking—the misperception that any one action alone will suffice to reduce zoonotic risks to an adequately low level. An example of such silver bullet thinking would be an overdependence on biosecurity and vaccinations. First, biosecurity practices alone are proving insufficient at lowering the risk of zoonotic disease to a manageable level, as demonstrated by indoor systems with good biosecurity that are still acquiring influenzas (11). To explain this, Kessler et al. (17, p. 10) state, "an exchange of pathogens with the environment can never be fully prevented." Moreover, they reject the assumption that huge, bio-contained holdings are necessary for controlling influenza viruses. Stevenson (11) (p. 138) highlights how optimized biosecurity may reduce the frequency of zoonoses arising within IIAF, but the nature of IIAF increases their severity when outbreaks do occur. Second, the nature and behavior of viruses and mutations they undergo (see antigenic drift and antigenic shift in section 3) are notoriously difficult to predict. Thus, there will always be gaps in knowledge, and humans will always be on the backfoot playing catch-up in relation to vaccine development. Nevertheless, any trade embargos on vaccinated animals should cease, so as to aid development in this arena (56).

Neither the authors nor the scientific literature suggest that optimizing biosecurity and research into vaccinations is redundant,

but given the multifactorial causes of zoonosis outbreaks described in section 4, it is clear that a more holistic approach is required. The very nature of IIAF unavoidably increases zoonosis risks. Accordingly, animal farming needs to (1) de-intensify and (2) adequately safeguard animal welfare. A reduced intensity of animal farming will necessarily mean a reduction in public consumption of animal products. Kessler et al. (17) and Stevenson (11) describe this vision of animal farms as "health-oriented" systems not dependent on routine antibiotic use. Such a vision aligns with the approaches of One Health and One Welfare that recognize the interdependency of human, animal, and planetary health (17, 34, 35) as well as the Sustainable Development Goals (35). Stevenson (11) and CIWF (56) frame such a vision as "radical restructuring" of poultry and pig farming.

Based on recommendations also from the EFSA, as a first key step, Stevenson (11) and CIWF (56) recommend reducing meat chicken stocking densities from an average of 38 kg/m$^2$ in the UK down to 11 kg/m$^2$ (around five chickens/m$^2$). These constitute estimated maximum densities at which highly motivated behaviors can still be fulfilled—which are essential for minimizing stress and safeguarding immune system health, and therefore, essential for minimizing zoonoses risks. Similarly, for pigs, Stevenson (11) calls for a reduction in density from over two pigs/m$^2$ down to one pig/1.5 m$^2$ (assuming a 100 kg pig). Stevenson (11) also calls for reducing clusters of operations to reduce transmission risks, as dust particles can carry viruses and be wind-carried between farms. There are also calls for improved breeding practices—away from a focus on maximizing productivity, and toward lowered susceptibility to infection, and optimal overall health and welfare (57).

Extant research typically also offers guidance for further research. For instance, Tomori and Oluwayelu (7) highlight that little is known about the number of zoonotic viruses that truly exist, and how this can lead to an *under*estimation of zoonotic effects. Thus, more general research is always required to advance understanding and knowledge of zoonoses, as also recommended by Guégan et al. (58). There is also room for methodological streamlining within future research. For instance, Abdelwhab and Mettenleiter (13) describe how direct comparisons between different studies are often not possible as different studies have used different methods to identify certain viral components. Finally, of note is that influenza viruses can survive for less time outdoors due to sunlight inactivating them [(36), p. 325]; hence, this is a benefit of small outdoor farms to consider and potentially examine further.[1]

## 6 Conclusion

The disease risks to humans from industrialized intensive animal farming, and especially mixed swine and poultry farms, are enormous, and must not be understated. Psychological and physiological stress in farmed animals needs to be substantially reduced to maintain their immunocompetence, and thus minimize disease risks. In particular, the latest scientific evidence concerning influenza viruses compounds the concerns about "mixing vessel" hosts; no longer are concerns about mixing vessels limited to pigs, but also several other animal species, and at a level of high probability—most notably chickens and humans. The authors therefore strongly discourage granting any planning applications for new or expanding industrialized intensive animal farms, especially poultry and pig farms or a mix thereof, and especially in areas with high existing concentrations of intensive animal farms. Instead, efforts should concentrate on supporting arable agriculture (or transitions toward this), and on de-intensifying remaining animal farms. This aligns with One Health and One Welfare approaches, which foreground the protection of both animal and human health and welfare as integral to any animal farm. As a recommended first step, stocking densities should be lowered to around five meat chickens/m$^2$ (11 kg/m$^2$) and to one pig/1.5 m$^2$ (assuming a 100 kg pig).

## Author contributions

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

1 Extensive farming has not been the focus of this review due to the dominance of and increasing global trend toward IIAF (59). Despite the reduced disease risks typically associated with more extensive farming types (e.g., stemming from lower stocking densities, more genetic diversity, higher welfare, less stress, and thus a more robust immune system), the authors acknowledge continued debate about potential higher enzootic disease risks stemming from some extensive farming types [e.g., (45, 60, 61), p. 65]. Moreover, without significantly lower consumer demand, extensive/higher-welfare farming methods will require even more land than IIAF, further increasing the interface between humans and animals [e.g., see (62, 63)]. Thus, the authors' recommendations are for both less intensity in animal farming *and* lower demand for animal products. More research and support is also required into enhanced means of lowering the general enzootic burden, which includes influenza viruses, especially in developing countries which are most impacted by these (64, 65).

## Publisher's note



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# Has avian influenza virus H9 originated from a bat source?

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Influenza A viruses are important pathogens that can cause diseases with high mortality in humans, animals, and birds; and wild birds are considered the primary reservoir of all subtypes in nature. After discovering the H9 influenza A viruses in bats, questions arose about their potential to serve as an additional natural reservoir and about the priority of the viral origin: Did the virus initially circulate in bats and then transmit to birds or vice versa? Influenza A viruses of the H9 subtype are of particular interest because fatal infections of humans caused by H5, H7, and H10 influenza viruses contained RNA segments from H9 viruses. Recently, a novel subtype of influenza A virus (H19) was reported and it was closely related to the H9 bat influenza A virus by its hemagglutinin structure. The genome of novel H19 has revealed a mixed characteristic genomic signature of both avian and bat influenza viruses. The time to most recent common ancestor (TMRCA) estimates have shown that the divergence time between the bat and avian H9-similar influenza virus occurred approximately at the end of the XVIII century. This article discusses the evolution and possible origin of influenza viruses of the H9 subtype isolated from bats and birds. The obtained data, along with the known data, suggest that the primary reservoir of the H9 influenza virus is wild birds, from which the virus was transmitted to bats. We hypothesize that the novel H19 could be a descendant of an intermediate influenza virus that was in the transition stage of spillover from avian to bat hosts.

KEYWORDS

**influenza A, subtype, bat, bird, Kazakhstan, Caspian**

## Introduction

Influenza A, as one of the most essential zoonotic infections, continues to pose a significant threat to human health and poultry production worldwide. Historically, wild birds were considered the natural reservoir for avian influenza viruses of all the known subtypes, serving as a central component for viral evolution and transmission to domestic birds and mammals (1). However, influenza A viruses (IAV) phylogenetically distant from avian isolates have been discovered in bats in South America (2, 3), raising questions about the existence of another reservoir in nature and the origin of influenza viruses from either bats or birds (4). In 2016 and 2017, influenza viruses of the H9 subtype were isolated from African bats, phylogenetically very close to the avian cluster (5, 6). It has been shown that despite the high degree of separation of bat populations, some genetic interaction between influenza viruses of the H9 subtype from birds and bats is possible.

Recently, a putatively novel subtype of influenza A virus (H19), closely related to the bat H9 influenza A virus, was reported from a wild duck in the Caspian seashore in Kazakhstan (7). Sequencing of its hemagglutinin (HA) gene (7) has shown the dual nature of its genome,

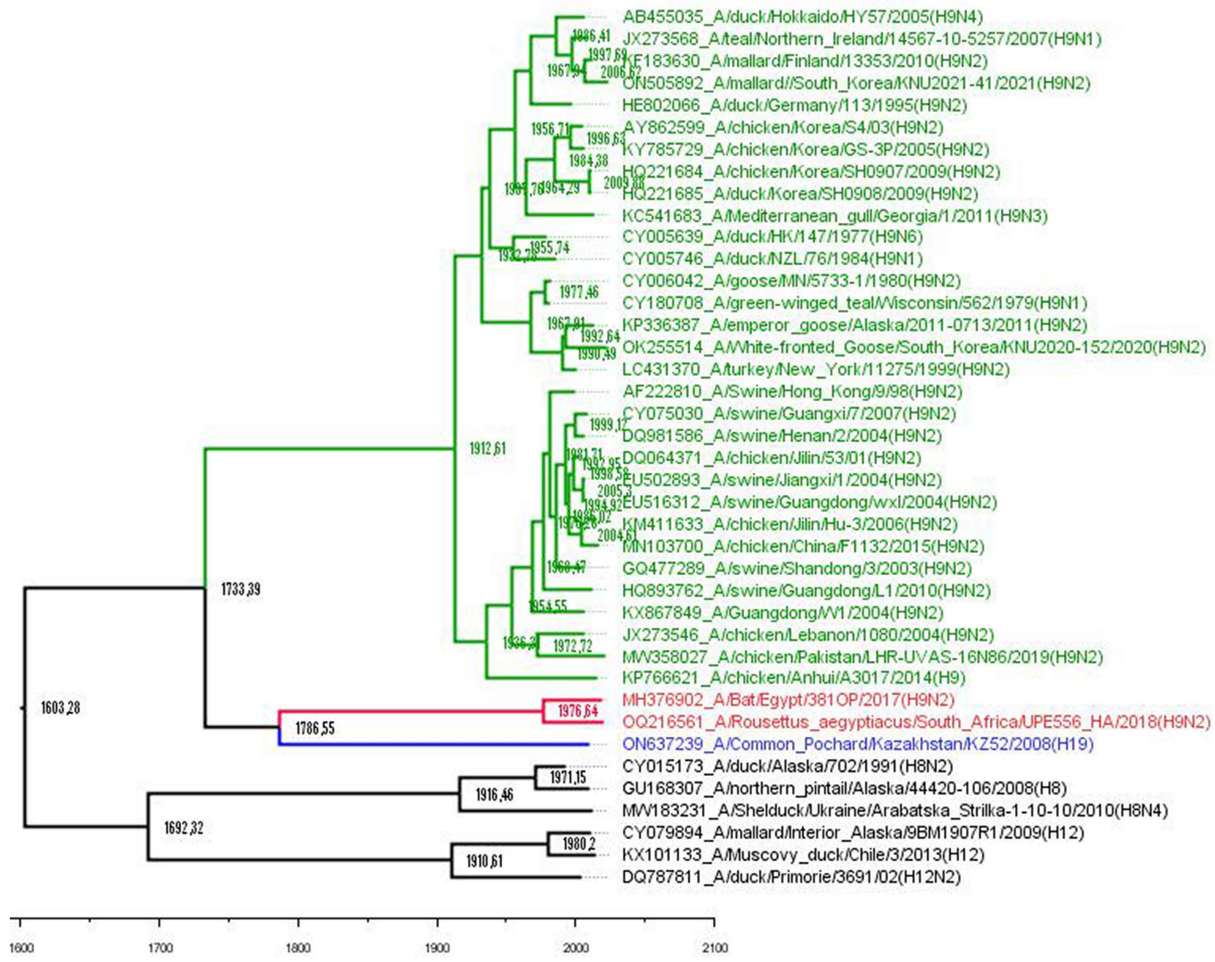


FIGURE 1
Phylogenetic tree based on the aligned H8, H9, H12, and H19 HA gene nucleotide sequences. Avian H9 subtype viruses are coded with green, bat H9 with red, and H19 with blue. Outgroup is black. A time-measured phylogeny using the BEAST v. 2.7.5 package based on the tip dates of each strain. The substitution model GTR with estimated base frequencies, four gamma categories, and invariant sites (GTR + F + I + G4) was applied. A fast relaxed clock with lognormal distribution was chosen, and the tree prior was set to Coalescent Skyline. The scale bar indicates the years before the most recent sampling time (2021).

characteristic of both avian and bat influenza viruses. The discovery of this virus has helped contribute to investigations into the genetic interaction between avian and bat influenza viruses and the origin of the H9 subtype from birds or bats.

## Materials and methods

A total of 34 HA gene sequences of H9 IAVs representing various hosts (avian, bat, swine, and humans) from Europe, Asia, New Zealand, and North America, along with the novel H19 subtype, were compiled from GenBank. The sequences of influenza viruses of H8 and H12 subtypes were used as outgroups (Figure 1). Nucleotide sequence alignments were created using the Clustal Omega incorporated in the Geneious software package (8). The most appropriate substitution estimation model for use in phylogenetic and molecular clock analyses was recommended by ModelTest, implemented in MEGA 11.0 (9), based on Akaike Information Criterion and Bayesian Information Criterion. Potential N-glycosylation sites (PGS) of the HA were predicted using the NetNGlyc 1.0 server.[1]

To be consistent with previous comparative molecular clock analyses of avian and bat H9 influenza viruses (10), the same models were used: GTR with four gamma categories and invariant sites along with the fast relaxed lognormal molecular clock. A Coalescent Skyline was set as a tree prior. Bayesian time-scaled analyses were conducted by the Bayesian Markov Chain Monte Carlo (BMCMC) method implemented in the BEAST v2.7.5 program (11) utilizing an HA gene sequence dataset. The tests were run for $10 \times 10^6$ generations, sampling every 1,000 steps. The convergence of output files was estimated in TRACER v1.7.2, where statistical uncertainty was provided by the 95% HPD values (Supplementary Table S1). The maximum clade credibility tree was generated using Tree Annotator v 2.7.5 and visualized in FigTree v1.4.4.

1 https://services.healthtech.dtu.dk/services/NetNGlyc-1.0

TABLE 1 Comparison of consensus amino acid sequences of H19 and avian, bat, and mammalian H9 HAs (H9 numbering).

| Host | Receptor-binding sites | | | | | | | | | | Bat influenza H9-specific sites | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 166 | 191 | 197 | 198 | 232 | 234 | 235 | 236 | 399 | | 5 | 14 | 15 | 17 | 18 | 28 | 32 | 35 |
| Bat | N | H | E | Q | G | Q/R | G | R | K | | K | Q | I | K/R | G | N | D | N |
| Avian H19 | N | N | E | Q | G | Q | G | R | K | | A | S | P | K | A | T | D | N |
| Avian H9 | N/G | H | E/V/A | Q | G | Q/I | G | R | K/R | | S/A/T | T/G | V/A/T | N | A | T | E | D |
| Swine | N | H | V | Q | G | Q | G | R | K | | S | T | V | N | A | T | E | D |
| Human | N | H | V | Q | G | Q | G | R | K | | P | T | A | N | A | T | E | D |
| Host | Bat influenza H9-specific sites | | | | | | | | | | | | | | | | | |
| | 38 | 40 | 46 | 47 | 52 | 53 | 54 | 58 | 61 | 62 | 63 | 64 | 66 | 69 | 71 | 72 | 74 | 75 |
| Bat | I | S | S | S | E | S | S | L | S | N | E | K | S | V | T | S/N | L | V |
| Avian H19 | I | N | Q | S | H | Q | E | L | S | T | S | K | S | E | D | K | K | I |
| Avian H9 | T/V/I | N/T | H | A/T | H | E | E | M | A | T | N/D | L/Q | R/H | I | D | T | T | I/V |
| Swine | T | N | H/Q | A | H | E | E | M | A | T | N | L | H | I | D | T | T | I |
| Human | T | N | Q | A | H | E | E | M | A | T | N | L | H | I | D | T | T | I |
| Host | Bat influenza H9-specific sites | | | | | | | | | | | | | | | | | |
| | 84 | 87 | 90 | 102 | 104 | 107 | 112 | 113 | 126 | 127 | 133 | 137 | 139 | 166 | 173 | 177 | 181 | 182 |
| Bat | Q | S | D/N | N | T | V | K | I | I | E | V | Q | V | T | E | R | G/K | H |
| Avian H19 | E | L | G | T | Q | I | N | I | S | S | V | P | V | S | E | K | S | D |
| Avian H9 | S | L | G | S | V/I | M/L | N/R | V | A/S | S/R | Q/L | P | I/T | N/S | D | T | G | K |
| Swine | S | L | G | S | V | M/L | N | V | A | S | Q | P | I | N | D | T | G | K |
| Human | S | L | G | S | V | M | N | V | A | S | Q | P | I | N | D | T | G | K |
| Host | Bat influenza H9-specific sites | | | | | | | | | | | | | | | | | |
| | 197 | 200 | 208 | 220 | 224 | 243 | 244 | 252 | 255 | 269 | 273 | 280 | 281 | 282 | 283 | 289 | 294 | 300 |
| Bat | S | N/S | S | S | A | G | I | K | T | K | Y | E | T | P | E | E | F | S |
| Avian H19 | S | E | T | S | T | G | I | K | T | R | H | A | AQ | P | L | E | K | S |
| Avian H9 | T | T/M | T | T/V | V | S | V | R | S | S | H | D | L | K/N/S | S | Q | K/R | T |
| Swine | T | T | T | T | L/V | S | V | R | S | S | H | D | L | N | S | Q | R | T |
| Human | T | T | T | T | V | S | V | R | S | S | H | D | L | N | S | Q | R | T |

*(Continued)*





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TABLE 1 (Continued)

| Host | Receptor-binding sites | | | | | | | | | | Bat influenza H9-specific sites | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 166 | 191 | 197 | 198 | 232 | 234 | 235 | 236 | 399 | | 5 | 14 | 15 | 17 | 18 | 28 | 32 | 35 |
| Host | Bat influenza H9-specific sites | | | | | | | | | | | | | | | | | |
| | 313 | 319 | 320 | 324 | 327 | 335 | 336 | 337 | 367 | 368 | 376 | 383 | 395 | 401 | 405 | 413 | 415 | 418 |
| Bat | Y | K | T | R | T | I | Q | T | A | E | Q | L | N | F | G | Q | I | S/L |
| Avian H19 | N | R | T | K | L | K | E | T | S | E | L | I | D | Y | G | T | I | M |
| Avian H9 | N/N | R/G | V | K | V | K/T/R/A | S | D/S/G | D | Q | R/K | V/I | D | Y | D | A/T | L | M |
| Swine | N | G | V | K | V | R | S/L | S | D | Q | R | I | D | Y | D | T | L | M |
| Human | N | G | V | K | V | R | S | S | D | Q | R | I | D | Y | D | T | L | M |
| Host | Bat influenza H9-specific sites | | | | | | | | | | | | | | | | | |
| | 426 | 429 | 438 | 454 | 458 | 463 | 466 | 469 | 472 | 477 | 479 | 483 | 485 | 496 | 498 | 501 | 518 | 519 |
| Bat | V | L | I | R/K | D | S | E | K | D | Q | L | N | E | I/M | S | E | E | N |
| Avian H19 | I | I | V | R | E | S | E | I | G | E | L | N | S | S | Y | S | S | N |
| Avian H9 | I | I | V | N | N | A | S | I/M/R/V | G | E | Y | D | Q | D/N | Q/R | Q/K | S | E |
| Swine | I | I | V | N | N | A | S | | G | E | Y | D | Q | N | R | K | S | E |
| Human | I | I | V | N | N | A | S | | G | E | Y | D | R | N | G | K | S | E |
| Host | Bat influenza H9-specific sites | | | | | | NGlyc sites | | | | | | | | | | Cleavage site | |
| | 525 | 538 | 545 | 546 | 557 | | 29 | 105 | 141 | 192 | 218 | 248 | 298 | 305 | 492 | | 333 | |
| Bat | M | T | F | I | T | | NSTD | – | NVTY | – | – | – | NTSL | NISK | NGTY | | PAIQTR | |
| Avian H19 | L | L | M | V | T | | NSTD | – | NVTY | NPSS | – | NQTL | NASL | NISK | NGTY | | PIKETR | |
| Avian H9 | L | A | M/L | F | I/N | | NSTE | NG(T/M/L)C | NV(T/S)Y | – | NRTF | – | NTTL | N(V/I)SK | NGTY | | PA(R/A)SDR | |
| Swine | L | A | L | F | N | | NSTE | – | NVSY | – | NRTF | – | NTTL | NVSK | NGTY | | PAR(S/L)SR | |
| Human | L | A | L | F | N | | NSTE | – | NVTY | – | NR(A/T)F | – | NTTL | NVSK | NGTY | | PARSSR | |





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## Results

The tMRCA estimates show that the divergence between avian and bat H9-like influenza virus subtypes occurred around 1733 CE (Figure 1), in agreement with a previous report that established the time of the MRCA for them as approximately 1700 CE (10). Then, the mixed bat H9-like precursor diverged to H9 bat and H9-like H19 avian influenza viruses around 1786 CE, which still circulate in their respective hosts. Maximum clade credibility trees revealed that H9, H8, and H12 have evolved from ancestors that existed approximately around the 1600s and these data are close to previously estimated data using a host-specific molecular clock (12).

To follow this evolutionary process, it was important to understand the differences in the amino acid structure of HA, related to the bird or bat host specificity. It was established that amino acid substitutions at positions E190D and G225D for H1N1 and Q226L with G228S for H2N2 and H3N2 (H3-numbering) play a key role in adaptation from avian to human host (13, 14). Both bat H9 and avian H19 have avian residues E190, G225, and G228, but avian H19 has a substitution Q226I, while Bat H9 has avian Q226. Analysis of the amino acid consensus sequences of HA from bat H9, avian H19, avian H9, swine, and human viruses revealed the presence of presumably bat-specific sites (highlighted in gray in Table 1).

A total of 103 putative bat-specific sites that distinguish bat and avian HAs were identified. Interestingly, avian H19 shared 34 bat-specific positions with bat H9, which confirms the mixed avian and bat nature of the H19 HA genome. The results of the analysis showed that the H19 subtype could have served as an intermediate link between avian and bat influenza virus hosts in the past.

Analysis of the receptor binding site (RBS) of H19, bat and other mammalian H9 HAs has shown their potential avian origin. Six potential PGSs at positions 29, 105, 141, 218, 298, and 305 in HA1 and one 492 in HA2 (H9-numbering) were identified in avian, swine, and human H9 viruses. Two PGSs at positions 192 and 248 were unique for the H19 virus. Some avian isolates had one more PGS at position 105 that was absent in bat and mammalian sequences as well as in the H19 subtype. The HA cleavage site of all avian and mammalian influenza viruses contained one or two basic amino acids that are typical mostly of low pathogenic strains. Interestingly, the cleavage site pattern of novel H19 was defined as PIKETR↓GLF and was identical to that of the bat influenza strain A (H18N11) isolated in South America (2).

## Discussion

Historically, wild birds have been considered the main and the only reservoir of IAV in nature (1). The bat H17 and H18 influenza A subtypes are thought to have diverged from avian lineages in the 14th century (10), and because of their huge difference from conventional IAVs, it is difficult to hypothesize their relationship with avian genotypes. On the contrary, the H9 bat IAVs turned out to be phylogenetically close to avian viruses (5, 6). Mortality cases in humans caused by H5, H7, and H10 IAV subtypes containing H9N2 segments in their genome were registered in Asia (15). The H9 subtype is known for its broad host range, including birds, mammals, and humans, and it is well-established that reassortment between isolates from different host species can generate viruses with pandemic potential (16). Due to the high host adaptability of this subtype, it possessed substantial potential to be transmitted to a new bat host. Obviously, a separate mammalian lineage of the H9 influenza A virus has emerged in bat populations. Interestingly, this H9 subtype lineage emerged in bats, and there is no evidence of similar events in human or pig populations, although it may potentially happen in the future. Extensive circulation of the bat lineage was serologically confirmed by the detection of neutralizing antibodies to H9 in several bat populations in Africa (5). The adaptation process in bats did not affect RBS as nearly all key genetic signature sequences retained avian features. It has been shown that although bats are resistant to avian H9 viruses, they are susceptible to specific bat H9 isolates (17).

The Caspian region is an important place where many wild bird migration routes intersect and different viral lineages and genotypes may potentially mix. In this region, subtypes of influenza A virus H14 (18) and H16 (19) were first discovered and reassortant American lineage H11 (20) and a novel paramyxovirus APMV-20 (21) were first identified. It is possible that mixed avian-bat viruses, such as H19, circulate in Africa, where H9 has been isolated from bats (5, 6), and spread to other places by migrating birds. A case of transmission of the H5 virus from Africa across the Caspian Sea by flamingo was previously described (22).

We assume that bat H9 viruses originated from avian H9 and the new avian H19 subtype could potentially be a descendant of an ancestor virus that further diverged into two lineages—bat and avian.

If we try to answer the question from the title of the article "Has avian influenza virus H9 originated from a bat source?," based on the findings obtained in this study and available data (5, 6), we are inclined to hypothesize the opposite, that the H9 bat influenza virus originated from an avian reservoir.

With the increasing infection of humans with H9N2 in Asia, a major concern arises that this subtype may be the cause of the next worldwide pandemic, although no human-to-human transmission has yet been reported (4, 23). Therefore, regular monitoring of circulation and evolution of the H9 subtype viruses in animal and human populations have great importance in ensuring preparedness for adverse pandemic scenarios.

## 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 study was approved by the Institutional Review Board: The SPC of Microbiology and Virology Local Ethics Committee (Approval number: 02–09-106). The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

KK: Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. AK: Resources, Supervision, Writing – review & editing. SF: 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.

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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.2023.1332886/full#supplementary-material

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# An overlooked poultry trade network of the smallholder farms in the border provinces of Thailand, 2021: implications for avian influenza surveillance

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**Introduction:** In Thailand, community-level poultry trade is conducted on a small-scale involving farmers and traders with many trade networks. Understanding the poultry movements may help identify different activities that farmers and traders might contribute to the spread of avian influenza.

**Methods:** This study aimed to describe the characteristics of players involved in the poultry trade network at the northeastern border of Thailand using network analysis approaches. Mukdahan and Nakhon Phanom provinces, which border Laos, and Ubon Ratchathani province, which borders both Laos and Cambodia, were selected as survey sites.

**Results:** Local veterinary officers identified and interviewed 338 poultry farmers and eight poultry traders in 2021. A weighted directed network identified incoming and outgoing movements of where the subdistricts traded chickens. Ninety-nine subdistricts and 181 trade links were captured. A self-looping (trader and consumer in the same subdistrict) feedback was found in 56 of 99 subdistricts. The median distance of the movements was 14.02km (interquartile range (IQR): 6.04–102.74km), with a maximum of 823.08km. Most subdistricts in the network had few poultry trade connections, with a median of 1. They typically connected to 1–5 other subdistricts, most often receiving poultry from 1 to 2.5 subdistricts, and sending to 1–2 subdistricts. The subdistricts with the highest overall and in-degree centrality were located in Mukdahan province, whereas one with the highest out-degree centrality was found in Nakhon Phanom province.

**Discussion:** The poultry movement pattern observed in this network helps explain how avian influenza could spread over the networks once introduced.

KEYWORDS

**avian influenza, poultry trade network, country border, local community, network analysis**

# 1 Introduction

Avian influenza (AI) is an infectious disease caused by type A influenza viruses. AI causes infections in birds, humans, and other mammals such as horses, pigs, and cats (1, 2). Different subtypes of avian influenza viruses have been observed globally (3), and AI viruses (AIVs) are generally not highly contagious to humans (3). However, the first reported evidence of animal-to-human transmission occurred when the highly pathogenic AI (HPAI) A(H5N1) virus was transmitted to humans in the Hong Kong Special Administrative Region in 1997 (4). In late 2003–2004, AI was detected in Thailand and neighboring countries. The Division of Epidemiology, Ministry of Public Health, Thailand, reported 25 human influenza A(H5N1) virus cases, including 17 deaths from 2004 to 2006. The last three persons with confirmed influenza A(H5N1) virus infection reported in Thailand occurred in 2006, and all three died (5).

According to the epidemiological data on the AI A(H5N1) virus, poultry is a primary source of human infection (6). The Thailand Ministry of Agriculture and Cooperative's Department of Livestock Development (DLD) has implemented various measures to control AI outbreaks and eliminate the disease. Presently, Thailand has had no confirmed reports of AI in poultry in over a decade. However, retrospective data from the World Organization for Animal Health show that AI outbreaks have continued to occur in Thailand's neighboring countries (7). Human and poultry movements across borders occur on a daily basis. The risk of disease reintroduction is not negligible, and poultry workers may be at greater risk for AI virus infections than the general public due to their daily poultry handling routines. For example, a study conducted in traditional markets in Taiwan suggested that market workers with a higher risk of AI infection seemed to be more careless in preventive behaviors compared to shoppers and those with lower risk (8).

Thailand has a variety of poultry farms, from smallholders to industrialized corporate farms. Thailand's poultry industry evolved to prioritize exports via vertically integrated companies controlling production chains through contracts. This concentrated system leaves independent smallholders and local markets as a smaller, potentially higher-risk sector for the spread of infectious diseases (9). Moreover, most smallholding farms have few biosecurity precautions, increasing their vulnerability to AI re-emergence and spread (9). Poultry production has increased due to growing export and domestic demand, driven by lower chicken prices (10). Increased consumption and the density of poultry populations could facilitate the spread of AIVs, as animals and farm workers are in close contact (11). An insight into the poultry trade networks is essential to better understanding potential AI transmission sources (12). A comprehension of poultry trade networks and designing surveillance systems can help identify and mitigate the risk of disease spread in poultry production chains, as well as inform more effective AI control measures by understanding local attitudes, knowledge, and beliefs (13, 14).

Social network analysis (SNA) has previously been used to describe how different livestock species are moved and traded in Thailand, including cattle (15), goats (16), pigs (17), and chickens (18). SNA can quantify the structure and geographical distribution of poultry trade networks in Thailand, helping policymakers direct resources to areas at the highest risk of AI transmission. This study explored poultry trade networks in Thailand and how their connectivity facilitates the spread of AI, especially in provinces bordering the Lao People's Democratic Republic (PDR) and Cambodia. These bordering provinces are at high risk of AI reintroduction as the disease is still prevalent across the borders (7). Focusing data collection on these areas may help strengthen regional AI surveillance.

# 2 Materials and methods

## 2.1 Study sites and target population

The Thailand governmental administrative system is divided into provinces, districts, subdistricts, and villages. In this study, we examined the local poultry trade network within specific subdistricts. The focus was on individuals engaged in trading activities or operating small farms. We herein defined a small or family-run farm as one managing 0.01–0.1 square kilometers of land (19). Smallholder farms in Thailand are characterized by being a part of family livelihood and integrative agriculture (20).

We conducted this study in 99 subdistricts in three districts located in the Nakhon Phanom, Mukdahan, and Ubon Ratchathani provinces (one district in each province). Nakhon Phanom and Mukdahan provinces border Lao PDR, and Ubon Ratchathani province borders both Lao PDR and Cambodia. Study sites located along the borders of Thailand are at high risk of AI outbreaks and are known areas of international poultry movement (21). We selected poultry farmers and poultry traders as the target population. All poultry farmers and traders aged 18 years and older who could speak and read Thai language and had lived in the study area for at least 1 year before participating were eligible. We excluded poultry farmers if they were not included in the Provincial Livestock Offices' registration database in 2019 and/or had not participated in poultry production for more than 1 month at enrollment. We excluded poultry traders if they had not been selling poultry for more than 1 month at enrollment.

We calculated the sample size of poultry farmers using the formula from the Tool 5 value chain sampling guidelines (22). We estimated the risk of AI infection at 50% (i.e., maximum uncertainty, which yields the largest sample size) of the total poultry farmers (23). We set precision at 7.5% with a z-score of 1.645. We estimated that we needed to sample 112 poultry farmers per district (336 total), with random sampling in each subdistrict. We sampled the farmers based on the 2019 District Livestock Office databases of poultry farmers in the three provinces using Epi Info Version 7 (24). Thai DLD has defined four size categories for poultry farms: backyard (fewer than 3,000 animals), small (3,000–10,000 animals), medium (10,000–50,000 animals), and large (more than 50,000 animals). We studied in rural areas near borders and then only included small-scale poultry farms. Due to the limited number of poultry traders in the study areas, we sampled all poultry traders in each district based on information in the Livestock District Office database. We invited all participants (farmers and traders) through a letter informing them of the requirements and requested they provide written informed consent. With our human ethical approval, we obtained informed consent and conducted in-person interviews with all participants, administered by trained health and veterinary officers using a standardized questionnaire

(Supplementary material S1). We collected demographic and risk factor information, including selling or receiving poultry practices from each participant.

## 2.2 Network analysis of the poultry trade network

We built a weighted directed network investigating poultry trading activities across subdistricts in the three study provinces. We defined a *node* as a subdistrict where the participants received and/or sold poultry. A *directed link* was trading activity and direction between the subdistricts. Each link was weighted with the cumulative frequency of the movements addressed by all participants, and a *self-loop* was defined as a link that occurred when both the trader and relevant consumer were identified in the same subdistrict. To identify essential nodes in the network, we measured the degree centrality and betweenness centrality of each node. Degree centrality measures the number of immediate neighbors a node has (25), while betweenness centrality measures how often a node is on the shortest path between two other nodes (26), helping identify the bridging property of each node (27). In degree centrality, the higher the degree centrality of a node, the more connected it is to other nodes in the network. In the context of poultry trade, this means that a node with a high degree centrality has more trading partners. For betweenness centrality, the higher the betweenness centrality of a node, the more important it is to the overall structure of the network. This means that a node with a high betweenness centrality is more likely to be involved in the flow of poultry between different subdistricts.

All network analyses and visualizations were performed with packages "igraph" (28), "dplyr" (29), "maps" (30), "sp" (31), and "leaflet" (32) in program R version 4.1.2 (33).

# 3 Results

## 3.1 Demographic data of participants

We interviewed 346 participants (100% response rate), consisting of 338 poultry farmers (97.7%) and eight poultry traders (2.3%). All poultry traders also reported raising poultry at home. The numbers of women and men were almost equal (184 women and 162 men). Study participants' ages ranged from 18 to 78 years. The mean and median age was 50 years. Most participants (335; 96.8%) had completed education up to the level of secondary school. The monthly incomes of 254 participants (73.4%) were less than 10,000 Thai Baht (approximately 300 USD). Totally, 120 (34.7%) participants raised mixed-type poultry, which comprised 103 farms with mixed backyard poultry, 54 farms with mixed ducks or geese, and 45 farms with mixed fighting cocks. The remaining 65.3% of poultry farmers raised specialized poultry, with 171, 47, and 8 farms raising only backyard poultry, fighting cocks, and ducks, respectively. In addition, over half (184; 53.2%) raised 2–40 free-range poultry in the household areas together with other animals such as dogs, cats, and cattle during the daytime. At night, these poultry sleep in the coops or small bamboo cages. Overall, 183 (52.9%) participants identified themselves as having over 10 years of experience in poultry farming or trading. There were only 10 participants who had less than 1 year of experience in the poultry sector. Most participants (251; 72.5%) reported having less than 1 h of close contact (feeding and taking care of) with poultry daily.

## 3.2 Poultry trade network

The network contained 99 nodes (subdistricts) and 181 links (trading activity between subdistricts). We found that the eight poultry traders identified in our survey lived in three subdistricts of Mukdahan and Ubon Ratchathani provinces (Figure 1A). Among the identified nodes, 28 were located in Nakhon Phanom province. An additional 20, 18, and 32 nodes were found in Mukdahan, Ubon Ratchathani, and other provinces, respectively. Interestingly, only one node was identified outside of Thailand, in Savanna Khet province in Lao PDR (Figure 1B). We found 56 self-loops, representing 56.6% of the nodes in this network.

Overall, in-degree and out-degree centrality values ranged from 1 to 18 (median = 1; interquartile range (IQR) = 1–5), 0–11 (median = 1; IQR = 1–2.5), and 0–8 (median = 1; IQR = 1–2), respectively. Of these, the highest overall and in-degree centralities were found in subdistricts located in Mukdahan province, while the subdistrict with the highest out-degree centrality was identified in Nakhon Phanom province (Table 1). Note that 46 subdistricts did not import poultry from other subdistricts, whereas 13 subdistricts did not export poultry to other subdistricts (detailed results of network measurement in Supplementary material S2).

## 3.3 Geographical distribution of the network

The poultry trade network covered 22 provinces, with most trading activity localized in the three participating provinces and their neighbors. Nearly three-quarters (72.3%) of the poultry movements were less than 50 km (Figure 2). The median distance of the movements was 14.02 km, with an IQR of 6.04–102.74 km. However, some traders moved their poultry far from their home locations. For example, poultry could be transported from the northern province of Lamphun to Mukdahan province (645.79 km away) or from the southern province of Prachuap Khiri Khan to Ubon Ratchathani and Mukdahan provinces with distances of 719.63 and 823.08 km, respectively (Figure 3).

# 4 Discussion

In this analysis, we describe the poultry trade network in three Thai border provinces using network measurement parameters. We found the poultry trade in the network to be localized, as evidenced by the self-looping pattern in over half of the nodes involved and that most of the trading activities occurred within each province.

Notably, the subdistricts with multiple trade partners (i.e., with a high degree centrality) were found in the three studied provinces. Network analysis may improve the effectiveness of disease control by focusing primarily on nodes with a high degree centrality (34). Identifying key nodes of trade within the network could help target

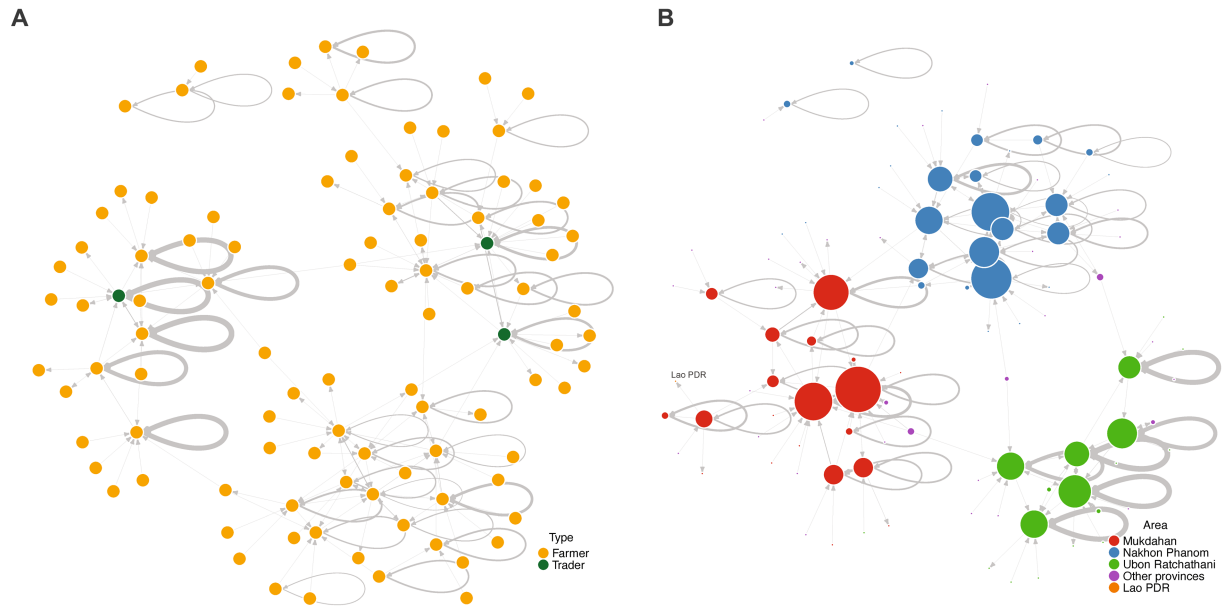


FIGURE 1
Sociograms of the poultry trade network in the three border provinces—Nakhon Phanom, Mukdahan, and Ubon Ratchathani—of Thailand and other provinces. A *node* refers to a subdistrict where farmers or traders resided at the time of the study, a *directed link* represents the direction of trading activities (buying or selling), and a loop demonstrates self-looping. The thickness of the links indicates the frequency of trading activities. **(A)** Node color shows whether the subdistrict contains only farmers (orange) or farmers and traders (green); **(B)** Node color depicts the provinces or areas where the subdistrict is located. The size of the nodes is proportional to their degree centrality (number of subdistrict trades with the nodes).

TABLE 1 Top five centrality values of the subdistricts identified in the poultry trade network in the three border provinces of Thailand.

| Province (geocode) | Overall-degree centrality | Province (geocode) | In-degree centrality | Province (geocode) | Out-degree centrality | Province (geocode) | Betweenness centrality |
|---|---|---|---|---|---|---|---|
| Mukdahan (490108) | 18 | Mukdahan (490101) | 11 | Nakhon Phanom (480107) | 8 | Mukdahan (490108) | 1,110 |
| Nakhon Phanom (480107) | 16 | Mukdahan (490108) | 11 | Ubon Ratchathani (342505) | 7 | Nakhon Phanom (480108) | 834 |
| Nakhon Phanom (480101) | 15 | Ubon Ratchathani (342502) | 8 | Nakhon Phanom (480101) | 7 | Nakhon Phanom (480112) | 818 |
| Mukdahan (490101) | 15 | Nakhon Phanom (480101) | 8 | Mukdahan (490108) | 7 | Mukdahan (490105) | 771 |
| Mukdahan (490105) | 14 | Nakhon Phanom (480107) | 8 | Ubon Ratchathani (342501) | 6 | Nakhon Phanom (480115) | 711 |

areas most likely to spread disease and aid in resource allocation decision-making. Strengthening poultry disease surveillance within communities at the subdistrict level and compiling the surveillance data at the provincial level may be the best approach. This surveillance strategy has the capacity to identify and track abnormal events quickly as the key nodes are readily identified with network analysis, allowing authorities to respond efficiently and effectively.

Betweenness centrality can also be used to identify the most influential spreaders of AI and other poultry diseases. It may also be useful in guiding policy decisions to identify areas more accurately in need of assistance in controlling the spread of infection (35). We found that almost three-quarters of the subdistricts included in this analysis did not bridge any trade pairs in the network (betweenness centrality = 0). This result reaffirmed that the poultry trade network in these provinces was distinctly localized. In contrast to those subdistricts with small or zero betweenness centrality values, we identified some subdistricts with high values of betweenness centrality, bridging many trade pairs. AI surveillance programs in these subdistricts could focus efforts on the nodes with high betweenness centrality, designating them as bridges between other nodes and sentinel sites for additional infectious diseases in poultry (36). Further research may identify factors affecting the high betweenness centrality values of these subdistricts to address the risks for relevant local populations.

As previously observed, animal movements can spread infection over long distances (17). Travel restrictions at the local, regional, and national levels are often among the first control policies enforced once a disease outbreak occurs (37). When examined provincially, poultry

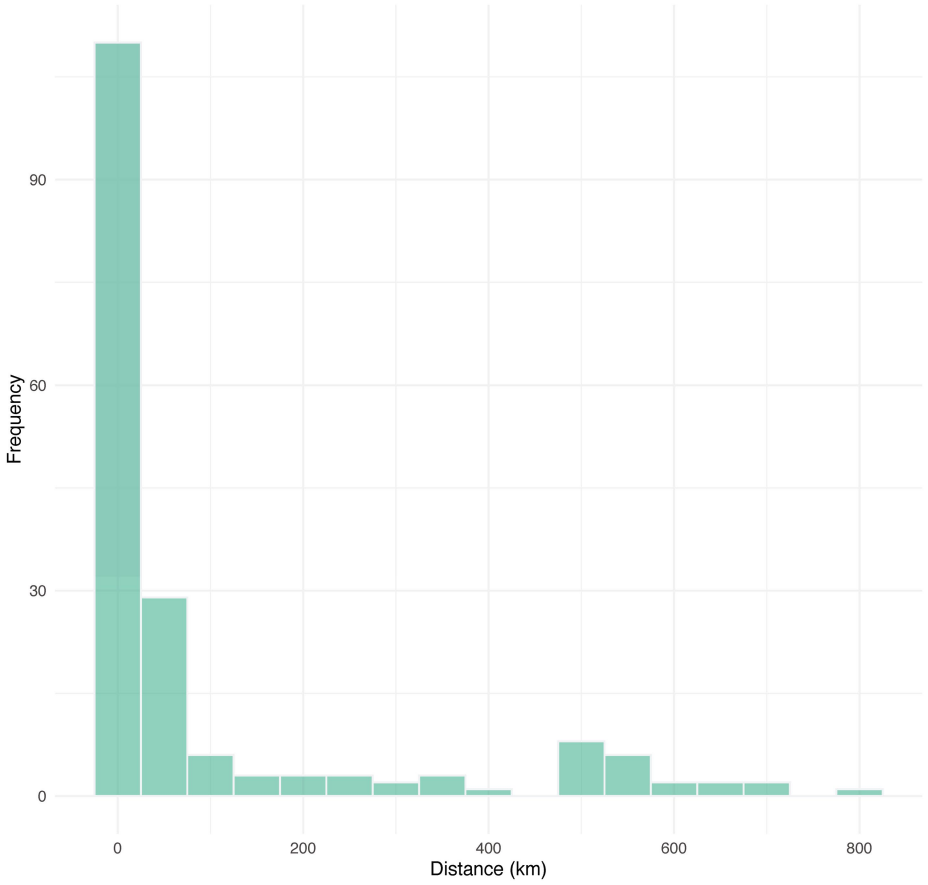


FIGURE 2
Distance distribution of the poultry movements in the poultry trade network in the three border provinces—Nakhon Phanom, Mukdahan, and Ubon Ratchathani—of Thailand and other provinces.

trade typically occurs only within the province where the trader lives, which is in line with a previous study in central Thailand (18). Our findings clearly showed the self-loops, particularly in Ubon Ratchathani province. In addition, poultry trades in Southeast Asia are often managed informally with little to no documentation, which is a part of local livelihood, as evidenced in previous studies across Thailand (18), Cambodia (38), and Vietnam (39). Rigorous surveillance encompassing these overlooked networks is critical to reducing the risk of AI transmission. Most of the participants in our study were older and had limited education and income, which may have limited their ability to trade outside their provinces. This pattern of poultry movement in the regions may reduce the risk of disease transmission. An additional study on the impacts of socioeconomic factors on poultry trade may provide insights into this aspect of the trade network. Indeed, we used our questionnaire results to investigate the impact of certain socioeconomic factors on risk perception in one of our prior studies (40). However, there was still some long-distance poultry trade, and our findings were consistent with previous studies on livestock movements in Thailand, which showed that most of the movements were managed locally with some remote translocations (15, 16). Future studies on long-distance movements of poultry trading may provide insight into trader motivations and their impacts on AI transmission. The central region of Thailand was the most frequent destination in the poultry trade beyond the three noted provinces. Approximately half of the country's chickens are raised in central Thailand, a relatively small but densely populated region, and most chicken exports come from farms in the region (41). Strengthening poultry disease surveillance and real-time information sharing across regions could be enhanced to improve early disease detection, especially in Thailand's central and northeastern regions.

In March 2021, Lao PDR reported that sentinel surveillance identified the first human AI A(H5N6) infection outside China (42). Afterward, an animal investigation in Lao PDR detected Muscovy ducks testing positive for the same AI subtype (42). Genetically, the virus originated from the reassortment of AI A(H5N1) and AI A(H6N6), which extensively circulates in ducks in China (43). In order to prevent the spread of AI and emerging infectious diseases in this region, integrated active surveillance with multi-sectoral collaboration that uses a One Health approach to balance the health of people, animals, and the environment (44) could focus on risk areas identified through social network analysis, especially along countries' borders. At the time of data collection, Thailand and other countries in the region were facing an increase in COVID-19 infections, which sparked regional border closures. Despite travel restrictions in the region, we identified a trade network outside the country in Savanna Khet province in Lao PDR. Nevertheless, the Thai government lifted lockdown measures to cope with the COVID-19 pandemic almost a year prior to our

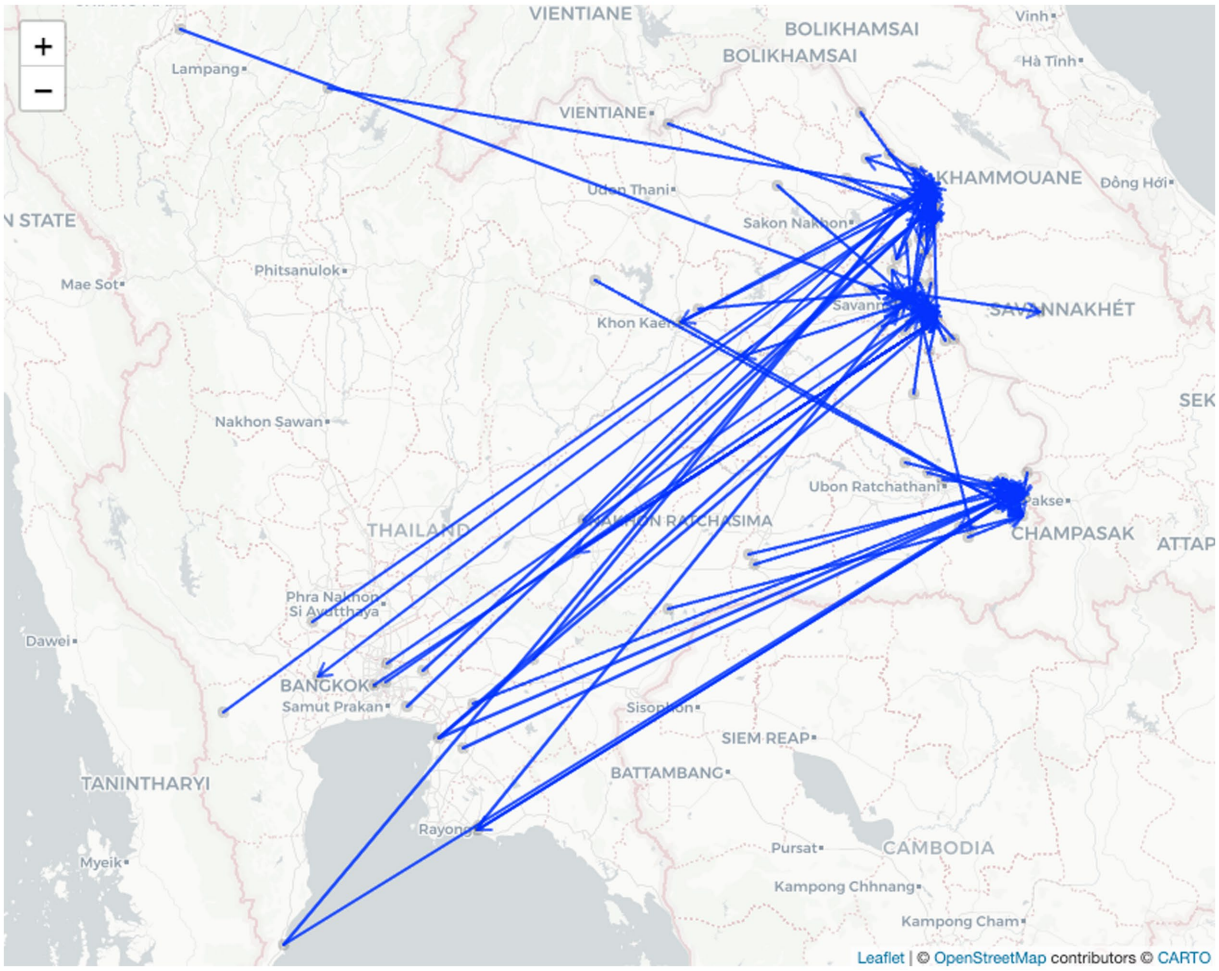


FIGURE 3
Geographic distribution of the poultry trade network in three Northeastern border provinces—Nakhon Phanom, Mukdahan, and Ubon Ratchathani—of Thailand and other provinces (zoomable version in Supplementary material S3).

study. Human and animal movements were proceeding normally within the country. However, border entry points and animal quarantine facilities were not operating as usual. Therefore, only one node outside Thailand was identified. Regardless, our questionnaire was designed to ask poultry farmers about their usual practices in the past, not just the current situation. This network demonstrates that despite the rising number of COVID-19 infections in the area and border closure mandates, small-scale international trade continued to occur but was less frequent than in pre-COVID-19 conditions. Human movement across the Thai-Myanmar border decreased substantially during regional COVID-19 border closures; however, movement between the two countries quickly rebounded after the relaxation of COVID-19 border closure policies (45). We expect the international poultry trade network to exhibit similar patterns at our study sites. A monitoring system for such dynamics could be implemented to enhance the cross-bordered surveillance system for AI. In addition, a recent review article suggested that AI was found more frequently and widely spread globally than it was before. Of those, almost half were classified as highly pathogenic AI A(H5N1) (46). Global surveillance of AI infections in both humans and animals should be rigorously maintained and strengthened, as evidenced in the present study, which shows that poultry trades around the border areas were still traditionally managed.

Limitations of this study include the impact of COVID-19 on study participants' typical trading and movement behaviors. For example, animal control points and international live bird markets were temporarily closed by order of the Thailand provincial Communicable Disease Committee to halt transmission of COVID-19 and, therefore, could not be included in our study. Additional data on the trade across borders could be collected post-pandemic to complete the whole network. Nevertheless, this study allowed us to observe the local poultry trade network under unusual circumstances, even though some unregistered producers were left out. Additionally, our study was totally carried out during the time of the COVID-19 pandemic. Hence, we could not directly compare our results with the non-COVID-19 situations. Our study strictly focuses on small-scale poultry networks; thus, large industrial farms were not included. A future network study covering all sectors could help identify different sectors' risks. Moreover, only three provinces were included in this study. Poultry farms in these three provinces accounted for only 6.25% of all poultry farms in Thailand (173,901/2,783,457 farms) in 2021 (47). A future study extending to cover a wider geographical area is suggested, as AI has

not been identified in Thailand for over a decade, even though AI surveillance activities have continuously been performed. Without any prevalence data, we need to maximize our sample size as calculated in the methods. In this study, we built a static network to describe the local poultry trade network cross-sectionally. A longitudinal data collection could be helpful in the future to capture additional changes over time. Finally, we holistically analyzed the network at the subdistrict level to describe the poultry trade patterns in the study area. A better insight into the demographic characteristics of the participants involved was previously addressed in our previous study (40).

Our findings indicate that the poultry trade in three border provinces of Thailand was relatively localized, as revealed by multiple self-loops. We identified subdistricts with high centrality values that reflect the substantial movement activities throughout the different study areas. Insights into the Thai poultry trade network provided by the SNA have important implications for identifying areas vulnerable to the re-emergence and spread of AI. Implementing a strengthened surveillance system with control measures in areas with extensive poultry trading could help mitigate the transmission of AI. Furthermore, SNA can greatly enhance risk communication and biosecurity measures, which can help to reduce the spread of disease across the entire value chain. SNA allows for targeted strategies, making disease risk reduction more effective and efficient.

## 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 studies involving humans were approved by Research in Human Subjects, Department of Disease Control, Ministry of Public Health, Thailand. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

## Author contributions

SH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Writing – original draft, Writing – review & editing, Supervision. PT: Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Writing – review & editing. JS: Data curation, Investigation, Resources, Writing – review & editing. CC: Data curation, Investigation, Resources, Writing – review & editing. WS: Data curation, Investigation, Resources, Writing – review & editing. YT: Data curation, Investigation, Resources, Writing – review & editing. OY: Data curation, Investigation, Resources, Writing – review & editing. OP: Funding acquisition, Investigation, Project administration, Writing – review & editing. SN: Funding acquisition, Investigation, Project administration, Writing – review & editing. PS: Data curation, Investigation, Resources, Writing – review & editing. JR: Data curation, Investigation, Resources, Writing – review & editing. CS: Validation, Writing – review & editing. AW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, 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.2024.1301513/full#supplementary-material

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# Epidemiology of *Salmonella enterica* subspecies *enterica* serotypes, isolated from imported, farmed and feral poultry in the Cayman Islands

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Non-typhoidal *Salmonellae (NTS)* are common foodborne pathogens throughout the world causing acute gastroenteritis. Compared to North America and Europe, there is little information on NTS in the Caribbean. Here we investigated the prevalence and characteristics of NTS present in the local poultry of the Cayman Islands to determine the public health risk. In total, we collected 156 samples. These were made up of boot swabs of 31 broiler farms and 31 layer farms (62 samples), paper bedding from 45 imported chick boxes, and 49 pooled cecum samples from feral chickens, each sample representing 10 individual chickens. *Salmonella* was isolated using the ISO 6579 protocol and isolates were characterized using Whole Genome Sequencing (WGS) analysis. Eighteen *Salmonella* isolates were obtained and comprised six *S. enterica* subspecies *enterica* serotypes and one subspecies *houtenae* serotype. Serotypes were: *S.* Kentucky ($n = 9$), *S.* Saintpaul ($n = 5$), *S.* Javiana ($n = 1$), *S.* Senftenberg ($n = 1$), *S.* Poona ($n = 1$) and *S.* Agona ($n = 1$). *S.* Kentucky strains were all ST152 and clonally related to poultry strains from the United states. *S.* Saintpaul ST50 strains showed clonality to North American strains. Over half of the strains ($n = 11$) contained resistance genes to at least two antibiotic groups and five strains were MDR, mainly those from imported day-old chicks. The $bla_{CMY-2}$ gene was found in *S.* Kentucky from day-old chicks. Strains from feral poultry had no acquired AMR genes. While serotypes from feral poultry have been identified in human infections, they pose minimal risk due to their low virulence.

KEYWORDS

***Salmonella enterica*, poultry, Cayman Islands, whole genome sequencing, public health, non-typhoidal salmonella**

# 1 Introduction

Non-typhoidal *Salmonellae* (NTS) cause over 93 million infections worldwide, with approximately 80.3 million attributed to food-borne transmission (1). The economic burden of salmonellosis in the United States alone has been estimated at $3.7 billion US dollars per year, ranking 1st among 15 other foodborne pathogens (2). NTS infections are one of the leading causes of death and hospitalizations amongst foodborne infections in the U.S (3). Symptoms are usually mild with fever, abdominal pain and diarrhea, but can becoming life-threating mainly in children under five years of age, immunocompromised and elderly people (4, 5). NTS infections usually do not require treatment, are self-limiting and patients can make a full recovery.

Human health is one of the three major components of the One Health concept, along with animal health and environmental health (6). The One Health concept emphasizes the close relationship between animal and human health as well as the environment and its ecosystems, to ensure optimal and sustainable balance between these three components. This is achieved by a collaborative, multi-discipline effort of different sectors to effectively address global health problems and create policies for long-term sustainability (7).

One critical threat that undermines the One Health concept is antimicrobial resistance (AMR), in which antimicrobials become ineffective, increased complications in treatment and the rise of 'superbug' microbes (8, 9). In 2019, AMR was declared among the top 10 threats to global health according to the World Health Organization. AMR is of great concern among NTS since it increases treatment difficulties. However, there is significant variation in the prevalence of AMR, depending on geographical location, time, and serotype (10). Of grave concern is the resistance against third-generation cephalosporins and fluoroquinolones (According to the World Health Organization's classification, these are deemed as Highest Priority Critically Important Antimicrobials), which are currently the preferred antibiotic treatment of severe human NTS infections in the both the United States and EU (11). In the USA there is a steady decline of AMR in NTS. In 2019, 78% of *Salmonella* isolated from clinical cases did not exhibit any antibiotic resistance (12), in comparison to previous years of 70% in 2014 and 76% in 2015 (13, 14). While ciprofloxacin resistance in the United States has remained steadily under 0.5% since 2018, decreased susceptibility to ciprofloxacin has been steadily rising starting from 0.2% of isolates in 1996 to 10.7% of isolates reported in 2019 (12). Resistance against ceftriaxone remained stable at 3% in 2019 (15), while in 2009, 20.8% of the *Salmonella* isolated from chickens were resistant to third generation cephalosporins. This contrasts to human clinical infections reported during those years, with ceftriaxone resistance consistently around 2.5% for the last decade (16).

Of the approximately 2,500 serotypes of *Salmonella*, only a small number of serotypes accounts for 99% of human and animal clinical cases. Common serotypes associated to human salmonellosis change over time, vary by geographical distribution and its ability to affect hosts (17). Currently, the most common serotypes implicated in human disease include *S. typhimurium* and *S. enteritidis* worldwide (18).

NTS are part of the commensal flora of the intestinal tract of cold-blooded animals such reptiles, turtles and amphibians (19) as well as some birds (20). These animals may infect domesticated animals when there are no rigorous biosecurity measures.

The Caribbean Public Health Agency (CARPHA), the major public health agency within the Caribbean comprised of 26 Caribbean nations, reported an average of 564 annual clinical cases of NTS infections during the years 2005–2012, but there has been a downward trend since 2010 (21). Between the years 2005–2012, a total of 146 different *Salmonella* serotypes were identified as causes of infections, with half of the infections attributed to *S. enteritidis* and *S. typhimurium* (21, 22).

NTS have been shown to be present in Caribbean wildlife including iguanas (23, 24), mongooses (25, 26), cane toads (27), crabs (28), sea turtles (29–31), snakes (32), albeit at low prevalence. Serotypes present varied between cold-blooded and warm-blooded animals. In farmed animals, the focus was mainly on poultry, and showed a prevalence rate range between 6.5–26.7% (33) and the prevalence in pigs in Surinam was 9% (34). Majority of poultry studies are reported from Trinidad and Tobago (35–40). In the Cayman Islands 10 different serotypes were found in native iguana and *S. enterica* serotype Saintpaul was the most frequently isolated serotype (23). In the Caribbean, biosecurity is not optimal as most animal houses are open houses allowing the entry of some of wildlife. Given that the majority of human infections often stem from poultry and eggs, the primary objective of this study was to assess the potential public health risk associated with salmonellosis in humans in the Cayman Islands, due to poultry.

Since most chickens in the Cayman Islands originate from imported embryonated eggs, we investigated whether *Salmonella* could be introduced in the local poultry by import. In addition, we looked whether feral chickens could be a source of salmonellosis for farmed poultry.

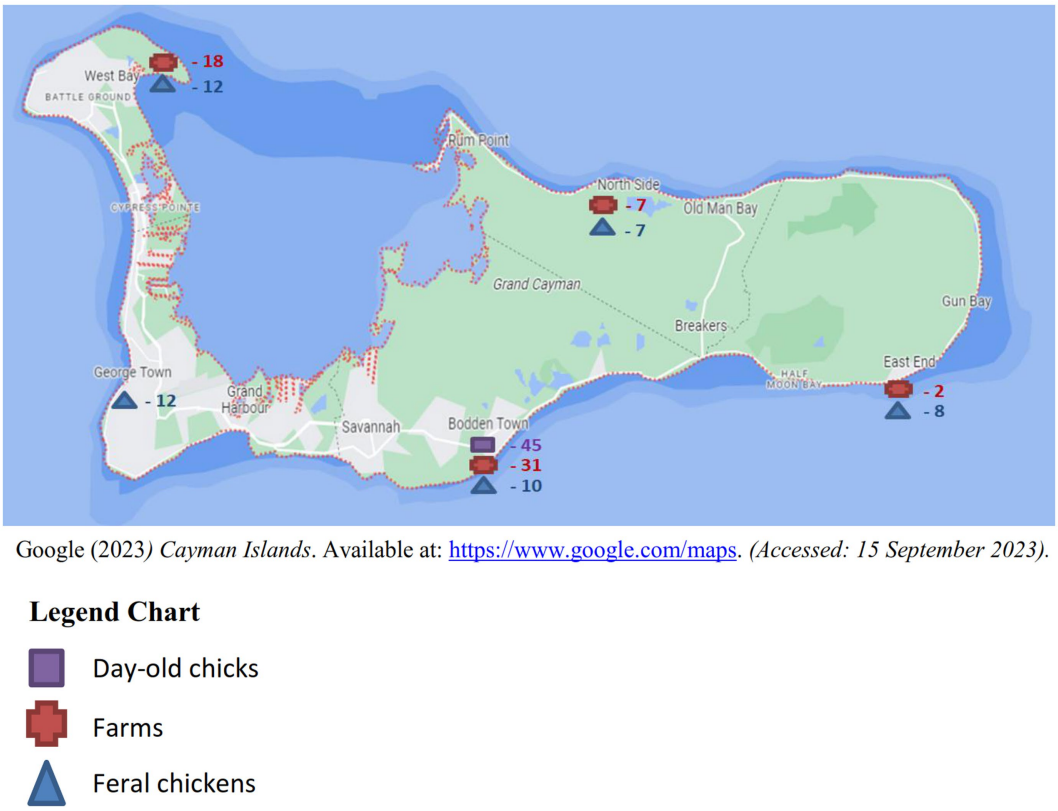
# 2 Methodology

## 2.1 Sampling

Between the years 2017 and 2018, sampling was conducted on a monthly basis totaling a minimum of four boot swabs, four pooled samples of 10 feral chickens per month and batches of imported day-old chicks as received. The samples originated from the five districts in Grand Cayman: West Bay, George Town, Bodden Town, East End and North Side (Figure 1), 3 samples (2 layer and 1 broiler farm) were taken from Cayman Brac and a single sample from Little Cayman (Figure 2).

For each monthly sampling event, a minimum of two pairs of boot swabs were sampled at each farm (broiler or layer), with a maximum of four boot swabs per farm depending on its size. Feral chickens of each district were captured, humanely euthanized and cecum contents were pooled from 10 feral chickens representing one sample. Lastly, paper bedding material from each chick-box of day-old chicks was sampled prior to distribution to farmers.

31 boot swab samples were taken in according to the Commission Regulation (EU) No. 517/2011 (41), from both broiler and layer farms. Since the population size of feral chickens in the Cayman Islands is undetermined, we collected 49 pooled cecum samples, each representing 10 feral chickens. 45 paper bedding samples composed




Google (2023) *Cayman Islands*. Available at: https://www.google.com/maps. *(Accessed: 15 September 2023).*

FIGURE 1
Number of samples of each sample type from the five districts of Grand Cayman.

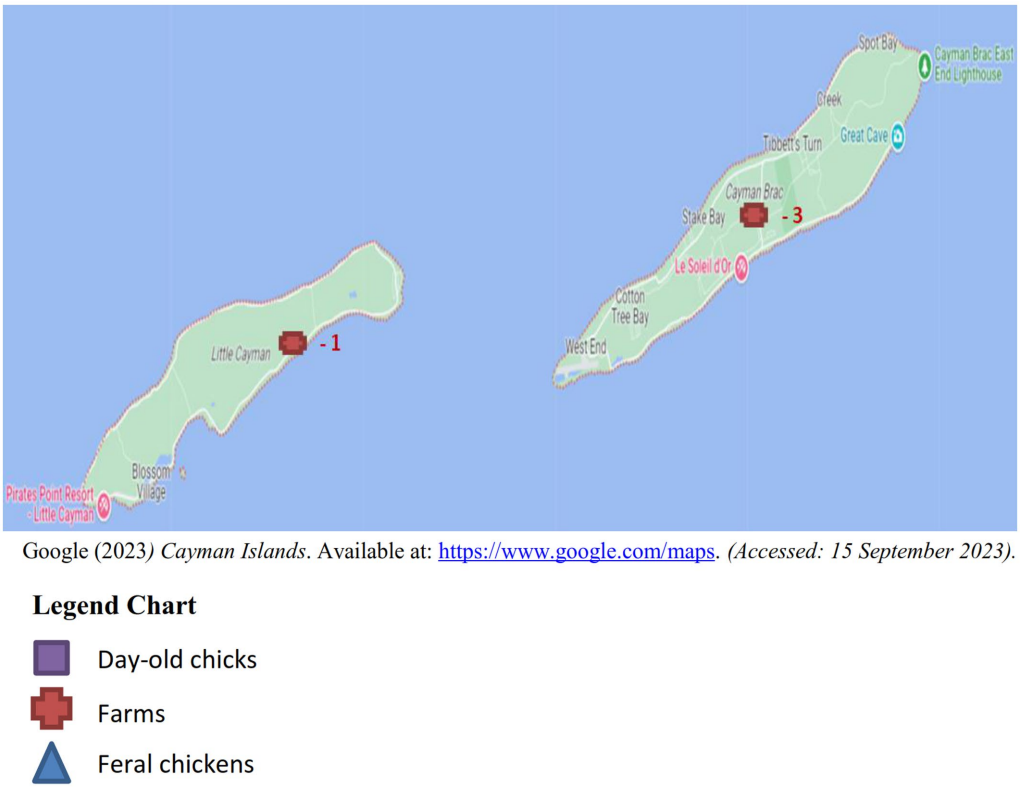
from 6 different shipments in 2018 were taken from the boxes in which the day-old chickens were imported.

*Salmonella* was isolated using ISO Standard 6,579 annex D (42). Briefly, a 1/10 (W/V) suspension of 25 g paper bedding, cecum content, or boot swabs were mixed with 225 mL Buffered Peptone Water (BD Life Sciences, Fanklin Lake, NJ, United States). After stomaching, they were aseptically incubated at 37 ± 1°C for 18 ± 2 h, then 0.1 mL was added to 10 mL Rappaport Vassiladis R10 (RV) broth (BD Life Sciences, Fanklin Lake, NJ, United States) and incubated at 41.5°C for 24 ± 4 h. One ml of resuscitation broth was added to 10 mL Tetrathionate broth (TB) base (Fisher Scientific, Waltham, MA, United States) and incubated at 37°C for 24 ± 4 h. Thereafter, 10 µL of enrichment broth was streaked on Xylose Lysine Deoxycholate (Fisher Scientific™, Waltham, MA, United States) and Brilliant Green Agar (Fisher Scientific™, Waltham, MA, USA) and incubated at 37 ± 1°C for 24 ± 4 h. Presumptive *Salmonella* colonies were inoculated on Blood Agar (Fisher Scientific™, Waltham, MA, United States) at 35 ± 2°C for 18 to 72 h. Suspected colonies were identified using API 20E test strips (bioMérieux, Marcy l'Étoile, France), which were incubated at 36 ± 2°C for 18–24 h and the *Salmonella* Express Plate (3M™ Petrifilm™, Maplewood, Minnesota, United States), incubated at 41.5°C for 24 ± 4 h. Lastly, *Salmonella* agglutination tests were conducted to determine serogroup using the Wellcolex Color Salmonella Rapid Latex Agglutination Test Kit (Thermo Fisher Scientific, Waltham, MA, USA). Serogrouped strains were selected for WGS.

## 2.2 Whole genome sequencing and sequence analysis

Purified strains were sent to Macrogen (Seoul, South Korea) for DNA extraction and sequencing on an Illumina platform using a TruSeq Babi DNA kit, 151 bp long paired end sequencing. Sequences were submitted to NCBI under bio-project PRJNA765319 with accession numbers: SAMN21553720 and SAMN22875821 - SAMN22875840. Quality control has been performed on the raw data using Phred and on the assembled data using Quast.

Illumina sequences were trimmed and assembled using SKESA (43) and annotated with PROKKA (44). *Salmonella* serotypes were identified using Seroseq (45) and SISTR (46). The Multi-Locus Sequence Typing (MLST) profile was determined using 'mlst 2.0' (47). Phylogeny was determined with strains from around the world using The Phylogenetic Tree Building Service on the PATRIC server (currently Bacterial and Viral Bioinformatics Resource Center) (48) *last accessed November 10th, 2022* and base differences between strains were quantified by CSIPhylogeny (49). The accessory genome was analysed using Resfinder (50) to identify antimicrobial resistance genes and associated antibiotic resistance of the strains. Multi-drug resistance (MDR) was defined as strains showing resistance to three or more different classes of antimicrobials (51). Plasmids were identified with PlasmidFinder 4.0 (52). PHASTER (53, 54) was used to identify prophage proteins present in bacterial sequences. Virulence genes were determined by Virulence Factor Database (VFDB), (55) and SPIFinder (56).




Google (2023) *Cayman Islands*. Available at: https://www.google.com/maps. *(Accessed: 15 September 2023).*

FIGURE 2
Number of samples of each sample type from Cayman Brac and Little Cayman.

TABLE 1 Number of samples collected, number positive for *Salmonella* and corresponding prevalence with confidence intervals.

| Origin | N* of samples | N* of positives | Prevalence | Confidence intervals |
|---|---|---|---|---|
| Broiler | 31 | 1 | 3.2% | 3.1–3.3% |
| Layer | 31 | 3 | 9.7% | 9.2–10.2% |
| Day-old chicks | 45 | 8 | 17.8% | 16.8–18.8% |
| Feral | 49 | 6 | 12.2% | 11.7–12.8% |
| Total | 156 | 18 | 11.5% | 11.2–11.8% |

*N-Number.

## 2.3 Statistical analysis

Confidence intervals to measure the prevalence of *Salmonella* in poultry, in the Cayman Islands were calculated using exact binomials in an Excel file. Differences between groups were determined using the chi square test.

# 3 Results

## 3.1 Prevalence of salmonella in poultry in the Cayman Islands

Out of 156 samples, 18 were found to be positive for *Salmonella*, resulting in an overall prevalence rate of 11.5% (confidence interval 11.2–11.8%) (Table 1). Each positive originating from a farm source, broilers or layers, were isolated from separate farms, resulting in four positives from four individual farms. With four farms positive for *Salmonella* of the 27 total farms sampled, the prevalence rate is 14.8% (confidence interval 13.8–15.8%). Six of the 49 pooled cecal samples of feral chickens were positive and eight of the 45 paper bedding samples of imported day-old chicks were positive. Broiler farms showed the lowest prevalence of 3.2% (confidence interval 3.1–3.3%) whereas imported day-old chicks showed the highest prevalence of 17.8% (confidence interval 16.8–18.8%).

## 3.2 Serotyping and MLST

We identified seven different *Salmonella* serovars, with *S.* Kentucky being the most frequently isolated. *Salmonella* found in paper bedding/transport material from day-old chicks was exclusively

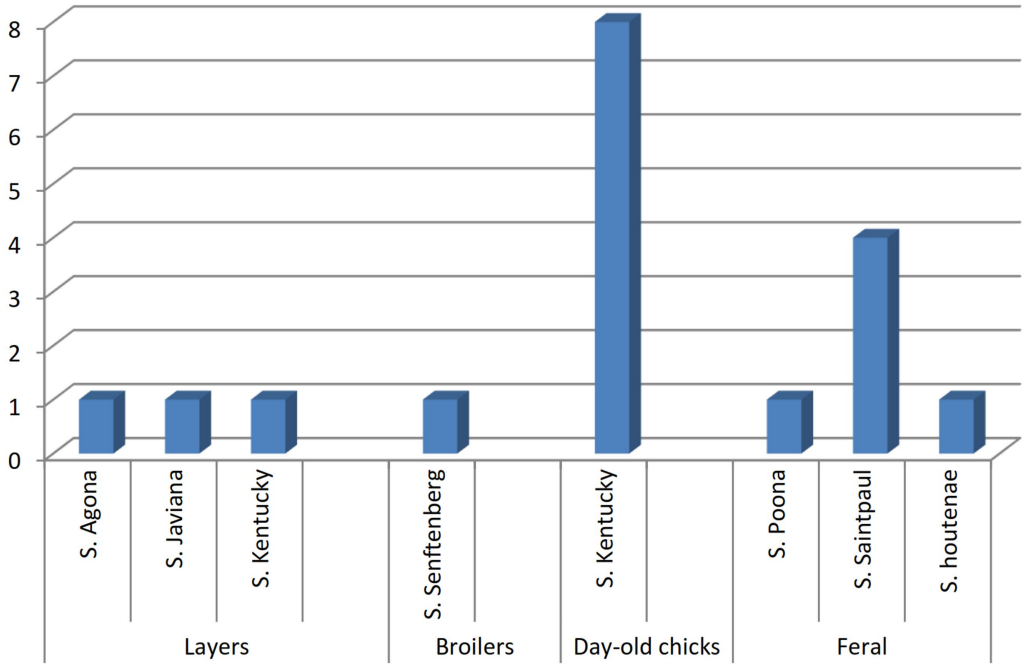



FIGURE 3
Distribution of *Salmonella* serotypes isolated from imported, farmed, and feral poultry, represented by sample origin.

*S.* Kentucky (Figure 3). Additionally, a *S.* Kentucky strain was isolated from a layer farm but none was found in feral poultry, where *S.* Saintpaul was the predominant serotype (Figure 4). Of the 9 *S.* Kentucky strains, eight were sequence type ST152 and one strain was ST2132, which is one allele (*purE*) different from ST152. *S.* Saintpaul strains ($n = 4$) were all sequence type ST50. The remaining strains ($n = 5$) were different *Salmonella* serotypes (Table 2).

## 3.3 Phylogenetic analysis

The single nucleotide polymorphism (SNP) analysis of the *S.* Kentucky strains, taken from imported day-old chicks and the layer farm, showed a variation ranging between 0 and 112 SNPs. When compared to ST152 *S.* Kentucky strains from around the world, the strains clustered closely with strains from North America (Figure 4). The S. Saintpaul strains showed less variation with 16–22 SNP differences, however, they formed a separate cluster from other ST50 strains from around the world (Figure 5).

## 3.4 Antimicrobial resistance genes

All 18 strains contained at least the *aac(6′)-Iaa* gene known to be present in the core genome of all *Salmonella* strains, giving resistance to amikacin and tobramycin (Table 3). Seven strains did not contain any other resistance genes. Only the serotypes *S.* Kentucky and *S.* Senftenberg contained more than one aminoglycoside resistance gene. Eleven strains showed additional resistance genes apart from those for aminoglycosides. All but one of these 11 strains were resistant to tetracyclines, mediated by the *tet*(B) gene and in one by the *tet*(A) gene. One strain was found to carry the *sul1* gene, encoding resistance to sulfonamide antibiotics, another was found to have chloramphenicol resistance gene, *catA3* and another strain to have fosfomycin resistance via *fosA7_1* gene. Lastly, two genes encoding resistance to β-lactam antibiotics were present: $bla_{\text{TEM-1B}}$ in the single *S.* Senftenberg and $bla_{\text{CMY-2}}$ in 4 *S.* Kentucky strains from one-day old chicks. Four *S.* Kentucky strains and one *S.* Senftenberg strain were multi-drug resistant.

## 3.5 Plasmids

Ten out of eighteen *Salmonella* strains contained at least one plasmid replicon (Table 3). A total of 6 different plasmids replicon sequences were found. Two colicin plasmids, ColpVC and Col156 and four different Inc. plasmids [IncFIB (AP001918), IncFII, IncX1 and Incl1-l (Alpha)] (Table 3). All eight Kentucky strains contained three Inc. plasmids (IncFIB, IncFII, and IncX1), three strains contained an additional plasmid (ColpVc) and three separate strains contained an additional Inc. plasmid (IncI1-I). The Col156 plasmid was present in the *S.* Senftenberg strain.

## 3.6 Prophages

All strains contained prophages, however many of those were incomplete prophages. In six strains we could not detect any intact prophage region. The remaining 12 strains had at least one complete prophage. Three had an additional prophage region (Table 4). Three of the 4 *S.* Kentucky strains contain the Escher_Lys12581Vzw prophage. The other *S.* Kentucky strain contains the prophage Escher_ArgO145. All the *S.* Saintpaul strains and the Javiana strain had the

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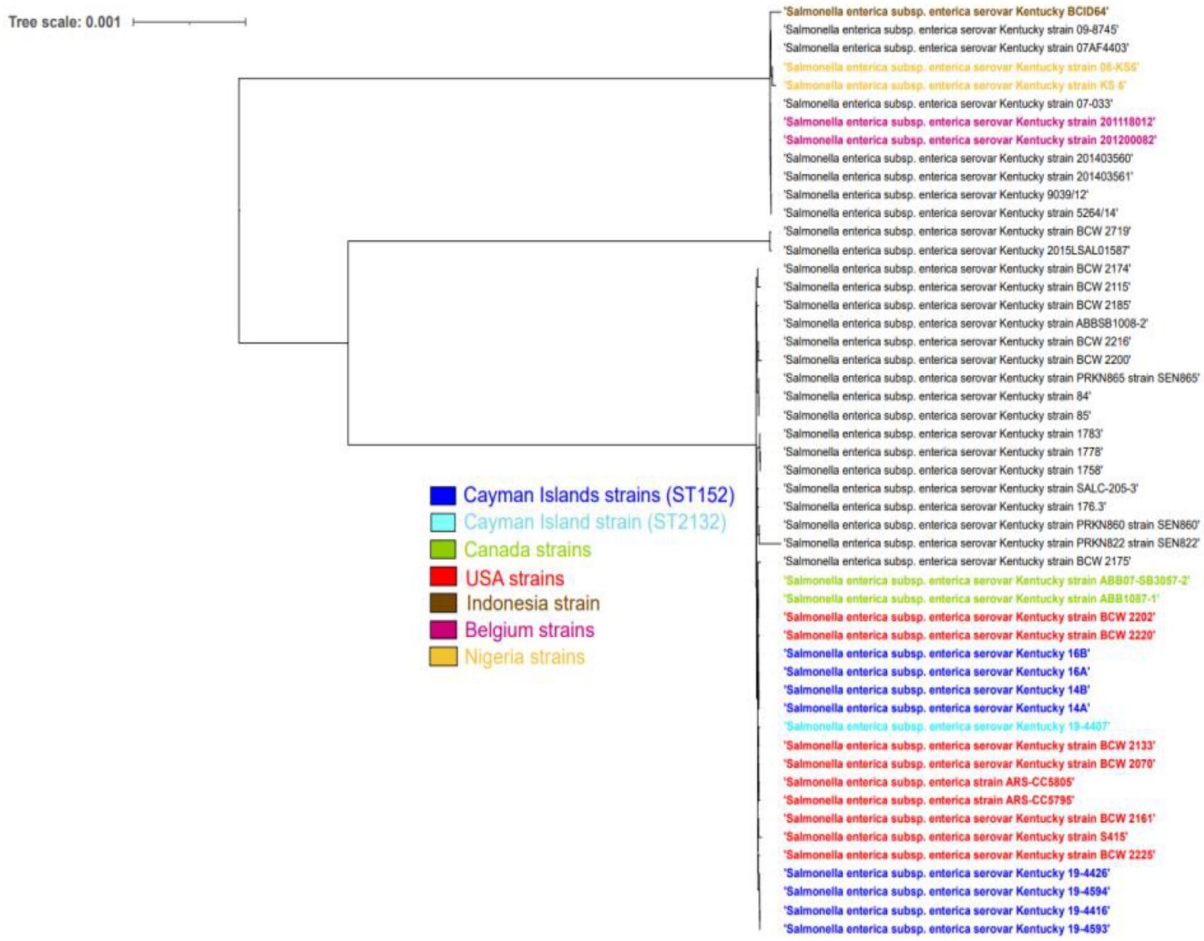


FIGURE 4
Phylogenetic tree analysis of Cayman Islands ST152 (dark blue) and ST2132 (light blue) strains and other worldwide ST152 strains (black) of *S.* Kentucky.

Gifsy-2 prophage. The *S.* Agona and *S.* Senftenberg strain contained the Salmon_SEN8 prophage.

## 3.7 Virulence gene content

All strains contained the highly conserved *Salmonella* Pathogencity Islands (SPI's) 1–3, with the exception of subspecies *houtenae* strain, which contained only SP1 (Table 5). The subspecies *houtenae* strain was the only strain to contain virulence genes *SpiC* and *SpvB* (Tables 6, 7). All strains with the exception of the 4 *S.* Saintpaul strains, contained Pathogenicity Island C63PI, an iron uptake system. All strains also contained the magnesium uptake system. Fimbrial adhesion genes varied slightly among serotypes. Only the *S.* Javiana strain contained six adhesion genes, whereas subspecies *houtenae* strain contained only two of those adhesion genes.

# 4 Discussion

This study is the first in the Cayman Islands, and only the second one in the Caribbean, to utilize Whole Genome Sequencing to determine the epidemiology of *Salmonella* from poultry sources (57). We could isolate *Salmonella* from broilers, layers, imported 1 day old chicks and feral chickens. While the prevalence differed between sample types, overall these differences in confidence intervals were statically insignificant when computed with chi square test. The different serotypes identified in each sample type were isolated with the exception of *S.* Kentucky present in two sample types and the largest diversity in serotypes was seen in samples from layer farms. Samples from bedding/transport material contained only *S.* Kentucky. In free roaming chickens, the dominant serotype was *S.* Saintpaul. This indicates that there is little exchange of strains between the sampled groups. *S.* Kentucky is commonly associated with poultry in North America (11, 18) and the ST152 is dominant sequence type (58, 59). *S.* Saintpaul strains were all ST50, an uncommon strain found in poultry, although it is frequently associated with outbreaks of foodborne infections in the United States (60–62). Our strains however formed a separate group of strains within the ST50, indicating a different epidemiology.

Interestingly, we identified a single *S. houtenae* strain among our feral poultry. This subspecies is commonly associated with cold-blooded animals, but it has also been found in few other animal species (63) and humans (64). It is likely that the strain originated

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TABLE 2 Characteristics and sample type from which the *Salmonella* strains were isolated.

| Sample ID | District | Origin | Serotype | MLST 2.0 |
|---|---|---|---|---|
| 14A | Bodden Town | Imported Day Old Chicks | Kentucky | ST 152 |
| 14B | Bodden Town | Imported Day Old Chicks | Kentucky | ST 152 |
| 16A | Bodden Town | Imported Day Old Chicks | Kentucky | ST 152 |
| 16B | Bodden Town | Imported Day Old Chicks | Kentucky | ST 152 |
| 19–4,407 | Bodden Town | Imported Day Old Chicks | Kentucky | ST 2132 |
| 19–4,416 | Bodden Town | Imported Day Old Chicks | Kentucky | ST 152 |
| 19–4,426 | Bodden Town | Imported Day Old Chicks | Kentucky | ST 152 |
| 19–4,593 | Bodden Town | Imported Day Old Chicks | Kentucky | ST 152 |
| 19–4,392 | Bodden Town | Broiler | Senftenberg | ST 14 |
| S25 | Bodden Town | Layer | Agona | ST 13 |
| 19–4,409 | North Side | Layer | Javiana | ST 371 |
| 19–4,594 | Bodden Town | Layer | Kentucky | ST 152 |
| 19–4,384 | Bodden Town | Feral | Saintpaul | ST 50 |
| 19–4,397 | George Town | Feral | Saintpaul | ST 50 |
| 19–4,417 | West Bay | Feral | Poona | ST 447 |
| 19–4,411 | East End | Feral | Saintpaul | ST 50 |
| 19–4,412 | George Town | Feral | Saintpaul | ST 50 |
| 19–4,422 | George Town | Feral | Subspecies *houtenae* | ST 162 |

TABLE 3 AMR genes and plasmid replicons detected in the *Salmonella* strains.

| Sample # | Serotype | Antimicrobial resistance genes | | | | | Plasmids |
|---|---|---|---|---|---|---|---|
| | | Amino-glyocide | Tetra-cycline | Sulfon-amide | Beta-Lactam | Fosfo-mycin | Plasmid replicons |
| 14A | Kentucky | *aph(6)-Id, aph(3″)-Ib, aac(6′)-Iaa* | *tet*(B) | | | | ColpVC, IncFIB, IncFII, IncX1 |
| 14B | Kentucky | *aph(6)-Id, aph(3″)-Ib, aac(6′)-Iaa* | *tet*(B) | | | | ColpVC, IncFIB, IncFII, IncX1 |
| 16A | Kentucky | *aph(6)-Id, aph(3″)-Ib, aac(6′)-Iaa* | *tet*(B) | | | | ColpVC, IncFIB, IncFII, IncX1 |
| 16B | Kentucky | *aph(6)-Id, aph(3″)-Ib, aac(6′)-Iaa* | *tet*(B) | | | | IncFIB, IncFII, IncX1 |
| 19–4,407 | Kentucky | *aph(6)-Id, aph(3″)-Ib, aac(6′)-Iaa* | *tet*(B) | | | | IncFIB, IncFII, IncX1 |
| 19–4,416 | Kentucky | *aph(6)-Id, aph(3″)-Ib, aac(6′)-Iaa* | *tet*(B) | | *blaCMY-2* | | IncFIB, IncFII, IncI1-I(Alpha), IncX1 |
| 19–4,426 | Kentucky | *aph(6)-Id, aph(3″)-Ib, aac(6′)-Iaa* | *tet*(B) | | *blaCMY-2* | | IncFIB, IncFII, IncI1-I(Alpha), IncX1 |
| 19–4,593 | Kentucky | *aph(6)-Id, aph(3″)-Ib, aac(6′)-Iaa* | *tet*(B) | | *blaCMY-2* | | IncFIB, IncFII, IncI1-I(Alpha), IncX1 |
| 19–4,594 | Kentucky | *aph(6)-Id, aph(3″)-Ib, aac(6′)-Iaa* | *tet*(B) | | *blaCMY-2* | | IncFIB, IncFII, IncI1-I(Alpha), IncX1 |
| 19–4,384 | Saintpaul | *aac(6′)-Iaa* | | | | | |
| 19–4,392 | Senftenberg | *aph(6)-Id, aph(3″)-Ib, aac(6′)-Iaa, aph(3′)-Ia, ant(3″)-Ia, ant(2″)-Ia, aadA2* | *tet*(A) | *sul1* | *blaTEM-1B* | | Col156 |
| 19–4,397 | Saintpaul | *aac(6′)-Iaa* | | | | | |
| 19–4,411 | Saintpaul | *aac(6′)-Iaa* | | | | | |
| 19–4,412 | Saintpaul | *aac(6′)-Iaa* | | | | | |
| 19–4,409 | Javiana | *aac(6′)-Iaa* | | | | | |
| 19–4,417 | Poona | *aac(6′)-Iaa* | | | | | |

*(Continued)*

TABLE 3 (Continued)

| Sample # | Serotype | Antimicrobial resistance genes | | | | | Plasmids |
|---|---|---|---|---|---|---|---|
| | | Amino-glyocide | Tetra-cycline | Sulfon-amide | Beta-Lactam | Fosfo-mycin | Plasmid replicons |
| 19–4,422 | subspecies *houtenae* | *aac(6′)-Iaa* | | | | | |
| S25 | Agona | *aac(6′)-Iaa_1* | | | | *fosA7_1_* | |

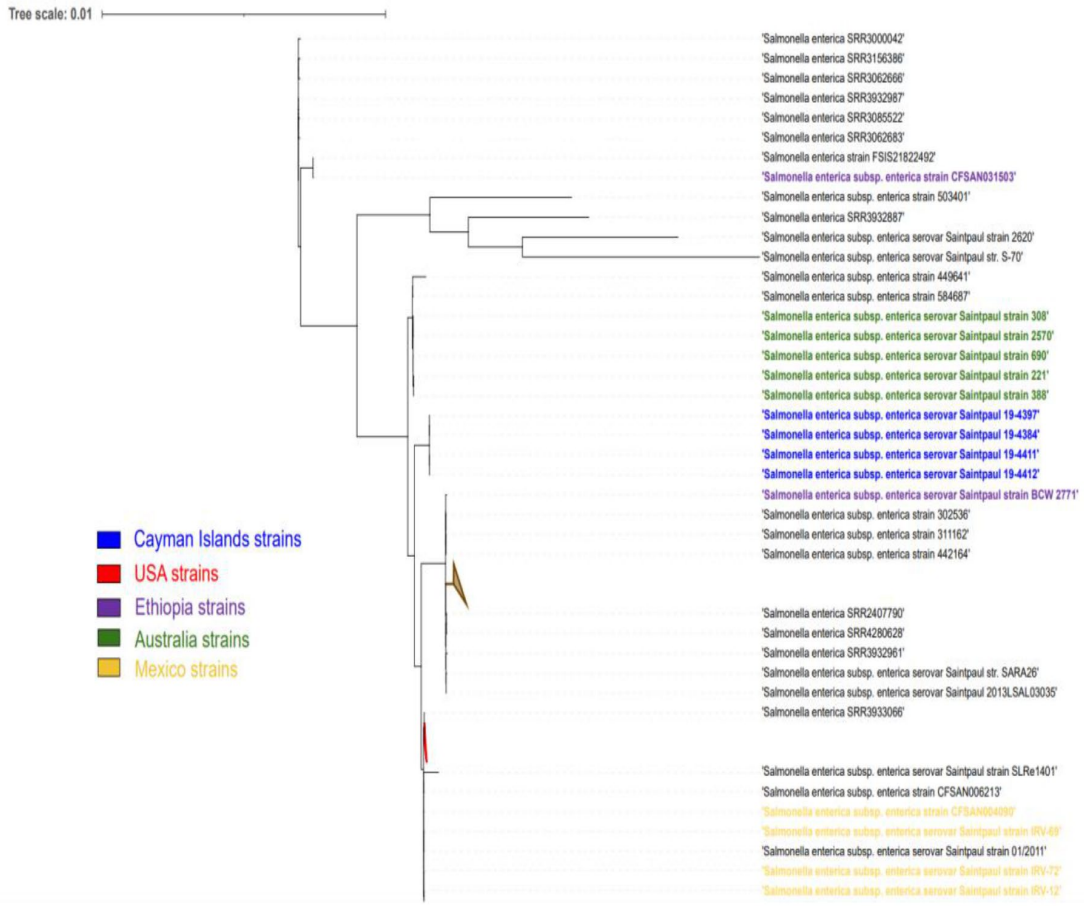


FIGURE 5
Phylogenetic tree analysis of Cayman Islands ST50 strains (blue), and other worldwide ST50 strains (black) of *S.* Saintpaul.

from cold blooded animals, which are common on the Cayman Islands. This is further substantiated by the fact that the other serotypes identified in feral chickens had previously been discovered in the endemic blue iguanas of Grand Cayman (23). Feral chickens thus likely share their *Salmonella* with cold blooded animals on the Cayman Islands.

The most frequently identified serotypes from human salmonellosis in the Caribbean were *S. typhimurium* and *S.* Enteriditis, similar to other parts of the world (65). However, in our samples, we did not detect these serotypes, indicating a low risk of Cayman reared poultry for human health. The isolated *S.* Kentucky strains were clonally related to the North American strains, which is not surprising as U.S hatcheries replenish poultry flocks in the Cayman Islands, under the Cayman Islands Department of Agriculture's (DOA) permission and regulation. This practice is not unique to the Cayman Islands, as it has been reported that approximately 91% of 27 Caribbean nations, import egg or egg products from a foreign source (66) and recent findings from Trinidad and Tobago also conclude close clustering of *S.* Kentucky strains to strains from a U.S hatchery (67).

All strains had at least the *aac(6′)-Iaa* gene, which is endogenous to the *Salmonella* genus (68). Five strains were MDR. Of those five strains, four were *S.* Kentucky strains and one from the single *S.* Senftenberg strain. These strains contained at least one resistance

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TABLE 4 Number of prophage regions and complete prophages detected in the *Salmonella* strains.

| Sample ID | Serotype | Prophage completeness quality | | | Complete prophage identified | GC content (%) |
|---|---|---|---|---|---|---|
| | | # of Questionable prophage regions | # of Incomplete prophage regions | # of Intact prophage regions | | |
| 14A | Kentucky | 3 | 5 | 1 | Escher_ArgO145_ NC_049918 | 49.19 |
| 14B | Kentucky | 3 | 5 | 1 | Escher_Lys12581Vzw_ NC_049917 | 49.19 |
| 16A | Kentucky | 3 | 5 | 1 | Escher_Lys12581Vzw_ NC_049917 | 49.19 |
| 16B | Kentucky | 3 | 5 | 1 | Escher_Lys12581Vzw_ NC_049917 | 49.19 |
| 19–4,407 | Kentucky | 2 | 5 | 0 | | |
| 19–4,416 | Kentucky | 2 | 4 | 0 | | |
| 19–4,426 | Kentucky | 1 | 6 | 0 | | |
| 19–4,593 | Kentucky | 2 | 5 | 0 | | |
| 19–4,594 | Kentucky | 1 | 6 | 0 | | |
| 19–4,384 | Saintpaul | 2 | 4 | 1 | Gifsy_2_NC_010393 | 51.07 |
| 19–4,397 | Saintpaul | 2 | 5 | 1 | Gifsy_2_NC_010393 | 51.11 |
| 19–4,411 | Saintpaul | 2 | 5 | 1 | Gifsy_2_NC_010393 | 51.11 |
| 19–4,412 | Saintpaul | 2 | 5 | 1 | Gifsy_2_NC_010393 | 51.11 |
| 19–4,392 | Senftenberg | 3 | 1 | 2 | (1) Salmon_SEN8_ NC_047753 (2) Salmon_SPN3UB_ NC_019545 | (1) 49.03 (2) 49.97 |
| 19–4,409 | Javiana | 3 | 2 | 2 | (1) Gifsy_2_ NC_010393 (2) Salmon_SP_004_ NC_021774 | (1) 51.71 (2) 52.23 |
| 19–4,417 | Poona | 1 | 3 | 0 | | |
| 19–4,422 | IV 6,7z4,z24 | 5 | 2 | 2 | (1) Salmon_118970_ sal3_NC_031940 (2) Salmon_SP_004_ NC_021774 | (1) 50.24 (2) 53.13 |
| S25 | Agona | 0 | 6 | 1 | Salmon_SEN8_ NC_047753 | 49.92 |

gene against aminoglycosides, tetracyclines and beta-lactams. The *S.* Senftenberg strain contained an additional resistance gene to sulfonamides. These findings are common in *Salmonell*a (69–71). The predominance of MDR was mainly observed in the day-old chicks imported from North America, a region where MDR is commonly identified in *S.* Kentucky strains (11, 72). More concerning is that in those strains, beta-lactam resistance was mediated by the $bla_{\text{CMY-2}}$, a plasmid mediated AmpC beta-lactamase which is of clinical importance as they infer resistance to third-generation cephalosporins (73). Plasmids on which this gene has been found are IncA/C and IncI1 plasmids (74). Although we could not locate the gene on a plasmid in our sequences, we suppose it was present on the IncI1 plasmid in our strains, as this plasmid was specifically present in all strains carrying $bla_{\text{CMY-2}}$.

Striking is also that *Salmonella* from feral chickens had no other AMR other than the *aac(6′)-Iaa* gene, which is present in all *Salmonella* (68), indicating there is no selection for resistance in this population, nor transfer of resistance from other sources. Fosfomycin resistance is rare and was first identified in Canada in *S.* Heidelberg from broilers (75) and is limited to a few other serotypes: *S.* Agona, *S.* Montevideo and *S.* Tennessee (75). This aligns with our findings, as the *fosA7* gene was only present in the single *S.* Agona strain. The *fosA7* gene has been identified outside of Canada, namely the United States (76), China (77), Nigeria (78) and Brazil (79). However, this is the first known reporting of Fosfomycin resistance in *Salmonella* from Caribbean poultry.

Apart from a single *S.* Senftenberg strain, plasmid replicons were only present in the *S.* Kentucky. The plasmids identified in our

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TABLE 5 *Salmonella* Pathogenicity Islands (SPI's) present among the *Salmonella* serotypes isolated.

| | Salmonella Pathogenicity Islands (SPI's) present | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Serotype (n) | 1 | 2 | 3 | 4 | 5 | 8 | 9 | 13 | 14 | CS54 | C63PI | *PPI (ssaD) |
| Kentucky ($n = 9$) | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 0 | 0 | 0 | 9 | 9 |
| Saintpaul ($n = 4$) | 4 | 4 | 4 | 4 | 4 | 0 | 4 | 4 | 4 | 4 | 0 | 4 |
| Javiana ($n = 1$) | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| Senftenberg ($n = 1$) | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 |
| Poona ($n = 1$) | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 |
| Agona ($n = 1$) | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 |
| subspecies *houtenae* ($n = 1$) | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| Total count | 18 | 17 | 17 | 16 | 18 | 11 | 18 | 6 | 7 | 4 | 14 | 17 |

*PPI(ssaD), Putative Pathogenicity Island protein (ssaD) gene.

TABLE 6 Virulence genes associated with host invasion and intercellular survival via *Salmonella* type three secretion systems (T3SS's), among the *Salmonella* serotypes.

| | SPI Island 1-T3SS1 | | | | | | SPI Island 2- T3SS2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Serotypes (n) | *sopA* | *sopB* | *sipA* | *SopE* | *SopE2* | *avrA* | *sifA* | *SpiC* | *SseI* | *SseF* | *PipB* |
| Kentucky ($n = 9$) | 9 | 9 | 9 | 0 | 7 | 9 | 9 | 0 | 0 | 9 | 9 |
| Saintpaul ($n = 4$) | 4 | 4 | 4 | 3 | 4 | 4 | 4 | 0 | 0 | 4 | 4 |
| Javiana ($n = 1$) | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| Senftenberg ($n = 1$) | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| Poona ($n = 1$) | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| Agona ($n = 1$) | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 |
| subspecies *houtenae* ($n = 1$) | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 |
| Total count | 17 | 18 | 18 | 4 | 16 | 17 | 18 | 1 | 0 | 18 | 17 |

TABLE 7 Non-T3SS's encoded virulence genes among the *Salmonella* serotypes.

| | Adhesion Genes | | | | | | Intracellular survival mechanisms | | |
|---|---|---|---|---|---|---|---|---|---|
| | Plasmid-encoded | | Fimbrial adherence | | Non-fimbrial | | Toxin | Serum-resistance | Stress survival |
| Serotypes (*n*) | *pefA* | *pefB* | *fimA* | *csgA* | *ShdA* | *ratB* | *SpvB* | *rck* | *SodCI* |
| Kentucky ($n = 9$) | 0 | 4 | 9 | 9 | 9 | 0 | 0 | 0 | 0 |
| Saintpaul ($n = 4$) | 0 | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 4 |
| Javiana ($n = 1$) | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| Senftenberg ($n = 1$) | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Poona ($n = 1$) | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| Agona ($n = 1$) | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| subspecies *houtenae* ($n = 1$) | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| Total count | 1 | 10 | 18 | 18 | 17 | 6 | 1 | 0 | 5 |

*S.* Kentucky strains are commonly found in North America and in general carry AMR genes (59, 72, 80, 81), which represents a risk for introducing plasmid carrying resistance genes. This indicates that few plasmids are present in *Salmonella* from feral chickens on the Cayman Islands. This explains also the lack of acquired resistance in these strains as many resistance genes are located on plasmids.

We found that our distribution of serotypes mostly differed from other parts of the world such as China (82, 83), but had similar AMR and virulence genes, particularly with our single *S.* Senftenberg strain (82) and our dominant serotype *S.* Kentucky strains (82, 83). Both serotypes exhibited MDR and AMR genes to beta-lactam antibiotics similarly to ours, but differed in the origin of those AMR genes; with the absence of harboring plasmid replicons in poultry found in China.

However, in these comparisons with our *S.* Kentucky ST152 strains isolated from the Cayman Islands, they differ in their sequence type to the *S.* Kentucky ST198 strains isolated from China. While they may be phenotypically similar in identified AMR and virulence genes, these two sequence types been demonstrated to form genetically distinct lineages (59, 84); ST198 is an international strain and ST152 is one primarily found in North America (84).

The majority of the strains contained at least one intact/functional prophage. Most of the prophage sequences were however not complete. This might be due to the sequencing and assembly. Typically, *S. enterica* subspecies *enterica* serovars contain on average $5 \pm 3$ prophage regions per genome (85). Gifsy-2 is a common prophage among *Salmonella* serotypes (85) and was represented in all the *S.* Saintpaul and *S.* Javiana strains. Gifsy prophages have been identified to encode virulence gene *SopE* (86), which allows bacterial entry into epithelial cells (87) thus making them more virulent. This virulence gene was present in 3 of the 4 *S.* Saintpaul strains. The other phages, apart from Phage Sen8, found in *S.* Senftenberg, do not contain any known *Salmonella* virulence genes (88).Virulence genes, *Mig-5* and *rck*, have been reported to be typically encoded on IncF plasmids; particularly IncFII (present in our *S.* Kentucky strains), but these genes were absent in our strains. Other major plasmid-encoded virulence genes also identified in IncFII plasmids such as: spv operon (*spvABCDR*) and pef operon (*pefABCD*) (80, 81), were absent in our strains. Only virulence gene, *pefB*, was present in four of the *S.* Kentucky strains.

All strains contained SPIs although they were composed differently. The SPIs and other virulence genes identified do not contribute greatly to the virulence of the strains in relation to infecting humans, as none of the strains are major pathogens for humans.

Due to the low incidence of human salmonellosis on the Cayman Islands, we are limited in assessing the public health risk of our findings. Only 24 cases were reported during 2010–2020. The two most frequently reported NTS serotypes from humans in the Cayman Islands were *S.* Saintpaul (14 cases) and *S.* Poona (5 cases) according to the Health Services Authority[1]. These serotypes were also present in the feral chickens, indicating that these may be a source of human infection together with iguanas. *S.* Saintpaul ST50 has been identified more commonly in clinical infections in humans. There were 2,616 *S.* Saintpaul ST50 strains in Enterobase (89) (*accessed June 25th, 2023*), 1,008 from human infections, whereas 176 strains were found in poultry, indicating a potential risk of those strains for human health.

The *S.* Kentucky found in our study is also reported in the United States, although infections with this serotype and the specific ST152 strains are rather rare (90). Probably this serotype does not pose a large public health burden. However, the AMR found in these strains is rather worrisome and are we limited in determining if these AMR genes and plasmids are actively transferred to the local poultry. This could be remedied with a longitudinal study of the life cycle of imported day-old chicks to adulthood, to determine if AMR dissemination occurs. Additionally, we could identify if the serotypes present in the local poultry may also be imported. This research showcases the need for strengthening local policies in food safety and collaborative efforts with all necessary stakeholders to prevent potential foodborne pathogens entering the food supply chain and the burden of illness to public health as envisioned by the One Health concept.

## 5 Conclusion

In conclusion, our study demonstrates that *Salmonella* in broilers and layers on the Cayman Islands are often introduced via the importation of day-old chicks, although other strains also contribute to the prevalence. The presence of *Salmonella* in feral chickens poses a potential risk, given that their serotypes are the ones most commonly isolated from humans in the Cayman Islands. However, due to the strains' low virulence and the absence of antimicrobial resistance (AMR) and plasmids, the overall health risk may be relatively small. Resistance against antibiotics including $bla_{\text{CMY-2}}$ gene, is of public health concern. This may warrant stricter import control measures via One Health ideology and also highlights the importance of *Salmonella* surveillance of imported 1 day old chicks and farmed poultry.

## Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/bioproject/; PRJNA765319.

## Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because the study involved fecal droppings from commercial animals and the samples from feral chickens were obtained from culled animals. These animals were culled by the Cayman Island Government instances (Ministry of Health) and we had no other relation to that apart from sampling from the already culled animals.

1 https://www.hsa.ky/

## Author contributions

SW: Investigation, Writing – original draft. FT: Supervision, Writing – review & editing. HL: Supervision, Writing – review & editing. AJ: Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing. PB: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, 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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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# Multidrug resistance in pathogenic *Escherichia coli* isolates from urinary tract infections in dogs, Spain

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*Escherichia coli* (*E. coli*) is a pathogen frequently isolated in cases of urinary tract infections (UTIs) in both humans and dogs and evidence exists that dogs are reservoirs for human infections. In addition, *E. coli* is associated to increasing antimicrobial resistance rates. This study focuses on the analysis of antimicrobial resistance and the presence of selected virulence genes in *E. coli* isolates from a Spanish dog population suffering from UTI. This collection of isolates showed an extremely high level of phenotypic resistance to 1st–3rd generation cephalosporins, followed by penicillins, fluoroquinolones and amphenicols. Apart from that, 13.46% of them were considered extended-spectrum beta-lactamase producers. An alarmingly high percentage (71.15%) of multidrug resistant isolates were also detected. There was a good correlation between the antimicrobial resistance genes found and the phenotypic resistance expressed. Most of the isolates were classified as extraintestinal pathogenic *E. coli*, and two others harbored virulence factors related to diarrheagenic pathotypes. A significant relationship between low antibiotic resistance and high virulence factor carriage was found, but the mechanisms behind it are still poorly understood. The detection of high antimicrobial resistance rates to first-choice treatments highlights the need of constant antimicrobial resistance surveillance, as well as continuous revision of therapeutic guidelines for canine UTI to adapt them to changes in antimicrobial resistance patterns.

KEYWORDS

**dog, *Escherichia coli*, multidrug resistance, urinary tract infection, virulence factors**

## 1 Introduction

Urinary tract infections are one the most common causes of primary care veterinary supervision in dogs and a treatment challenge due to their high recurrence and therapeutic implications. *Escherichia coli* is the most common bacterium isolated in UTIs in dogs and humans (1–3). In addition, *E. coli* bacteremia in humans (the most common cause of bacteremia in high-income countries) is caused by urinary tract infections in more than 50% of cases (4). *Escherichia coli* has also been associated with an increase in antimicrobial

resistance (2, 5). Evidence suggests that dogs act as a reservoir of human infections with uropathogenic *E. coli* (UPEC) and are a source of spread of antimicrobial resistance (6).

*Escherichia coli* is classified into various pathotypes based on the presence of virulence factors. Uropathogenic *E. coli* is included within the group of extraintestinal pathogenic *E. coli* (ExPEC) and are characterized by specific virulence factor. Some of these virulence factors include P-fimbriae (*papC*), α-haemolysin (*hlyA*) and cytotoxic necrotizing factor type 1 (*cnf1*) (7). *Eae*, the gene that codifies for intimin and is associated with diarrheic strains, can also be found in uropathogenic strains (8). Other relevant *E. coli* virulent factors include Shiga toxins (*Stx*), also known as verotoxins and characteristic of Shiga toxin-producing *E. coli* (STEC), which are related to hemorrhagic diarrhea and hemolytic uremic syndrome, and have been described in several species, although natural infections are rarely described in dogs (9, 10). There is a potential zoonotic risk associated with the presence of these genetic elements in companion animals and other species (11, 12). Hybrid strains have gained recent attention, especially those that harbor several virulence factors traditionally associated with different pathotypes. These new types of strains are considered "heteropathogen" or hybrid, such as STEC/UPEC strains (2), and are considered as able to produce both outcomes, diarrhea, or UTI (13).

Virulence genes are encoded by plasmids, bacteriophages, or pathogenicity islands (PAI). Pathogenicity islands are mobile and unstable fragments of DNA present in pathogenic strains, but absent in the related non-pathogenic strains, which can be shared by horizontal transmission. *PapC*, *hlyA* and *cnf1*, among other virulence genes, are usually encoded simultaneously within PAIs in UPEC (14, 15). P-fimbriae, encoded by *papC* gene, plays an important role in kidney adherence and the inflammatory response (16). α-Haemolysin is a toxin known to produce renal injuries, and even though the mechanism is still unclear, *cnf1* does not play a major role in the severity of the disease but it is usually associated with other virulence genes (15).

It has been previously demonstrated that some canine UPEC isolates are clonal with those isolated from humans, suggesting their zoonotic potential. It has also been proposed that dogs could act as a reservoir of this *E. coli* pathotype, hence the importance of the study of the potential implications of UTI in this animal species (17, 18).

In the last few decades there has been a rising concern about the increase in the number of *E. coli* isolates presenting a multidrug resistant (MDR) profile (19, 20). It has been described that the ownership of companion animals could be a risk factor in the spread of pathogenic *E. coli* strains between humans and pets, also favoring the dissemination of antimicrobial resistance in the community (21–23).

It is common to find antibiotic resistance in *E. coli* isolates from cases of UTI, which highlights the importance of monitoring the strain susceptibility to the antibiotic treatment, even if an experimental treatment has already been implemented. In fact, UPEC strains isolated from dogs have been described as MDR reservoirs in several countries (24, 25) and as carriers of extended-spectrum beta-lactamase (ESBL) genes (5). ESBL-producing *E. coli* have been previously found in cats and dogs, and human-dog co-carriage in the same household has also been demonstrated in fecal samples (26–28). In general, ESBL and the presence of other antibiotic resistance mechanisms can difficult the treatment of infectious diseases and, therefore, result in complicated chronic infections.

Although microbiological culture and susceptibility testing are recommended before any antimicrobial therapy is established, empiric treatment is frequently established and the most common recommendations to treat these infections in companion animals include amoxicillin (without clavulanic acid) and trimethoprim-sulfonamides as a first approach (29). Therefore, updated information about antimicrobial susceptibility patterns is highly needed.

The aim of this study was to determine the presence of *E. coli* in urine samples from a Spanish dog population presenting clinical signs of urinary tract infections and to characterize the isolates according to selected virulence factors and their antimicrobial resistance pattern, a research field scarcely investigated in Spain.

## 2 Materials and methods

### 2.1 Collection of *Escherichia coli* isolates

This study was conducted on a total of 52 *E. coli* isolates. This collection of isolates came from urine samples from dogs diagnosed with UTI. Samples were aseptically collected by cystocentesis as part of the daily activity of private veterinary practitioners in Zaragoza, Spain. The criteria followed to diagnose UTI were those used in everyday clinic, which include frequent urination, pain during urination, fever or vomiting, among others. The sampling period ranged from 2017 to 2019, and all urine samples were taken before any treatment was established. Mean age of the individuals was 8.97 years old (95% CI: 4.11–13.83%). In regard to the gender of these individuals, 21 of them were male and 31 were female.

Isolates were identified using VITEK® (bioMérieux, France) and those confirmed as *E. coli* were then stored at −20°C for further analysis.

### 2.2 Virulence gene detection

DNA was extracted by boiling 3–5 colonies from pure cultures and then conventional PCR for the detection of virulence-related genes was performed. These genes included *eae* (intimin), *Stx1* and *Stx2* (Shiga toxins 1 and 2), *papC* (P-fimbriae), *hlyA* (α-haemolysin) and *cnf1* (cytotoxic necrotizing factor type 1). Primers used in this study were those described in Table 1. PCR was performed in a BiometraTRIO 48 thermocycler (Analytik Jena, Germany), and PCR products were analyzed under UV light in 1.5% agarose gels stained with GelGreen® (Biotium, United States).

CECT 4783 strain was used as positive control for *eae*, *Stx1* and *Stx2* genes; C136b strain was the positive control for *hlyA* and *cnf1* genes, kindly provided by Dr. J. A. Orden, University Complutense of Madrid, Spain. A canine strain previously isolated by our research group (Pe8 strain, GenBank accession number MK034302) was used as positive control for *papC* gene.

*Escherichia coli* isolates were classified in pathotypes according to the presence of the virulence factor genes analyzed. Enterohemorrhagic *E. coli* (EHEC) are described as those *E. coli* strains that harbor both intimin and Shiga toxins (12). When only one of these virulence factors was present, isolates were classified as enteropathogenic *E. coli*

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TABLE 1 Primers used in conventional PCR performed in this study.

| Gene | Primer | Sequence (5′ → 3′) | Annealing temperature (°C) | Amplicon size (bp) | Reference |
|---|---|---|---|---|---|
| *eae* | eae-common-F | CCCGAATTCGGCACAAGCATAAGC | 55 | 881 | (30) |
| | eae-common-R | CCCGGATCCGTCTCGCCAGTATTCG | | | |
| *Stx1* | EC-vt1_2-F | CGTCTTTACTGATGATTGATAGTGGC | 58 | 637 | (31) |
| | EC-vt1_2-R | CGCGATGCATGATGATGAC | | | |
| *Stx2* | EC-vt2_2-F | TACCACTCTGCAACGTGTCG | 58 | 297 | (31) |
| | EC-vt2_2-R | CGATACTCCGGAAGCACATT | | | |
| *papC* | pap1 | GACGGCTGTACTGCAGGGTGTGGCG | 55 | 328 | (32) |
| | pap2 | ATATCCTTTCTGCAGGGATGCAATA | | | |
| *hlyA* | hlyA-F | AACAAGGATAAGCACTGTTCTGGCT | 55 | 1,177 | (33) |
| | hlyA-R | ACCATATAAGCGGTCATTCCCGTCA | | | |
| *cnf1* | cnf1-A | GAACTTATTAAGGATAGT | 54 | 543 | (32) |
| | cnf1-B | CATTATTTATAACGCTG | | | |

(EPEC) or STEC, respectively. If any of the other virulence factor genes analyzed were found, that is *hlyA, PapC* and/or *cnf1*, isolates were classified as extraintestinal pathogenic *E. coli* (ExPEC) (34).

## 2.3 Antimicrobial susceptibility testing

Susceptibility to 74 different antimicrobials was determined using VITEK® (bioMérieux, France). The antimicrobial agents selected to test each isolate susceptibility depended on VITEK® guidelines, as clinically relevant antimicrobials recommended by VITEK® varied during the period in which the study was performed. Antimicrobials were classified in 12 categories: aminoglycosides, amphenicols, carbapenems, fluoroquinolones, nitrofurans, other β-lactams, penicillins, tetracyclines, sulfonamides, 1st–2nd generation cephalosporins, 3rd generation cephalosporins and 4th–5th generation cephalosporins, as shown in Table 2. All isolates were tested against at least one antibiotic of each category, except for 4th and 5th generation cephalosporins, which were added in the middle of the study. For those isolates having no information regarding 4th–5th generation cephalosporins, neither susceptibility nor resistance was included, and they were thus excluded from the prevalence analysis for that group. Resistance to a category of antimicrobials was defined as resistance to at least one of the agents in that category. MDR isolates were defined as those isolates with non-susceptibility to three or more antimicrobial categories (35).

Additionally, VITEK 2 ESBL test (bioMérieux) was used in these isolates for rapid detection of extended-spectrum β-lactamase (ESBL) production, which is based on simultaneous assessment of the inhibitory effects of cefepime, cefotaxime, and ceftazidime, alone and in the presence of clavulanate.

Breakpoints for the interpretation of minimal inhibitory concentration (MIC) results were applied according to the criteria stablished by bioMérieux for small animals (AST-GN97, bioMérieux, France), which include natural resistance and breakpoints from the Clinical and Laboratory Standards Institute (36).

Intermediate resistance category provides a flexible information in clinical practice. However, *E. coli* isolates have been previously found to harbor resistance genes (37) For this reason, when they had to be categorized into dichotomic variants they were assessed as resistant.

## 2.4 Whole genome sequencing

Those isolates showing the highest rate of phenotypic resistance, that is resistance to six or more antimicrobial categories, were selected for further characterization through whole genome sequencing (WGS). A total of ten *E. coli* isolates were cultured for 24 h in Nutrient Agar (Oxoid, United Kingdom) and DNA was then extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, United States). Quality parameters for DNA were checked both on Qubit 4 (Invitrogen) and gel electrophoresis. Genome sequencing was performed on an Illumina Miseq platform with a paired-end read length of 150 bp. Sequences were trimmed on Galaxy (Version 0.3.8.1) and assembled with Unicycler (Galaxy version 0.5.0 + Galaxy 1). All sequencing data have been submitted to NCBI Genome Database under BioProject PRJNA1031085, and individual accession numbers are the following: SAMN37924970 (isolate 258.883), SAMN37926527 (isolate 262.947), SAMN37926528 (isolate 263.715), SAMN37926529 (isolate 266.493), SAMN37926530 (isolate 267.252), SAMN37926531 (isolate 269.901), SAMN37926532 (isolate 271.550), SAMN37926533 (isolate 271.758), SAMN37926534 (isolate 271.811) and SAMN37926535 (isolate 271.960).

Antibiotic resistance genes, virulence factors, serotypes and sequence types (ST) were assigned to these sequenced genomes using tools that included ResFinder 4.1 (38–40), PathogenFinder 1.1 (41), VirulenceFinder 2.0 (39, 42), MLST 2.0 (*E. coli* #1 and #2) (39, 43–48), cgMLSTFinder 1.2 (42, 49), MGE v1.0.3 (39, 50–52) and SeroTypeFinder 2.0 (53). Visualization of the genomic data was carried out using Proksee (54). A phylogenetic tree was created with Roary pipeline (55) based on Prokka annotation (56), and followed by use of IQ-TREE software (57).

## 2.5 Statistical analysis

Prevalence was calculated with 95% confidence intervals (CI). To test simple relationship between virulence factors and antibiotics,

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TABLE 2 Antimicrobials tested in *E. coli* isolates and category classification.

| Antimicrobial categories | Antimicrobials included in each category | | | |
|---|---|---|---|---|
| Aminoglycosides | Amikacin | Gentamicin | Neomycin | |
| | Isepamycin | Netilmicin | Tobramycin | |
| Amphenicols | Chloramphenicol | | | |
| Carbapenems | Doripenem | Ertapenem | Imipenem | Meropenem |
| 1st and 2nd generation cephalosporins | Cephalexin | Cephalothin | Cefadroxil | |
| | Cefradine | Cefaclor | Cefonicid | |
| | Cefamandole | Cefotiam | Cefuroxime | |
| | Cefmetazole | Cefotetan | Cefoxitin | |
| 3rd generation cephalosporins | Cefpodoxime | Ceftiofur | Cefsulodin | |
| | Cefditoren | Cefixime | Cefoperazone | |
| | Cefotaxime | Ceftazidime | Ceftizoxime | |
| | Ceftriaxone | Cefoperazone/Sulbactam | Ceftazidime/Avibactam | |
| | Cefpirome | Cefcapene | Cefdinir | |
| | Latamoxef | Cefmenoxime | Cefteram | |
| | Cefovecin | | | |
| 4th and 5th generation cephalosporins | Cefepime | Cefozopran | Ceftobiprole | Ceftolozan/Tazobactam |
| Fluoroquinolones | Enrofloxacin | | Marbofloxacin | |
| | Pradofloxacin | | Ciprofloxacin | |
| Nitrofurans | Nitrofurantoin | | | |
| Other beta-lactams | Loracarbef | Faropenem | Aztreonam | |
| Penicillins | Ampicillin | Temocillin | Oxacillin | |
| | Ampicillin/sulbactam | Carbenicillin | Amoxicillin | |
| | Amoxicillin/clavulanic acid | Mecillinam | Ticarcillin | |
| | Ticarcillin/clavulanic acid | Piperacillin | Piperacillin/Tazobactam | |
| | Azlocillin | Mezlocillin | Benzylpenicillin | |
| Sulfonamides | Trimethoprim/Sulfamethoxazole | | | |
| Tetracyclines | Doxycycline | Tetracycline | Minocycline | |

Fisher 's Exact Test was used, and the *p*-values determined, considering them statistically significant when value of $p \leq 0.05$. Numeric values were calculated using Pearson's coefficient. Isolates showing intermediate antibiotic resistance were considered as resistant for statistical comparisons. All the analyses and calculations were performed using R version 4.1.1 and RCommander 2.7–1.

# 3 Results

## 3.1 Virulence factor analysis

According to the virulence factor analysis performed, the prevalence of the virulence-related genes was as follows: 1.92% for *eae* (95% CI: 0–5.66%), 1.92% for *Stx2* (95% CI: 0–5.66%), 59.62% for *papC* (95% CI: 46.28–72.95%), 53.85% for *hlyA* (95% CI: 40.30–67.4%) and 32.69% for *cnf1* (95% CI: 19.97–45.44%). However, *Stx1* gene was not found in this study.

Regarding *E. coli* pathotype classification, 82.69% (95% CI: 79–87%) of isolates were classified as ExPEC, and around 20% (9/43) of them simultaneously harbored the three extraintestinal virulence factors analyzed. Additionally, 1.92% (95% CI: 0–5.66%) of isolates were defined as EPEC, and the same value was found for STEC. However, no EHEC isolates were detected. None of the virulence factors analyzed in this study were found in 13.46% (95%CI: 3–23%) of the isolates.

## 3.2 Prevalence of phenotypic antimicrobial resistance

According to the antimicrobial resistance profiles observed, only one out of 52 (95% CI: 0–5.66%) *E. coli* isolates was susceptible to all the antimicrobials tested. Also, all the antimicrobial categories presented resistant isolates, although in a variable percentage.

According to antimicrobial resistance levels defined by the European Food Safety Authority (58), an extremely high resistance level was found for the categories of 1st–2nd and 3rd generation cephalosporins, followed by very high resistance to penicillins and fluoroquinolones. These isolates also displayed a high resistance level to amphenicols (Figure 1). A low resistance level was found in 5 out

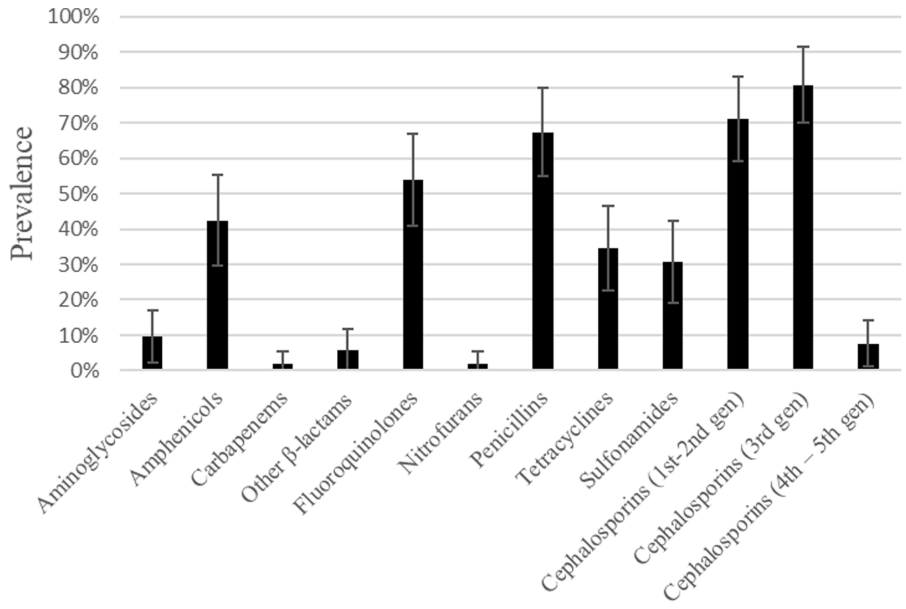


FIGURE 1
Prevalence of resistance to different antimicrobial categories found in *E. coli* isolates from dog urine.

of the 12 categories: carbapenems, nitrofurans, other β-lactams, 4th–5th generation cephalosporins and aminoglycosides.

Several isolates showed resistance to antibiotics which are considered critically important antimicrobials and are listed in category A (59). For example, three (out of 30) isolates were resistant to the β-lactam aztreonam, and there were several others found resistant to category A antibiotics from the penicillin group: three (out of 17) isolates were resistant to carbenicillin, 18 (out of 31) to ticarcillin, one (out of 33) to piperacillin and three (out of three) to mezlocillin. There was also one isolate showing intermediate resistance, and thus classified as resistant, to an agent from the carbapenem category (imipenem).

Apart from that, 13.46% (95% CI: 4.17–22.73%) of the isolates were considered ESBL-producers, and almost 60% (4/7) of them showed resistance to 9 or more out of the 12 antibiotic categories tested.

## 3.3 Multidrug resistant profiles

A total of 71.15% (95% CI: 58.84–83.46%) of the studied isolates were described as MDR.

Two main profiles of MDR, with a prevalence of 7.69% (95% CI: 0.45–14.93%) each of them, were observed. The isolates included in one of these profiles showed resistance to the following antimicrobial categories: penicillins, 1st–2nd and 3rd generation cephalosporins; while the other profile comprised those isolates resistant to amphenicols, fluoroquinolones, penicillins, and 1st–2nd and 3rd generation cephalosporins.

## 3.4 Genomic analysis of selected isolates

*In silico* molecular typing was performed in the sequenced genomes from those selected phenotypically resistant isolates (Table 3). Three different nomenclatures for sequence typing were assigned to each isolate according to Achtman's MLST scheme, Pasteur MLST scheme and core genome (cg)-MLST.

TABLE 3 Sequence types (ST) and serogroups of sequenced *E. coli* isolates.

| Isolate ID | Serogroup | ST (Achtman) | ST (Pasteur) | cgMLST |
|---|---|---|---|---|
| 267.252 | O15H1 | 393 | 494 | 163,945 |
| 258.883 | O5H20 | 6,448 | 901 | 174,146 |
| 271.960 | O4H31 | 372 | 490 | 135,819 |
| 262.947 | O5H20 | 6,448 | 901 | 174,146 |
| 263.715 | O5H20 | 6,448 | 901 | 174,146 |
| 266.493 | O9aH30 | 224 | 479 | 143,321 |
| 269.901 | O9H17 | 88 | 74 | 11,260 |
| 271.550 | O8H25 | 58 | 24 | 207,634 |
| 271.758 | O8H9 | 90 | 66 | 202,038 |
| 271.811 | O183H18 | 117 | 48 | 187,123 |

Three of these isolates, that is 258.883, 262.947 and 263.715, shared the same serotype (O5H20), and the corresponding sequence type (Pasteur ST 901 / Achtman ST 6448) and core genome sequence type (cg-ST 174146), making this *E. coli* type the most prevalent one among the studied isolates. The rest of the isolates presented unique molecular types, although isolates 271.758 and 269.901 belonged to the same clonal complex (CC ST23) and were paired together in the phylogenetic tree (Figure 2). Annotated comparison of the isolates (Figure 3) showed no major missing regions.

Several genes and mutations associated with resistance to different antimicrobial categories were detected in the sequenced isolates and are detailed in Table 4. In addition, some of these genes were

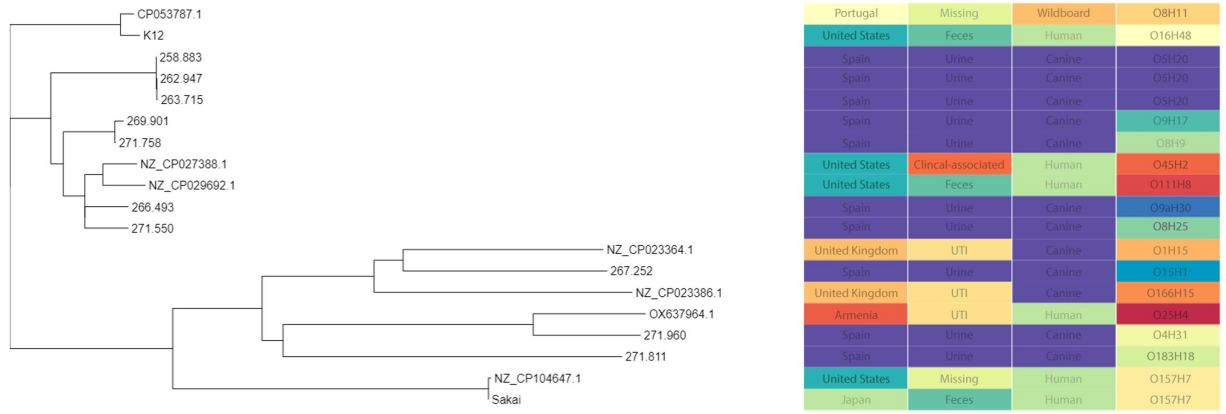


FIGURE 2
Phylogenetic tree including the sequenced *E. coli* isolates. Metadata was added using Phandango web application (60).

associated to mobile genetic elements (MGE), which are described in Table 5.

Interestingly, there were two MGE of particular interest due to its association with important resistance genes or a high number of them, that is IS*6100* and IS*Ec9*, which can be seen in Figure 4.

## 3.5 Association between antimicrobial resistance and virulence factors

When testing for simple relationships between phenotypic antimicrobial resistance and presence of virulence factors in these isolates, the carriage of *cnf1* gene showed a significant association with resistance to several penicillins, as well as with the penicillin category itself (Value of $p = 0.01$). *Cnf1* gene also showed a significant association (Value of $p = 0.019$) with MDR category. Apart from that, age of individuals was significantly associated with *E. coli* isolates showing resistance to various cephalosporins and to the 4th–5th generation cephalosporin category (Value of $p = 0.034$). Gender was associated with aminoglycoside resistant isolates (Value of $p = 0.007$).

ESBL production showed association with resistance to five out of the 12 antimicrobial categories tested (amphenicols, other β-lactams, 4th–5th generation cephalosporins, sulfonamides and fluoroquinolones). No significant relationship between the number of virulence-related genes and ESBL production was observed, however a negative relationship between the number of antimicrobial categories to which isolates showed resistance and number of virulence-related genes was found (Pearson coefficient = 0.33 value of – = 0.014).

# 4 Discussion

This study has evaluated both phenotypic and genotypic antimicrobial resistance as well as the presence of selected virulence genes in *E. coli* isolates from Spanish dogs with UTI and has shown that dogs may be reservoirs of resistant uropathogenic strains of *E. coli*.

Comparing with results obtained in a previous study (61) in which samples were collected in a similar time and geographical location, although they had a different origin (feces), we found that *E. coli* isolates from urine were more susceptible to aminoglycosides than those obtained from dog feces (phenotypic resistance found in 9.62% vs. 40% of isolates). In general, the prevalence of antimicrobial resistances found are similar to those found in *E. coli* isolated from UTI patients (62, 63), except for the penicillin group, which was slightly higher in this study (67.31% vs. ~45–50%). Such high prevalence is also contrary to the decreasing trend of penicillin resistance in *E. coli* isolates in Europe (20). Some hypotheses for this phenomenon could be the trend of increase of antibiotic resistance year to year or the fact that more antibiotics from the penicillin group were studied in this work.

*Escherichia coli* aminoglycoside resistance was the only type of resistance linked to the gender of the animal, being found exclusively in male individuals. In fact, male gender has previously been associated with aminoglycoside resistance in Gram-negative bacteria (64).

When taking into account some of the antibiotics considered clinically important for human and animal health by the European Medicines Agency (59), it is worth mentioning that *E. coli* isolates showing resistance to several of these antibiotics were detected in this study, even to antibiotics from category A ("Avoid," it includes antibiotics not authorized in veterinary medicine in the European Union), such as certain penicillins or carbapenems. Another relevant antibiotic category is category B ("Restrict"), which includes those listed as highest priority critically important antimicrobials (HP-CIAs) by the World Health Organization categorization, e.g., 3rd generation cephalosporins or fluoroquinolones. Indeed, as much as 80.77 and 53.85% of these isolates were considered resistant to 3rd generation cephalosporins and fluoroquinolones, respectively. The high amount of overall resistance found among all the categories could be biased by the fact that complicated UTI are more often requested for culture and antibiogram testing than simpler cases of UTI.

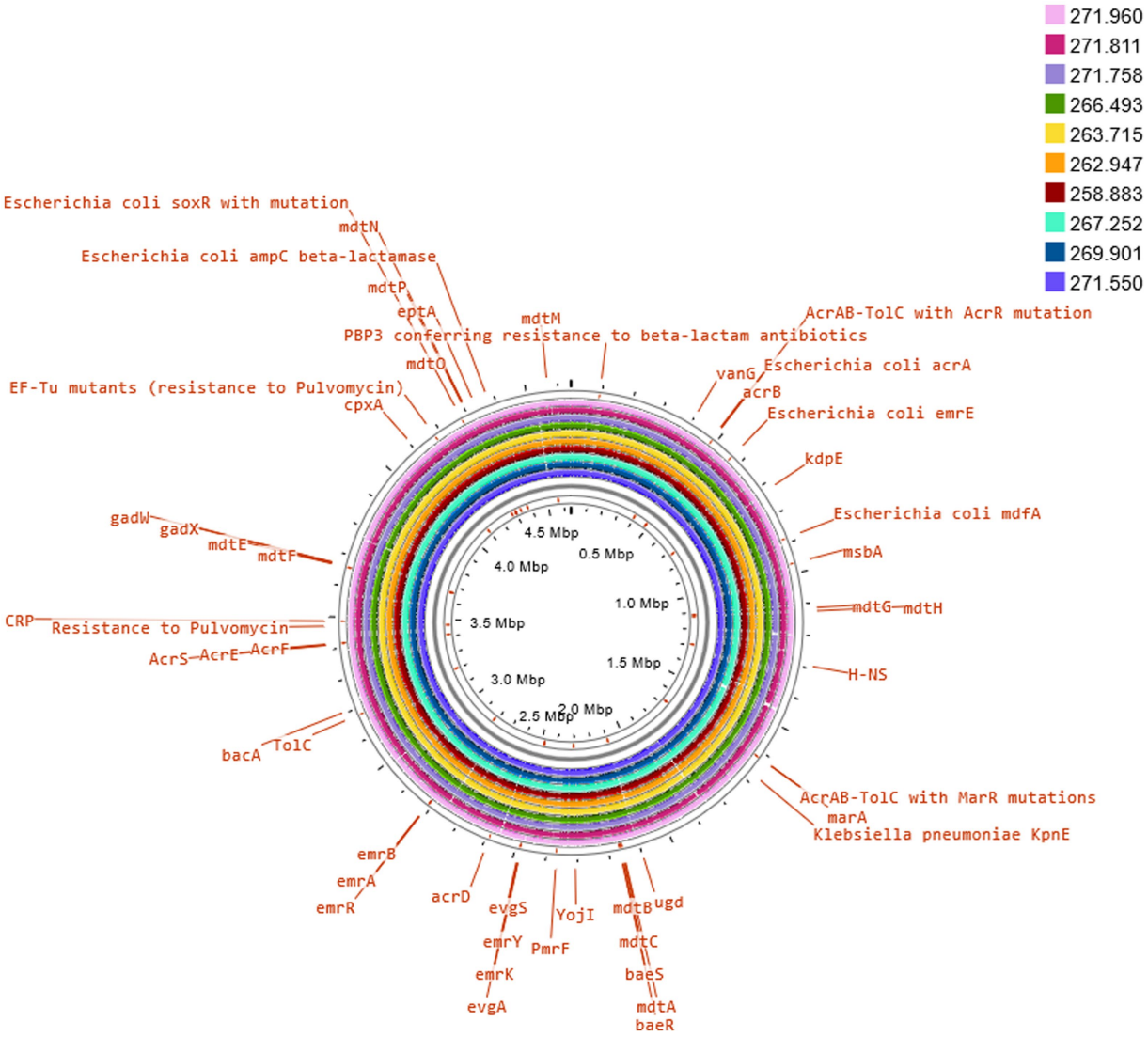


FIGURE 3
Comparison of *E. coli* K12 reference genome with sequenced isolates. The annotation of selected antimicrobial resistance genes was carried out on Proksee Server from the Stothard Research Group (University of Alberta, Canada) that uses BLAST analysis to illustrate conserved and missing genomic sequences (available online: https://proksee.ca/).

The treatment with amoxicillin or trimethoprim-sulfonamides as first-line agents is currently recommended for the management of bacterial UTI in dogs (29). However, considering these results, it seems that the use of these antimicrobials may be ineffective in a high percentage of cases, since 58.33% of isolates were found to be resistant to amoxicillin and 30.77% to trimethoprim-sulfonamides. Before suggesting any change in current guidelines for antibiotic treatment in canine UTI, it should be noted that *E. coli* is not the only pathogen responsible for UTI and that the data analyzed in this study might be overestimating the baseline resistance, mostly because of the selection of the patients. In any case, the use of antibiotic resistance testing as a routine allows not only the monitoring of the epidemiology of antibiotic resistance profiles but also the faster implementation of a treatment in case of failure of the empiric one.

It is worth mentioning the high percentage of MDR isolates found (71.15%). Among the MDR isolates found, more than 80% (30/37) were classified as ExPEC, and one of them corresponded to EPEC pathotype. This kind of strains possesses a potential zoonotic risk and can also serve as a reservoir of resistance genes (18), further contributing to the dissemination of antibiotic resistance and limiting the options for the treatment of infectious diseases in both humans and animals.

High antibiotic resistance has been associated with MGE in *Enterobacteriaceae*. Bacteria harboring these mobile elements can become a reservoir for antibiotic resistances and be transmitted then from pets to their owners or the environment. This phenomenon poses a serious health problem due to the spread of resistance and failure of current antibiotic treatments (65).

Of special interest is the presence of transposon Tn6009 carrying *tet*(M) gene in isolate 263.715. This element has been previously described in other Gram-negative bacteria, such as *Enterococcus fecalis* (66), and is associated with tetracycline resistance due to the presence of *tet*(M) (Figure 4E). This non-composite conjugative transposon is of clinical importance in Gram-positive bacteria and has a potential

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TABLE 4 Antimicrobial resistance genes and mutations found in the sequenced genomes of the selected *E. coli* isolates, as well as the corresponding phenotypic resistance pattern showed in the susceptibility testing assay.

| Isolate ID | Aminoglycosides | Phenicols | Beta-lactams | Fluoroquinolones | Tetracyclines | Sulfonamides | Other categories | Phenotypic resistance |
|---|---|---|---|---|---|---|---|---|
| 258.883 | *aph*(6)-Id, *aph*(3″)-Ib, *aadA5* | *floR* | $bla_{CTX-M-55}$ | parC;p.S80I, gyrA;p.S83L, gyrA;p.D87N | *tet*(A) | *dfrA17* | | A-AN-FL-P-S-CP |
| 262.947 | *aadA1*, *aadA2b*, *aadA5* | *cmlA1* | $bla_{CTX-M-55}$, $bla_{TEM-1B}$ | parC;p.S80I, gyrA;p.S83L, gyrA;p.D87N | *tet*(A) | *dfrA17*, *sul2*, *sul3* | *qacL* | AN-B-FL-P-T-S-CP |
| 263.715 | *aph*(6)-Id, *aph*(3″)-Ib, *aadA5* | *florR* | $bla_{CTX-M-55}$, $bla_{Z}$ | parC;p.S80I, gyrA;p.S83L, gyrA;p.D87N | *tet*(A), *tet*(M) | *dfrA17* | | AN-B-FL-P-T-S-CP |
| 266.493 | *aadA5* | | | parC;p.S80I, gyrA;p.S83L, gyrA;p.D87N, parE;p.S458A | | *dfrA17*, *sul1* | *erm(B)*, *mph(A)*, *qacE*, *sitABCD* | AN-C-FL-N-P-T-S-CP |
| 267.252 | *aph*(6)-Id, *aph*(3″)-Ib | | $bla_{TEM-1B}$ | parC;p.S80I, gyrA;p.S83L, gyrA;p.D87N, parC;p.S57T, parE;p.L416F | *tet*(B) | *sul2* | *sitABCD* | AN-B-FL-P-T-S-CP |
| 269.901 | *aadA1*, *ant(2″)*-Ia, *16S_rrsC;g.926_926del* | *floR*, *catA1* | $bla_{OXA-1}$ | parC;p.S80I, gyrA;p.S83L,gyrA;p.D87N, parE;p.S458A | *tet*(B) | *dfrA36*, *sul 1*, *sul2* | *qacE* | A-AN-FL-P-T-S-CP |
| 271.550 | *aph*(6)-Id, *aph*(3″)-Ib, *aph*(3′)-Ia | | $bla_{TEM-1B}$ | gyrA;p.S83L | *tet*(A) | *dfrA5*, *sul2* | *sitABCD* | A-FL-P-T-S-CP |
| 271.758 | | | | parC;p.S80I, gyrA;p.S83L, gyrA;p.D87N, parE;p.S458A | | | | AN-FL-P-T-CP |
| 271.811 | *aph*(6)-Id, *aph*(3″)-Ib | | $bla_{TEM-1B}$ | | *tet*(B) | *dfrA8*, *sul2* | *sitABCD* | AN-FL-P-T-S-CP |
| 271.960 | *aph*(6)-Id, *aph*(3″)-Ib, *aadA1* | *cat86*, *catA1* | | parC;p.S80I, gyrA;p.S83L, gyrA;p.D87G, parE;p.I355T | *tet*(A) | *dfrA1*, *sul1*, *sul2* | *qacE*, *sitABCD* | AN-FL-T-S-CP |

A, Aminoglycosides; AN, Amphenicols; C, Carbapenems; B, Beta-lactams; FL, Fluoroquinolones; N, Nitrofurans; P, Penicillins; T, Tetracyclines; S, Sulfonamides; CP, Cephalosporins.

role in the dissemination of resistance (67, 68). Resistance to beta-lactams was encoded by $bla_{CTX-M-55}$ gene in three isolates, which was located in a IS*Ec9* insertion sequence (IS*1380-like*). IS*Ec9* region has been previously associated with ESBL genes (69) and has been described in other bacteria such as *Vibrio vulnificus* (70). In all cases, the gene and the insertion sequence were 46 bp away. One of them (isolate 263.715) also harbored a $bla_{Z}$ gene, and another one (isolate 263.715) had a copy of $bla_{TEM-1B}$. Beta-lactamase-encoding genes such as $bla_{TEM-1B}$ and $bla_{OXA-1}$ were also identified in other isolates. For example, $bla_{TEM-1B}$ was present in two isolates that displayed no phenotypic resistance to beta-lactams (isolates 271.550 and 271.811), and in one that did (isolate 267.252). There was only one isolate (269.901) containing a $bla_{OXA-1}$ gene. As expected, this isolate was resistant to several antibiotics in the penicillin group (ampicillin, amoxicillin + clavulanic acid, amoxicillin), and it was negative in the ESBL production test $Bla_{OXA-1}$ has been found in ST131 or associated with other genes in plasmids (71). Despite this gene being originally described in MGE (72), we only identified IncI1 plasmid in this isolate, and it was not associated with any antibiotic resistance gene. Sulfonamide resistance genes (*sul 1* or *2*) were found in most of the sequenced genomes (7/10), and in five of them these genes were located in a MGE (IS*6100* and IncQ1 for *sul1*, and IS*Vsa3*, IncQ1 or IncFII for *sul2*). All these MGE also harbored other resistance genes, including those linked to streptomycin (*aadA5*, *aph*(6)-Id, *aph*(3″)-Ib *and aadA1* in IS*6100,* IncFII and IncQ1) (73–76), trimethoprim (*dfrA17* and *dfrA1* in IS*6100* and IncQ1) (77, 78), antiseptics (*qacL* and *qacE* in IS*6100* and IncQ1, although all antiseptic resistance genes were incomplete) (79), erythromycin (*mph*(A) in IS*6100*) (80), doxycycline and tetracycline (*tet*(A) in IncQ1) (81) and chloramphenicol (*catA1* in IncQ1) (82).

The IS*Vsa3* transposase was found in one of the sequenced isolates (262.947) and contained the *sul2* gene, which has also been identified in other enteropathogens (83–85). Isolate 266.493 harbored *dfrA17-aadA5-qacEdelta1*-IS*6100-mph*(A)-*sul1* integron structure, which is commonly identified in ExPEC pathotype (86).

Regarding the plasmids identified, IncFII plasmid has been previously documented in Spain as frequently linked to ESBL production (87). In this study, the plasmid was only identified in one isolate, although it was classified as non-ESBL producer.

Another plasmid identified was IncQ1, commonly found in *E. coli* and with ability to transfer between different bacterial species and strains, which facilitates the dissemination of antibiotic resistance in bacterial populations (88). This plasmid was detected in the genomes of three isolates (267.252, 271.550 and 271.960) and was found close to resistance genes linked to aminoglycoside resistance (*aph(6)-Id* and *aph(3″)-Ib*). All these three isolates were distant in the phylogenetic tree, which suggests that the plasmid has been likely acquired independently. One of these isolates harbored nine more resistance genes close to the detected plasmid (Figure 4D), indicating a potential hotspot for antibiotic resistance dissemination.

When studying antibiotic resistance genes in sequenced isolates, there was in general a consistent correlation between phenotypic and genetic resistance. However, there were two significant exceptions to this pattern. When examining aminoglycosides, several isolates exhibited susceptibility to this category despite carrying resistance genes related to both streptomycin and spectinomycin, which are included in this antimicrobial category. The second exception was observed with tetracycline, where the relationship between resistance

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Table 5 Presence of mobile genetic elements (MGE) in sequenced isolates, and antibiotic resistance and associated virulence genes.

| Isolate ID | MGE | Type | Coverage (%) | Identity (%) | Associated resistance and virulence genes |
|---|---|---|---|---|---|
| 258.883 | IS*Ec9* | IS | 100 | 100 | $bla_{CTX-M-55}$ |
| | MITEEc1 | MIR | 100 | 98.88 | *terC, yehB, yehD, yehA, yehC* |
| | IS*Ec1* | IS | 99.77 | 96.74 | *fdeC* |
| | IS*26* | IS | 100 | 100 | - |
| | IS*421* | IS | 99.78 | 99.7 | - |
| 262.947 | IS*Vsa3* | IS | 100 | 100 | *sul2* |
| | IS*Ec9* | IS | 100 | 100 | $bla_{CTX-M-55}$, *terC* |
| | MITEEc1 | IS | 100 | 97.56 | *yehB, yehD, yehA, yehC* |
| | IncFIC | PL | 100 | 100 | *traT, anr* |
| | IS*102* | IS | 100 | 92.72 | *cma, cba* |
| | IS*Ec1* | IS | 99.77 | 96.74 | *fdeC* |
| | IS*640* | IS | 99.91 | 98.36 | - |
| | IS*421* | IS | 99.78 | 99.7 | - |
| 263.715 | IS*Ec9* | IS | 100 | 100 | $bla_{CTX-M-55}$ |
| | Tn*6009* | ICE | 100 | 99.89 | *tet*(M) |
| | MITEEc1 | MIR | 100 | 97.56 | *terC, yehB, yehD, yehA, yehC* |
| | IS*Ec1* | IS | 99.77 | 96.74 | *fdeC* |
| | IS*421* | IS | 99.78 | 99.7 | - |
| | IS*26* | IS | 100 | 100 | - |
| 266.493 | IS*6100* | IS | 100 | 100 | *mph(A), qacE, dfrA17, sul1, aadA5* |
| | MITEEc1 | MIR | 100 | 100 | *terC, nlpl, terC, yehB, yehD, yehA, yehC, csgA, hlyE* |
| | IS*5* | IS | 100 | 99.75 | *irp2, gad, fyuA* |
| | IncFII | PL | 98.85 | 95.06 | *traT* |
| | IncFIB | PL | 100 | 98.93 | - |
| | IncFIA | PL | 100 | 99.74 | - |
| | IncX1 | PL | 100 | 94.92 | - |
| 267.252 | IncQ1 | PL | 65.83 | 100 | *aph*(6)-Id, *aph*(3″), *sul2* |
| | IncFII | PL | 99.62 | 96.95 | *anr* |
| | IncFIB | PL | 100 | 99.22 | - |
| | IncX4 | PL | 100 | 98.88 | - |
| | IncFIA | PL | 100 | 99.74 | - |
| | IS*Ec45* | IS | 100 | 99.86 | *iucC, papA, papC, iutA, sat, iha* |
| | IS*Ec46* | IS | 100 | 99.94 | *fyuA, irp2* |
| | IS*Ec1* | IS | 100 | 98.06 | *csgA, ompT* |
| | MITEEc1 | MIR | 99.19 | 97.56 | *terC* |
| 269.901 | Incl1 | PL | 100 | 100 | *cia* |
| | IS*Ec78* | IS | 99.84 | 98.97 | *fyuA, irp2* |
| | MITEEc1 | MIR | 100 | 98.37 | *yehD, iss, fdeC* |
| 271.550 | IncQ1 | PL | 65.83 | 100 | *aph*(6)-Id, *aph*(3″), *sul2* |
| | IncFIB | PL | 100 | 98.39 | *cia, iroN, iss, mchF, etsC, cvaC, etsC, ompT, hlyF* |
| | IncFII | PL | 100 | 100 | *traT, anr* |
| | IS*Ec31* | IS | 99.28 | 92.73 | *terC* |
| | MITEEc1 | MIR | 99.19 | 94.26 | *iss, fdeC, terC* |
| | IS*Ec38* | IS | 100 | 94.6 | *fyuA, irp2* |

*(Continued)*

Table 5 (Continued)

| Isolate ID | MGE | Type | Coverage (%) | Identity (%) | Associated resistance and virulence genes |
|---|---|---|---|---|---|
| 271.758 | IncFII | PL | 100 | 100 | - |
| | IncFIA | PL | 100 | 99.74 | - |
| | IS3 | IS | 100 | 99.92 | *hlyE, csgA* |
| | MITEEc1 | MIR | 100 | 97.56 | *yehB, yehD, yehA, yehC, terC, nlpl* |
| | IS*Ec1* | IS | 100 | 97.91 | *fdeC* |
| 271.811 | IncFII | PL | 100 | 100 | *sul2, aph*(6)-Id, *aph*(3″)-Ib, *anr* |
| | Incl1 | PL | 100 | 100 | - |
| | Col(MG828) | PL | 98.85 | 95.38 | - |
| 271.960 | IncQ1 | PL | 66.46 | 100 | *dfrA1, aadA1, aph*(6)-Id, *qacE, tet*(A), *aph*(3″)-Ib, *sul1, dfrA1, catA1, sul2, dfrA1* |
| | IncFII | PL | 98.85 | 98.05 | *traT, anr, traJ* |
| | IncHI2A | PL | 100 | 99.52 | - |
| | IncHI2 | PL | 100 | 100 | - |
| | IS*Kpn37* | IS | 99.68 | 97.3 | *hlyA, cnf1* |
| | MITEEc1 | MIR | 100 | 100 | *terC* |
| | IS*Ec38* | IS | 99.94 | 97.16 | *cea* |

PL, plasmid; IS, insertion sequence; MIR, miniature inverted repeat; ICE, Integrative Conjugative Element.

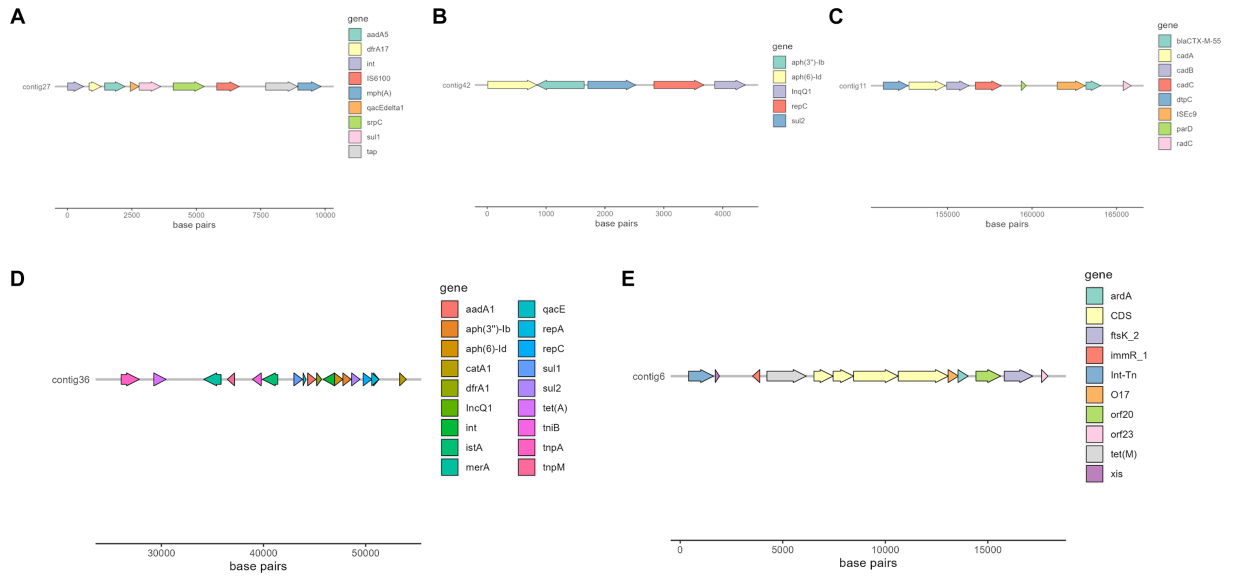


FIGURE 4
Selected contigs of different isolates containing antimicrobial resistance genes as well as MGE annotated with Prokka (56). **(A)** Organization of a fragment of contig 27 in isolate 266.493, which contains *aadA5*, *dfrA17*, *qacE*, *sul1*, and *mphA* genes. **(B)** Organization of contig 42 in isolate 269.901, which contains *aph*(6)-Id, *aph*(3″)-Ib and *sul2* genes. **(C)** Organization of a fragment of contig 11 in isolate 258.883, which contains $bla_{CTX\text{-}M\text{-}55}$ gene. **(D)** Organization of a fragment of contig 36 in isolate 271.960, which contains several resistance genes, such as *aadA1*, *sul2* and *tet*(A). **(E)** Organization of a fragment of isolate 263.715, which contains *tet*(M) resistance gene.

genes and phenotypical resistance did not consistently align. This discrepancy in isolate 258.883 may be attributed to a nucleotide substitution at position 924 within the *tet*(A) gene (position 3,323, GenBank: AF534183.1), specifically transitioning from cytosine (C) to thymine (T). Because of this alteration, there is a shift in the protein composition from alanine (Ala) at position 118 to threonine (Thr). Although these are not the first *E. coli* isolates that harbor this gene mutation (89), to the authors knowledge our study is the first that associates this mutation in *tet*(A) to a failure in phenotypic response. Also, in more than 40% (4/9) of the isolates considered phenotypically resistant to amphenicols, no resistance gene associated with this antimicrobial category was found.

The phylogenetic tree (Figure 3) showed that canine isolates clustered together, except isolate 271.960 (ST 372), which was grouped

with a human isolate (assembly reference OX637964.1) that belongs to ST131. ST 131 is one of the predominant sequence types within the ExPEC pathotype worldwide (71, 90). In fact, *E. coli* O25b:H4/ST131 was described as a prevalent clone in Spanish human population. In accordance with bibliography, this canine isolate was not associated with ESBL resistance and had a similar resistance profile to human strains (91, 92).

According to the virulence factors analyzed, most of the *E. coli* isolates found in urine samples were categorized in the ExPEC pathotype, as expected. The most frequently detected virulence factors were *papC* and *hlyA*, followed by *cnf1*. *Cnf1* prevalence in these isolates was similar to that found in isolates from both dogs and humans, while papC and *hlyA* prevalence were higher in this study (18, 93–97). However, most of the sampled populations in these studies include healthy animals, which could lower the prevalence of *E. coli* virulence factors. The prevalence of these three virulence factors were higher in dog isolates than in those found in humans (95, 98–100). Some of these factors were found in MGE (Table 5), which highlights their potential of spread to other strains.

Almost 20% of these ExPEC isolates displayed in combination with the three extraintestinal virulence-related genes analyzed (*papC*, *hlyA* and *cnf1*), likely due to the presence of a PAI (101). This type of virulence factors are frequently found in *E. coli* strains causing extraintestinal disease in both humans and dogs, being thus this animal species a possible reservoir for the ExPEC pathotype (102).

Apart from that there was one Stx2-positive isolate, that did not harbor any other virulence factor studied. Shiga toxin 2 is believed to be associated with the development of HUS (11) and is better produced when it is found in combination with other strains or bacteria (103). However, there are also some descriptions of Shiga toxin-producing *E. coli* isolates associated with UTI cases, and it has been proposed that Shiga toxins can bind to receptors from urinary bladder epithelial cells and damage them (104, 105). Additionally, an EPEC isolate was also detected. It is not the first time that an *eae*-positive isolate has been found among UTI-associated strains, although its frequency seems to be quite low as well (8, 106, 107). The role of this gene product (i.e., intimin) in UTI pathogenesis is not fully understood and its significance remains to be studied (8).

Taking into consideration that fecal *E. coli* population might have a relationship with UTI pathogenesis (108), it may be suggested that certain diarrheagenic pathotypes also have potential to cause UTI, although uncommon. However, the role of these strains in UTI development and the molecular and pathogenic causes behind it are still poorly understood, and more research in this field is needed in order to comprehend the mechanisms and epidemiological causes. Nevertheless, the ability of such strains to cause an extraintestinal infection in the host is not only dependent on their virulence-related genes but also on risk factors such as age or immunosuppression (106).

It is also important to note that a wide variety of extraintestinal-associated virulence traits has been described in the literature. Thus, apart from these virulence genes typical of diarrheagenic strains, these two isolates might be also harboring some other extraintestinal virulence factors different from those analyzed in this study. In this regard, some *E. coli* strains have been recently classified as hybrids for harboring virulence factors usually associated with various pathotypes, e.g., STEC/UPEC strains (109). The genome plasticity of this microorganism promotes the exchange and combination of both intestinal and extraintestinal virulence determinants, resulting in an heteropathogenic potential (106, 107). The possible emergence of hybrid pathotypes not only in humans but also in animals should therefore be surveilled.

The finding of eight isolates (15.38%) considered neither intestinal nor extraintestinal pathogenic isolates could be explained by the fact that only a selection of virulence factors was tested. Thus, these *E. coli* might harbor other different virulence-related genes not analyzed in this study. However, another explanation could be that the causal agent for UTI in these dogs was different from *E. coli*, or even a non-infectious cause. It is also worth noting that the detection of virulence factor genes does not mean that they are phenotypically expressed, so the severity of the disease could not be only assessed with this information. Nevertheless, it is known that the severity of the disease is not caused by a single virulence factor but a combination of them (110, 111).

The most commonly isolated serotype in this study was O5H20. In this regard, O5:H(−) has been associated with STEC strains, and Shiga toxins have been also described in *E. coli* strains causing UTI (112, 113). However, these isolates did not harbor any Shiga toxin gene. The rest of *E. coli* serotypes are distributed along different STs and antibiotic resistance patterns, showing a heterogenic distribution.

Interestingly, low antibiotic resistance patterns were linked to a higher number of virulence factors. There is some literature (114, 115) that suggests a positive relationship between virulence factors and MDR. However, in isolates from this study only *cnf1* carriage showed a significant association with MDR, while a high virulence factor carriage was associated with low resistance profiles. The reason for this mechanism is still unclear, but it is hypothesized that the acquisition of MDR is "sacrificed" in exchange for virulence factors, or that the low presence of virulence factors facilitates the acquisition of antibiotic resistance (116, 117). When analyzing correlation between all virulence factors found and the presence of antibiotic resistance genes in whole genome sequenced isolates, the relationship was non-significant ($p = 0.14$). However, there was a bias in selection of isolates, as only the more resistant ones were chosen.

## 5 Conclusion

Based on these data, a very high percentage of *E. coli* isolates found in urine samples from dogs suffering from UTI was considered MDR, the majority of them being classified as ExPEC. Phenotypic antimicrobial resistance to first-lines agents recommended in UTI management was also frequently observed, which could be associated with a treatment failure. Furthermore, several antimicrobial resistance genes, some of them contained in MGE, were identified in the genome of selected resistant isolates. The use of WGS could identify some of the genetic mechanisms underlying antimicrobial resistance, although there were a few discordances between phenotypic resistance and genes found. Combining both phenotypic and genetic data enhances our understanding of antibiotic resistance and improves treatment selection efficiency.

Overall, these findings are of concern for both animal and public health, since dogs could act as reservoirs of MDR pathogenic *E. coli* and contribute to the spread of antimicrobial resistance. Surveillance of antimicrobial resistance and revision of therapeutic guidelines

should be therefore continuously addressed in clinical veterinary settings.

## Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/bioproject; PRJNA1031085.

## Ethics statement

Ethical approval was not required for the studies involving animals in accordance with the local legislation and institutional requirements because samples were collected as part of the daily activity of private veterinary practitioners. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

AA-F: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. ES: Data curation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. AO: Investigation, Writing – original draft. IM-B: Formal analysis, Supervision, Writing – review & editing. BM: Resources, Supervision, Writing – review & editing. MM: Conceptualization, Resources, Supervision. RB: Conceptualization, Funding acquisition, 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.

## Publisher's note



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# Clinical validation of circulating immune complexes for use as a diagnostic marker of canine leishmaniosis

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**Introduction:** Canine leishmaniosis (CanL) is a systemic disease that affects dogs. When multiplication of the parasite cannot be controlled, dogs consistently show high levels of antigen and IgG antibodies, which lead to the formation of circulating immune complexes (CIC). Timely intervention to reduce the parasite load and CIC levels is crucial for preventing irreversible organ damage. However, a diagnostic test to quantify CIC levels is currently lacking.

**Methods:** In this real-world study, we aimed to examine the performance of a new ELISA to measure CIC levels in dogs naturally infected with *Leishmania infantum*. Thirty-four dogs were treated according to their clinical condition and followed for 360 days. Before (day 0) and after treatment (days 30, 90, 180, 270, and 360), all dogs underwent a physical examination, and blood samples were obtained for CBC, biochemical profile, serum protein electrophoresis and IFAT. Serum PEG-precipitated CIC were determined by ELISA.

**Results:** Our results indicate higher CIC levels in dogs in advanced disease stages showing higher antibody titres ($p < 0.0001$, $r = 0.735$), anemia ($p < 0.0001$), dysproteinemia ($p < 0.0001$), and proteinuria ($p = 0.004$). Importantly, dogs responding well to treatment exhibited declining CIC levels ($p < 0.0001$), while in poor responders and those experiencing relapses, CIC were consistently elevated. CIC emerged as a robust discriminator of relapse, with an area under the curve (AUC) of 0.808. The optimal cut-off to accurately identify relapse was an optical density of 1.539.

**Discussion:** Our findings suggest that declining CIC levels should be expected in dogs showing a favorable treatment response. Conversely, in dogs displaying a poor response and recurrent clinical relapses, CIC levels will be high, emphasizing the need for vigilant monitoring. These findings suggest that CIC could serve as a valuable biomarker for disease progression, treatment efficacy, and relapse detection in CanL. Our study contributes to enhancing diagnostic approaches for CanL and underscores the potential of CIC as a complementary tool in veterinary practice. As we move forward, larger studies will be essential to confirm these findings and establish definitive cut-offs for clinical application.

KEYWORDS

***Leishmania infantum*, immune complexes deposition, canine leishmaniosis, biomarker, PEG-ELISA**

# 1 Introduction

Canine leishmaniosis (CanL), a life-threatening zoonotic disease caused by an obligate intracellular protozoan *Leishmania infantum*, is transmitted to humans and other animals by blood-sucking phlebotomine sand flies. Dogs are considered the main peridomestic reservoir of infection (1, 2).

*Leishmania* may infect any organ, tissue or biological fluid, determining that clinical signs of CanL are non-specific and include lethargy, loss of appetite, weight loss, cutaneous lesions, onychogryphosis, generalized lymphadenomegaly, polyuria and polydipsia, ocular lesions, epistaxis, lameness, and other less common clinical signs like vomiting and diarrhea (3). However, many dogs show no apparent clinical signs or clinical-pathological abnormalities for several months or years after infection is confirmed. These dogs are defined as clinically healthy infected dogs. As for sick dogs, a classification system proposed by the LeishVet group helps guide practitioners in the clinical management of these animals and offers prognostic information (3). In this system, four clinical stages are described according to disease severity based on serological status, clinical signs, and laboratory findings.

After infection, clinically healthy infected and sick dogs show different immune responses to the parasite. Healthy dogs mount a robust Th1 response, which is crucial to halt parasite multiplication and for intracellular parasite elimination. In contrast, sick dogs mostly show a Th2 response, which leads to immune suppression and parasite proliferation (4, 5). In these animals, *Leishmania* antigens and non-specific IgG antibodies gradually build-up in the bloodstream as the parasite multiplies, leading to the formation of circulating immune complexes (CIC). These large molecules are deposited in the endothelium of several organs including the skin, eyes, joints, and renal glomeruli, and cause severe, often irreversible, organ damage. When this occurs, dogs can develop immune-mediated polyarthritis, vasculitis, indolent ulcers, and chronic uveitis, which can lead to blindness requiring the enucleation of one or both eyes. In LeishVet stages III or IV, CIC deposition in renal glomeruli can lead to chronic kidney disease (CKD) with life-threatening consequences (6–8).

The use of antimonials or miltefosine in combination with allopurinol is the standard treatment for CanL. In some cases, steroids are also needed to control immune-mediated clinical signs (9, 10). Prompt treatment is vital to reduce the parasite load and CIC levels, and thus mitigate the risk of irreversible organ damage. However, a diagnostic test to quantify CIC levels before and after treatment is currently lacking. Parody et al. (11) developed a method to quantify serum levels of CIC in dogs with CanL that was recently laboratory validated (12). In their work, Parody et al. (11) confirmed a clear association between disease progression and increasing CIC levels, suggesting that CIC could serve as a valuable biomarker for diagnostic purposes and also to track disease progression and assess treatment efficacy (13). Validating these findings in a real-world study is the first step toward making this method available to practitioners. The aim of the present study was to examine the performance of this new biomarker in a clinical setting by measuring CIC levels in dogs naturally infected with *L. infantum*.

# 2 Materials and methods

## 2.1 Study design

This longitudinal cohort study was conducted at the Infectious Diseases Unit of the Veterinary Teaching Hospital, Universidad Complutense de Madrid (UCM), Madrid, Spain. The study protocol was approved by this University's Research Ethics Committee. Participants were dogs brought to the hospital by their owners over the period May 2021 to April 2022 for evaluation after a first diagnosis of CanL, or follow-up for check-ups or due to a clinical relapse. Dogs were considered eligible if naturally infected with *L. infantum* [positive serology for *Leishmania* by indirect fluorescent antibody test (IFAT >1:100, positive cytology and/or positive PCR result)] and their disease was then graded as LeishVet stages I–IV. Before enrolment, we informed all dog owners about the study protocol, including the option to withdraw their dogs from the study at any time, and obtained their written consent.

## 2.2 Study protocol

Dogs were included in the study on day 0 (Visit 1 enrolment pre-treatment) and thereafter followed for 12 months. Appropriate treatment was started shortly after enrolment. Visits to our service were then scheduled for post-treatment onset days 30, 90, 180, 270, and 360 (Figure 1).

Dogs underwent a thorough physical examination in each visit. Data were compiled on clinical signs including signs of CIC deposition (Figure 2). Each clinical sign was graded from 0 to 3 (low to high severity) to give an overall clinical score for each animal (maximum 68) (Table 1).

At each visit, blood samples were collected to determine serum CIC concentrations by PEG ELISA (11) (Figure 1). On days 0, 30, 180, and 360, routine laboratory tests were also performed to determine the clinical condition of the dog, which included complete blood count (CBC), biochemical profile (creatinine, urea, and alanine aminotransferase), serum protein electrophoresis, and anti-*Leishmania* immunofluorescence antibody test (IFAT) (15). Serial dilutions from 1:50 to 1:6400 were used and seropositivity defined by a cut-off ≥1:200. Urinalysis, including urine density, sediment analysis and protein/creatinine ratio (UPC), were also undertaken on days 0, 30, 180, and 360 on urine samples obtained preferably by cystocentesis, or by free catch into a sterile container. Quantitative PCR was also performed to confirm the infection on day 0, and to quantify parasite load at 6 and 12 months post enrolment. Based on physical examination and clinical-pathological findings, dogs were assigned to LeishVet clinical disease stages I–IV (3).

## 2.3 CIC isolation and quantification

A modified precipitation method with polyethylene glycol (PEG; Sigma-Aldrich, St. Louis, MO, United States) was used to separate free antigens and antibodies from CIC (11). PEG-precipitated CIC were pelleted by centrifugation, then reconstituted in 0.01 M phosphate-buffered saline (PBS) and stored at −80°C for further use.

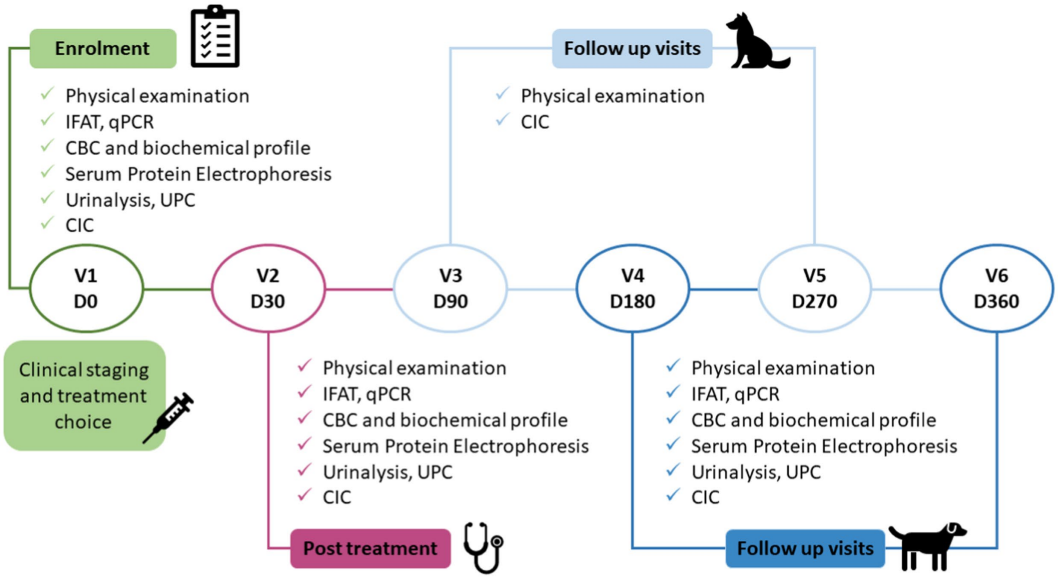


FIGURE 1
Study protocol. The clinical study consisted of six visits, enrolment (pre-treatment), after completing 30 days of treatment, and four follow-up visits. IFAT, indirect fluorescent antibody test; CBC, complete blood count; UPC, urinary protein/creatinine ratio; CIC, circulating immune complexes; qPCR, quantitative polymerase chain reaction.

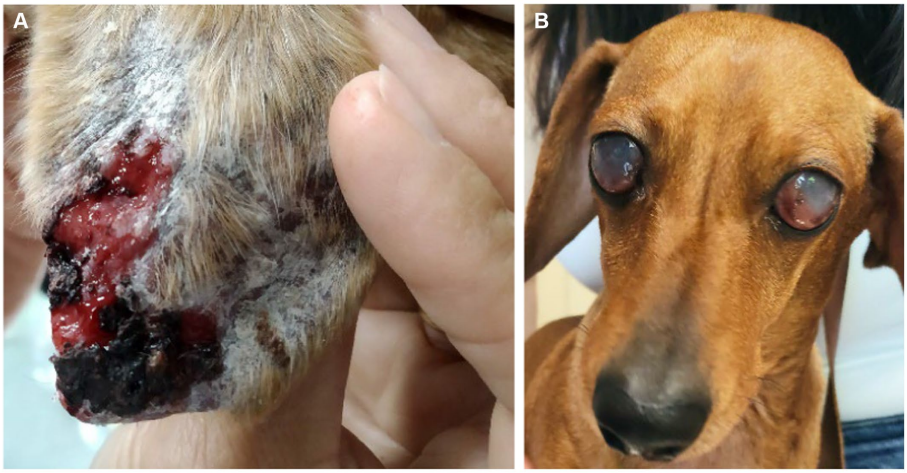


FIGURE 2
Clinical signs of CIC deposition in dogs with CanL. **(A)** Ulcer in the pinna associated with vasculitis. **(B)** Blindness associated with bilateral uveitis.

CIC were quantified using a *Leishmania*-specific ELISA method as described by Parody et al. (11). All samples were tested in duplicate, and the mean value was recorded. A mean OD of 0.274 + 3 standard deviations of negative samples was considered as the cut-off value; any sample exhibiting absorbance above the cut-off value was considered positive.

## 2.4 Treatment

The treatment for each dog was determined by one of the authors (a veterinarian specialist in infectious diseases) and was based on clinical history, physical examination, and laboratory findings. Clinically healthy infected dogs or those at stage I received no treatment or received only allopurinol for 6–12 months. Dogs in stages II or III were treated with a combination of meglumine antimoniate (MGA) (50 mg/kg subcutaneously q12h for 28 days) and allopurinol (10 mg/kg orally q12h for 6–12 months). When considered necessary, prednisone (0.5 mg/kg orally q12h for 3–4 weeks) was added to the treatment regimen in dogs in stage III to control signs of CIC deposition (10). Proteinuric dogs were treated following International Renal Interest Society Guidelines for CKD in dogs (16).

Relapses were also recorded and a second course of leishmanicide was administered if needed. Disease relapse was defined as significant

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TABLE 1 Clinical scoring system used in this study.

| Clinical signs | Score | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| Apathy | Absent | Mild (unable to properly run) | Severe (intolerant to exercise) | Prostration (refuses to walk) |
| Appetite | Normal | Slightly reduced (> half normal intake) | Markedly reduced (< half normal intake) | Anorexia |
| Weight loss | Absent | Mild (<10%) | Moderate (10-20%) | Severe (>20%) |
| Fever | Absent | Temperature 1°C above normal | Temperature 2°C above normal | Temperature ≥ 3°C above normal |
| Pale mucous membranes | Absent | Mild | Moderate | Severe (white mucous membranes) |
| Adenopathy | Absent | 1 or 2 enlarged lymph nodes | More than 2 enlarged lymph nodes | Generalized lymphadenomegaly |
| Splenomegaly | Absent | – | Present | – |
| Oral ulcers | Absent | Small focal ulcers | Multiple medium-size ulcers | Multiple oral ulcers, mastication impaired |
| Onychogryphosis | Absent | – | Present | – |
| Seborrhoeic dermatitis | Absent | <10% body surface | <25% body surface | Generalized seborrhoeic dermatitis |
| Hyperkeratosis | Absent | <10% body surface | <25% body surface | >25% body surface |
| Conjunctivitis | Absent | Mild unilateral | Severe unilateral or mild or moderate bilateral | Severe bilateral |
| Uveitis | Absent | Mild, resolved with topical treatment | Moderate, requiring oral treatment | Severe risk of blindness |
| Epistaxis | Absent | Occasional bleeding | Frequent bleeding | Uncontrolled bleeding |
| Vasculitis | Absent | Mild lesions | Moderate lesions | Severe lesions |
| Lameness | Absent | Mild (no impact on movement) | Moderate lameness (mild pain or discomfort) | Severe lameness (movement impaired) |
| Ulcers | Absent | Small focal ulcers | Multiple medium-sized ulcers | Severe ulcerated lesions |
| Polyuria | Absent | – | Present | – |
| Polydipsia | Absent | – | Present | – |
| Haematuria | Absent | Blood on sediment analysis | Visible hematuria | Red urine |
| Dysuria | Absent | – | Present | – |
| Urinary incontinence | Absent | – | Present | – |
| Vomiting | Absent | Occasional | Frequent | Vomit bloody |
| Diarrhea | Absent | Occasional | Frequent | Diarrhea with blood/melena |

Adapted from Miró et al. (14).

clinical worsening and/or laboratory abnormalities prompting a new course of treatment with antimonials.

The clinical response to treatment was assessed by examining clinical records, changes produced in clinical scores and laboratory variables over time, and the number of relapses produced during the study period. Good responders were dogs that showed improvement in clinical signs and laboratory abnormalities and no relapses in the 12 months following treatment onset. Dogs were considered poor responders if they experienced one or more relapses during the study or showed no improvement in clinical-pathological variables by the end of the study.

## 2.5 Statistical analysis

All statistical tests were performed using the software packages SAS (SAS Institute Inc., Cary, NC, United States) version 9.4, and SPSS Statistics version 28 (SPSS Inc., Chicago, IL, United States). Graphs were created using GraphPad Prism 10. Significance was set at $p \leq 0.05$.

Some variables were categorized for analysis: age (2–4 years: young adult, 5–8 years: mature adult, ≥9 years: senior) (17), IFAT titer according to cut-off values established by the laboratory (<200 negative, 200–400 low positive, 800–1,600 medium positive, >1,600

high positive) (3, 15, 18, 19), and albumin/globulin ratio (A/G) (>0.8 normal, 0.7–0.8 mild dysproteinemia, 0.5–0.69 moderate dysproteinemia, <0.5 severe dysproteinemia) (19–21).

As CIC values within groups were not normally distributed (Kolmogorov–Smirnov test, $p < 0.001$), non-parametric tests were used. The Wilcoxon test was used to compare categorical variables between two established groups and the Kruskal–Wallis test with Bonferroni correction when there were more than two groups. The association between CIC levels and the clinical signs from the scoring system used in this study was evaluated using the Chi-Square test, specifically analyzing the 2×2 contingency table formed by the variables. From this analysis, relative risks (RR) for each clinical sign were calculated, accompanied by their respective 95% confidence intervals (CI). These measures serve to quantify the strength and direction of the association between CIC and the clinical signs, providing valuable insights into their relationship.

To assess changes in CIC levels over time, we used the Friedman test for repeated measures also with Bonferroni correction. Spearman's correlation test was used to examine possible correlations between CIC levels and continuous variables. We also assessed the capacity of CIC level to accurately identify relapse by calculating the area under the receiver operating characteristic (ROC) curve (AUC).

# 3 Results

## 3.1 Study population

Of the 44 dogs enrolled, 23 were male and 21 female. Fourteen dogs were classified as young adults (2–4 years), 18 as mature adults (5–8 years), and 11 as senior (mean age 6.6 years; 95% CI: 1.6–14.64). During the study, three dogs died of a comorbidity (2 lymphoma, 1 carcinoma), and seven were lost to follow-up as owners failed to return for the re-visits. Even though only 34 dogs completed the 12 months of the study, data for all 44 dogs enrolled were included in the analysis until the loss of follow-up.

The CIC levels were significantly higher in young adult dogs compared to senior ($p < 0.001$), and low negative correlation was found between CIC level and age ($r = -0.273$). No differences in CIC levels were detected between mongrels and pure breeds ($p = 0.107$) or between male and female dogs ($p = 0.416$).

## 3.2 CIC and clinical scores, and LeishVet stages

The mean clinical score awarded to the 34 dogs that completed the 12 months of follow up gradually decreased throughout the study (Figure 3), and clinical scores and CIC levels showed positive correlation (Figure 3) ($r = 0.5135$, $p < 0.0001$). Dogs showing clinical signs were also found to have a greater risk of having elevated CIC levels. Table 2 displays the relative risks (RR) for various clinical signs evaluated in association with the presence of CIC in dogs. Interestingly, 10/44 dogs (22.72%) had a clinical score of five or less, but high CIC levels (≥2 OD).

At enrolment, 15 dogs were classified as LeishVet stage I, 20 as stage II, 7 as stage III and 2 as stage IV. Owing to the small number of animals at stage IV, data for stages III and IV were combined for analysis. Dogs at stage I showed significantly lower CIC levels than those classified as having CanL stages II and III ($p < 0.0001$) (Figure 4). No differences were found between dogs staged as II or III ($p = 0.485$). Moreover, stage I dogs consistently maintained low CIC levels throughout the study (Figure 4). In stage II patients, CIC levels decreased after treatment and remained stable, whereas in stage III patients, they increased after 90 and 360 days of treatment (Figure 4).

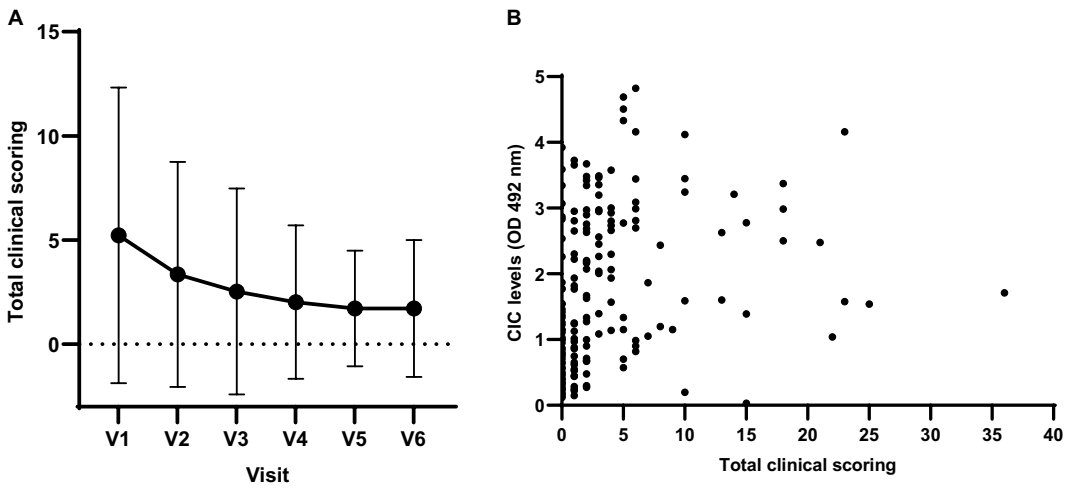


FIGURE 3
**(A)** Means and SDs of total clinical scores recorded in each visit. **(B)** Correlation between total clinical scores and CIC levels (Spearman correlation, $r = 0.5135$). V1 (Day 0), V2 (Day 30), V3 (Day 90), V4 (Day 180), V5 (Day 270), and V6 (Day 360).

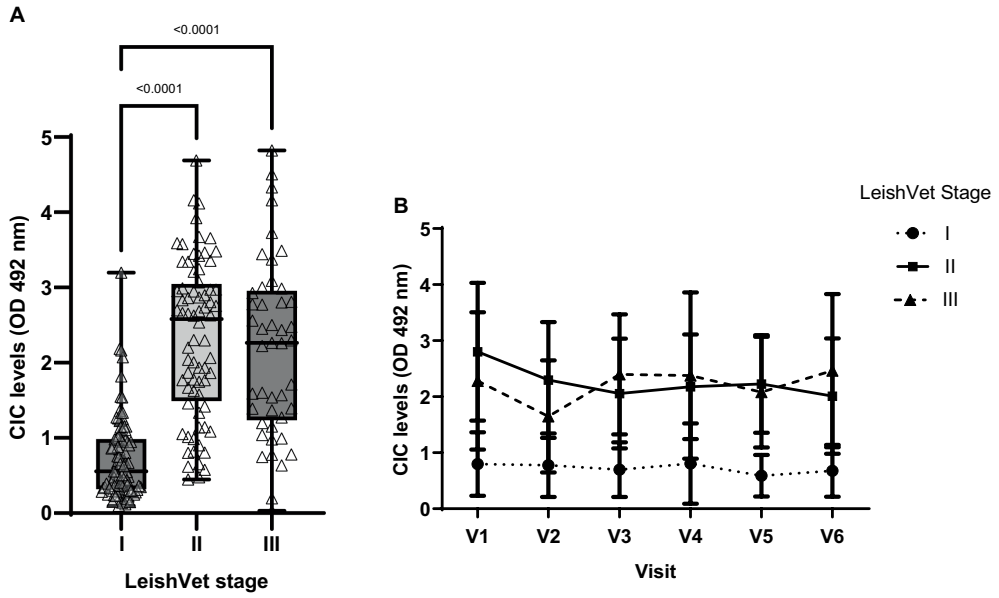


FIGURE 4
**(A)** CIC levels recorded in the dogs assigned to different LeishVet stages (non-parametric Kruskal–Wallis test). **(B)** Changes in mean CIC levels recorded over the 12-month study period in dogs at different LeishVet stages. V1 (Day 0), V2 (Day 30), V3 (Day 90), V4 (Day 180), V5 (Day 270), and V6 (Day 360).

TABLE 2 **Relative risk of clinical signs associated with a significant risk of elevated circulating immune complexes (CIC).**

| Clinical sign | | Elevated CIC | | Relative risk [95% CI] | *p*-value |
|---|---|---|---|---|---|
| | | Yes | No | | |
| Apathy | Yes | 17 | 1 | 2.58 [2.08–3.20] | <0.0001 |
| | No | 72 | 125 | | |
| Weight loss | Yes | 12 | 7 | 1.60 [1.09–2.36] | 0.0437 |
| | No | 77 | 119 | | |
| Adenopathy | Yes | 43 | 13 | 2.65 [1.99–3.52] | <0.0001 |
| | No | 46 | 113 | | |
| Pale mucous membranes | Yes | 20 | 2 | 2.54 [2.01–3.20] | <0.0001 |
| | No | 69 | 124 | | |
| Uveitis | Yes | 20 | 3 | 2.41 [1.89–3.09] | <0.0001 |
| | No | 69 | 123 | | |
| Lameness | Yes | 26 | 9 | 2.12 [1.60–2.80] | <0.0001 |
| | No | 63 | 117 | | |
| Hyperkeratosis | Yes | 20 | 7 | 2.01 [1.50–2.70] | 0.0002 |
| | No | 69 | 119 | | |
| Seborrhoeic dermatitis | Yes | 20 | 11 | 1.72 [1.24–2.37] | 0.0047 |
| | No | 69 | 115 | | |
| Ulcers | Yes | 15 | 8 | 1.69 [1.20–2.39] | 0.0141 |
| | No | 74 | 118 | | |

Repeated measures were treated as independent measures for relative risk calculation. CI, confidence interval.

## 3.3 CIC and laboratory findings

Leukopenia was the most frequent abnormality detected in the CBC, occurring in 27.78% of dogs, followed by thrombocytopenia (22.62%) and non-regenerative anemia (18.23%). However, significant differences in CIC levels were only found for anemic dogs ($p < 0.0001$) (Figure 5), which not only had higher CIC levels but also experienced a gradual increase in these levels as early as 6 months after treatment

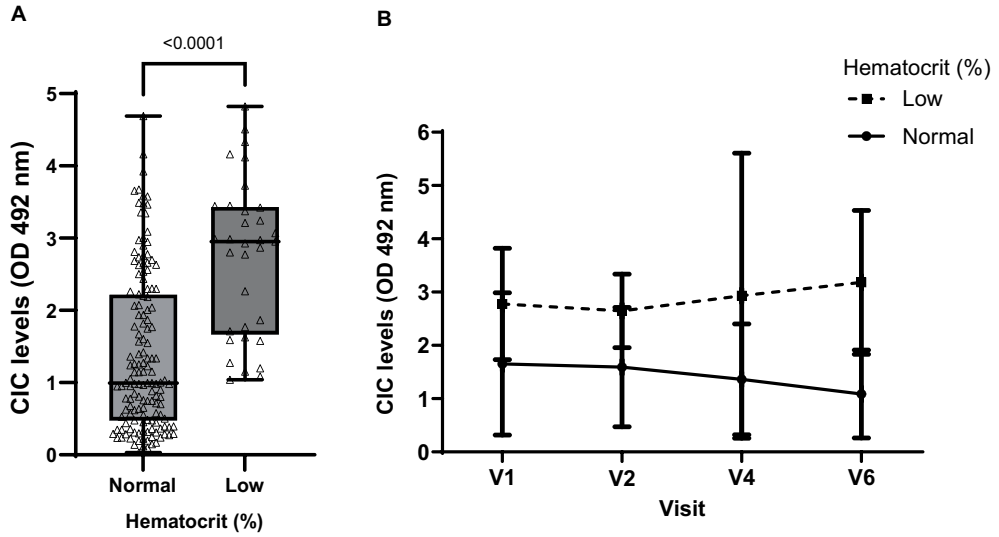


FIGURE 5
**(A)** CIC levels recorded in dogs with or without anemia (non-parametric Wilcoxon test). **(B)** Changes in CIC means produced during the study in dogs with or without anemia. Reference value hematocrit: 37–55%. V1 (Day 0), V2 (Day 30), V3 (Day 90), V4 (Day 180), V5 (Day 270), and V6 (Day 360).

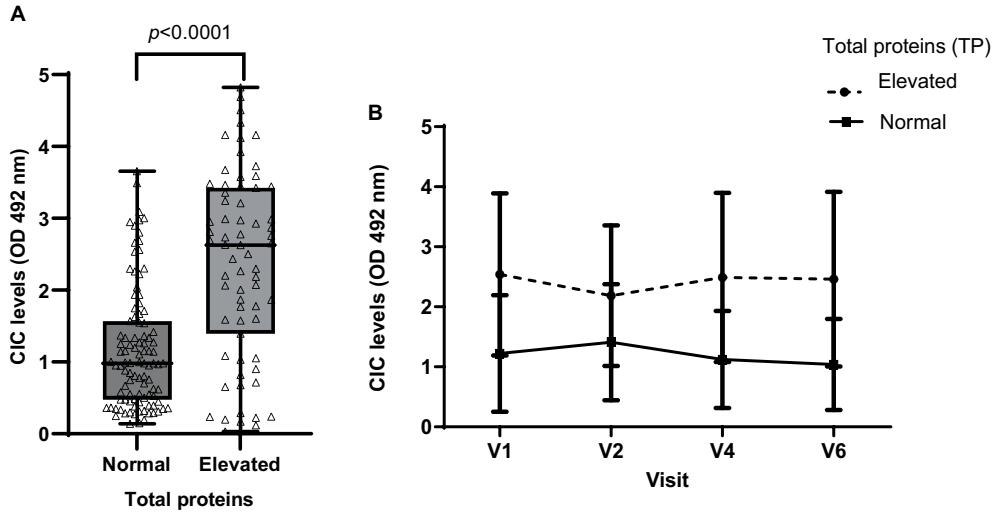


FIGURE 6
**(A)** CIC levels recorded in dogs with or without hyperproteinemia (non-parametric Wilcoxon test). **(B)** Changes in mean CIC levels produced during the study in dogs with or without hyperproteinemia. Normal reference value total proteins: 5,8–7,5 g/dL. Frequencies: Normal: 57.78%; hyperproteinemia: 37.22%. V1 (Day 0), V2 (Day 30), V4 (Day 180), and V6 (Day 360).

cessation (Figure 5). Finally, we observed moderate negative correlation between hematocrit and CIC level ($r = -0.547$).

Among the biochemical variables measured, creatinine was found elevated in only 8.57% of dogs, whereas alanine aminotransferase (ALT) was increased in 11.49%. No significant differences were found in CIC levels between dogs with normal or elevated creatinine ($p = 0.789$, $r = -0.327$) or ALT ($p = 0.434$, $r = -0.216$) levels.

Hyperproteinemia was detected in 37.22% of visits. Dogs displaying hyperproteinemia had significantly higher CIC levels ($p < 0.0001$) (Figure 6). At visit 3, which was the first follow up visit after the end of treatment, both groups showed reduced CIC levels. However, in dogs with dysproteinemia, these levels subsequently increased, while dogs without dysproteinemia consistently maintained lower CIC levels (Figure 6). Additionally, we found moderate positive correlation between CIC and total protein levels ($r = 0.533$, $p < 0.0001$).

Hypoalbuminemia was present in 22.02% of patients. Dogs with hypoalbuminemia displayed significantly more elevated CIC levels ($p = 0.02$) (Figure 7), and low negative correlation was found between CIC and albumin levels ($r = -0.306$).

Among the globulins, gamma globulin was the most affected with hypergammaglobulinemia present in 31.1% of animals, followed by hyperbetaglobulinemia (9.82%). Dogs with these abnormalities showed significantly higher CIC levels ($p < 0.0001$) (Figure 8), and we found positive correlation between CIC and beta ($r = 0.444$) and

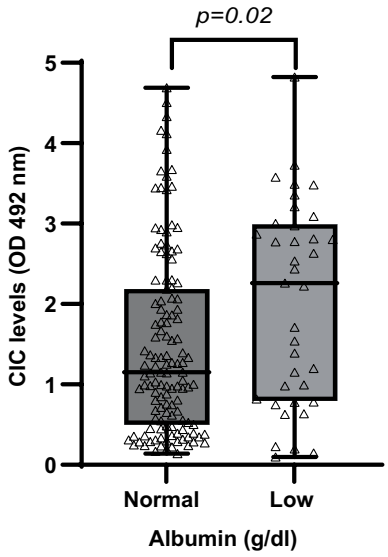


FIGURE 7
CIC levels recorded in dogs according to albumin level (non-parametric Wilcoxon test). Normal reference value albumin: 2.4–3.9 g/dL.

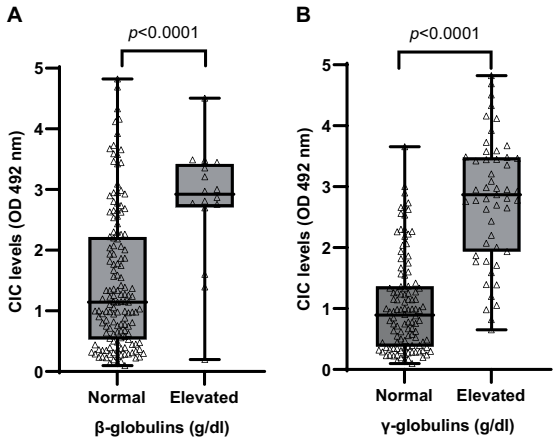


FIGURE 8
**(A)** CIC levels recorded in dogs according to the presence of beta-or **(B)** gamma-hyperglobulinemia (non-parametric Wilcoxon test). Normal reference values beta-globulins: 1.3–2.7 g/dL; gamma-globulins: 0.5–2 g/dL.

gamma ($r = 0.720$) globulin levels. Elevated alpha-1 and alpha-2 globulins were noted in 2.48 and 8.18% of dogs, respectively, but no differences in CIC levels were detected between dogs with normal or high blood alpha-1 globulins ($p = 0.960$). Dogs with elevated alpha-2 globulins had higher CIC levels ($p = 0.022$). No correlation was found between CIC levels and alpha-1 and alpha-2 globulins ($r = 0.021$; $r = -0.022$, respectively).

Among all variables examined, dysproteinemia was the most common laboratory finding, detected in 41.66% of animals. Dogs with dysproteinemia showed significantly higher CIC levels ($p < 0.0001$), and high negative correlation was found between CIC level and the A/G ratio ($r = -0.618$). When we assessed the effect of the level of dysproteinemia on CIC level (Figure 9), we found no differences between dogs with mild ($p = 1.0$), moderate ($p = 0.419$) or severe ($p = 0.121$) dysproteinemia. Throughout the study, CIC levels remained below 1.5 OD in dogs without dysproteinemia, while levels were consistently over 2.5 OD in those with severe dysproteinemia (Figure 9).

A large proportion (36.18%) of dogs showed a normal UPC ratio. Borderline proteinuria and proteinuria were recorded in 26.32 and 36.18% of the patients, respectively. Dogs with borderline proteinuria (UPC = 0.2–0.5) had higher CIC levels than dogs without proteinuria (UPC < 0.5) ($p = 0.035$, $r = 0.172$). However, no differences in CIC were found between dogs with borderline proteinuria or proteinuria ($p = 0.69$).

## 3.4 CIC and IFAT titres

The IFAT titres ranged from negative to 1:6400, with the cut-off set at >1/100. Significant differences in CIC levels were observed among groups showing negative, low, medium, or high antibody titres ($p < 0.0001$) (Figure 10), and there was strong positive correlation between CIC levels and IFAT titres ($r = 0.735$; $p < 0.0001$). Further, throughout the study, CIC levels remained consistently low in clinically healthy infected dogs returning negative IFAT results (Figure 10).

## 3.5 CIC and treatment

As CanL is a chronic disease, at the time of enrolment some dogs were already receiving treatment with allopurinol ($n = 15/44$), domperidone ($n = 1/44$) (22–24), nucleotides ($n = 5/44$) (25–28), prednisone ($n = 1/44$) or the angiotensin-converting enzyme inhibitor benazepril ($n = 2/44$). The treatment regimens of the dogs after enrolment in this study are detailed in Table 3.

The CIC levels were significantly higher in dogs treated with meglumine antimoniate ($p = 0.0036$) or allopurinol ($p = 0.015$) and in those with proteinuria treated with benazepril ($p = 0.0039$). Dogs treated with meglumine antimoniate or allopurinol or that received benazepril to control proteinuria were almost two times more likely to have high CIC levels (Table 4). In contrast, no differences were detected according to treatment with nucleotides ($p = 0.0519$) or prednisone ($p = 0.2312$).

## 3.6 CIC and treatment response

To assess the impacts of treatment in sick dogs, we also examined CIC levels over time. As the data were not normally distributed, we employed the non-parametric Friedman test. Nine of the 44 dogs, each missing one visit, were excluded from this part of the analysis. Our results show that mean CIC levels gradually decreased after treatment and remained relatively stable throughout the study period (Figure 11). Nevertheless, significant differences in CIC levels were only detected between the first (D0) and last visit (D360) (Friedman test $p = 0.022$) (Figure 11).

Of the 44 dogs initially included in our study, 28 were classified as good responders, 11 as poor responders and 5 could not be classified,

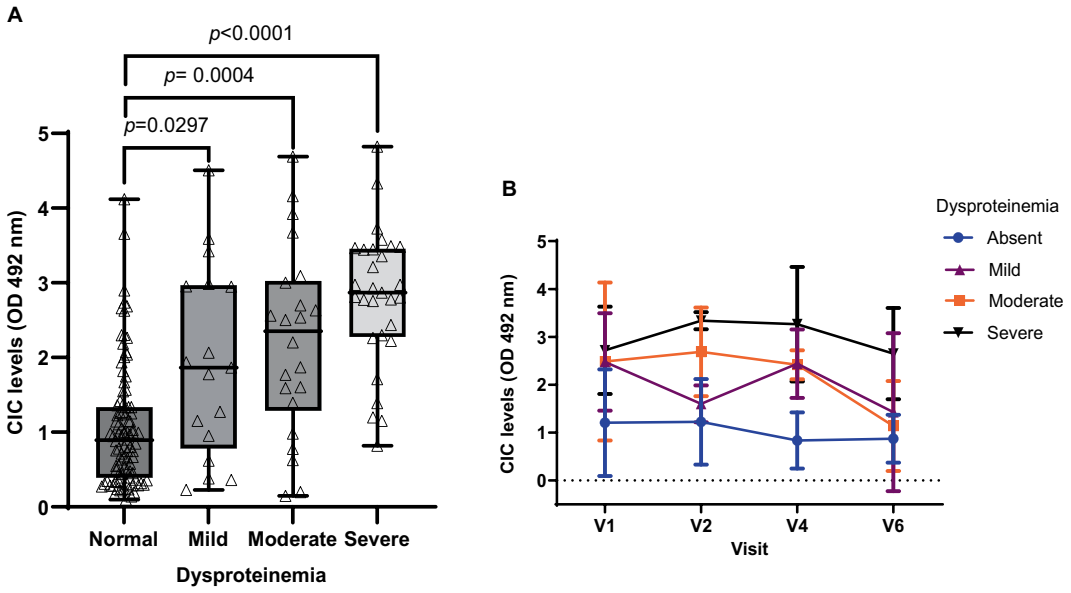


FIGURE 9
**(A)** CIC levels in dogs with a normal *A/G* ratio and different degrees of dysproteinemia (non-parametric Kruskal–Wallis test). **(B)** Changes in CIC means detected in dogs with a normal *A/G* ratio and different degrees of dysproteinemia. Normal *A/G* ratio > 0.8; mild dysproteinemia: 0.7–0.8; moderate dysproteinemia: 0.6–0.5; severe dysproteinemia: *A/G* < 0.5. V1 (Day 0), V2 (Day 30), V4 (Day 180), and V6 (Day 360).

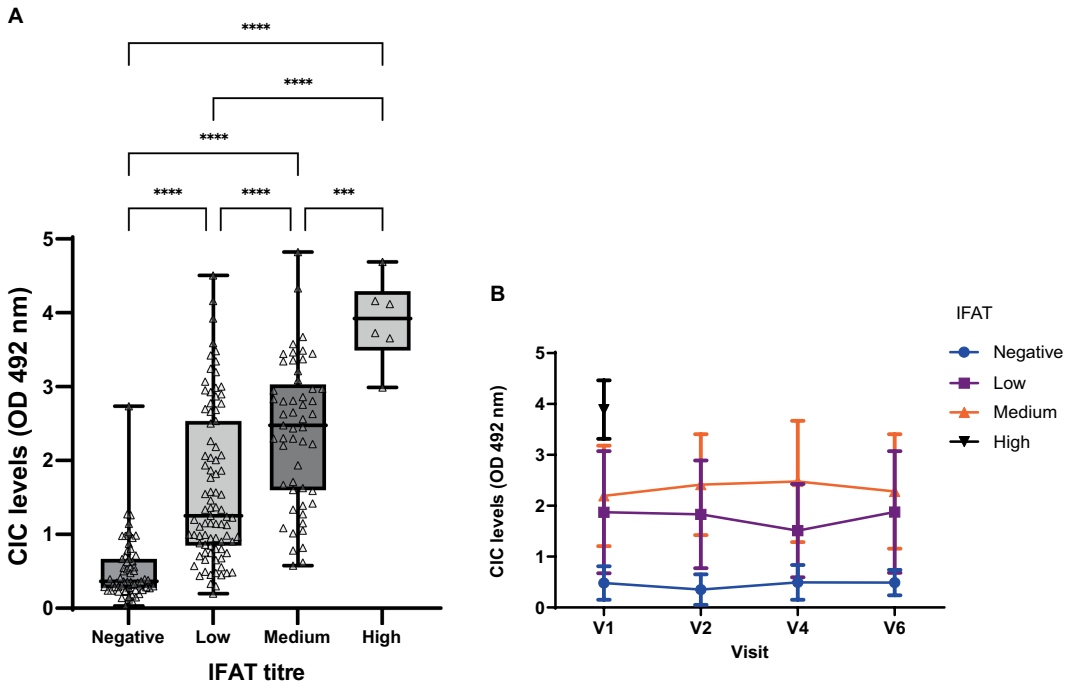


FIGURE 10
**(A)** CIC levels recorded in dogs showing different titres of antibodies against *L. infantum*. IFAT < 200 negative, 200–400 low, 800–1600 medium, >1600 high (non-parametric Kruskal–Wallis test; ***$p < 0.0005$, ****$p < 0.0001$). **(B)** Changes in CIC means produced during the study in dogs showing different antibody titres against *L. infantum*. High titres are not displayed due to the low number detected. V1 (Day 0), V2 (Day 30), V4 (Day 180), and V6 (Day 360).

as they were lost to follow-up. Good responders showed significantly lower CIC levels than poor responders ($p < 0.0001$) (Figure 12). Moreover, good responders showed lower CIC levels and they declined throughout the study (Figure 12). In poor responders, while CIC reduction was observed after treatment, levels progressively increased as early as 90 days later (Figure 12).

TABLE 3 Treatment regimens of dogs included in this study.

| Treatment post-enrolment | | Number of dogs |
|---|---|---|
| Meglumine antimoniate + allopurinol $n = 23/44$ (lost = 3) | + prednisone | 4/23 |
| | + nucleotides[1] | 7/23 |
| Allopurinol $n = 11/44$ (lost = 4) | + prednisone | 1/11 |
| | + nucleotides[1] | 4/11 |
| | + domperidone[2] | 1/11 |
| Nucleotides $n = 2$ (lost = 1) | | 2/44 |
| No treatment $n = 8$ (lost = 2) | | 8/44 |

[1]Impromune® (Bioiberica). [2]Leisguard® (Ecuphar).

TABLE 4 Relative risk of treatment regimens associated with a significant risk of elevated circulating immune complexes (CIC).

| Treatment | | Elevated CIC | | Relative risk [95% CI] | *p*-value |
|---|---|---|---|---|---|
| | | Yes | No | | |
| MGA | Yes | 16 | 6 | 1.90 [1.39–2.60] | 0.0018 |
| | No | 74 | 120 | | |
| Allopurinol | Yes | 64 | 72 | 1.44 [1.00–2.08] | 0.0361 |
| | No | 26 | 54 | | |
| Benazepril | Yes | 14 | 6 | 1.80 [1.28–2.52] | 0.007 |
| | No | 76 | 120 | | |

Repeated measures were treated as independent measures for relative risk calculation. MGA, meglumine antimoniate; CI, confidence interval.

## 3.7 CIC and relapses

Relapses were detected in 23.18% of the animals. At inclusion, 18 of the 44 dogs had had a previous diagnosis of CanL and were referred for treatment due to clinical relapse. Over the 12-month follow-up period, six dogs had one relapse and two had two relapses (Table 5).

Dogs experiencing relapse had significantly higher CIC levels compared to dogs not experiencing relapse ($p < 0.0001$) (Figure 13); these were elevated consistently in all visits (OD > 2) (Figure 13).

Interestingly, we detected markedly increased CIC levels in V4 (D180) (Figure 14), persisting into V5 (D270) and V6 (D360) and coinciding with the relapses recorded in these dogs (Table 5). In contrast, dogs not relapsing had CIC levels below 2 OD during the entire study period (Figure 14).

Importantly, through ROC curve analysis we were able to determine that CIC was a good discriminator of relapse (AUC = 0.808, $p < 0.0001$; 95% CI: 0.736–0.881) (Figure 15). Our results indicate that the optimal cut-off to correctly identify a relapse was 1.539 OD (sensitivity 82.4%; specificity 70.9%).

# 4 Discussion

To our knowledge, this is the first study to clinically validate the use of CICs as biomarkers of the progression of canine leishmaniosis.

TABLE 5 Number of relapses detected during the study and their time points.

| Dog ID | No of relapses | Time-point | Treatment |
|---|---|---|---|
| 01 | 2 | V4 (D180), V6 (D360) | MGA and allopurinol |
| 04 | 1 | V6 (D360) | MGA and allopurinol |
| 05 | 2 | V4 (D180), V6 (D360) | MGA and allopurinol |
| 06 | 1 | V6 (D360) | MGA and allopurinol |
| 20 | 1 | V4 (D180) | MGA and allopurinol |
| 29 | 1 | V6 (D360) | MGA and allopurinol |
| 32 | 1 | V5 (D270) | Initiation of allopurinol |
| 33 | 1 | V5 (D270) | MGA and allopurinol |

MGA, meglumine antimoniate.

Dogs in advanced stages of this disease show higher CIC levels (11) and a higher risk of multiple organ damage, which can significantly impair quality of life and dramatically reduce survival (6–8, 29). Currently, diagnostic methods for quantifying CIC are lacking. Parody et al. (11) introduced a non-invasive *Leishmania*-specific method of measuring CIC levels in serum samples from dogs with leishmaniosis. This method was recently laboratory validated following the recommendations of the NIH Biomarkers group (12). The method's specificity and robustness suggest this new biomarker could be useful not only for a diagnosis of CanL but also for tracking disease progression and potentially assessing treatment efficacy (13). In the present real-world study, we sought to clinically validate the performance of this new biomarker.

In our study, 34 dogs naturally infected with *L. infantum* at different LeishVet stages were followed for 12 months. Our data show that CIC levels are significantly higher in young adult dogs than senior dogs ($p < 0.001$) while no differences were detected according to sex or breed. The influence of sex, breed, and age on susceptibility to acquiring the infection has been explored in numerous studies with contrasting results (30–34). Nonetheless, the authors of two epidemiologic studies involving a large number of dogs, which took into account the presence of clinical signs of CanL, described that the age distribution for affected animals was bimodal, with a first peak corresponding to animals of around 3 years of age and a second, less evident, peak representing animals around 8 years old (30, 31). Age also seems to be an important risk factor associated with visceral leishmaniasis (VL) in humans according to two systematic reviews (35, 36) such that children are more susceptible to both infection and illness. Our results point to younger dogs controlling the disease less effectively and thus being more likely to have formed CIC.

Mean clinical scores progressively declined during the study and significant differences were detected in total clinical scores between visits. Moreover, we found positive correlation between clinical score and CIC levels ($r = 0.5135$, $p < 0.0001$). These results suggest that a decrease in clinical score will be accompanied by a drop in CIC levels. Nevertheless, some dogs had a low clinical score (≤5) despite having high levels of CIC (≥2 OD). The only clinical signs observed in these dogs were lameness, uveitis, skin ulcers, or polyuria/polydipsia. Hence, measuring CIC levels could be useful in dogs that do not have

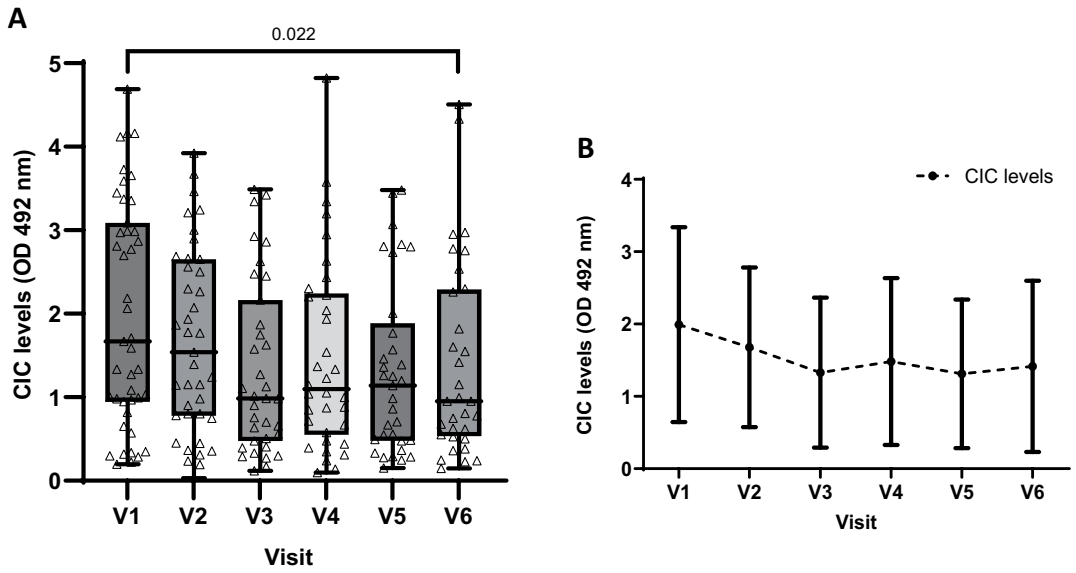


FIGURE 11
Box chart **(A)** and **(B)** line graph displaying changes in mean CIC levels recorded during the study. **(A)** Non-parametric Friedman test.

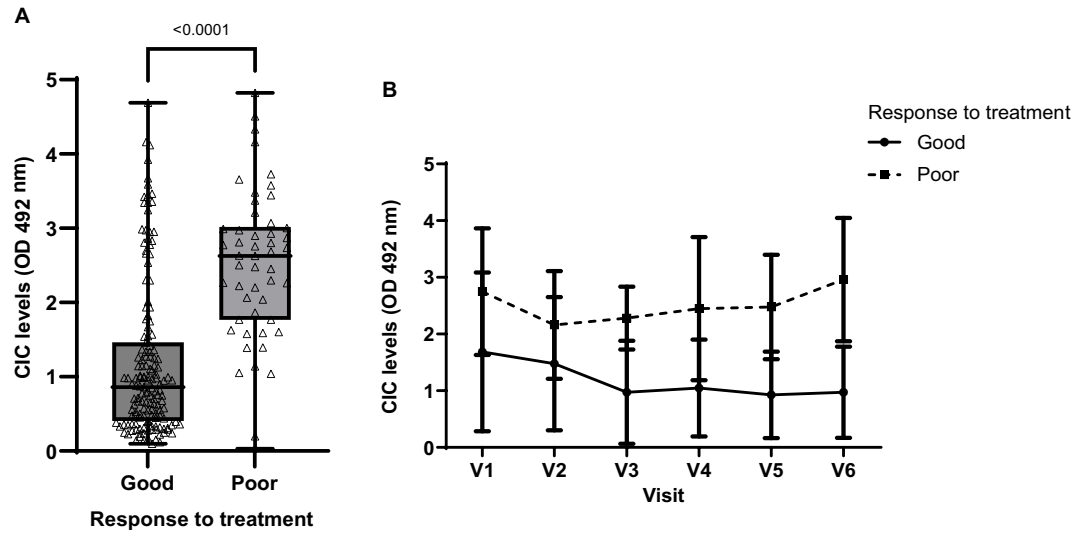


FIGURE 12
**(A)** CIC levels recorded in dogs showing a good or poor response to treatment (non-parametric Wilcoxon test). **(B)** Changes in CIC means produced during the study in dogs showing a good or poor response to treatment. V1 (Day 0), V2 (Day 30), V3 (Day 90), V4 (Day 180), V5 (Day 270), and V6 (Day 360).

an overall picture of generalized CanL yet show clinical signs of immune complex deposition. Future studies will bring light to the relationship between clinical scores and CIC levels.

The CIC levels were significantly lower in stage I dogs compared to stages II and III, consistent with the findings of Parody et al. (11), who reported a clear association between CIC levels and disease progression. In our study, we examined trends in CIC levels over 12 months in dogs at different clinical stages of CanL. Our results indicate that dogs in early disease stages have low CIC levels that remain low for long periods. Conversely, in advanced stages, dogs consistently show high levels of immune complexes, and increases may be observed as early as 90 days after initiating specific treatment. These results highlight the importance of close and frequent monitoring of CIC deposition in dogs in advanced stages of CanL, and suggest this new biomarker could be used to detect relapses.

Non-regenerative anemia is one of the most frequent clinical-pathological abnormalities reported in dogs with CanL. Some authors describe correlation between anemia or the severity of clinical signs (21, 37, 38) and parasite load (38–40). Dysproteinemia is also frequently observed in dogs with CanL (19–21). Protein

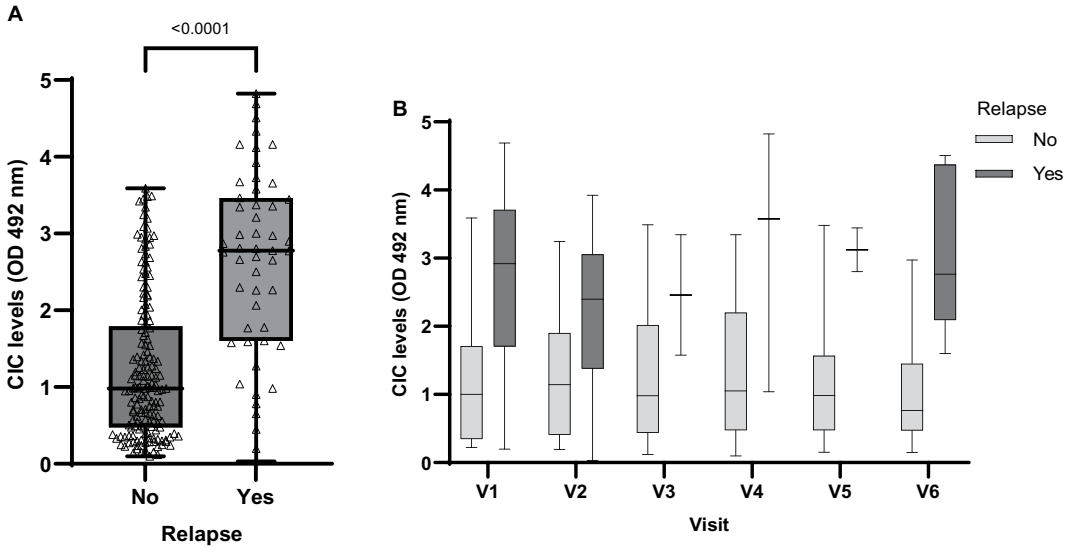


FIGURE 13
**(A)** CIC levels recorded in dogs experiencing or not experiencing relapse (non-parametric Wilcoxon test). **(B)** CIC levels recorded over the study period in dogs experiencing or not experiencing relapse. V1 (Day 0), V2 (Day 30), V3 (Day 90), V4 (Day 180), V5 (Day 270), and V6 (Day 360).

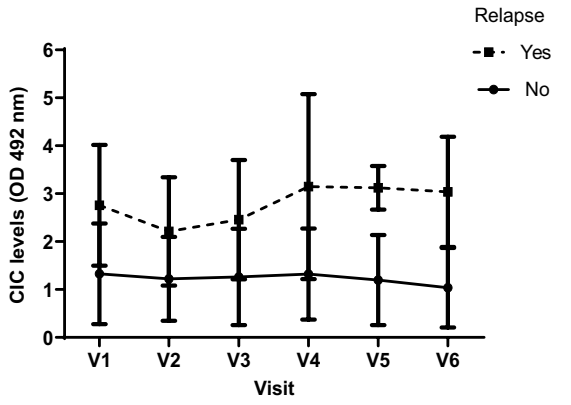


FIGURE 14
Changes in CIC means recorded over the study in dogs experiencing or not experiencing relapse. V1 (Day 0), V2 (Day 30), V3 (Day 90), V4 (Day 180), V5 (Day 270), and V6 (Day 360).

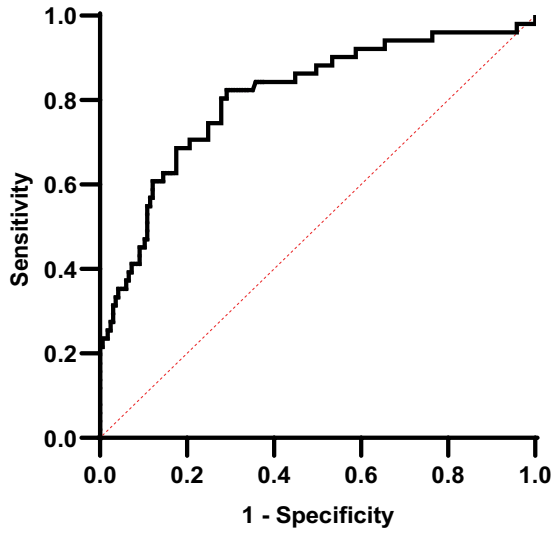


FIGURE 15
ROC curve analysis of relapse (AUC = 0.8085, $p < 0.0001$).

electrophoresis has proven extremely useful for the diagnosis and monitoring of CanL and is routinely performed in clinical practice (41–44). Indeed, several studies have shown that this technique may show abnormalities very early during the course of disease, even before the onset of overt clinical signs (21, 41, 42, 45, 46). In our study, we observed strong correlation between CIC and hematocrit ($r = 0.524$), total proteins ($r = 0.507$), beta- ($r = 0.426$) and gamma globulins ($r = 0.673$), and the $A/G$ ratio ($r = -0.647$). Further, our results also show that dogs with non-regenerative anemia and/or dysproteinemia have significantly higher CIC levels. When we examined the kinetics of these immune complexes, we noted that dogs with anemia and/or dysproteinemia showed higher CIC levels throughout the study despite specific treatment. Taken together, our findings point to the high reliability of this new biomarker, as it correlates with other tools used for the diagnosis and clinical management of CanL. Moreover, they confirm that dogs with anemia or dysproteinemia are at a higher risk of CIC deposition in specific organs.

The IFAT is one of the most used quantitative serologic techniques for the detection of anti-*Leishmania* antibodies and is considered the gold standard test for *Leishmania* infection. Extensive research has identified correlation between IFAT results and clinical signs (47–51), becoming more evident in severe clinical forms of CanL (51). In human VL, high anti-*Leishmania* antibodies also correlate with disease progression (4). Studies have also shown that IgG antibodies not only fail to protect against this intracellular parasite but also contribute to disease progression by reversing the inflammatory

cytokine profile of immune cells and inducing the production of high IL-10 levels via the receptor FcγRIII (4, 52). Here, significant differences in CIC levels were found among dogs showing negative, low, medium or high antibody titres, and high positive correlation was observed between CIC and IFAT results ($r = 0.735$). These findings are in line with those described by Parody et al. (11), who also noted high correlation between these two variables ($r = 0.754$). Thus, dogs with high antibody titres have significantly higher CIC levels and are thus at greater risk of their deposition in target organs.

Mean CIC levels dropped after treatment and remained under 1.5 OD throughout the study, although significant differences were only found between the first and the last visits. Likely reasons for this are: (1) the small number of animals included in the study, and thus reduced statistical power when using the Friedman test with Bonferroni correction; and (2) in dogs showing a poor response to treatment, CIC levels can remain elevated for several months. In this study, sick dogs were treated with antimonials in combination with allopurinol, which is the standard treatment for CanL in Europe (3). Four dogs treated with antimonials, and one treated with allopurinol also received prednisone for 1 month to control clinical signs of CIC deposition. Dogs treated with antimonials or allopurinol, or those that received benazepril to control proteinuria, had significantly higher CIC levels and were up to two times more likely to have elevated CIC levels in serum. This is not surprising as, except for allopurinol, these medications are used in more advanced stages of the disease. Due to the small number of animals that received treatment with prednisone, no conclusions can be made regarding the kinetics of CIC levels in these dogs. Further studies are needed to examine the optimal use of this new biomarker in tailoring and monitoring corticosteroid use in dogs experiencing CIC deposition.

Good responders had significantly lower CIC levels compared to poor responders ($p < 0.0001$), which progressively decreased over the study course. In poor responders, although a CIC reduction was observed after treatment, levels progressively increased as early as 90 days post-treatment. These results suggest that measuring CIC levels before and after treatment could serve to assess treatment effectiveness.

Dogs experiencing relapse after treatment had significantly higher CIC levels ($p < 0.0001$); over 2 OD in all visits. During the study, six dogs had one relapse and two had two relapses. Most relapses occurred in visit 6, 1 year after treatment. Nevertheless, three dogs had a relapse as early as at 6 months post-treatment. Studies have shown that the presence of immune complexes contributes to the establishment of chronic infections in murine models and human VL (4, 52). One key mechanism is their ability to stimulate macrophages and other immune cells to produce IL10. This cytokine reduces the expression of inducible nitric oxide synthase (iNOS) and the intracellular production of nitric oxide, which are both vital for eliminating intracellular *Leishmania* parasites. Moreover, IL-10 downregulates the Th1-associated IFN-γ response, which is essential to activate nitric oxide production within infected cells and eliminate the parasite. Consequently, dogs that relapsed had higher CIC levels, which may have been responsible for their inability to control the infection despite receiving a specific leishmanicidal treatment.

Importantly, our ROC curve analysis revealed that CIC were a good discriminator of relapse (AUC = 0.808) and that the optimal cut-off for accurately identifying relapse was 1.539 OD. This is relevant as the cut-off for positivity adopted in the study by Parody et al. (11) was 0.274 OD, which may be too low to be associated with clinical disease. In fact, CIC levels ranged between 0.8 and 1.0 OD in clinically healthy infected dogs, while sick dogs had levels between 1.9 and 3.1 OD. Further larger studies are needed to confirm our findings and establish definitive cut-offs to distinguish between healthy infected and sick dogs.

It is important to mention that our study did not include a formal sample size calculation due to the lack of prior data concerning expected effect sizes for the novel biomarker under investigation. However, our study yielded statistically significant results, providing valuable insights into the association between CIC clinical signs and clinicopathological parameters related to CanL. Moving forward, sample size calculations in future studies could enhance the robustness of our findings and contribute to a deeper understanding of CIC's role in the progression of the disease.

## 5 Conclusion

Our findings indicate elevated serum CIC concentrations in both young adult dogs and/or those in advanced stages of CanL with anemia and/or dysproteinemia. Dogs showing a favorable response to treatment showed declining CIC levels. Conversely, dogs displaying a poor treatment response and recurrent clinical relapses consistently exhibited high CIC levels. In these dogs, vigilant monitoring is essential to enable prompt and targeted treatment and prevent irreversible organ damage due to CIC deposition.

While studies in larger populations of dogs are still needed to confirm our findings, the use of CICs as a complementary biomarker to track disease progression in CanL is promising. Further studies using this new biomarker may provide insight into specific organ tropism and relapse mechanisms in non-responders. Such studies will also improve our understanding of long-lasting cell-mediated immunity in resistant dogs, and aid in the development of *Leishmania* vaccines.

## 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 Ethical committee Universidad Complutense de Madrid. 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

JS: Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing. NP: Investigation, Validation, Writing – review & editing. AM: Resources, Writing – review & editing. CC-L: Investigation, Validation, Writing – review & editing. JB: Resources,

Writing – review & editing. RC: Resources, Writing – review & editing. MD: Resources, Writing – review & editing. JC: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. GM: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

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## Conflict of interest

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## Publisher's note



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# Brucellosis in camel, small ruminants, and Somali pastoralists in Eastern Ethiopia: a One Health approach

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Brucellosis is a neglected bacterial zoonotic disease with economic and public health importance in pastoral communities of sub-Saharan Africa. A cross-sectional study was conducted from December 2021 to April 2022, to estimate the prevalence and identify the associated risk factors causing brucellosis in animals and associated with occupational diseases in humans from three selected districts of "the Somali Pastoral region," Eastern Ethiopia. In this study, 1,000 serum samples were screened for anti-*Brucella* spp. antibodies using Rose Bengal Plate Test (RBPT) and further confirmed using a competitive enzyme-linked immunosorbent assay (cELISA). A structured questionnaire was used to collect the biodata of tested animals and animal attendants to test the association between explanatory and outcome variables. The overall animal level prevalence was 5% (95% CI, 6.1–7.2.0) in small ruminants, 2.9% (95% CI, 1.5–4.9) in camels, and 2.0% (95% CI, 0.2–3.7) in occupationally linked humans. Herd size and herd history of retained fetal membranes were risk factors associated with *Brucella* spp. seropositivity in animals ($p < 0.05$). Disposing of retained fetal membranes was significantly associated ($p < 0.05$) with *Brucella* spp. seropositivity in humans. Evidence of brucellosis in various livestock species and associated seropositivity in humans indicates the need for a coordinated One Health approach, considering sociocultural dynamics of pastoral communities in controlling brucellosis to safe guard public health and increase livestock productivity.

KEYWORDS

**animals, brucellosis, occupationally linked humans, seroprevalence, Somali region**

## Introduction

Brucellosis is one of the re-emerging bacterial diseases that posing public and animal health problems in many pastoral settings. Currently, 12 *Brucella* spp. are included in the genus *Brucella* (1), of which, *B. abortus* in cattle, *B. melitensis* in goats and camel, *B. suis* in pigs, *B. ovis* in sheep, *B. canis* in dogs, *and B. neotomae* in rats are considered as classical (2). The disease can be transmitted between animals and from animals to humans by direct contact or indirect contact with contaminated materials. Due to close physical contact with animals and the tradition of consumption of unpasteurized milk, pastoralists are at highest risk of contracting the disease (3).

Currently, three classical species, *B. abortus*, *B. melitensis*, and *B. suis*, have an essential impact on public health. Being a public health concern that poses economic losses, brucellosis is a devastating disease that lacks pathognomonic symptoms in humans (4), making it difficult to differentiate from febrile conditions including malaria (5). Annually, approximately 500,000 human infections have been reported every year in low-income and middle-income countries (LMICs), where livestock is their mainstay. In LMICs, the disease is endemic and remains neglected, with huge public and animal health-associated problems (6).

The risk factors that influence the transmission, maintenance, and/or control of animal brucellosis are related to livestock management practices, animal movements, environmental factors, pastoralist behaviors and practices, lack of veterinary control measures, socioeconomic factors, genetic content of the animal host population, and biology of *Brucella* spp. (7). Risk factors for human brucellosis include, but are not limited to, the handling of infected animals and ingestion of contaminated animal products such as unpasteurized milk and milk products (including cow, goat, and camel milk) and meat (8).

In humans, the disease can lead to long-term complications, disability, and reduced productivity, resulting in potential income loss. It also has a negative impact on livestock production, reducing milk production, causing infertility, abortion, and poor growth rates, leading to decreased profitability in the agricultural industry. The correct diagnosis of brucellosis presents difficulties as its symptoms in humans are non-specific and can resemble other diseases. Laboratory tests may yield false-positive or false-negative results, delaying proper diagnosis and treatment initiation (9).

*Brucella* spp. infection causes huge financial losses and community health concerns in countries around the world. Globally, the economic losses due to brucellosis are substantial. According to the Food and Agriculture Organization (FAO), the estimated annual economic losses caused by brucellosis in livestock production, including cattle, goats, and sheep, can range from USD 200 to 600 million. These losses result from decreased productivity, increased veterinary costs, trade restrictions, and losses in animal products. In addition to livestock-related losses, severe health-related problems in humans, including life-threatening conditions, should be taken into account when dealing with brucellosis socioeconomic impacts. These include healthcare costs, such as hospitalization, medication, and follow-up care, as well as productivity losses due to morbidity, disability, and potential income loss (10).

In Ethiopia, brucellosis is one of the top five prioritized zoonotic diseases in Ethiopia (11). The animal brucellosis was first reported in the 1970s (12). Since then, many seroepidemiological studies from different regions of the country showed a prevalence report that ranges from 1.5 to 22.2%. Most of these reports were either from limited livestock species or relatively confined in a single environmental setting. There are few studies conducted on the seroepidemiology of brucellosis in Somali pastoral regions, and those involving epidemiology of brucellosis and its public health significance at the human–animal interface are scarce. In addition, the magnitude of the disease in different livestock species sharing the same environmental settings is not well studied. Therefore, understanding the epidemiology of the disease in mixed livestock populations and pastoralists in Somali region is of paramount importance. Hence, the objectives of this study are (i) to estimate the seroprevalence; (ii) to identify brucellosis-associated risk factors for the disease in camel, small ruminants, and pastoralist herders; (iii) to assess knowledge, attitude, and exposure risks of the herders toward the disease.

# Materials and methods

## Description of the study area

This study was conducted in selected districts of the Somali region: Goro Baqaqsa, Guradamole, and Dolo Ado of Liban Zone (Figure 1). The Liban zone is 887 km away from Addis Ababa, Ethiopia. The communities are pastoralists, rearing livestock as a livelihood, and means of income. The climate varies from arid to semi-arid, which is characterized by regular water and fodder shortages, forcing pastoralists to seasonal migration with their animals. The altitude ranges from 250 to 1,500 m above the sea level and is located between 6°00′N 43°45′E. The area experiences average annual rainfall of 600–700 mm. The main rainy season, known as "Gu," lasts from March to May, followed by the short dry season, known as "Xagaa," which lasts from June to August. The short rainy season "Dayr" occurs between September and November, and the long dry season "Jilaal" occurs between December and March (13).

Pastoralists own large, mixed livestock species of herds, on which their daily livelihood depends from a social, economic, and dietary point of view. Many pastoralists in Ethiopia migrate seasonally with their animals in search of grazing land and water and share pastures and watering points. The livestock production system in the region is influenced by traditional pastoralist practices and the dry environment. Pastoralists lead to a nomadic lifestyle, constantly moving their animals to find food and water. Cattle, camels, sheep, and goats are the main livestock species, well adapted to the dry climate, and provide meat, milk, and income for pastoralist communities. However, the livestock production system faces challenges, such as inadequate market infrastructure, long travel distances to reach markets, and environmental and socioeconomic issues such as recurring droughts, land degradation, limited access to water, insufficient veterinary services, and conflicts over resources (14).

## Study design and study population

A cross-sectional study was undertaken from December 2021 to April 2022 to estimate the prevalence of brucellosis in animals and occupationally linked humans in the Somali pastoral region of Ethiopia. Animal-level bio-data were collected using a structured questionnaire that included sex, age, herd size, animal movement, parity, herd history of abortion and retained fetal membrane (RFM), and physiological status of the animals. Age was categorized into young (<6 months in goats and sheep and < 4 years in camels) and old (≥6 months in sheep and goats and ≥4 years in camels), herd size was considered as small (<50) and large (>50), animal migration (yes or no), number of parity (Null, ≤3 and > 3), herd history of abortion and RFM (Yes or No), and finally, physiological status of the animal was classified as dry, lactating, and pregnant. Furthermore, to assess the public health impact and estimate the magnitude of the disease in occupationally associated humans, blood samples were also collected from the owners of the animals, and information such as gender, age,

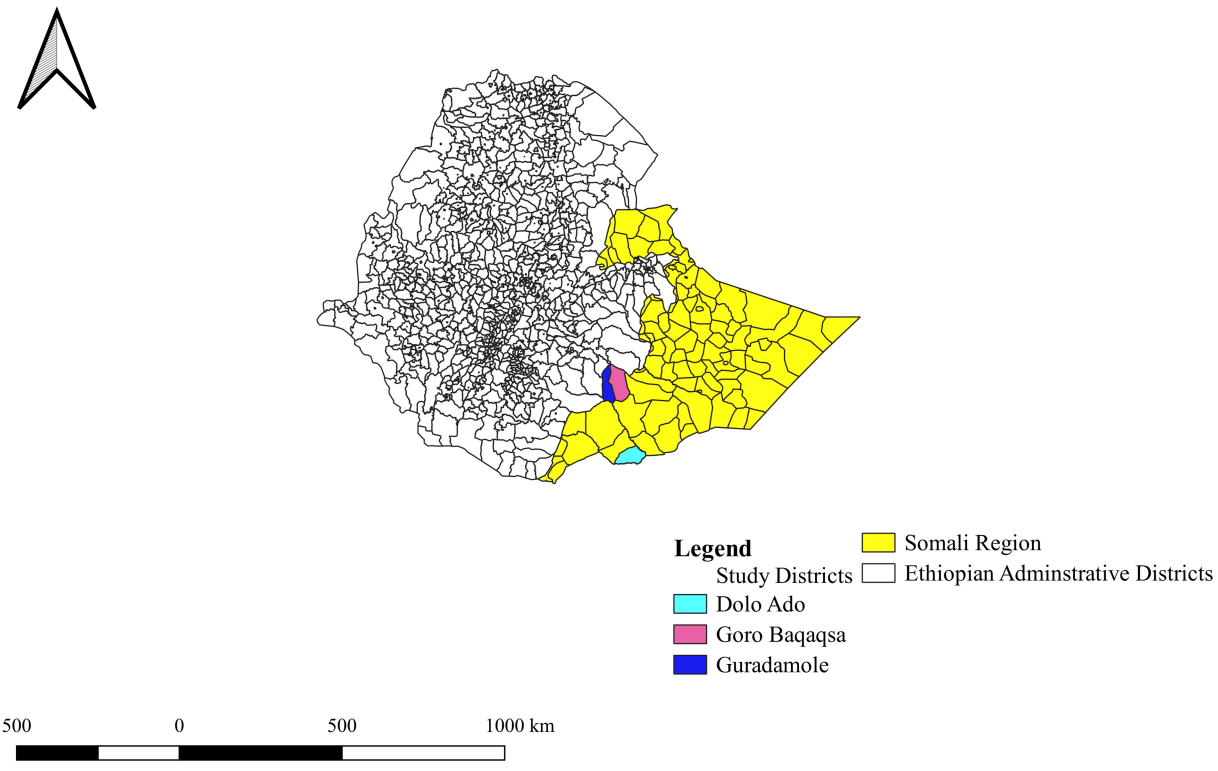


FIGURE 1
Map of the study areas. This map is extracted from Ethiopian shape file using QGIS version 3.20.0. Reproduced with permission of Ethiopian Mapping Agency.

the habit of consuming raw milk, assisting calving/birthing, and disposing of aborted fetuses and fetal material was recorded.

## Sampling method and sample size determination

In this study, a multistage sampling combined with the convenient sampling strategy was employed for sampling of individual animal species. Three districts were purposively selected based on livestock populations and proximity to the road. Two pastoralist associations (PAs) were randomly selected from each district, resulting in six PAs being included in the study. Within each selected PA, households were then selected based on the presence of more than two livestock species per household. This method ensured that the households were chosen more likely to have a diverse range of livestock. As a result, 60 households were included in the study. To gather data from these households, a further sampling process was conducted. From each household, a minimum of four people were randomly selected and sampled. Furthermore, a lottery system was used to select an individual animal from a herd, by assigning a number 1 (to be selected) or 0 (not to be selected) to an animal.

The number of animals in each household was considered as a herd and was sampled using a systematic random sampling technique. Sample size was determined using the expected prevalence of 7.5% in camels (11), 9.7% in sheep (15), and 16.5% in humans (12), by considering a 5% desired precision at a 95% confidence interval according to the formula previously published (16). Accordingly, 450 samples from camel, 300 samples from small ruminants, and 250 samples from humans were collected from the three districts.

## Sample collection and laboratory analysis

### Blood sample collection

To minimize error, a bar code system was developed for both human and animal samples. The code is an abbreviation that consists of the first letter of the region, zone, district, and PAs (SLGBB0001). A specific label was fixed to the vacutainer tube after blood collection. After restraining the animals properly and having disinfected the area of venipuncture with 70% alcohol, 10 mL and 4–5 mL of blood were drawn from the jugular veins of camels, sheep, and goats, respectively. The blood samples were then labeled and left tilted overnight at room temperature to allow for clotting. Sera were later decanted into sterile cryovials. For human samples, approximately 5 mL of blood was drawn by a qualified nurse at the PAs via venipuncture of the medium cuboidal vein using a plain vacutainer tube. The sera samples were then transported to Jigjiga Regional Veterinary Diagnostic and Research Laboratory in an ice box and stored at −20°C for further processing.

### Serological test

#### RBPT

The serum samples were screened for anti-*Brucella* antibodies using RBPT, according to the standard procedure described by Nielsen (17). Any visible agglutination was considered positive. Based on the

level of clumping, the results were read as weak, moderate, and strong agglutinations. For interpretations of the results, both positive and negative control sera were used as recommended by OIE (18). For the test, 30 µL of RBPT was used in camel. To improve the sensitivity of RBPT, one volume of antigen and three volumes of serum (e.g., 25 µL with 75 µL) were used in sheep and goats as recommended by Garin-Bastuji et al. (19). The antigen and test serum were thoroughly mixed using a plastic applicator for 4 min, and the result (presence of agglutination or not) was read immediately (18).

### Competitive ELISA

All the RBPT-positive animal and human sera samples were further tested at Jigjiga Veterinary Diagnostic and Research Laboratory using a commercial cELISA (Abbexa Ltd., Cambridge Science Park, and Cambridge, CB4 0EY, United Kingdom) and an IgG ELISA (Abbexa LTD, Cambridge, UK), respectively, following the manufacturer's protocol.

### Case definition

A sample was considered seropositive when it tested positive for RBPT and cELISA methods. A flock or herd was considered seropositive when at least one animal tested positive for both tests. Since vaccination against brucellosis is not practiced in Ethiopia, seropositivity in this study was considered to be due to natural infection.

## Data analysis

The data from the field and laboratory were entered into Microsoft Excel and analyzed using R software version R-3.3.0. Univariate logistic regression model was used to determine putative risk factors associated with *Brucella* spp. seropositivity. Multiple logistic regression was used to model the relationship between a binary dependent variable (Result) and multiple independent variables (age, sex, species, RFM, parity, abortion, physiological status, flock/herd size, and migration). The process involved data preparation, model specification, model estimation using maximum likelihood estimation, and interpretation of results through estimated coefficients and $p > 0.2$. As some variables that are individually insignificant but could potentially be significant in multivariable analysis, a cutoff value of $p$ was inflated to a value $\leq 0.2$. Multiple logistic regression allows for understanding the relationships between the dependent variable and multiple independent variables, accounting for interactions and potential confounding effects. For variables that showed strong colinearity ($p < 0.05$), one of the two variables was excluded based on biological plausibility to *Brucella* infection. Stepwise backward elimination procedure was employed for the selection of variables in the final model. The strength of association of exposure variables with seropositivity of the disease was assessed using odd ratios.

# Results

## Descriptive statistics of seroprevalence

A total of 450 samples from camel, 300 samples from small ruminants, and 250 samples from humans collected from the three districts were tested for anti-Brucella antibodies. The overall seroprevalence was 5% (95% CI = 6.1–7.2.0) in small ruminants, 2.9% (95% CI = 1.5–4.9) in camel, and 2.0% (95% CI = 0.2–3.7) in occupationally linked humans. The highest seroprevalence of 6.5% (95% CI = 3.5–10.8) was observed in goats compared with camels 2.9% (95% CI = 1.5–4.9) and sheep 2.0% (95% CI = 0.2–7.1). Regarding districts, the overall seroprevalence of the disease in livestock was lowest in Dolo Ado (3.2%) compared with the other two districts with 4% in each.

The distribution of seroreactor animals and humans varied among the three districts. Goro Baqaqsa district had the highest proportion of seropositive sheep and goats (6, 95% CI = 2.2–10) and humans (2.8, 95% CI = 0.3–9.6) compared with the other districts. Dolo Ado had the highest (3, 95% CI = 1.1–7.6) seropositive camels compared with the other two districts. However, it had the lowest seroprevalence in humans (1, 95% CI = 0.02–5.4) and sheep and goat (3, 95% CI = 0.6–8.5). When pastoral village was considered, seropositive animals were found in 83% (5/6) of the villages. Village level seropositivity was more frequently detected in sheep and goats (5%) than in camel (2.9%). The seroprevalence ranges from 0 to 9.8% in sheep and goats and from 0 to5.9% in camels (Supplementary Table S1).

## Risk factors for *Brucella* spp. seropositivity in sheep and goats

The major variables that were considered in the univariable logistic regression analysis include district, sex, species, age, herd size, animal mobility or migration, parity, physiological status, history of abortion, and retained fetal membranes. The result showed that herd history of RFM was significantly associated with *Brucella* spp. seropositivity ($p < 0.05$; Table 1).

In the final multivariable logistic regression model, all variables with a value of p less than or equal 0.2 on the univariate analysis were included. The result indicated that small ruminants from a large herd were 5.01 times more likely to acquire *Brucella* spp. infection compared with those kept in a small herd (OR: 5.01, 95% CI: 1.2–21.4, $p = 0.02$). Similarly, sheep and goats with a history of retained fetal membranes were more likely to be seropositive for *Brucella* spp. infection than sheep and goats without a history of retained fetal membranes (OR: 9, 95% CI: 1.9–42; Table 2).

## Risk factors for *Brucella* spp. seropositivity in camels

The univariable logistic regression analysis indicated that seropositivity in camels was significantly associated with pregnancy (OR = 3.3, 95% CI: 1.4–15, $p < 0.05$), history of abortion (OR = 3.9, 95% CI: 1.2–13, $p < 0.05$), and RFM (OR = 22, 95% CI: 5.9–8, $p < 0.05$; Table 3). Multivariable logistic regression model using variables with value of $p \leq 0.2$ from univariate analysis indicated that history of RFM had a significant association with *Brucella* spp. seropositivity (95% CI: 12.7–60, $p < 0.05$; Table 4).

## Serological survey for human brucellosis

Higher seroprevalence was observed in female individuals, 2.5% ($n = 4$) compared with male, 1.1% ($n = 1$). Participants from households

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TABLE 1 Univariable logistic regression analysis for brucella seropositivity in small ruminants.

| Variables | Category | Samples | No positive (%) | OR (95% CI) | Value of *p* |
|---|---|---|---|---|---|
| | Dolo Ado | 100 | 3 (3) | Ref | |
| Districts | Goro Baqaqsa | 100 | 6 (6) | 2.0 (0.5–8.5) | 0.32 |
| | Guradamole | 100 | 6 (6) | 1 (0.4–3.3) | 1.00 |
| Species | Ovine | 99 | 2 (2) | Ref | |
| | Caprine | 201 | 13 (6.5) | 0.9 (1–21) | 0.21 |
| Sex | Male | 137 | 3 (2.5) | Ref | |
| | Female | 163 | 12 (7.4) | 3.5 (1.0–12.8) | 0.05 |
| Age | Young | 121 | 4 (3.3) | Ref | |
| | Adult | 179 | 11 (6.1) | 2 (0.6–7) | 0.27 |
| Flock size | Small | 156 | 5 (3.2) | Ref | |
| | Large | 144 | 10 (6.4) | 3.14 (1–11) | 0.05 |
| Migration | No | 177 | 8 (6.8) | Ref | |
| | Yes | 83 | 7 (3.8) | 0.96 (0.3–3.0) | 0.94 |
| Parity | >3 | 59 | 3 (5.1) | Ref | |
| | ≤3 | 104 | 9 (8.7) | 1.43 (0.3–4.6) | 0.53 |
| Abortion | Not aborted | 128 | 4 (3.1) | Ref | |
| | Aborted | 35 | 8 (23) | 3 (0.9–9.7) | 0.08 |
| RFM | No | 134 | 2 (1.5) | Ref | |
| | Yes | 29 | 10 (33.3) | 8.2 (2.4–29) | **0.00*** |
| PS | Lactating | 38 | 2 (5.2) | Ref | |
| | Dry | 84 | 6 (7.1) | 0.4 (0.02–2.0) | 0.40 |
| | Pregnant | 41 | 4 (9.8) | 1.4 (0.4–4.6) | 0.46 |

Reference category: *Statistically significant. PS, physiological status; RFM, retained foetal membrane.

TABLE 2 Multivariable logistic regression analysis for brucella seropositivity in small ruminants.

| Variable category | Category | Samples | No positive (%) | OR (95% CI) | Value of *p* |
|---|---|---|---|---|---|
| Flock size | Small | 156 | 5 (3.2) | | |
| | Large | 144 | 10 (6.4) | 5.01 (1.2–21.4) | **0.02*** |
| Abortion | Not aborted | 128 | 4 (3.1) | Ref | |
| | Aborted | 35 | 8 (23) | 3 (0.9–9.7) | 0.08 |
| RFM | No | 134 | 2 (1.5) | | |
| | Yes | 29 | 10 (33.3) | 8.2 (2.4–29) | **0.00*** |

*Statistically significant.

with seropositive animals had seven times more risk of being seropositive for *Brucella* spp. infection than those without seropositive animals (OR = 7, 95% CI: 0.2–13.7, $p = 0.72$). Participants who consumed raw milk had 5.7 times higher odds of *Brucella* spp. seropositivity compared with those who consumed pasteurized milk (OR = 5.7; 95% CI = 0.9–35, $p = 0.06$); however, this was not statistically significant. Individuals who assisted during calving had higher odds of *Brucella* spp. seropositivity than those who had not higher odds of *Brucella* spp. seropositivity (OR: 3.6, 95% CI: 0.58–22, $p = 0.16$), and this was not statistically significant. The multivariable logistic regression analysis indicated that handling and disposing of aborted fetal materials was significantly associated with *Brucella* spp. seropositivity in humans (95% CI: 4.0–45, $p = 0.001$; Tables 5, 6).

## Discussion

This study showed an overall brucellosis seroprevalence of 5% (95% CI: 6.1–7.2) in camel, sheep, goats, and humans in three districts of Somali region, Ethiopia. Two tests were used serially to rule out false-positive cross-reactions and maintain maximal specificity (12, 13). A combination of RBPT and c-ELISA was used to test camel and human sera, whereas modified mRBPT and c-ELISA were used to test sera samples from sheep and goats. RBPT was used as a screening test because of its high sensitivity (20). Competitive ELISA was used by its high specificities to exclude false-positive cross-reactions. False-positive serological reactions in RBT could be due to cross-reactions with smooth lipopolysaccharide (S-LPS) antigens of other

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TABLE 3 Univariable logistic regression analysis for brucella seropositivity in camel.

| Variables | Category | Samples | No positive (%) | OR (95% CI) | *p*-value |
|---|---|---|---|---|---|
| Districts | Goro Baqaqsa | 150 | 4 (2.7) | Ref | |
| | Guradamole | 150 | 4 (2.7) | 1.1 (0.2–4) | 0.73 |
| | Dolo Ado | 150 | 5 (3.3) | 1.2 (0.3–4.8) | 1.0 |
| Sex | Male | 151 | 1 (0.7) | Ref | |
| | Female | 299 | 12 (4.0) | 0.2 (0–1.2) | 0.07 |
| Age | Young | 256 | 5 (2.0) | Ref | |
| | Adult | 194 | 8 (4.1) | 2.2 (0.7–6.7) | 0.18 |
| Herd size | Small | 162 | 3 (1.9) | Ref | |
| | Large | 288 | 10 (3.5) | 1.9 (0.5–7) | 0.33 |
| Migration | No | 185 | 5 (2.7) | Ref | 0.84 |
| | Yes | 265 | 8 (3.0) | 1 (0.4–3.5) | |
| Parity | Null | 159 | 4 (1.9) | Ref | |
| | ≤3 | 124 | 7 (5.6) | 2.3 (0.6–8) | 0.18 |
| | ≥3 | 16 | 1 (6.3) | 2.6 (0.3–24) | 0.40 |
| Abortion | Not Aborted | 250 | 7 (2.8) | Ref | |
| | Aborted | 49 | 5 (10.2) | 3.9 (1.2–13) | **0.02*** |
| RFM** | No | 285 | 7 (2.5) | Ref | |
| | Yes | 14 | 5 (35.7) | 22 (5.9–8) | **0.00*** |
| Physiology | Dry | 200 | 4 (2) | Ref | |
| | Pregnant | 51 | 5 (9.8) | 5.3 (0.7–20) | **0.01*** |
| | Lactating | 48 | 3 (6.3) | 3.3 (1.4–15) | 0.13 |

*Statistically significant. **Retained fetal membranes.

TABLE 4 Multivariable logistic regression for brucella seropositivity in camel brucellosis.

| Variables | Category | Samples | No positive (%) | OR (95% CI) | *p*-value |
|---|---|---|---|---|---|
| Age | Young | 256 | 5 (2.0) | | |
| | Adult | 194 | 8 (4.1) | 1.7 (0.1–31) | 0.709 |
| Parity | Null | 159 | 4 (1.9) | | |
| | ≤3 | 124 | 7 (5.6) | 0.4 (0.0–3.2) | 0.350 |
| | ≥3 | 16 | 1 (6.3) | | |
| Abortion | Not aborted | 259 | 7 (2.8) | | |
| | Aborted | 49 | 5 (10.2) | 0.7 (0.1–5.6) | 0.80 |
| RFM | No | 285 | 7 (2.5) | | |
| | Yes | 14 | 5 (35.7) | 35 (12.7–60,) | **0.004*** |
| PS | Lactating | 48 | 3 (6.3) | | |
| | Dry | 200 | 4 (2) | | |
| | Pregnant | 51 | 5 (9.8) | 1.6 (0.5–4.6) | 0.43 |

*Statistically significant.

gram-negative bacteria. As there has never been history of vaccination in Ethiopia, seropositivity in all cases is due to natural infection (15, 21).

In this study, the animal level seroprevalence of 5% detected in small ruminants was comparable with the report by Traoré et al. (22) in Mali, who reported a prevalence of 4.1%. However, the prevalence estimated in the current study is higher than a prevalence with the report of 3.33% by Dosa et al. (23) from Southern Nation Nationalities and People (SNNP) region in Kolme and Abala Abaya districts, 0.24% by Geletu et al. (24) from Eastern Hararge, Oromia Region, and 0.9% by Girmay et al. (25) from sheep export farm in Adama, and 0.4% by Yeshwas et al. (26) from Bahir Dar. On the other hand, a higher prevalence of 12.35 and 13.7% than the present study was reported by Tegegne et al. (27) and Tedeg et al. (28) in Afar pastoral region, respectively. The observed differences in seroprevalence might be due to variation in the

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TABLE 5 Univariable logistic regression analysis for brucella seropositivity in humans.

| Variables | Categories | Samples | No positive (%) | OR (95% CI) | *p*-value |
|---|---|---|---|---|---|
| Districts | Dolo Ado | 100 | 1 (1) | Ref | |
| | Goro Baqaqsa | 72 | 2 (2.7) | 1.4 (0.2–10.0) | 0.74 |
| | Guradamole | 78 | 2 (2.6) | 0.6 (0.1–7.0) | 0.50 |
| Gender | Male | 89 | 1 (1.1) | Ref | |
| | Female | 161 | 4 (2.5) | 7.5 (0.8–68) | 0.07 |
| Age | 18–60 years | 177 | 1 (0.6) | Ref | |
| | <18 years | 37 | 1 (2.7) | 0.2 (0.1–3) | 0.25 |
| | >60 years | 36 | 3 (8.3) | 5.0 (0.3–87) | 0.33 |
| Seropositive animals at household | No | 196 | 2 (1.0) | Ref | |
| | Yes | 54 | 3 (1.9) | 7 (0.2–13.7) | 0.72 |
| Consume raw milk | No | 59 | 1 (1.7) | Ref | |
| | Yes | 191 | 4 (2.1) | 5.7 (0.9–35) | 0.06 |
| Ass. Calving | No | 210 | 3 (1.4) | Ref | |
| | Yes | 40 | 2 (5) | 3.6 (0.58–22) | 0.16 |
| Dispose RFM | No | 234 | 2 (0.9) | Ref | |
| | Yes | 16 | 3 (19) | 26 (4.0–45.6) | **0.001*** |

*Statistically significant.

TABLE 6 Multivariable logistic regression analysis for brucella seropositivity in humans.

| Variables | Categories | Samples | No positive (%) | OR (95%CI) | *p*-value |
|---|---|---|---|---|---|
| Districts | Goro Baqaqsa | 72 | 2 (2.7) | | |
| | Guradamole | 78 | 2 (2.6) | 2.6 (0.4–14) | 0.26 |
| | Dolo Ado | 100 | 1 (1) | | |
| Gender | Male | 89 | 3 (3.4) | | |
| | Female | 161 | 2 (1.2) | 14 (0.8–25.5) | 0.06 |
| Consume | No | 159 | 2 (3.7) | | |
| Raw milk | Yes | 191 | 3 (1.5) | 2.5 (0.9–68) | 0.56 |
| Assist birthing or | No | 210 | 3 (1.4) | | |
| Calving | Yes | 40 | 2 (5) | 8 (0.6–127) | 0.11 |
| Dispose RFM | No | 234 | 2 (0.8) | | |
| | Yes | 16 | 3 (23) | 37.4 (5.1–18.5) | **0.001*** |

*Statistically significant.

sensitivity and specificity tests used, geographic location, and sample size.

In this study, larger herd/flock size was found to have a higher seroprevalence (3.5%) than a smaller herd size (2.5%). This is in agreement with the findings by Traoré et al. (29) who reported 6.9 and 4.8%, respecively. However, this is inconsistent with the study by Rob et al. (30) who reported 19.35% in smaller and 6.45% in larger herds. This variation in the prevalence could be attributed to an increase in stock density in large herd sizes, which facilitates transmission of *Brucella* spp. infection during calving or abortion. Furthermore, this variation could be influenced by fluctuations in disease prevalence at the overall animal level and the herd size during the study period.

There was a significant association ($p < 0.001$) between *Brucella* spp. seropositivity and a history of RFM as previously reported (20, 29, 31). On the contrary, Deddefo et al. (32) reported that a history of RFM had no association with *Brucella* spp. seropositivity. The difference could be due to variations in physiological status of the studied population. However, this study is in agreement with Weken et al. (33) who reported that a history of retained fetal membranes was significantly associated with *Brucella* spp. seropositivity in small ruminants ($p = 0.04$). When abortion is caused by *Brucella* spp. infection, the placenta is frequently retained, and there is inflammation of the uterine wall (metritis). This explains that retained fetal membrane is a sequel of brucellosis (34).

In the current study, the overall animal level seroprevalence of 2.9% was detected in camels. This is similar to the reports of previous studies (23–26) conducted in similar agroecology in Ethiopia. Conversely, Bekele et al. (35) and Hadush et al. (36) reported a higher animal-level prevalence of 5.4 and 4.1%, respectively, in Afar pastoral region. The observed differences in seroprevalence could be due to

differences in herd size, absence or presence of infectious foci, such as *Brucella*-infected herds, sample size, and sensitivity and specificity of tests used.

This study also showed that female camels had higher seroprevalence of brucellosis (4%) than males (0.7%). The same trend of a higher prevalence was observed in the report by Waktole et al. (37) with prevalence of 9.2 and 7% in female and male animals, respectively. This could be explained by the longer period in which female camels are kept in herds for breeding purposes compared with male camels. In the latter cases, camels are usually fed and sold off, except for few individuals that are kept for breeding purposes, haulage, and transport purposes (38). However, in this study, the findings are inconsistent with the report by Bekele et al. (35), who reported a higher prevalence in male camels than females. The differences in the proportion of male and female animals sampled could also contribute to the observed variations in the seroprevalence of camel brucellosis in different sexes.

In natural hosts, brucellosis is characterized by reproductive losses such as infertility, abortion, and birth of weak offspring (30, 39). In this study, seropositivity to *Brucella* spp. infection was significantly associated with RFM and history of abortion of camels, as previously reported in Ethiopia (24, 40). Abortion due to brucellosis is linked to the ability of the bacterium to adapt to the intracellular replicative niche typically characterized by low pH and reactive oxygen intermediates (41).

In general, seropositivity to *Brucella* spp. infection varied among different districts, animal species, and pastoral villages. This could be attributed to the difference in the herd size and sample size tested per visited households. Somali pastoralists move their livestock to different villages, districts, or even cross-national and international borders by travelling several kilometers in search of better pasture and watering points during short drought cycles driven by climate changes. This results in concentration of animals in specific areas, a factor that facilitates spillover of *Brucella* spp. from infected animals to susceptible populations. This, in turn, results in an emergence of new infectious foci contributing to the variability in seroprevalence of brucellosis among different villages and districts.

Wildlife and domestic animal population sharing same ecology in a traditional livestock production system was reported to be an important risk factor for transmission of brucellosis. The transmission of brucellosis between wildlife and domestic animals in a traditional livestock production system could occur by direct contact, as infected wildlife and the domestic counterparts may come into contact when sharing the same grazing areas. Additionally, environmental contamination can take place when infected wildlife shed *Brucella* spp. through bodily secretions such as urine, feces, or placental fluids, thus exposing domestic animals that come into contact with these contaminated sources (14, 33). In this study, at least two seroreactor animal species were identified in villages and households visited. Though the possibility of host-switching of *Brucella* spp. cannot be ruled out (42), particularly when different animal species mix so freely, the findings of the current study may suggest that *B. abortus* and *B. melitensis* circulate in this pastoralist population (43). This warrants more research studies, particularly molecular detection in the study areas to determine the prevalent *Brucella* spp. strains, which will also be essential before embarking on any vaccination program.

The overall prevalence of 2.0% was recorded in occupationally linked humans in the study area ($n = 5/250$; CI = 0.0–0.04), indicating the public health importance of the disease in this pastoral setting. This is comparable with the findings by Edao et al. (44), who reported 2.6% in Borana, and Ibrahim et al. (13), who reported 2.8% in the Somali region. However, Tschopp et al. (45) reported a higher prevalence of 48.3% in Afar and 34.9% in Somalia region. The difference observed could be attributed to the degree of endemicity of the disease in the livestock population, degree of exposure, sample size, the difference in location, variability related to the type of diagnostic test used, and the different time period when the studies were conducted.

Older participants had higher prevalence (8.3%) than middle aged (2.7%) and young (0.6%) people. This finding is in agreement with Yapi et al. (22) from Mali, who reported 9.5% in old age, 6% in middle age, and none in young people. The increase in seropositivity of participants in the old age group could be due to an increasing exposure risk associated with an increase in age (46). Because *Brucella* spp. are known to prefer the reproductive organs of female animals, particularly the placenta and aborted tissue, it is reasonable to assume that improper disposal and handling of aborted fetuses and fetal membranes would increase the risk of transmission (47). Individuals who had close contact with RFM while disposing had a 26-fold higher risk of acquiring brucellosis compared with those who did not. This finding fairly disagrees with the report by Edao et al. (44) probably because the number of respondents and the level of awareness in the study areas were different.

Brucellosis in humans was reported to be associated with the consumption of unpasteurized milk (29, 48). The practice of consumption of raw milk is common among Somali pastoral communities ascribed to a belief that milk would lose its nutritional contents when pasteurized. This study indicated that 76.4% ($n = 191$) of participants had consumed raw milk; however, this practice was not significantly associated with seropositivity. The large proportion of participants who consumed raw milk could therefore indicate a potential risk of acquiring zoonotic infections including brucellosis. Reproductive organs such as placenta are known to be a predilection site for *Brucella* spp. Assisting animals during calving or birthing could therefore increase the risk of infection (47). Multivariate logistic regression model indicated a significant association with *Brucella* spp. seropositivity practice of assisting during calving or birthing (OR = 8; 95% CI = 0.6–127). This finding is in agreement with previous studies conducted in Northern Tanzania by Cash-Goldwasser et al. (49) and in Kenya by Muturi et al. (50) that showed assisting calving or birthing would increase the risk of infection.

The source of infection of humans with *Brucella* spp. is often a close contact with infected animals (21, 25, 26). In light of this, the present study revealed that seropositivity in humans was seven times higher in households with seropositive animals compared with those without seropositive animals. This is in agreement with the report by Osoro et al. (47) in Kenya and Edao et al. (44) in Ethiopia.

In conclusion, the current study revealed that antibodies against *Brucella* spp. in camels, sheep, and goats, sharing the same ecological zone and occupationally linked pastoralists in Somali Region, Ethiopia. Herd size and history of RFM were found to be risk factors for brucellosis in animals. Contact with RFM was significantly associated with *Brucella* spp. seropositivity in humans. The recurrent drought in the region triggered by climatic changes that contributes to the mobility of mixed livestock population in search of feed, and water will likely continue to enhance the endemicity of the disease in the area. Extensive epidemiological studies involving One Health approach need to be undertaken to isolate and characterize circulating

*Brucella* spp. among humans and livestock in the study area. This would help to identify the transmission dynamics of *Brucella* spp. among the traditional mixed livestock production system. In this study, existence of close contact between humans and animals in the pastoral community and wide prevalence of brucellosis in various livestock species remarkably indicated the potential risk of public health. Therefore, feasible control strategy of the disease in respect of pastoral community and the sociocultural status through One Health approach is highly recommended.

## Limitations

In some villages of the study area, pastoralists refused to consent to allow blood sample collection from their herds contending that this practice could impede productivity of their animals. Therefore, the desired sample size has not been reached, particularly human and camel sample. The number of districts and villages surveyed was limited to areas with less security concerns. Hence, the results are non-generalizable.

## 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 studies involving humans were approved by Somali Region Health Bureau with certificate (ref: SRHB-18-7738/2022). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. The animal studies were approved by the Research Ethical Review Committee of Addis Ababa University College of Veterinary Medicine and Agriculture (AAU-CVMA) with certificate (ref: VM/ERC/21/02/142022). 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

AA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. BM: Conceptualization, Data curation, Supervision, Writing – review & editing. BE: 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.2024.1276275/full#supplementary-material

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# Isolation, genome analysis and comparison of a novel parainfluenza virus 5 from a Siberian tiger (*Panthera tigris*)

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Paramyxoviruses are important pathogens affecting various animals, including mammals and humans. Parainfluenza virus 5 (PIV5)—a member of the family *Paramyxoviridae*—is a major threat to the health of mammals and humans. However, studies on terrestrial wild animals infected with PIV5 are scanty. In this study, we utilized reverse transcription PCR to detect PIV5 infection in the visceral organ tissues of a Siberian tiger (*Panthera tigris* ssp. *altaica*) with vomiting, diarrhea, and dyspnea before its death. A novel PIV5 (named SR strain) with a slowly progressive cytopathic effect was isolated in Vero cells and validated using a transmission electron microscope. Full-length sequencing and analysis revealed that the whole genome of the PIV5 SR strain contained 15,246 nucleotides (nt) and seven non-overlapping genes (3′-N-V/P-M-F-SH-HN-L-5′) encoding eight proteins. Phylogenetic analysis of three PIV5 strains identified in the same zoo confirmed that PIV5 strains SR and ZJQ-221 shared the closest genetic relationship as they were clustered in the same branch, while the recently found Siberian tiger strain SZ2 kept a certain distance and formed a relatively unique branch. Furthermore, mutations of nt and amino acids (aa) between strains ZJQ-221, SR, and SZ2 were identified. In summary, we report the identification and genomic characterization of a novel PIV5 strain SR isolated in a Siberian tiger, which may help future research on interspecific transmission mechanisms.

KEYWORDS

**Siberian tiger, parainfluenza virus 5, etiology, isolation, genome analysis, transmission electron microscopy, phylogenetic analysis, interspecies transmission**

## Highlights

- A novel PIV5 strain (named SR) was isolated from a Siberian tiger (*Panthera tigris* ssp. *altaica*) with clinical symptoms.
- PIV5 SR strain infection was diagnosed by molecular biology and caused a slowly progressive cytopathic effect in Vero cells, the virions of which were imaged using a transmission electron microscope.
- Full-length sequencing and analysis of the PIV5 SR strain genome were performed for alignment and phylogenesis.

- Mutations of nucleotides and predicted viral proteins were found in ZJQ-221, SR, and SZ2 isolated from the same zoo, which might help to explore the potential pattern of evolution and interspecies transmission.

# Background

Parainfluenza virus 5 (PIV5)—originally known as canine parainfluenza virus (CPIV) and simian virus 5 (SV5) for its first identification in primary monkey kidney cells in 1956 (1)—is a member of the genus *Rubulavirus* in the family *Paramyxoviridae* (2, 3). With a virion diameter of 50–200 nm, PIV5 has a non-segmented negative-sense single-stranded RNA of ~15,246 nucleotides (nt), which contains seven genes and encodes eight proteins (NP, V/P, M, F, SH, HN, and L) (4–7). The V and P proteins of PIV5 share the same genomic encoding region and are encoded by a specific RNA editing mechanism (8).

*Paramyxoviruses* (PVs) represent important zoonotic pathogens with implications for the central nervous system, encephalitis, and respiratory systems, posing risks for both animal and human health (9–12). They are members of the *Paramyxoviridae* family and exhibit a global prevalence in various animal populations, including PIV5 in Korean porcine (13) and in Switzerland cattle populations (9). In addition, peste des petits ruminant viruses have been identified in the Comoros Archipelago (14), canine distemper virus (CDV) in captive Siberian tigers and red pandas (15), and novel PVs of the *Jeilongvirus* genus in bats from China (16). Notably, Nipah and Hendra viruses were predicted to have the potential to cause the next zoonotic pandemic (10). Zoonotic pathogens, such as PVs, have demonstrated the ability to cross species boundaries, leading to zoonotic outbreaks and posing a public health risk over the past two decades. Particularly, PVs exhibit a wide range of common hosts, suggesting a heightened potential for interspecies transmission. The role of the tick as an intermediate host has been proposed, promoting the transmission of PVs among mammals (17). Nonetheless, the evolutionary dynamics of PVs during interspecies transmission remain inadequately studied. This knowledge gap underscores the need for further research to enhance our understanding of the mechanisms underlying the evolution of PVs in the context of interspecies transmission.

To investigate the etiology of the death of the Siberian tiger (*Panthera tigris* ssp. *altaica*) exhibiting symptoms of vomiting, diarrhea, and dyspnea, we utilized RT-PCR, virus isolation, and electron microscopy, ultimately confirming PIV5 infection. Subsequently, we conducted phylogenetic and evolutionary analyzes and compared nt and amino acid (aa) mutations of the successive PIV5 strains SR, SZ2, and ZJQ-221 identified in the zoo. These findings might provide valuable insights into the prevalence and the interspecies transmission mechanisms of PIV5.

# Materials and methods

## Samples

A 12-year-old male Siberian tiger (~230 kg) died in 2015 at a zoo in Guangdong province in southern China after vomiting, diarrhea, and dyspnea for approximately a month. Lobular pneumonia was observed after necropsy, and tissue samples of the livers, spleen, lungs, and kidneys were collected and stored at −80°C.

## Reverse transcription PCR (RT-PCR)

Tissue samples of the Siberian tiger were tested for the possible presence of PIV5, feline parvovirus (FPV), feline coronavirus (FCoV), and CDV using RT-PCR according to previous studies (18). Total RNA of tissue homogenates was extracted using a RNeasy Lipid Tissue Kit (Qiagen, CA, United States) and reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' recommendations. PCR assays were carried out to detect viral nt using a pair of detection primers named PIV5-1-F/R, and full-length genome sequences were amplified using a set of 12 pairs of primers following our previous study (19). Complementary DNA (cDNA) samples as templates were added to a total volume of 25 μL using PrimeSTAR® Max DNA Polymerase (TaKaRa, Japan) according to the manufacturer's protocol. PCR reactions were performed using the following conditions: 95°C for 4 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s to 2 min; and final extension of 72°C for 10 min.

## Gel electrophoresis, sequencing, and similarity analysis

To separate the indicated DNA fragments, the prepared PCR products were added to a 1% agarose gel with Golden View™ (TaKaRa) and electrophoresed with DL2000 DNA marker (TaKaRa) at 120 V for approximately 25 min, as we reported (20). PCR band products were visualized using a Gel Doc™ EZ imaging system (Bio-Rad, CA). Positive PCR products were purified (MiniBEST Agarose Gel DNA Extraction Kit, TaKaRa), tailed with "A"-overhang tails (DNA A-Tailing Kit, TaKaRa), and cloned into the pMD19-T vector (TaKaRa). Positive recombinant plasmids were sequenced by Ruibiotech Co., Ltd. (Beijing, China). Nucleotide sequences were blasted against sequences deposited in GenBank using the basic local alignment search tool, nt (BLASTn)[1] for similarity analysis.

## Virus isolation

Tissue samples were homogenized in sterile phosphate-buffered saline (PBS, pH 7.4) in a ratio of 1/10 (w/v) and centrifuged at 5,000 × g for 10 min at 4°C. Then, supernatants filtered with 0.22-μm membrane were inoculated onto 90% monolayer Vero cells with Dulbecco's

1 https://blast.ncbi.nlm.nih.gov/Blast.cgi

minimal essential medium (DMEM, Gibco) and incubated at 37°C under 5% $CO_2$ for 1 h. Cells were then maintained in DMEM containing 3% fetal bovine serum (FBS, Gibco) and 1% Pen-Strep (21, 22) at 37°C under 5% $CO_2$ and monitored daily for the cytopathic effect (CPE). Cell supernatants were collected after a freeze–thawing cycle three times when the CPE reached ~70% and stored at −80°C as virus stock.

### TEM scanning

To observe virus particles in infected cells, cytopathic Vero cells infected with virus stock were scraped gently and fixed using 2.5% glutaraldehyde fixation fluid at 4°C for 4 h. Finally, ultrathin sections of the infected cell aggregate samples were stained with 2% uranium acetate and 2.6% lead citrate and then scanned on a Hitachi TEM system (Hitachi H-7000FA, Japan).

### Sequence alignment and phylogenetic analysis

Nucleotide sequence alignment based on different rubulavirus species genome sequences was performed using DNAStar Lasergene 7.10 (Madison, WI, United States), and phylogenetic analysis was performed using MEGA 7 (23) with the maximum likelihood algorithm. Bootstrap values were calculated with 1,000 replicates. Geneious Prime (Version 2021.1.1) was used to compare sequences of nt and related encoding proteins of PIV5 SR, ZJQ-221, and SZ2 strains isolated in the same zoo, and then the results of nt and aa mutations were organized using Adobe Photoshop CC 2019.

## Results

### Isolation and identification of PIV5 from Siberian tiger

A Siberian tiger in a zoo of southern China died after vomiting, diarrhea, and dyspnea, and tissue samples of which were collected during necropsy and tested for virus infection. RT-PCR revealed the positive presence of PIV5 nt in the Siberian tiger tissue samples (Figure 1A), which were negative for FCoV, FPV, or CDV (data not shown). The supernatants of tissue homogenates were prepared for virus isolation by inoculating Vero cells with a 90% monolayer for several passages. Compared with uninfected cells, Vero cells inoculated with virus stocks from tissue homogenates for 5 days post-infection (dpi) showed loose attachment, roundness, and random orientation, which indicated a slowly progressive CPE (Figure 1B) of this PIV5 strain. PIV5 infection in Vero cells was further confirmed by TEM, which exhibited spherical particles with a diameter of 50–200 nm (Figure 1C). Thus, the PIV5 strain from the Siberian tiger was isolated and named SR.

### Genome characterization of PIV5 SR strain

A set of 12 pairs of primers was designed to amplify the full-length amplification of genome sequences from Siberian tiger tissue samples (Figure 2) as previously reported. After sequencing, alignment, and high similarity analysis, a full genome of the PIV5 SR strain with 15,246 nt was obtained, encompassing a 3′ leader sequence (55 nt), a non-overlapping encoding area (15,160 nt), and a 5′ trailer sequence (31 nt). The encoding area was predicted to encode seven viral proteins, including the NP gene (position: 152–1,681), V/P gene (position: 1,850–2,518), M gene (position: 3,141–4,274), F gene (position: 4,530–6,185), SH gene (position: 6,303–6,437), HN gene (position: 6,584–8,281), and L gene (position: 8,414–15,181), and six non-coding interval sequences between each gene. The complete genome sequence of the PIV5 SR strain was deposited in GenBank under the accession number KY685075.

### Sequence alignments and phylogenetic analysis of PIV5 SR strain

Whole-genome sequence alignment showed that the PIV5 SR strain had the lowest nt similarity (~97.13%) with the D277 strain of canine-origin (GenBank: KC237065) from South Korea and the highest nt similarity (~99.76%) with the ZJQ-221 strain of lesser panda (GenBank: KX100034) isolated from the same zoo. Phylogenetic analysis further confirmed the close genetic relationship between SR and ZJQ-221, as they were clustered in the same branch (Figure 3). Interestingly, the PIV5 SZ2 strain (GenBank: OQ939949.1), recently isolated from another dead Siberian tiger in the same zoo using Metavirome sequencing, kept a distance from the branch of SR and ZJQ-221 strains and formed an individual branch, indicating that SZ2 might have been evolved in a different way.

### Nt and aa comparisons of PIV5 ZJQ-221, SR, and SZ2 strains

Since Siberian tiger strains SR and SZ2, as well as lesser panda strain ZJQ-221, were all found in the same zoo, these three strains might be helpful in exploring the potential pattern of evolution and interspecies transmission of PIV5. Thus, ZJQ-221, SR, and SZ2 sequences were analyzed using Geneious Prime (Version 2021.1.1), and mutation sites of nt and aa between strains are listed in Supplementary Table S1. The three PIV5 strains had several sense mutations in viral encoding areas (Figure 4). Intriguingly, no sense mutation in F or SH was found between ZJQ-221 and SR, while the newly found PIV5 strain SZ2 had 6 and 5 sense mutations in each viral protein, respectively. Furthermore, compared with ZJQ-221 and SR strains, three aa mutations of the PIV5 SZ2 strain in SH (13: Ala to Thr), HN (447: Ser to Asp), and L (2,229: Val to Met) resulted from two nt mutations in each codon (Figure 4).

## Discussion

The *Paramyxoviridae* family boasts a broad spectrum of viral reservoirs and is implicated in various diseases, including mumps and measles in humans, Newcastle disease in poultry, and distemper in carnivorous animals (24–26). Since its identification in 1954, PIV5 has evolved into a globally infectious agent over the past half century. Research has revealed that PIV5 can infect a diverse array of

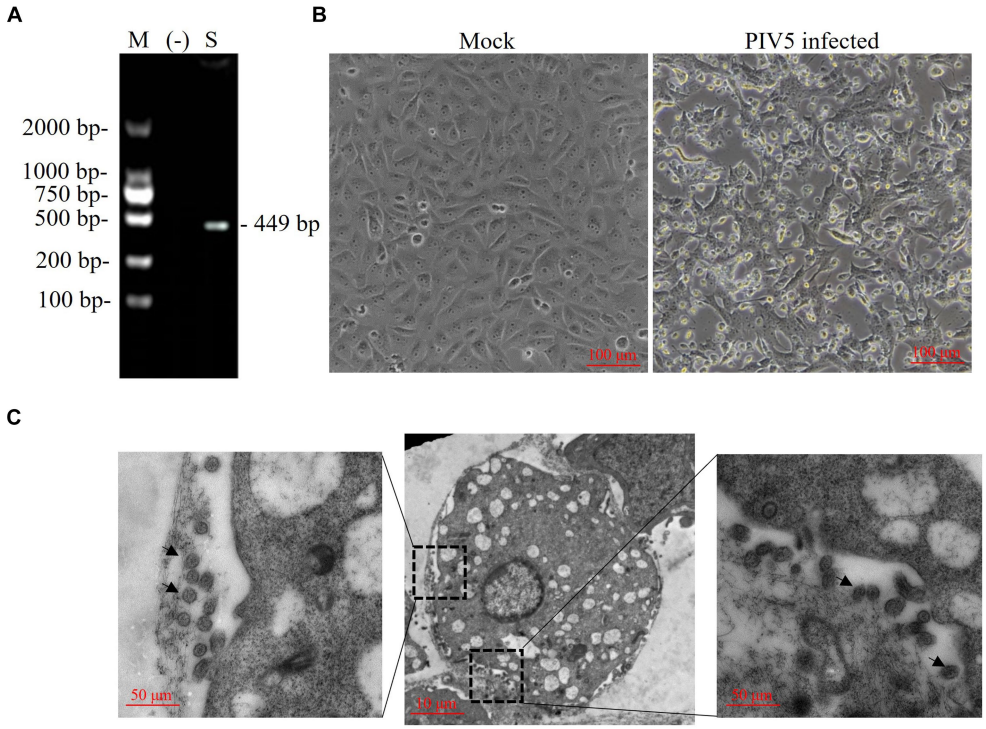


FIGURE 1
Identification of a paramyxovirus isolated from a Siberian tiger. **(A)** PIV5 viral nucleotides (nt) were detected in a Siberian tiger sample using reverse transcription PCR (RT-PCR). M, DL2000 DNA marker; –, negative control; S, Siberian tiger sample. **(B)** Vero cells of mock infection (left) and PIV5 infection (right) using an inverted microscope. Bar, 100 μm. **(C)** Transmission electron microscopy images of Vero cells inoculated with PIV5 virus stock. Bar, 10 μm or 50 μm; black arrow, viral particles.

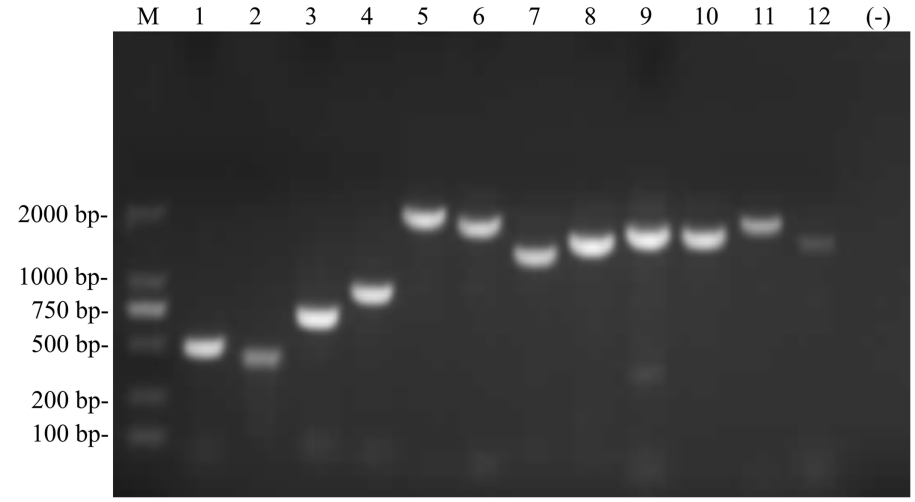


FIGURE 2
Full-length genome sequence of the PIV5 SR strain was amplified using RT-PCR. M, DL2000 DNA Marker; 1–12, 12 pairs of primers were used to amplify the full-length of the PIV5 SR strain.

mammals, including humans, dogs, pigs, rats, rabbits, foxes, and cats, across different countries and regions. Despite the extensive knowledge of the infectivity of PIV5 in various species, the evolutionary dynamics of PVs during interspecies transmission remain elusive. While the virus has demonstrated its ability to traverse species boundaries, the mechanisms and factors influencing its evolution in the context of interspecies transmission are yet to be fully elucidated. This knowledge gap underscores the need for further investigations to unravel the intricacies of the evolutionary pathways of PVs during interspecies transmission.

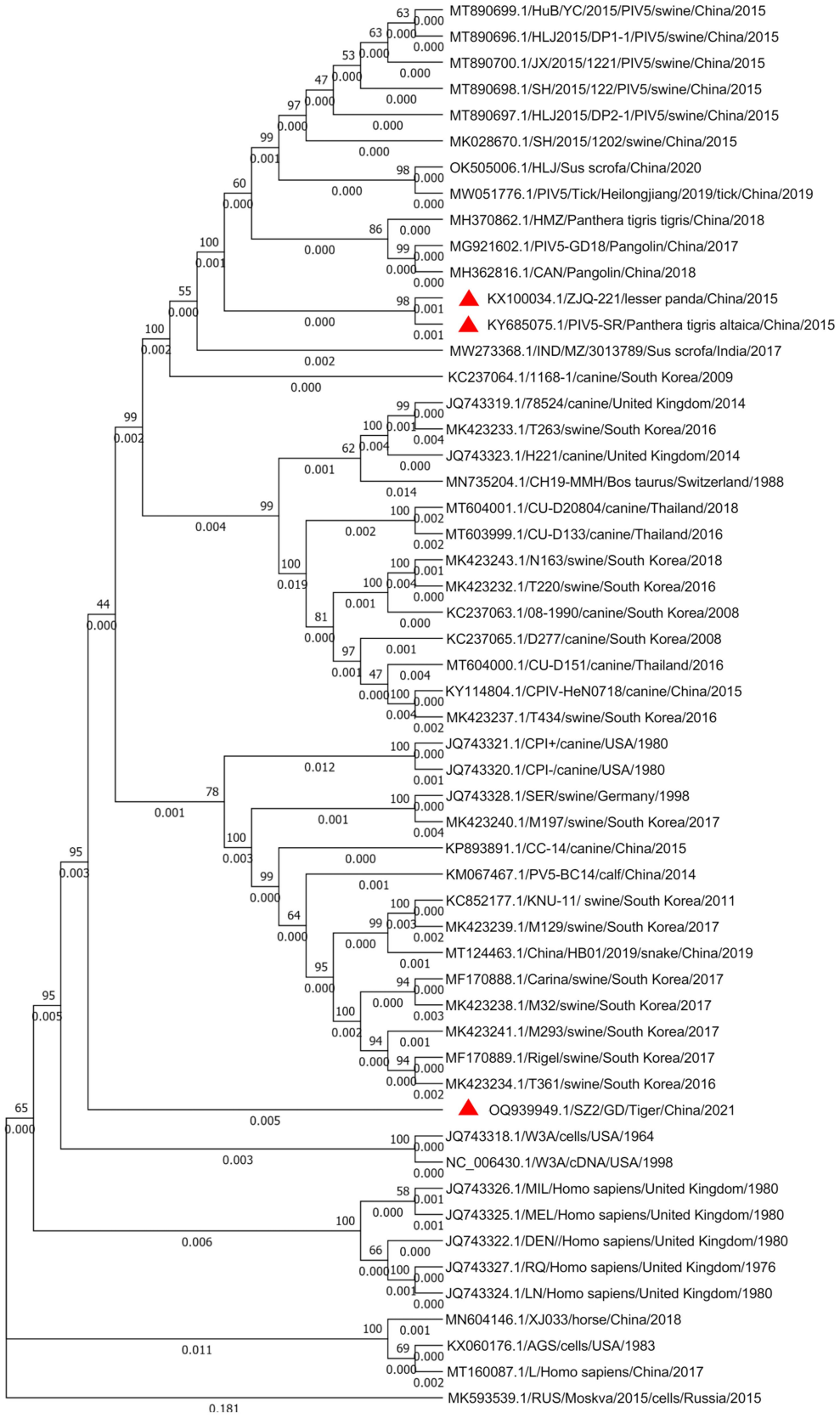


FIGURE 3
Phylogenetic analysis of the PIV5 SR strain based on the nucleotide sequences of the complete viral genome sequence. The nucleotide sequences of various parainfluenza virus strains were aligned using MEGA 7. One thousand bootstrap replicates were subjected to nt sequence distance and neighbor-joining analyzes, and a consensus phylogenetic tree was generated. Red triangles indicate strains found in the same zoo.

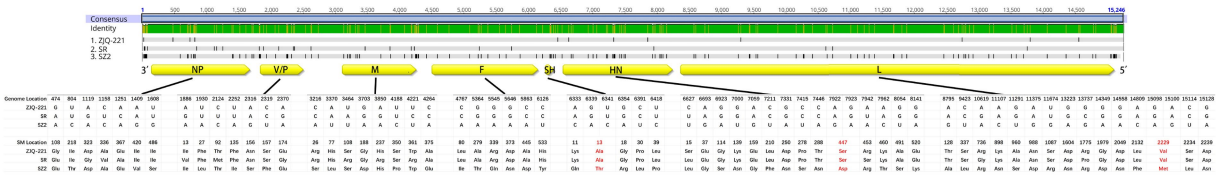


FIGURE 4
Sense mutations of lesser panda PIV5 strain ZJQ-221 and Siberian tiger PIV5 strains SR and SZ2 identified from the same zoo. Amino acid (aa) sequences of ZJQ-221, SR, and SZ2 strains were analyzed using Geneious Prime (Version 2021.1.1). The schematic diagram of the PIV5 genome structure and aa mutations was illustrated. Aa sense mutations resulting from two nt site mutations in one codon are shown in red. Yellow box, viral protein encoding area and direction in the genome. NP, nucleocapsid protein. V/P, V protein/phosphoprotein. M, Matrix protein. F, fusion protein. SH, small hydrophobic protein. HN, hemagglutinin-neuraminidase protein. L, large protein, or RNA-dependent RNA polymerase.

In this study, a novel PIV5 strain named SR, with a full length of 15,246 nt and exhibiting spherical particles with a diameter of 50–200 nm consistent with previously reported studies (7, 13), was isolated from a captive Siberian tiger with clinical symptoms. The isolated PIV5 SR strain conforms to Koch's postulates (Supplementary Table S1). Meanwhile, alignment and high similarity analysis demonstrated that this novel PIV5 SR strain shared the same genome structure and had the highest nt similarity (~99.76%) with the ZJQ-221 strain of lesser panda (GenBank: KX100034) from the same zoo. Additionally, the PIV5 SR strain was predicted to encode a small hydrophobic (SH) protein, which is a type II membrane protein of 44 aa residues, including a 5-aa C-terminal ectodomain, a 23-aa transmembrane domain, and a 16-aa N-terminal cytoplasmic region (13, 27). The SH protein was absent in PIV5 strains isolated from pigs, dogs, calves, and cells (19), implying that this protein might be dispensable in the lifecycle of PVs (28–31). However, the absence of SH protein in PIV5 induced apoptosis of infected cells by activating tumor necrosis factor-alpha expression (32). Interestingly, the role of SH protein in the inhibition of apoptosis, which contributes to the virulence of PV members, was further confirmed in the J paramyxovirus (JPV), mumps virus, and respiratory syncytial virus (RSV) (33). Our study identified the presence of the SH protein in tiger PIV5 strains SR and SZ2. The presence of this protein is speculated to play a role in reducing apoptosis and contributing to virulence throughout the lifecycles of these strains, thereby facilitating transmission among diverse host species.

One specific mutation in proteins might play an important role in the lifecycle, pathogenesis, evolution, and even interspecies transmission of viruses (34). It has been previously reported that mutations in the F fusogenic peptide (G3A) and near the F transmembrane domain (S443P) not only enhanced viral fusion activity but also increased viral susceptibility to antibody-mediated neutralization (35). A point mutation of glutamine at position 202 of the RNA-binding domain of human parainfluenza virus type 2 (hPIV2) nucleocapsid protein (NP) enhanced polymerase activity by approximately 30-fold, whereas a recombinant hPIV2 possessing the NP Q202A mutation could not be recovered from cDNA (36). Moreover, leucine at position 302 of the M protein of hPIV3 played a crucial role in viral RNA synthesis by regulating inclusion body formation (12). Hemagglutinin-neuraminidase (HN) of PV is a multifunctional protein mediating hemagglutination, neuraminidase, and fusion promotion activities. A study of PIV5 HN ectodomain structure revealed that V81T and L85Q mutations in the stalk region significantly impaired cell–cell fusion, while the D398L mutation within the head domain showed reduced fusion activity (37). A second receptor binding site on hPIV3 HN contributed to the activation of the fusion mechanism during host cell invasion (38). However, the creation of a second receptor binding site by site-specific mutagenesis at residue 523 on hPIV1 HN did not significantly affect the growth or fusion activity of the recombinant virus (39). pH-dependent (acid-activated) channel activity of human RSV SH proteins in transiently expressing HEK 293 cells was abolished when both $His^{22}$ and $His^{51}$ residues of the SH protein were mutated, but not when either was present (40). An additional mutation in E1658D of the PIV5 L protein might enhance virus replication in Vero cells when PIV5 without the conserved C-terminal of the V protein was inserted with hemagglutinin from the H5N1 Influenza A virus between the HN and L genes in the genome (41). Post-translation modification of specific residues of viral proteins also plays a vital role in virus transmission in hosts. The phosphoprotein status of PIV5 viral phosphoprotein (P) acted as a replication switch during virus replication (42), while SUMOylation played a key role in the growth of PIV5. Mutation of the P protein at 254 lysine to arginine (K254R) reduced PIV5 minigenome activity and the SUMOylation level of the P protein (43). The present study compared nt and protein mutations, which may help in exploring mechanisms underlying the evolution and interspecies transmission of PIV5.

In the current study, a novel PIV5 strain, designated SR strain, causing slowly progressive CPEs in Vero cells was isolated from a dead Siberian tiger with clinical symptoms including vomiting, diarrhea, and dyspnea. Virions of the PIV5 SR strain in infected cells were imaged using a transmission electron microscope (TEM). The full genome of the SR strain showed a classical PIV5 genome structure characteristic and the closest genetic relationship with a lesser panda strain, ZJQ-221, isolated in the same zoo. Furthermore, mutations of nt and aa in SR, SZ2, and ZJQ-221 strains were identified. Our study findings provide insight into the epidemiology and genomics of PIV5 and highlight the urgent need to control PIV5 in zoo animals to avoid interspecies transmission. The occurrence of PIV5 mutations in these wild animals might provide potential candidates for future research on the molecular mechanisms underlying virus evolution and interspecies transmission.

## 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.

The data presented in the study are deposited in the NCBI repository, SRA accession number: SRR27458479

## Ethics statement

The animal study was approved by Guangzhou Zoo Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

## Author contributions

NZ: Project administration, Writing – original draft, Writing – review & editing. LC: Project administration, Writing – original draft. CW: Data curation, Formal analysis, Writing – original draft. MeL: Project administration, Writing – original draft. FS: Investigation, Methodology, Writing – original draft. WL: Methodology, Project administration, Writing – original draft. YW: Investigation, Methodology, Writing – original draft. XD: Methodology, Writing – original draft. JF: Data curation, Writing – original draft. MiL: Data curation, Writing – original draft. MS: Methodology, Writing – original draft. JC: Data curation, Writing – original draft, Writing – review & editing. JZ: Writing – original draft, Writing – review & editing. WC: 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.2024.1356378/full#supplementary-material

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# Re-emergence of foot-and-mouth disease in the Republic of Korea caused by the O/ME-SA/Ind-2001e lineage

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The O/ME-SA/Ind-2001e foot-and-mouth disease virus (FMDV) lineage is a pandemic strain that has recently become dominant within East and Southeast Asia. During May 2023, this viral lineage spread to the Republic of Korea, where 11 outbreaks were detected on cattle and goat farms located in Cheongju and Jeungpyeong. Infected animals displayed typical FMD signs including vesicular lesions with drooling and anorexia. Molecular diagnostic testing and genetic analysis (VP1 sequencing) showed that the causative FMDVs belonged to the O/ME-SA/Ind-2001e lineage and shared the closest nucleotide identity (97.95–99.21%) to viruses that have been collected from Mongolia and South-East Asian countries. Phylogenetic analyses showed that these sequences were distinct to those collected from the previous Korean O/ME-SA/Ind-2001e lineage outbreaks in 2019, demonstrating that these cases are due to a new incursion of the virus into the country. Prompt implementation of emergency vaccination using antigenically matched serotype O vaccines (r1 value: 0.74–0.93), together with intensive active surveillance on farms surrounding the infected premises has successfully prevented further spread of FMD. These recent FMD outbreaks reinforce the importance of research to understand the risks associated with transboundary pathways in the region, in order to reduce the possibility of a further reintroduction of FMD into the Republic of Korea.

KEYWORDS

**foot-and-mouth disease virus, outbreak, ME-SA, Ind-2001e, Korea, vaccination**

## 1 Introduction

Foot-and-mouth disease (FMD) is one of the most important animal diseases. FMD is highly contagious to cloven-hoofed animals and causes severe economic losses due to its devastating impact on trade and reduced animal productivity (1). The causative agent is the foot-and-mouth disease virus (FMDV), which belongs to the genus *Aphthovirus* of the family *Picornaviridae*. FMDV is divided into seven genetically and immunologically distinct serotypes [O, A, Asia 1, C, Southern African Territories (SAT) 1, SAT 2 and SAT 3] including multiple topotypes and genetic lineages (2, 3). FMDV strains circulating throughout the world have been geographically divided into seven regional virus pools (1, 4). Serotype O, A, and

Asia 1 viruses circulate in the Asian pools (Pools 1, 2, and 3), which pose threats to livestock industries in countries such as the Republic of Korea.

Serotype O is the most widely distributed of the seven FMDV serotypes. In Southeast Asia and East Asia (Pool 1), serotype O viruses are responsible for over 80% of the officially reported outbreaks. Type O viruses belonging to the SEA (O/SEA/Mya-98), ME-SA (O/ME-SA/PanAsia, O/ME-SA/Ind-2001) and CATHAY topotypes are endemic in this region. The O/ME-SA/Ind-2001 lineage has been classified into five sublineages, namely, a, b, c, d, and e. Since 2020, there has been an upward trend in the dominance of the O/Ind-2001e sublineage in Southeast Asian countries (5).

The Republic of Korea was free from FMD from 1934 until 2000. However, since 2000, FMD has been introduced into the country on multiple occasions. FMD outbreaks in 2010 caused significant economic damage estimated at more than US$ 3.6 billion (6). As a consequence, a national program that ensured that all FMD-susceptible animals were vaccinated began in early 2011. However, despite this strong nationwide vaccination policies, FMD recurred every year from 2014 to 2019, recording a total of 11 incursions since 2000 caused by both FMD type O and A viruses (7, 8). The genotypes of FMDV that have been detected in the Republic of Korea are O/ME-SA/PanAsia, O/ME-SA/Ind-2001e, O/SEA/Mya-98 and A/ASIA/Sea-97. During 9 days in May 2023, new FMD clinical cases were reported on ten cattle and one goat farms located in two regions, Cheongju and Jeungpyeong of Chungbuk. The FMD-reported animals showed typical clinical signs and lesions, such as anorexia, vesicular fluids, erosion, and drooling. In this study, genetic characterization of the causative virus and vaccine matching test using a virus isolated from clinical samples were conducted to investigate (i) the epidemiological relationship with contemporary FMD viruses circulating in neighboring regions, and (ii) the antigenic relatedness of commercial vaccine strains used for domestic compulsory vaccination. These data help to understand the risk pathways by which FMDVs enter the Republic of Korea and provide data to support the national vaccination control strategy.

# 2 Materials and methods

## 2.1 Clinical samples and virus isolation

A total of 54 clinical samples (epithelium, saliva, and tissue) were collected from cattle and goats exhibiting clinical signs of FMD in FMD-affected farms located in one province of Republic of Korea. The samples were transported to the laboratory in transport media (BD Diagnostics). Tissue samples were processed by homogenization of 50 mg of tissue in DMEM media (without Fetal bovine serum) using a bead-based homogenizer. The LFBK-$\alpha\nu\beta_6$ cell lines (supplied by the Plum Island Animal Disease Center, United States) were cultured using DMEM media (Gibco) supplemented 1% antibiotic-antimycotic (Gibco) and 10% fetal bovine serum (Atlas) for viral isolation (9). Inoculated cells were harvested when 90% of cytopathic effect (CPE) were observed, the cells and supernatant were then frozen and thawed three times, harvested and stored at −80°C until use in aliquot. If no CPE was observed, the cells were frozen and thawed, and the supernatant was used to be inoculated onto new fresh cells, which were examined for CPE for another 48 h.

## 2.2 Lateral flow strip test for rapid detection of FMDV serotypes A, O and Asia1

Clinical samples collected from 11 farms with suspect FMD cases were tested using the VDRG® FMDV 3Diff/PAN Ag Rapid Kit (Median Diagnostics, Korea). Fresh vesicular fluid was collected using a syringe and added to a test tube containing 250 μL of sample dilution buffer. Tissue extracted fluid was added to 1 mL of sample dilution buffer to the tissue extraction vial. Then, after adding the tissue sample to the extraction vial, cut the tissue into pieces using scissors and grind it using the pestle and sand included in the kit. Remove the VDRG® FMDV 3Diff/PAN Rapid Test Device (LFD) from the foil pouch, place it on a flat surface, and slowly add 100 μL of the treated sample to the "S1" and "S2" positions. The results were read after exactly 15 min. Specimen inlet S1 shows the three test lines O (Type O), A (Type A), and AS (Type Asia1) and the control line (C) position, while specimen inlet S2 display test line PAN (FMDV common) and the control line (C) position.

## 2.3 RNA extraction and real-time RT-PCR

Viral RNA was extracted from clinical samples and cell-isolated viruses using Nextractor® NX-48 s viral NA kit (Genolution, South Korea) according to the manufacturer's instructions. Extracted samples were tested for the presence of FMDV RNA using primers and probes directed toward the conserved 3D gene (10). PowerChek™ FMDV Multiplex Real-time PCR kit (3D/IRES) (Kogenebiotech, South Korea) was used for the amplification of 3D gene, according to the manufacturer's instruction. The VDx FMDV O genotyping qRT-PCR set (Median Diagnostics, Korea) was used to differentiate lineages (O/Ind-2001, O/PanAsia, O/Mya-98, and O/CATHAY) by VP1 gene amplification according to the manufacturer's protocol.

## 2.4 Sequencing and phylogenetic analysis

The VP1 coding region was amplified using universal primers as detailed in (11). DNA Sanger sequencing was performed for the purified PCR product using Big Dye Terminator v3.1 Cycle sequencing kit (Thermofisher, United States) and run on an ABI 3730 DNA analyzer (Thermofisher, United States). FMDV VP1 coding sequences ($n = 267$) were retrieved from both the Animal and Plant Quarantine Agency (APQA) database and WRLFMD database of FMDV sequences,[1] which also includes FMDV genomes deposited in GenBank. These sequences were aligned using CLC workbench 23 (Qiagen, United States) and the homology of the nucleotide (NT) or amino acid (AA) sequences among the viral isolates was determined. VP1 coding sequences were subjected to phylogenetic trees including those of Korean field FMDV viruses ($n = 11$) isolated from 2017 to 2023, field isolates collected from

1 www.fmdbase.org

neighboring countries from 2016 to 2022 ($n = 221$), and also by including FMDV prototype strains ($n = 35$). The phylogenetic tree was constructed using the Maximum Likelihood method based on the GTR (generalized time reversible) model (12), bootstrapping over 1,000 replicates using MEGA software version X (13).

Bayesian analyses were performed using BEAST version 1.10.4 (14). The evolution of FMDV was modelled by parameterizing the process of nucleotide substitution model using gamma-discretized among-site rate variation GTR model (GTR+ $\Gamma_4$). Time to most recent common ancestor (TMRCA) for lineages were estimated in BEAST v1.10.4 employing a log-normal distributed relaxed molecular clock (15) and the non-parametric Skygrid coalescent population prior with 100 transition points (16). The posterior trajectories were acquired by running a Markov chain Monte Carlo (MCMC) for 200 million iterations, while recovering samples at intervals of 20,000 states. Mixing and convergence of the MCMC chain was assessed using Tracer 1.7.3 (17), ensuring sufficient sampling was achieved. The Maximum Clade Credibility (MCC) tree was generated by TreeAnnotator from 9,000 posterior trees, following the elimination of the initial 10% of sampled trees as burnin.

### 2.5 Two-dimensional virus neutralization test for strain differentiation

To identify a suitable FMD vaccine, FMD viruses isolated in South Korea were sent to the World Reference Laboratory for FMD (WRLFMD, Pirbright, United Kingdom). Virus neutralization test (VNT) was performed according to the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (18) on IB-RS2 cells. VNT was performed to determine the serological relationships ($r$-value) between field isolates recovered during the May 2023 Korean outbreak and reference FMDV serotype O vaccine strains of three commercial vaccines used for compulsory vaccination in the Republic of Korea.

## 3 Results

### 3.1 Sample collection and diagnosis

Between the 10th and the 18th of May 2023, a total of 11 cases of FMD were confirmed in two regions (Cheongju and Jeungpyeong) of Chungbuk in the Republic of Korea (Figure 1A). These were identified following four reports of FMD suspicion and screening animals. All livestock on the 11 farms were clinically examined, and samples (both epithelium and saliva) were collected from animals with suspected FMD clinical signs. Samples such as epithelial fluid were tested for FMD using LFD which confirmed the FMDV positive (serotype O) status of samples from 8 farms. A total of 36 samples were collected from suspected animals on 11 farms and subjected to 3D rRT-PCR, which detected the FMDV in 40 samples from all 11 farms. These samples had Ct values ranging from 16.08 to 38.59. Of the 40 FMDV positive samples, samples from eight farms ($n = 31$) were sequenced for the VP1 coding gene, and samples from three farms ($n = 9$) where the VP1 coding gene was not amplified were identified as O/ME-SA/Ind-2001 lineage by genotyping rRT-PCR (Table 1). Twenty FMDV positive samples from 11 farms were inoculated into cells, and nine FMDVs from 8 farms were isolated.

### 3.2 Sequence homology and phylogenetic tree analysis

Sequencing the VP1 coding gene (1D) using samples that were positive using the FMDV 3D real-time RT-PCR confirmed the O/ME-SA/Ind-2001e lineage in eight farms. However, it was not possible to amplify the VP1 region for samples collected from the remaining three farms. The VP1 sequences analyzed in this study have been submitted to GenBank under accession numbers PP156917-PP156919.

For the 2023 Korean isolates, the nucleotide identities of the VP1 coding region varied from 99.68 to 100% and all the sequences were 100% identical to each other at the amino acid level. Based on the virus isolated from the first farm in 2023, the VP1 coding sequence was 95.73 and 94.79% identical to viruses isolated in 2017 (Boeun) and 2019 (Anseong) from the Republic of Korea, respectively, whilst percentage of identity values between 97.95 and 99.2% were estimated from FMDV isolates collected from neighboring Asian countries during 2019 and 2022 (Table 2).

A phylogenetic analysis was performed using FMDV sequences ($n = 267$) encoding the VP1 region (alignment of 633 nt in length) of the O/ME-SA/Ind-2001e lineage collected from East and Southeast Asia regions, including the ones from the Republic of Korea. The resulting topology clustered the O/ME-SA/Ind-2001e lineage into two main groups, here named as G1 and G2 (Figure 1). G1 included field viruses isolated from FMDV endemic countries in Pool 2, such as India, Bangladesh, and Bhutan, and Pool 1 such as Myanmar, Thailand, Vietnam, Russia, and China collected between 2016 and 2019. In addition, this group also contained field viruses isolated from Indonesia in 2022 as well as those collected from the Republic of Korea in 2017 and 2019. The G2 phylogenetic cluster included field viruses from Southeast Asia region (Vietnam, Cambodia, Laos, Thailand, Malaysia) and some more recent FMDV isolates (2019–2022) from outbreaks reported in Mongolia, Russia, and Kazakhstan. Thus, FMD viruses causing the 2023 outbreak in the Republic of Korea were assigned to G2 and shared a common ancestor with the FMDV isolates from Mongolia in 2021, with its MRCA dated Aug-2020 (95% BCI March 2020 to January 2021).

### 3.3 Vaccine matching

The antigenic relationship between a single field isolate (O/JP2/SKR/6/2023) and the serotype O FMDV vaccine strains (O3039, O Manisa, O1 Campos) used in the Republic of Korea was evaluated in 2D-VNT using bovine vaccine serum raised against the vaccine strains. The r1 values between the field viruses and vaccine strains were all higher than the 0.3 cut-off for expected protection (Table 3).

## 4 Discussion

There had been no FMD outbreaks detected in livestock of the Republic of Korea after the 11th FMD incursion reported in Anseong during January 2019. In the recent outbreaks of May 2023, a lateral flow device was used to rapidly detect FMDV in samples collected from animals exhibiting FMD clinical signs. Further testing using 3D and VP1 real-time RT-PCR confirmed FMD and serotype of FMDV, and sequencing of VP1 coding region revealed that the FMDV strain

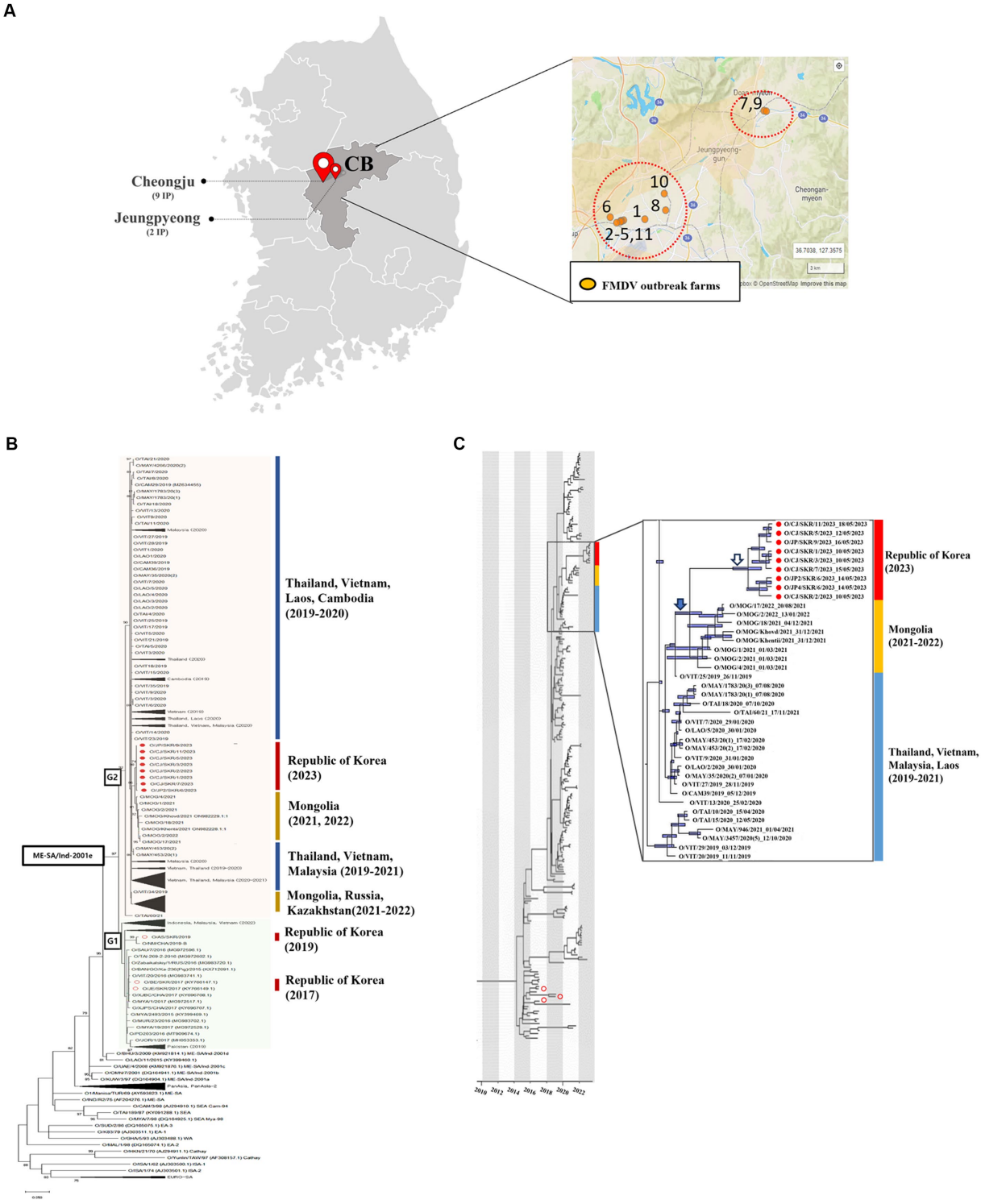


FIGURE 1
**(A)** Location and number of farms infected. **(B)** Maximum likelihood tree showing the FMDV VP1 coding sequence of the O/ME-SA/Ind-2001e lineage that caused an outbreak in the Republic of Korea in 2023 (filled red circles) and 2017 and 2019 (empty red circles) and its relationship to other recent FMD type O viruses circulating in the surrounding region. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. **(C)** The MCC tree displays the isolates identified during the 2023 outbreak in the Republic of Korea (filled red circles) and the 2017 and 2019 outbreaks (empty red circles). It also indicates their correlation with other O/ME-SA/Ind-2001e lineages prevalent in the neighboring countries of the Republic of Korea. 95% High Posterior Density (HPD) intervals for the timing of node ancestry are represented by slate blue bars in the MCC tree. The enlarged box contains isolates from neighboring countries that have an MRCA with the FMDV isolated in the Republic of Korea in 2023. Also included in this box is the MRCA of FMDV isolated in the Republic of Korea in 2023 (empty arrow), the MRCA of FMDV isolated in the Republic of Korea in 2023, and FMDV isolated in Mongolia in 2021-2022 (filled arrow).

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TABLE 1 Details of the clinical samples obtained from the 11 FMD-positive farms reported during May 2023 in the Republic of Korea.

| Farm No. | Category | Date | City | Species | No. of samples | LFD (Pan/Type) | Clinical sign | Sample type | rRT-PCR (3D/IRES) | Genotype |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Report | 10 May | CJ | Cattle | 12 | +/O | Vesicle | epithelium, saliva | Positive | O/ME-SA/Ind-2001e |
| 2 | Report | 10 May | CJ | Cattle | 2 | +/O | Vesicle | tissue, saliva | Positive | O/ME-SA/Ind-2001e |
| 3 | Surveillance | 10 May | CJ | Cattle | 2 | +/O | Salivation | tissue, saliva | Positive | O/ME-SA/Ind-2001e |
| 4 | Surveillance | 11 May | CJ | Cattle | 1 | −/− | Salivation, erosion ulcer | tissue, saliva | Positive | O/ME-SA/Ind-2001 (by rRT-PCR) |
| 5 | Surveillance | 12 May | CJ | Cattle | 3 | +/O | Salivation, ulcer | tissue, saliva | Positive | O/ME-SA/Ind-2001e |
| 6 | Report | 14 May | JP | Cattle | 4 | +/O | ulcer | epithelium, tissue | Positive | O/ME-SA/Ind-2001e |
| 7 | Report | 15 May | CJ | Cattle | 3 | +/O | Salivation, ulcer | tissue | Positive | O/ME-SA/Ind-2001e |
| 8 | Surveillance | 16 May | CJ | Cattle | 3 | −/− | Salivation | saliva | Positive | O/ME-SA/Ind-2001 (by rRT-PCR) |
| 9 | Surveillance | 16 Ma | JP | Cattle | 4 | +/O | Salivation | saliva | Positive | O/ME-SA/Ind-2001e |
| 10 | Surveillance | 16 Ma | CJ | Goat | 1 | −/− | Salivation | saliva | Positive | O/ME-SA/Ind-2001 (by rRT-PCR) |
| 11 | Surveillance | 18 Ma | CJ | Cattle | 3 | +/O | Salivation, ulcer | epithelium, saliva | Positive | O/ME-SA/Ind-2001e |

*CJ, Cheongju; JP, Jeungpyeong.
**Samples from three farms (No. 4, 8, 10) tested negative for FMDV in LFD had Ct values above 24 in 3D rRT-PCR.

introduced into the Republic of Korea belonged to O/ME-SA/Ind-2001e sub-lineage. A national livestock standstill with culling and disinfection of the infected farms was implemented. Furthermore, emergency vaccination was conducted for all FMD-susceptible animals nationwide. These measures have been successful since no further FMD-suspected case has been identified since the last case reported the 18th of May 2023.

The thorough national epidemiological investigation suggested FMDV introduction possibly from neighboring countries via illegal animal products, based on the following assumptions: (i) for the past 4 years after the last FMD outbreak of 2019, a strict FMD reporting system and a post-vaccination monitoring (PVM) program involving 1.2 millions of animals have been implemented for all FMDV susceptible livestock in the country; these systems have not detected any FMD-suspected cases nor evidence of FMDV infection from non-negative NSP-positive animals identified during surveillance; finding which were confirmed by real-time RT-PCR testing of clinical samples of the NSP-non-negative animals as well as other animals from these farms; (ii) FMDV and 5 antibody test using antisera ($n = 8,681$) of wild animals (wild boar, water deer, roe deer, etc.) during the last 5 years from 2019 to June 2023 showed no FMDV infection in wild animal populations of the Republic of Korea (data not shown); (iii) the FMDV isolates genetically characterized from the 2023 outbreak belonged to the O/ME-SA/Ind-2001e, clustering in a phylogenetic group that was distinct from those viruses of the same lineage that caused the outbreaks reported in 2017 and 2019, with 5–6% of nucleotide difference in the FMDV sequences of the VP1 coding region. From the phylogenetic tree, the 2023 FMD viruses were closely related to field viruses (O/ME-SA/Ind-2001e) isolated between 2022 and 2021 from Mongolia and other Southeast Asian countries (Laos, Vietnam, and Cambodia) with ~1% of nucleotide sequence difference, clustering within the same group. This FMDV lineage, is the predominant strain circulating within FMD endemic Pool 1, as was reported during 2020-2023 (19, 20); (iv) Inspections of illegal livestock products found about 60 cases/day in the luggage of travelers visiting neighboring countries and about 70 cases/day in overseas express cargo. In addition, inspections of foreign grocery stores in cities and counties adjacent to the FMD outbreak areas confirmed the illegal sale of animal products.

It was confirmed by vaccine-matching tests that type O FMDV strains of the commercial vaccines used for compulsory systemic vaccination policy in the Republic of Korea were well matched (r1 value >0.3) against the FMD field isolates (Table 3), including those FMDV isolates causing outbreaks in 2017 and 2019. Therefore, it is believed that the emergency vaccination should be highly effective to contain the FMD outbreaks within limited areas (two regions in a province) for a short period of time (9 days), as demonstrated in swift control of FMD outbreaks during the past two outbreaks (2017 and 2019) reported in the Republic of Korea. The effect of emergency vaccination as one of major control measures in emergency FMD outbreaks (18, 21) was already proven by previous studies (22).

During the nationwide vaccination policy, post-vaccination monitoring (PVM) showed that over 90% of FMD-susceptible livestock had seroconverted (23), satisfying 80% population herd immunity on average, which was also confirmed in the recent PVM carried out between January and June of 2023 (data not shown). Nevertheless, the likely cause of FMDV introduction and transmission within a systematically vaccinated population might be occur if the levels of protective immunity are low as observed on some farms. As shown in a previous study (23), around 0.3–0.79% of farms were found to have less than 80% of herd

TABLE 2 VP1 coding sequences of O/ME-SA/Ind-2001e FMDVs generated in this study.

| Name | Collected date | Country | Host | Id (%) | Accession no. | References |
|---|---|---|---|---|---|---|
| CJ/SKR/1/2023 | May-23 | Republic of Korea | Cattle | Ref | PP156917 | This study |
| CJ/SKR/2/2023 | May-23 | Republic of Korea | Cattle | 100 | – | This study |
| CJ/SKR/3/2023 | May-23 | Republic of Korea | Cattle | 100 | – | This study |
| CJ/SKR/5/2023 | May-23 | Republic of Korea | Cattle | 100 | – | This study |
| CJ/SKR/7/2023 | May-23 | Republic of Korea | Cattle | 99.84 | PP1569179 | This study |
| JP2/SKR/6/2023 | May-23 | Republic of Korea | Cattle | 99.68 | PP1569178 | This study |
| JP/SKR/9/2023 | May-23 | Republic of Korea | Cattle | 100 | – | This study |
| CJ/SKR/11/2023 | May-23 | Republic of Korea | Cattle | 100 | – | This study |
| JE/SKR/2017 | February-17 | Republic of Korea | Cattle | 96.37 | KY766149.1 | – |
| BE/SKR/2017 | February-17 | Republic of Korea | Cattle | 95.73 | KY766147.1 | – |
| AS/SKR/2019 | January-19 | Republic of Korea | Cattle | 94.79 | n/a | APQA |
| MOG/1/2021 | March-21 | Mongolia | n/a | 99.21 | n/a | WRLFMD |
| MOG/4/2021 | March-21 | Mongolia | n/a | 99.05 | n/a | WRLFMD |
| LAO/3/2020 | January-20 | Lao PDR | Cattle | 98.89 | n/a | WRLFMD |
| TAI/4/2020 | May-20 | Thailand | Cattle | 98.89 | n/a | WRLFMD |
| VIT/7/2020 | January-20 | Vietnam | Pig | 98.89 | n/a | WRLFMD |
| VIT/28/2019 | November-19 | Vietnam | Water buffalo | 98.89 | n/a | WRLFMD |
| MAY/35/2020 (2) | January-20 | Malaysia | Cattle | 98.89 | n/a | WRLFMD |
| CAM36/2019 | October-19 | Cambodia | Cattle | 98.89 | n/a | APQA |
| CAM39/2019 | December-19 | Cambodia | Cattle | 98.89 | n/a | APQA |
| LAO1/2020 | January-20 | Lao PDR | Cattle | 98.89 | n/a | APQA |
| VIT1/2020 | January-20 | Vietnam | Cattle | 98.89 | n/a | APQA |
| MOG/2/2021 | March-21 | Mongolia | | 98.74 | n/a | WRLFMD |
| MOG/Khentii/2021 | December-21 | Mongolia | Cattle | 98.74 | ON982228.1 | Viktor et al. (27) |
| CAM30/2019 | January-19 | Cambodia | Cattle | 98.74 | MZ634456.1 | Ryoo et al. (28) |
| MAY/453/20 (2) | February-20 | Malaysia | – | 98.74 | n/a | WRLFMD |
| LAO/1/2020 | – | Lao PDR | – | 98.74 | n/a | WRLFMD |
| MAY/127/2020 | – | Malaysia | – | 98.74 | n/a | WRLFMD |
| MAY/9/2020 | – | Malaysia | – | 98.74 | n/a | WRLFMD |
| VIT/23/2019 | – | Vietnam | – | 98.74 | n/a | WRLFMD |
| VIT/22/2019 | – | Vietnam | – | 98.74 | n/a | WRLFMD |
| VIT/21/2019 | – | Vietnam | – | 98.74 | n/a | WRLFMD |
| MOG/17/2021 | – | Mongolia | – | 98.74 | n/a | WRLFMD |
| MOG/2/2022 | – | Mongolia | – | 98.74 | n/a | WRLFMD |
| CAM29/2019 | January-19 | Cambodia | Cattle | 98.54 | MZ634455.1 | Ryoo et al. (28) |
| MOG/Khovd/2021 | December-21 | Mongolia | Cattle | 98.26 | ON9822298.1 | Viktor et al. (27) |
| CAM22/2019 | January-19 | Cambodia | Cattle | 98.10 | MZ634454.1 | Ryoo et al. (28) |
| MOG/Sukhbaatar/2021 | December-21 | Mongolia | Cattle | 97.95 | ON982230.1 | Viktor et al. (27) |
| Orenburg/RUS/2021 | December-21 | Russia | Cattle | 97.16 | ON982231.1 | Viktor et al. (27) |
| KAZ/2022 | January-22 | Kazahstan | Cattle | 96.37 | ON982227.1 | Viktor et al. (27) |

Includes FMDV VP1 coding sequences from neighboring East and Southeast Asian countries with more than 95% sequence identity to the CJ/SKR/1/2023 isolate from Chungbuk province.

immunity, despite the effective vaccination policy, with presence of livestock farms having low herd immunity was still observed during the recent PVM carried out in 2023. In general, the level of herd immunity depends on the reproduction ratio ($R_0$) as well as effectiveness of vaccination. 75–80% coverage of population herd immunity is usually referenced as the level of immunity required to block the spread of FMDV (24, 25), when $R_0$ of an FMD outbreak is estimated to be below 5 and vaccination is 100%

TABLE 3 Antigenic relationship ($r_1$ values) of FMD viruses from the Republic of Korea against the currently used FMDV vaccine strains, as determined by 2D-VNT assay.

| | Vaccine strain | | |
|---|---|---|---|
| Field isolates | O3039 | O Manisa | O1 Campos |
| O/JP/SKR/2/2023 | 0.93 | 0.74 | 0.88 |
| O/SKR/1/2019 | 0.62 | 0.52 | ND* |
| O/SKR/1/2017 | 0.48 | 0.43 | ND |
| O/SKR/2/2017 | 0.59 | 0.38 | ND |

Data for the FMDV isolate collected during the 2023 outbreak is compared to previous outbreaks in 2017 and 2019 against with the currently used FMDV vaccine strains, as determined by 2D-VNT assay (*ND: not done, cattle not vaccinated with O campos in 2017 and 2019).

effective (26). SP/O ELISA was conducted in all the 11 farms reported of being infected by FMD in Chungbuk Province, where results revealed a positive rate between 24 and 75% for 7 farms (data not shown). Understanding these results is important because even if the vaccine is well matched and high potency, any non-compliance with vaccination procedures prescribed by manufacturer or the Republic of Korea Government guidelines may lead to immunity gaps on farms. Testing of samples from the remaining four FMD-positive farms revealed over 80% SP/O ELISA positive rate, which might be results of anamnestic responses induced by viral infection following vaccination.

After a period of 4 years where no FMD outbreaks have been detected, this report highlights the reintroduction of the O/ME-SA/Ind-2001e FMDV lineage into the Republic of Korea during May of 2023. The active circulation of this lineage in neighboring countries suggest a transboundary origin for these cases, which was further supported by the close genetic relatedness between the field viruses of the same FMDV lineage isolated from other East Asian countries between 2021 and 2022. It was noted that the outbreaks occurred on farms with a low level of serotype O-specific antibodies despite the systemic compulsory vaccination policy. These findings reinforce the importance of maintaining the protective immune status against FMDV to prevent the occurrence of FMD in geographic situations where FMDV may be introduced.

## 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

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because No ethical approval was required for this study as sample collection was performed using standard diagnostic procedures with no harm to the animals. Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

SR: Conceptualization, Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. HK: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. D-RL: Data curation, Formal analysis, Visualization, Writing – original draft. J-MK: Project administration, Writing – review & editing. YW: Investigation, Writing – review & editing. JK: Investigation, Writing – review & editing. DK: Data curation, Formal analysis, Writing – review & editing. AN: Writing – review & editing, Data curation, Formal analysis. S-HC: Conceptualization, Data curation, Formal analysis, Supervision, 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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# Sero-epidemiological investigation and cross-neutralization activity against SARS-CoV-2 variants in cats and dogs, Thailand

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Epidemiological data on SARS-CoV-2 infection in companion animals have been thoroughly investigated in many countries. However, information on the neutralizing cross-reactivity against SARS-CoV-2 variants in companion animals is still limited. Here, we explored the neutralizing antibodies against SARS-CoV-2 in cats and dogs between May 2020 and December 2021 during the first wave (a Wuhan-Hu-1-dominant period) and the fourth wave (a Delta-dominant period) of the Thailand COVID-19 outbreak. Archival plasma samples of 1,304 cats and 1,795 dogs (total = 3,099) submitted for diagnosis and health checks were collected at the Prasu-Arthorn Veterinary Teaching Hospital, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom. A microneutralization test was used to detect neutralizing antibodies against the ancestral Wuhan-Hu-1 and the Delta variants. A plasma sample with neutralizing titers ≥10 was considered positive. Our results showed relatively low seroprevalence with seropositive samples detected in 8 out of 3,099 individuals (0.26, 95% CI 0.11–0.51%). Among these cases, SARS-CoV-2 neutralizing antibodies from both the ancestral Wuhan-Hu-1 and the Delta variants were found in three out of eight cases in two cats ($n = 2$) and one dog ($n = 1$). Furthermore, neutralizing antibodies specific to only the ancestral Wuhan-Hu-1 variant were exclusively found in one cat ($n = 1$), while antibodies against only the Delta variant were detected in four dogs ($n = 4$). Additionally, the neutralizing cross-activities against SARS-CoV-2 variants (Alpha, Beta, and Omicron BA.2) were observed in the seropositive cats with limited capacity to neutralize the Omicron BA.2 variant. In summary, the seropositivity among cats and dogs in households with an unknown COVID-19 status was relatively low in Thailand. Moreover, the neutralizing antibodies against SARS-CoV-2 found in the seropositive cats and dogs had limited or no ability to neutralize the Omicron BA.2 variant. Thus, monitoring SARS-CoV-2

infection and sero-surveillance, particularly in cats, is imperative for tracking virus susceptibility to the emergence of new SARS-CoV-2 variants.

KEYWORDS

cat, dog, SARS-CoV-2, neutralizing antibodies, Thailand

# 1 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first detected in the Wuhan province of China in late 2019. It has rapidly spread worldwide (1) and the viral genome has acquired new mutations, resulting in the emergence of SARS-CoV-2 variants of concern (VOC) (2). SARS-CoV-2 originated from wild animals, in particular, horseshoe bats (*Rhinolophus affinis*) and Malayan pangolins (*Manis javanica*) (3, 4). SARS-CoV-2 transmission is mainly human-to-human. However, several animal species have been infected with SARS-CoV-2 (5, 6).

Companion animals in close contact with humans (mainly cats and dogs) were thought to be at increased risk of infection (7). SARS-CoV-2 infection in cats and dogs has been investigated to better understand their role in the epidemiology of the disease. SARS-CoV-2 viral isolates (8, 9), SARS-CoV-2 RNA (8–13), and specific SARS-CoV-2 antibodies (8–28) have been detected in cats and dogs in different countries. Previous studies showed that the SARS-CoV-2 virus detected in companion animals corresponds to concurrent locally dominant lineages circulating in the human population (8, 26). The antibody response to the SARS-CoV-2 virus in cats in the United Kingdom followed circulating SARS-CoV-2 variants in humans (28). It indicated that long-term multiple contact between humans and companion animals could cause pathogen spillover. However, it is not clear whether they could become reservoirs for the SARS-CoV-2 virus.

Several studies investigated the role of neutralizing antibodies in viral clearance and protection against SARS-CoV-2 infection/re-infection in cats and dogs, both experimentally and naturally. The seropositive cats demonstrated a robust neutralizing antibody response, effectively preventing re-infection by SARS-CoV-2 (29). In dogs, evidence indicated seroconversion along with neutralizing antibody activities (29). The vaccinated cats had an antibody response that could neutralize the ancestral Wuhan-Hu-1 and the Delta variants at comparable levels (30). Cats and dogs naturally infected with SARS-CoV-2 have produced neutralizing antibodies against the ancestral Wuhan-Hu-1, Alpha, Beta, Delta, and Omicron BA.1 variants, with lower neutralizing antibody titers against the latest variant (26). A similar study was also conducted in Mexico, and the neutralizing antibodies against the ancestral Wuhan-Hu-1 strain found in cats and dogs had a lower capacity to neutralize Omicron BA.1 (31). In humans, the neutralizing antibodies generated in response to SARS-CoV-2 vaccines, based on the ancestral Wuhan-Hu-1 strain, are less effective against the Delta and Omicron variants (32–35). According to a recent animal experiment conducted on Syrian hamsters, it was found that the Omicron BA.2 variant showed a significant level of resistance (2.9-fold) against the convalescent hamster sera previously infected with the Omicron BA.1 variant (36). This highlights the need to investigate the effectiveness of SARS-CoV-2 neutralizing antibodies in naturally infected cats and dogs against other variants of SARS-CoV-2, such as the Omicron BA.2.

To provide the sero-epidemiological data, we investigated SARS-CoV-2 ancestral Wuhan-Hu-1 and Delta variants neutralizing antibodies in 3099 plasma samples from domestic cats and dogs brought for any treatment or health check at the Prasu-Arthorn Veterinary Teaching Hospital, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom during the period from the first to fourth COVID-19 outbreak waves in Thailand, spanning from May 2020 to December 2021. Additionally, samples that tested positive for either or both the ancestral Wuhan-Hu-1 and the Delta variants, were evaluated for cross-reactive neutralizing activity against SARS-CoV-2 variants, including the Alpha, Beta, and Omicron BA.2.

# 2 Materials and methods

## 2.1 Ethical approval

The Faculty of Veterinary Science, Mahidol University-Institute Animal Care and Use Committee (FVS-MU-IACUC) approved the use of animal samples with Animal Ethics No. MUVS-2022-01-01. The study protocol involving the SARS-CoV-2 virus was approved by the Institutional Biosafety Committee of Mahidol University (IBC#2022-050).

## 2.2 Sample collection

The samples used in the study consisted of plasma from cats ($n = 1,304$) and dogs ($n = 1,795$) taken during routine healthcare visits to the Prasu-Arthorn Veterinary Teaching Hospital, Faculty of Veterinary Science, Mahidol University between May 2020 and December 2021. The animals were from 12 provinces in the Central and Western regions of Thailand. Information on species, identity, collection date, sex, age, breed, and location of each animal was recorded (Supplementary Table S1). However, information on the COVID-19 status of their originating households was unavailable. All EDTA blood samples used were residual plasma after routine diagnostic testing. Approximately 1 mL of blood was collected from each animal, and the plasma was separated by centrifugation and stored at −20°C until use. Dog and cat sera ($n = 40$) from 2014 to 2019 (pre-COVID-19 cohort) were from the Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals (MoZWE) serum bank, Faculty of Veterinary Science, Mahidol University.

## 2.3 SARS-CoV-2 viruses

The ancestral Wuhan-Hu-1 and Delta SARS-CoV-2 variants were used to detect the neutralizing antibodies in all tested samples. These two strains were selected to match the SARS-CoV-2 strains circulating

in humans when the animal blood was collected. Two isolates were included: hCoV-19/Thailand/MUMT-4/2020, representing the ancestral Wuhan-Hu-1 strain (GISAID accession number EPI_ISL_493139), and hCoV-19/Thailand/Nan_SEQ7413/2021, representing the Delta variant (GISAID accession number EPI_ISL_3797061). Three additional SARS-CoV-2 variants were used to detect cross-neutralizing antibodies in the seropositive samples. These were the Alpha, Beta, and Omicron BA.2 variants (hCoV-19/Thailand/Ranong_SEQ4773/2021, GISAID accession number EPI_ISL_3797062.2; hCoV-19/Thailand/Songkhla_SEQ8178/2021, GISAID accession number EPI_ISL_3407848; and hCoV-19/Thailand/NIC_BKK_SEQ4804/2022, GISAID accession number EPI_ISL_9611330). The virus stock was titrated in serial half-log10 dilutions (from 0.5 log to 7 log) to obtain 50% tissue culture infectious dose (TCID50) on 96-well microtiter plates of Vero cells. The infection experiments were performed in a biosafety level-3 (BLS-3) laboratory.

## 2.4 Microneutralization test

All plasma samples were assayed for SARS-CoV-2 ancestral Wuhan-Hu-1 and Delta variant neutralizing antibodies. The in-house microneutralization test (MNT) followed a previously described method (37, 38). An equal volume (60 µL) of serial 2-fold dilutions of heat-inactivated plasma (56°C, 30 min) and 100 TCID50 of SARS-CoV-2 virus were mixed and incubated at 37°C with 5% $CO_2$ for 1 h and transferred onto Vero cell monolayers ($2 \times 10^4$ cells/well). This was maintained for 3 days in Eagle's minimum essential medium supplemented with 2% fetal bovine serum at 37°C with 5% $CO_2$. Each sample was observed for evidence of a cytopathic effect (CPE). To ensure optimal testing results, the viral antigen used in each run was back-titrated, and a positive and negative serum control obtained from a COVID-19 vaccinated and non-vaccinated individual was included in each plate. Each plasma sample was tested in duplicate in 96-well plates. The sample titers were recorded as the reciprocal of the highest serum dilution that neutralized 100% of the tested virus, determined by CPE visualization. Samples with neutralizing antibody titers ≥10 were considered positive. The geometric mean titer (GMT) was estimated for each virus strain in cats and dogs, with neutralizing antibody titers of <10 assigned 5 and ≥ 320 assigned 320.

## 2.5 Statistical analysis

Microsoft Office Excel 2019 was used for data management, and the programming language R version 4.2.2 (R Foundation for Statistical Computing, Vienna, Austria) was used to analyze the GMT and for data analysis.

# 3 Results

The study revealed that seropositive samples were detected in 8 out of 3,099 individuals (0.26%) (Table 1; Figure 1). The seropositive samples were obtained from three cats (0.23%, 3/1304) and five dogs (0.28%, 5/1795) living in five provinces of Thailand (Table 2; Figure 2). Testing for both pre-COVID-19 sera/plasma from animal samples and those from the first wave of Thai human infections (January–December 2020) and the third wave (April 2021–May 2021) yielded negative results (Figure 1). However, plasma collected from one cat (0.08%) during the second wave (mid-December 2020–March 2021), and five dogs and two cats during the fourth wave (June 2021–December 2021) showed positive results for neutralizing antibodies (Figure 1). Sero-epidemiological studies demonstrated that the highest seropositivity (87.5%, 7/8) was found in the Central region of Thailand, specifically in Samut Sakhon ($n = 3$), Bangkok ($n = 2$), Nakhon Pathom ($n = 1$), and Nonthaburi ($n = 1$) provinces, while only one case (12.5%, 1/8) was found in the Western region, specifically in Phetchaburi province (Table 2; Figure 2).

Of eight seropositive samples, three had antibodies against both Wuhan-Hu-1 and Delta variants, including: (1) two seropositive cats (ID 1495 and ID 2893) with neutralizing antibody titers ranging from 40 to 320 (GMT of the ancestral Wuhan-Hu-1 strain = 80 and GMT of the Delta variant = 113) and (2) one seropositive dog (ID 2597) had the neutralizing antibody titers of 20 (GMT of the ancestral Wuhan-Hu-1 strain = 20 and GMT of the Delta variant = 20) (Table 2; Supplementary Table S1). In comparison, we detected neutralizing antibodies specific to only one strain of the SARS-CoV-2 virus in the other five animals. One seropositive cat (ID 2622) (0.08%, 1/1304) had the neutralizing antibodies against only the ancestral Wuhan-Hu-1 strain with titers of 20 (GMT = 20), while four seropositive dogs (ID 2332, ID 2903, ID 2980, and ID 2981) had the neutralizing antibodies against only the Delta variant with titers ranging from 10 to 40 (GMT = 20) (Table 2; Supplementary Table S1).

We further investigated the cross-neutralizing activity of antibodies from seropositive cats and dogs ($n = 8$) against SARS-CoV-2 variants, including the Alpha, Beta, and Omicron BA.2 variants. Our results demonstrated that the seropositive cat (ID 1495) had neutralizing antibody titers of 40 against the ancestral Wuhan-Hu-1, Alpha, Beta, and Delta variants but none against the Omicron BA.2 variant. The seropositive cat (ID 2893) had neutralizing antibody titers of 160, 40, 40, 320, and 20 against the ancestral Wuhan-Hu-1, Alpha, Beta, Delta, and Omicron BA.2 variants, respectively. In contrast, the seropositive dog (ID 2597) had neutralizing antibody titers of 20 against the ancestral Wuhan-Hu-1 and Delta variants but none against the Alpha, Beta, and Omicron BA.2 variants. The other five seropositive animals, including cats (ID 2622) and dogs (ID 2332, ID 2903, ID 2980, and ID 2981), had no neutralizing antibody titers against the tested variants (Table 2).

# 4 Discussion

SARS-CoV-2 virus continues to evolve and adapt to the human population, leading to the emergence of new variants (2). Moreover, the SARS-CoV-2 virus has been transmitted to several animal species, some of which may serve as viral reservoirs (39). Cats and dogs are among the animal species frequently in close contact with humans. Therefore, it is essential to provide the sero-epidemiological data and the cross-reactive neutralizing activity against SARS-CoV-2 variants in these species. This study is a large-scale investigation of SARS-CoV-2 neutralizing antibodies against the ancestral Wuhan-Hu-1 and Delta variants in cats and dogs performed over a prolonged period before the Thailand Omicron variant outbreak.

Our study indicated a relatively low seroprevalence, similar to the other large-scale study in cats and dogs from households with

TABLE 1 Screening for the neutralizing antibody titers against SARS-CoV-2 viruses in cats and dogs by microneutralization assay.

| Species | Total | Number of positive samples with the neutralizing antibody titers of ≥ 10, and percentage with 95%CI | | | |
|---|---|---|---|---|---|
| | | Wuhan-Hu-1 | Delta (B.1.617.2) | Wuhan-Hu-1 and Delta (B.1.617.2) | Total |
| Cat | 1,304 | 1, 0.07% [95% CI 0.009–0.42] | 0, 0.00% [95% CI 0.00–0.28] | 2, 0.15% [95% CI 0.01–0.55] | 3, 0.23% [95% CI 0.04–0.67] |
| Dog | 1,795 | 0, 0.00% [95% CI 0.00–0.20] | 4, 0.22% [95% CI 0.06–0.56] | 1, 0.05% [95% CI 0.001–0.31] | 5, 0.27% [95% CI 0.09–0.64] |
| Total | 3,099 | 1, 0.03% [95%CI 0.0008–0.17] | 4, 0.12% [95% CI 0.03–0.33] | 3, 0.09% [95% CI 0.01–0.28] | 8, 0.26% [95% CI 0.11–0.51] |

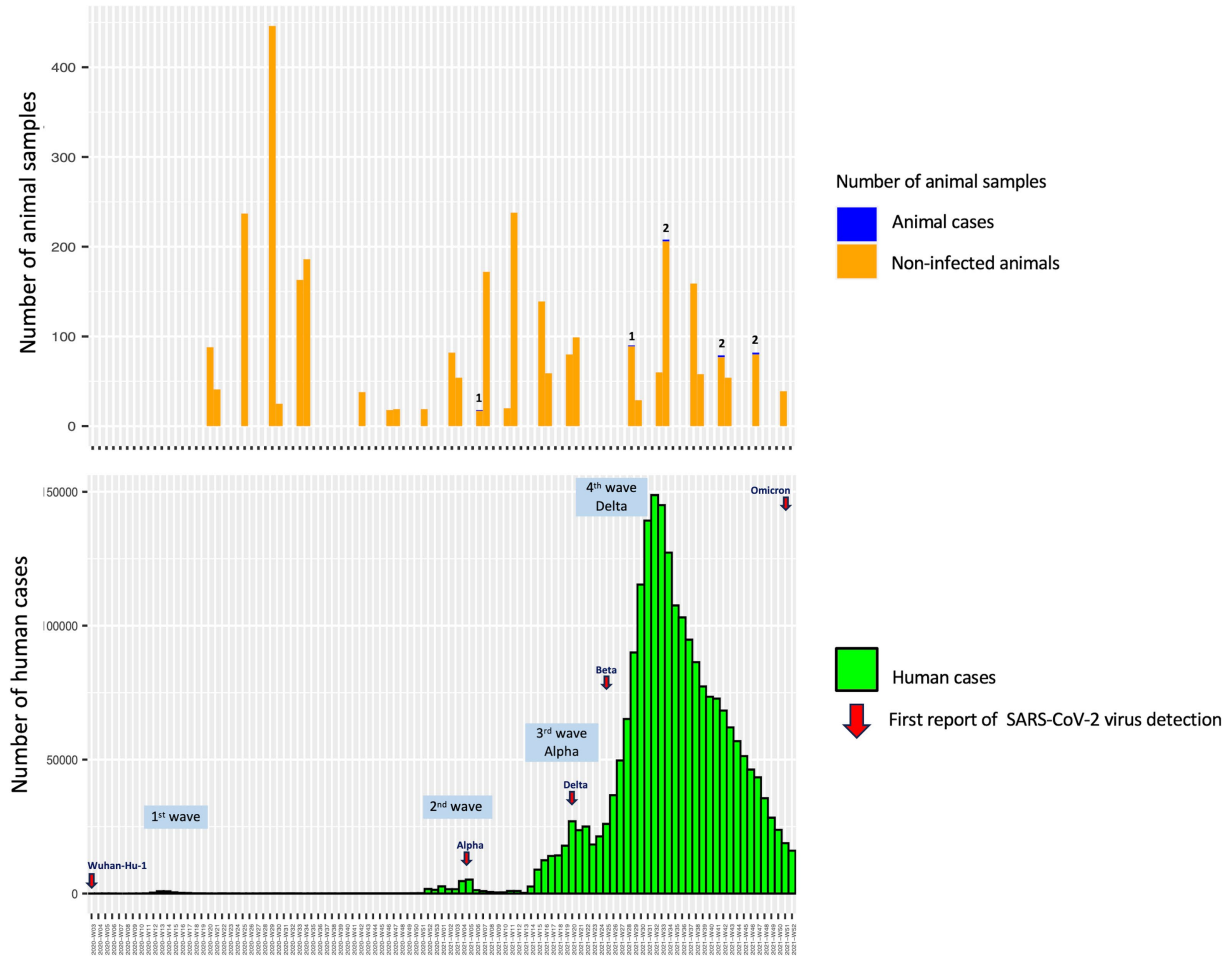


FIGURE 1
Animal sampling duration and human COVID-19 case detection in Thailand between May 2020 and December 2021.

unknown COVID-19 status (14, 17, 23). However, several studies demonstrated that the seropositivity was significantly greater in cats and dogs from COVID-19-positive households compared to those with owners of COVID-19-negative households or unknown status (8, 15, 18, 20, 26).

Spillover of the SARS-CoV-2 virus from humans to pet animals has been continuously documented (8–28). Several studies have demonstrated that SARS-CoV-2 viruses detected in companion animals correspond to concurrent locally dominant lineages circulating in the human population (8, 26). In this study, we found that the prevalence of antibodies in these cats and dogs was also in line with that found in the human population of Thailand, consistent with a previous report (28). Previous sero-epidemiological studies have yielded data indicating a low infection rate of SARS-CoV-2 in Thai people in various risk groups in Bangkok and Chiang Mai Province of Thailand during the first year of the COVID-19 pandemic, which spanned the first two waves and part of the third wave of the COVID-19 outbreaks in Thailand (40). It is consistent with the negative results obtained from animal samples collected during those same periods (Figure 1). Among the seropositive samples, one cat (0.08%) was identified in Samut Sakhon province of Thailand during the second wave, which was considered an epicenter of COVID-19 outbreaks in the country (41). The study by Jairak et al. (42) demonstrated that cats and dogs in Samut Sakhon province of

TABLE 2 Demographic information and neutralizing antibody titers of cats and dogs with SARS-CoV-2 seropositivity.

| ID | Species | Date of collection (2021) | Age | Sex | Breed | Location | Neutralizing antibodies titer | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Wuhan-Hu-1 | Alpha (B.1.1.7) | Beta (B.1.351) | Delta (B.1.617.2) | Omicron (BA.2) |
| 1495 | Cat | 10 February | 8 M | Female | Thai | Samut Sakhon | 40 | 40 | 40 | 40 | <10 |
| 2332 | Dog | 29 July | 11 Y 9 M | Male | German Shepherd Mix | Samut Sakhon | <10 | <10 | <10 | 40 | <10 |
| 2597 | Dog | 26 August | 2 Y | Male | Thai | Nakhon Pathom | 20 | <10 | <10 | 20 | <10 |
| 2622 | Cat | 30 August | 3 Y 1 M | Male | Thai | Phetchaburi | 20 | <10 | <10 | <10 | <10 |
| 2893 | Cat | 7 October | 5 M | Male | Persian | Bangkok | 160 | 40 | 40 | 320 | 20 |
| 2903 | Dog | 11 October | 13 Y 2 M | Male | Golden Retriever | Bangkok | <10 | <10 | <10 | 20 | <10 |
| 2980 | Dog | 4 November | 7 Y 3 M | Female | Thai Ridgeback | Samut Sakhon | <10 | <10 | <10 | 20 | <10 |
| 2981 | Dog | 4 November | 16 Y 4 M | Male | Shih Tzu | Nonthaburi | <10 | <10 | <10 | 10 | <10 |
| NC | Human | 28 December | 42 Y 4 M | Female | – | Nakhon Pathom | <10 | <10 | <10 | <10 | <10 |
| PC | Human | 27 July | 32 Y 6 M | Female | – | Samut Prakan | 320 | 20 | <10 | 20 | <10 |

ID, identity; NC, negative control; PC, positive control; M, month; Y, year.

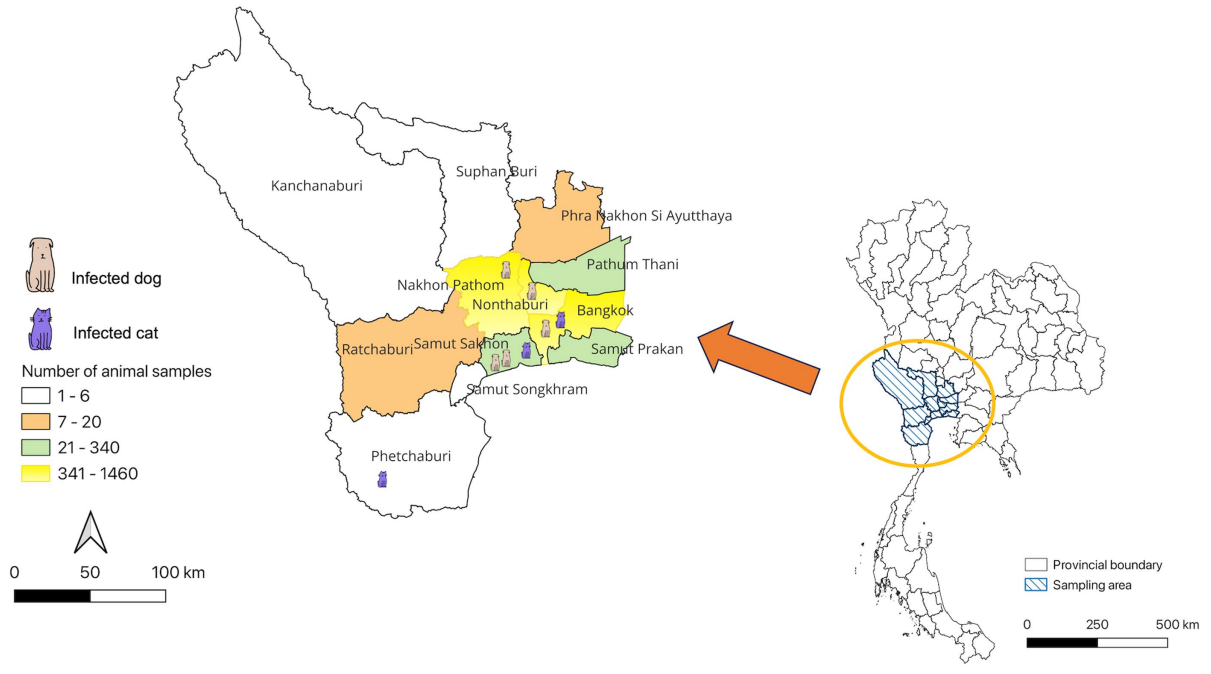


FIGURE 2
Distribution of SARS-CoV-2 seropositivity cases in cats and dogs, Thailand.

Thailand during the second wave had anti-N-IgG antibodies against SARS-CoV-2, with a prevalence of 3.14% (5/159) as determined by indirect multispecies ELISA. In our study, the highest seroprevalence found in cats and dogs in the fourth wave of COVID-19 outbreaks in Thailand could be linked to the locally dominant viral lineages circulating in the Thai human population, which are primarily driven

by the Delta variant (43). It has been reported that the Delta variant is approximately twice as transmissible as the ancestral strain from Wuhan in the human population (44). The study by Jairak et al. (45) demonstrated that cats and dogs in Bangkok and the vicinities of Thailand during the fourth wave were infected with the Delta variant. After viral RNA detection, SARS-CoV-2 antibodies could be detected in both dogs (day 9) and cats (day 14) (45). Although the source of the SARS-CoV-2 infection in cats and dogs in our study is unknown, it is most likely that they contracted the virus from their owners, who were infected during close contact activities.

We further investigated the neutralizing cross-activities against SARS-CoV-2 variants in seropositive cats and dogs. The timing of animal blood sample collection, during which the animal may have been exposed to owners infected with SARS-CoV-2 variants, could impact antibody titers. For instance, the seropositive cat with ID 1495 displayed neutralizing antibody titers of 40 against the ancestral Wuhan-Hu-1, Alpha, Beta, and Delta variants while exhibiting titers of less than 10 against the Omicron BA.2 variant. This cat was discovered to be seropositive in February 2021, during the second wave of SARS-CoV-2 infections in the human population (43). Given that this period coincided with the ancestral Wuhan-Hu-1 variant in the COVID-19 wave, it is possible that the animal was also exposed to this strain. Another seropositive cat with ID 2893 was sampled in October 2021, during the period when the Delta variant was predominant among humans in Thailand (43). The seropositive cat exhibited the highest neutralizing antibody titers of 320 against the dominant Delta variant, compared to other variants (Table 2; Supplementary Table S1). It is important to consider the timing of animal blood sample collection in relation to potential exposure to owners infected with the Delta variant. This seropositive cat showed neutralizing cross-reactivity with similar neutralizing antibody titers (range 40–160) against the ancestral Wuhan-Hu-1, Alpha, and Beta variants and lower neutralizing antibody titers (equal 20) against the Omicron BA.2 variant. Our data are consistent with previous reports showing that cats and dogs were found to produce neutralizing antibodies against the ancestral strain (31) and the Alpha, Beta, Delta, and Omicron BA.1 variants, with lower neutralizing antibody titers against the latter (26, 31).

It is worth noting that the seropositive dog (ID 2597) was sampled in August 2021, during the Delta variant's dominant period (43). This seropositive dog exhibited neutralizing antibody titers of 20 against both the ancestral Wuhan-Hu-1 and Delta variants, showing no cross-reactivity against the Alpha, Beta, and Omicron BA.2 variants (Table 2). These findings suggest that this dog was previously exposed to the Delta variant.

Several studies demonstrated the difference in SARS-CoV-2 susceptibility experimentally in cats and dogs, which may explain the differences in humoral immune responses against SARS-CoV-2 variants seen in this study. Only SARS-CoV-2-infected cats exhibited viral shedding and tissue tropism, which suggests that cats are highly susceptible to SARS-CoV-2. However, dogs have relatively low susceptibility to SARS-CoV-2 (29, 46). Cats have a higher presence of ACE2 host cell receptors in the respiratory tract than dogs, which may account for more efficient viral replication (47). Younger cats are more susceptible to SARS-CoV-2 than older ones (46). In our study, young cats developed a robust humoral immune response with neutralizing antibody titers ranging from 40 to 320 in two seropositive cats at the age of 5 months (ID 2893) and 8 months (ID 1495). In contrast, the seropositive cat aged 3 years (ID 2622) exhibited low neutralizing antibody titers of 20 (Table 2; Supplementary Table S1). The seropositive cats had neutralizing antibody titers ranging from 20 to 320, while seropositive dogs had antibody titers ranging from 10 to 40. In this study, we found that cats had higher neutralizing antibody titers than dogs, consistent with previous reports (15, 16, 19, 21, 31).

Our study had some limitations: First, all plasma samples were obtained from households with an unknown COVID-19 status. Therefore, we could not determine the impact of disease transmission between humans and animals. Second, more data and positive samples will help determine the accurate level of neutralizing antibodies that cross-react against SARS-CoV-2 variants in cats and dogs. Finally, this was a cross-sectional study relying on single blood samples. Conducting a longitudinal study would be beneficial for gaining a deeper understanding of the persistence of SARS-CoV-2 neutralizing antibodies in cats and dogs under natural conditions. As time passes, neutralizing antibody levels might decrease or disappear in some animals.

In conclusion, the investigation of SARS-CoV-2 neutralizing antibodies has enhanced our understanding of sero-epidemiological data in cats and dogs over an extended period preceding the outbreak of the Omicron variant in Thailand. It was observed that the neutralizing antibodies against SARS-CoV-2 in the seropositive cats and dogs demonstrated limited or no ability to neutralize the Omicron BA.2. Disease surveillance in companion animals, particularly cats, should be maintained due to the possibility of increased susceptibility to new SARS-CoV-2 variants. This could lead to the creation of potential viral reservoirs and transmission between humans and animals.

## 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 animal studies were approved by the Faculty of Veterinary Science, Mahidol University-Institute Animal Care and Use Committee (FVS-MU-IACUC). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was not obtained from the owners for the participation of their animals in this study because All EDTA blood samples were residual samples obtained after routine diagnostic testing. The Institute Animal Care and Use Committee (IACUC) does not require informed consent. However, researchers must submit a protocol for the exemption review when using secondary samples.

## Author contributions

SS: Conceptualization, Writing – original draft, Writing – review & editing, Formal analysis, Visualization, Data curation, Project administration. NK: Writing – review & editing, Investigation, Methodology, Validation, Formal analysis. MT: Methodology,

Validation, Investigation, Formal analysis, Writing – review & editing. SC: Methodology, Validation, Investigation, Formal analysis, Writing – review & editing. ST: Project administration, Resources, Investigation, Writing – review & editing. WW: Project administration, Conceptualization, Writing – review & editing. PJ: Data curation, Resources, Investigation, Software, Writing – review & editing. WT: Data curation, Resources, Investigation, Software, Writing – review & editing. SB: Resources, Investigation, Software, Writing – review & editing. WS: Resources, Project administration, Visualization, Writing – review & editing. PO: Project administration, Resources, Writing – review & editing. PP: Project administration, Resources, Writing – review & editing. WP: Project administration, Writing – original draft, Conceptualization, Formal analysis, 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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## Supplementary material

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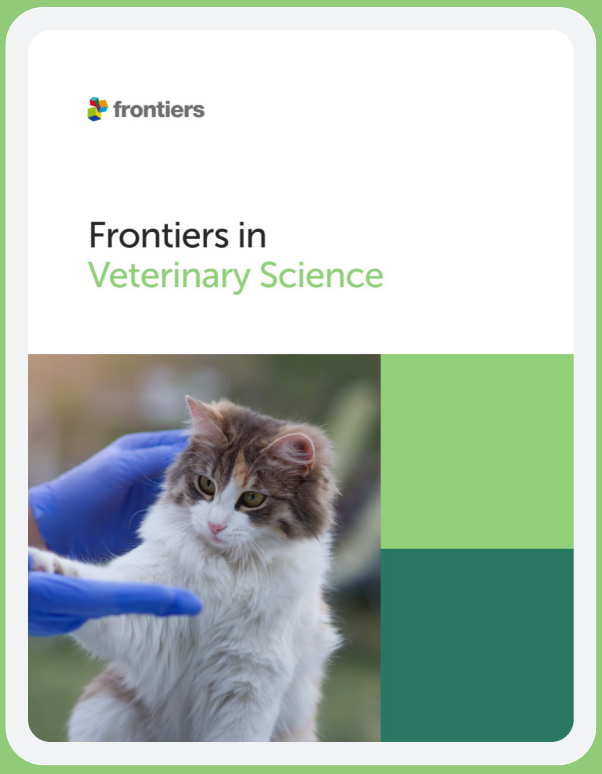


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