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Advancements in understanding zoonotic parasitic diseases

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Advancements in understanding zoonotic parasitic diseases

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Editorial: Advancements in understanding zoonotic parasitic diseases

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Editorial on the Research Topic
Advancements in understanding zoonotic parasitic diseases

The field of zoonotic parasitic diseases, which encompasses infections transmissible between animals and humans, is currently undergoing significant advances, underscoring their growing importance in global public health. These diseases, caused by diverse range of pathogens including protozoa, helminths and ectoparasites, exhibit a broad spectrum of symptoms and severities, some of which result in severe acute illness or even death. Transmission of these parasites can occur through a variety of sources, including contaminated food and water, direct contact with domestic and wild animals, and vector-borne pathways involving insects or other arthropods. Despite the critical impact of zoonotic parasitic diseases, their detection, treatment, and control are often hindered by incomplete understanding and fragmented knowledge of their complex life cycles, host interactions, and environmental reservoirs (1, 2).

These challenges are further exacerbated by environmental and societal factors. Global warming is altering the habitats and distribution of both vectors and hosts, leading to the emergence and re-emergence of zoonotic diseases in previously unaffected regions (3). Additionally, the globalization of trade and travel facilitates the rapid movement of animal and human reservoirs, introducing parasites and vectors into new geographic areas and increasing the risk of outbreaks. These factors call for a comprehensive and integrated approach to the management of zoonotic parasitic diseases (4).

The primary aim of this Research Topic is to enhance the understanding of zoonotic parasitic diseases, especially those that are emerging and that have recently come to widespread public attention. This includes deepening the knowledge of their biology, epidemiology, and the local, regional, national or global advances in their diagnosis, treatment and control. Additionally, the Research Topic aims to facilitate the exchange of updated information on these diseases, particularly in relation to their proteomics, immunology and molecular biology, as well as new vaccines and diagnostic tools.

This Research Topic has gathered 15 manuscripts—12 original research papers, one short research paper, one case report and one opinion paper—with contributions from 104 authors. These papers focus on zoonotic parasitic diseases and related areas, covering Research Topics such as the biology and epidemiology of these diseases; advances in their diagnosis, treatment and control; studies on parasite-host relationships; research in proteomics, immunology and molecular aspects; the development of new vaccines and diagnostic tools; and illustrative clinical cases of these conditions.

The team of Rondón et al. has contributed to the detection of *Iodamoeba bütschlii, Dientamoeba fragilis*-like, *Giardia* sp., *Balantidium/Buxtonella* sp., *Capillaria* sp., *Trichuris* sp., strongyliform larvae, and *Oesophagostomum* sp. potentially zoonotic parasites, in primates from a zoo in Italy. The results provide important and necessary information that justifies the generation of adequate safety measures for both visitors and animal keepers. Another study, by Cancino-Faure et al., searched for the presence of zoonotic filarial nematodes in mosquitoes [*Aedes* (*Ochlerotatus*) *albifasciatus* and *Culex pipiens*] and dogs in a previously unstudied semi-rural area of Central Chile, finding *Acanthocheilonema reconditum* and *Setaria equina*; although the authors did not detect the presence of zoonotic parasites, they did stress the importance of continuous surveillance, especially in areas that are not regularly monitored.

de Andrade Vieira et al. analyzed the presence of *Dirofilaria immitis* in an area of Rio de Janeiro (Brazil) showing the expansion of the disease and highlighting the importance of the use of prophylactic measures and awareness of both health personnel and dog owners to interrupt the spread and establishment of heartworm disease. On the other hand, Esteban-Mendoza et al., through the application of molecular and morphological characterization techniques, demonstrated the importance of their use in the detection of microfilaremic dogs infected by *D. immitis* and *A. reconditum,* and their usefulness in making an accurate diagnosis to establish an appropriate treatment for each filarial species. Likewise, and in relation to the study of diagnostic techniques, the study carried out by Albasyouni et al. underlines the need to use molecular techniques to describe intestinal coccidian parasites (*Eimeria* spp.) together with morphological tools in birds (pigeons).

The research of Liu et al. presented a study in which molecular techniques were applied to detect several zoonotic species of *Cryptosporidium* spp. and others adapted to wild rodents in a province of China. Similarly, Uakhit et al. identified *Baylisascaris* spp. by molecular and phylogenetic analyses in wild carnivores from different regions of Kazakhstan, highlighting their potential risk of infection to humans. These findings underscore the importance of a multidisciplinary "One Health" approach to prevent the spread of such pathogens.

Also, Bandelj et al. presented, for the first time, a case report describing the presence of *Gongylonema pulchrum*, a potentially zoonotic parasite, in a Slovenian roe deer using morphological and molecular techniques. Moreover, Elshahawy et al. conducted a study to molecularly characterize leukocytozoonosis in pigeons in Egypt, with the aim of developing more effective control and prevention strategies to limit the spread of infection to other birds.

Rodríguez-Escolar, Balmori-de la Puente et al. and Rodríguez-Escolar, Hernández-Lambraño et al. conducted two studies focused on controlling vector-borne zoonotic diseases, developing infection risk maps for *Dirofilaria* spp. in Serbia and canine leishmaniasis in Spain and Portugal. To this aim, they took into account the habitat suitability map for their main vectors, the weighting of these maps with the parasite behavior in these vectors and their validation with the geolocation of infected dogs. This approach offers high predictive accuracy, providing an excellent tool for the control and prevention of these diseases.

In addition, Raw et al. highlight the relevance of conducting efficient and effective deworming programs in dogs for *Ancylostoma caninum* that can be administered regularly without the need for veterinary supervision in Australian Aboriginal communities where veterinary visits may be limited. In reference to leishmaniasis, Cavalera et al. report that the seasonality of anti-*L. infantum* titres in dogs should be considered in the design of clinical trials to evaluate treatments and preventive measures for canine leishmaniasis, which would enhance the efficacy of control strategies.

In relation to parasite-host relationships and the resulting immune response, Chai et al. presented data on the T cell-mediated immune response in the maintenance of intestinal immune homeostasis and the impact of *Moniezia benedeni* infections in sheep, particularly in altering immune cell densities. In addition, the role of a recombinant protein (rEg.P29) from *Echinococcus granulosus* as a potential epitope peptide vaccine is explored by Yang et al., emphasizing its relevance given the zoonotic significance of this parasite.

We would like to express all our gratitude to all 104 researchers who have contributed to this Research Topic by sharing their valuable studies on zoonotic parasitoses from the "One Health" perspective. We also extend our thanks to the reviewers and staff of *Frontiers in Veterinary Science*, whose efforts have ensured the successful completion of this Research Topic.

Author contributions

RM: Writing – original draft, Writing – review & editing. SG: Writing – review & editing. LC: Writing – review & editing. EN: Writing – review & editing. EC: Writing – review & editing.

Conflict of interest

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

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Filarial nematodes in domestic dogs and mosquitoes (Diptera: Culicidae) from semi-rural areas in Central Chile

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Climate change, competent vectors, and reservoir animals are the main factors for developing vector-borne zoonotic diseases. These diseases encompass a significant and widespread category of pathogens (e.g., viruses, bacteria, protozoa, and helminths) transmitted by blood-feeding arthropods, including ticks, fleas, lice, triatomines, mosquitoes, sandflies, and blackflies. In Chile, several studies have explored the role of dogs as reservoirs of vector-borne pathogens; however, there is a lack of research investigating the presence of pathogens in arthropods. Specifically, within the order Diptera, limited knowledge exists regarding their roles as carriers of pathogens. This study aimed to examine the presence of zoonotic filarial nematodes in mosquitoes and dogs within a previously unstudied semi-rural area of Central Chile. Two hundred samples of dog blood and seven hundred and twenty-four mosquitoes were collected during 2021–2022 and studied for filarial nematodes by PCR. The prevalence of microfilaremic dogs detected by Knott's test was 7.5%, with *Acanthocheilonema reconditum* being the only species identified. *Aedes* (*Ochlerotatus*) *albifasciatus* was the most abundant mosquito species collected, and 15 out of 65 pools were positive for filarial nematodes. Among these pools, 13 tested positive for *Acanthocheilonema reconditum*, and two tested positive for *Setaria equina* through PCR. Additionally, five *Culex pipiens* specimens were positive for *Acanthocheilonema reconditum*. Despite the absence of zoonotic filarial species, these findings underscore the significance of monitoring pathogens in mosquitoes and animal hosts and continued research into the dynamics of vector-borne diseases, particularly in unexplored regions.

KEYWORDS

Acantocheilonema reconditum, dogs as reservoirs, vector-borne diseases, parasitic infections, mosquito surveillance, climate change

1 Introduction

Global changes (climate change, biodiversity loss, land use changes, biological invasion), competent vectors, and reservoir animals are the main factors for developing vector-borne zoonotic diseases (1). These comprise a relevant and globally distributed group of disease agents (i.e., viruses, bacteria, protozoa, and helminths) transmitted by hematophagous arthropods, such as ticks, fleas, lice, triatomines, mosquitoes, sand flies, and black flies (2, 3). The increased mobility and worldwide distribution of domestic dogs has contributed to the geographic expansion of some vector-borne pathogens (4). Additionally, migration of pet-owners from endemic areas has resulted in an overall increase in vector-borne diseases in previously non-endemic areas (5, 6). All the factors mentioned above are present in Chile. Furthermore, the number of free-roaming dogs, considered an important factor for parasite or pathogen transmission (7), has increased in Chile (8, 9). This is especially the case regarding roaming dogs with an owner, where irresponsible ownership practices persist, and preventive measures such as antiparasitic treatments are absent (10).

In Chile, several studies have examined the role of dogs as reservoirs of vector-borne pathogens (11–13). However, there has been limited exploration of arthropods as vectors, especially within the Diptera order. Knowledge is scarce on whether Diptera serves as a carrier of pathogens or not.

A recent study conducted by Cevidanes et al. (13) in the Metropolitan region of Chile discovered that 75% of the surveyed dogs harbored at least one pathogen in their blood, with 34% showing coinfection by two or more pathogens. The most prevalent pathogens were bacteria and protozoa (*Anaplasma platys*, Candidatus *Mycoplasma haematoparvum*, *Mycoplasma haemocanis*, *Trypanosoma cruzi*, and *Leishmania* sp.), followed by the filarial nematode *Acanthocheilonema* spp. (Nematoda: Onchocercidae), although at a low percentage. Unlike in Europe, filarial nematodes such as *Thelazia callipaeda*, *Onchocerca lupi*, and *Cercopithifilaria* spp., have not been documented in Chile.

It is worth noting that *Dirofilaria immitis* has not been considered endemic in Chile, with only one documented case involving an infected dog imported from Venezuela and living for two years before the finding in Santiago, Chile (5). Additionally, López et al. (12) discovered microfilariae resembling those of *D. repens* in a semi-rural area of the Metropolitan region in Chile. The authors considered these to represent a new *Dirofilaria* species or a variant closely related to *D. repens*. However, no further attempts were made to characterize the nematode.

The role of mosquitoes (Diptera: Culicidae) as vectors remains relatively understudied in Chile. Mosquito populations notably increase during warmer months, particularly in rural and semi-rural areas, leading to discomfort for both humans and animals and presenting potential as vectors. Collao et al. (14) researched on Rapa Nui, and Cancino-Faure et al. (15) studied the extreme north and central parts of Chile, focusing on the presence of *Flavivirus* in mosquitoes. None of the studies found medically significant flaviviruses in the studied mosquito species. However, there is still a need for further comprehensive studies in this field. Hence, this study aimed to explore the presence of zoonotic filarial nematodes in mosquitoes and dogs within a previously unexplored semi-rural region of Central Chile.

2 Materials and methods

2.1 Sampling area

This study was conducted in two specific, unexplored locations within the Región del Maule: Villa Alegre (35°40′00″S 71°45′00″W) and San Clemente (35°33′00″S 71°29′00″W), as depicted in Figure 1. Despite being unexplored, these areas were chosen because of their potential for endemic vector-borne disease. This possibility arose from their rural nature and observed inadequate dog ownership practices. Both areas feature vast expanses of plantations, where irrigation is characterized by either flooding or furrows, allowing for the conducive development of mosquitoes and the likely presence of vector arthropods that could act as potential disease vectors and reservoirs.

2.2 Dogs and mosquitoes sampling

To calculate the sample size, the study by Alcaíno et al. (16) was used as a reference, and a maximum difference of 10% from the prevalence was estimated using the study by Lopez et al. (12) as a reference. In addition, a significance level (α) of 5% and a study power of 90% were considered, resulting in a sample size of 200 dogs proportionally distributed in both study localities.

Blood samples were obtained from 200 dogs of at least two years in age between 2021 and 2022, with 100 dogs corresponding to Villa Alegre and 100 to San Clemente (Table 1). Data regarding sex, age (years), and presence of pruritus, alopecia, dermatitis, cardiopulmonary disease, or chronic cough were recorded. Owners voluntarily took their dogs for blood sampling to test for this study. Three milliliter of blood from the cephalic vein were collected in EDTA tubes. The plasma was removed after centrifugation and frozen at −20°C; the remaining sample was kept at 2–8°C until the analysis.

The entomological surveys for this study were conducted during the summer months (December to March) in 2021 and 2022 near the areas where the dogs lived. Adult mosquitoes were captured at the collection sites using an entomological net and suction tube through human landing, specifically during the most active biting period, from 20:00 to 23:00, for three days at each point. Subsequently, the captured mosquitoes were euthanized by freezing at −80°C for 20–30 min and later identified using taxonomic keys from Darsie and González et al. (17, 18) Female mosquitoes were grouped, and some

FIGURE 1
Geographical map of the Región del Maule in central Chile highlighting the specific localities of Villa Alegre and San Clemente, where dogs and mosquitoes were sampled in the present study.

individuals were placed in separate tubes based on the collection point and species. Male mosquitoes were not included in the study and were discarded.

2.3 Parasitological and molecular study of blood

Blood samples were processed within the first 72 h after collection. One milliliter of each centrifuged blood sample was used to perform the modified Knott's test (19). In the case of a positive sample, ten microfilariae were measured under microscopic examination using Leica Application Suite 3.4.0 software.[1] DNA was isolated from an aliquot of 250 µL from each positive blood sample by the modified Knott's test using the EZNA Tissue DNA Kit (Omega Bio-Tek),[2] according to the manufacturer's instructions. All the mosquitoes were ground (by pools or individually) with a pestle using 600 µL of SKP buffer plus β−mercaptoethanol and then extracted using NORGEN RNA/DNA purification kit (NORGEN Biotek Corp.,)[3] according to the manufacturer's instructions. The DNA was used as a template for the PCRs with the GoTaq DNA Polymerase (Promega),[4] amplifying a section of the 12S rRNA with the primers 12S-F GTTCCAGAATAATCGGCTA – 12S-R ATTGA CGGATGRTTTGTACC to determine the presence of nematodes in both blood and mosquito samples (20). A second PCR was performed on the same samples to amplify the regions 5.8S-ITS2-28S of filarial nematodes using the primers DIDR-F1 AGTGCGA ATTGCAGACGCATTGAG and DIDR-R1 AGCGGGTAATCA CGACTGAGTTGA for blood samples (21). The initial amplification

1 https://www.leica-microsystems.com

2 https://www.omegabiotek.com
3 https://norgenbiotek.com
4 https://www.promega.com

TABLE 1 Profile of dogs with filarial nematode presence in their blood, from two localities in Región del Maule.

Sample ID	Breed	Sex	Age (years old)	Symptoms associated	Locality		Coordinates	Sleeps
12	Mixed	Male	10	subcutaneous nodules	San Clemente	Corralones	35.5671987, −71.4041283	Outdoors
59	Mixed	Male	4	No		San Clemente	35.5825446, −71.4478778	Outdoors
63	Mixed	Male	2 1/2	No		Corralones	−35.5525588, −71.3589958	Outdoors
64	Mixed	Male	10	No		Corralones	−35.5525588, −71.3589958	Outdoors
65	Mixed	Male	10	No		Corralones	−35.5525588, −71.3589958	Outdoors
66	Mixed	Male	3	No		Corralones	−35.5520321, −71.3529779	Outdoors
91	Mixed	Female	6	No		Corralones	−35.5698906, −71.4333679	Outdoors
99	Mixed	Male	2 1/2	No		Corralones	−35.5468424, −71.4188117	Outdoors
147	Mixed	Male	8	No	Villa Alegre	Estación Ferrocarril	−35.6963739, −71.6806805	Outdoors
139	Mixed	Male	3 1/2	No		Estación Ferrocarril	−35.6963739, −71.6806805	Outdoors
145	Mixed	Female	8	No		Estación Ferrocarril	−35.6963739, −71.6806805	Outdoors
177	Mixed	Male	15	No		Avenida Certenejas	−35.6975914, −71.7320145,	Outdoors
183	Mixed	Male	18	No		Avenida Certenejas	−35.6975914,-71.7320145,	Outdoors
185	Mixed	Female	5	No		Avenida Certenejas	−35.6975914,-71.7320145,	Outdoors
186	Mixed	Female	5	No		Avenida Certenejas	−35.6975914,-71.7320145,	Outdoors
127	Mixed	Female	7	No		Los Conquistadores	−35.6724134,-71.7420647	Indoors

conditions were obtained from Rishniw et al. (21). Both PCR protocols were modified to improve the specificity and sensitivity of the reaction through a touchdown PCR. This modification was necessary because many of the samples, particularly those from mosquitoes, produced two or more bands of different sizes (22). Finally, DNA from both types of samples was used as a template in a third PCR using primers COIint-F TGATTGGTGGTTTTGGTAA and COIint-R ATAAGTACGAGTATCAATATC to detect *Dirofilaria immitis*, following the conditions of the technique described by Casiraghi et al. (23). Refer to the Supplementary material (Supplementary Table 1) for detailed information on the PCR protocol conditions.

DNA corresponding to *D. immitis* adult and *Acanthocheilonema reconditum* microfilariae were used as a positive control, and non-template DNA was included in each run as a negative control. Electrophoresis was performed in a 2% agarose gel. Amplification products from positive canine and mosquito samples were sent to MacroGen Chile for sequencing. The sequences obtained were edited in the BioEdit v.7.0.5.3 software suite (24), and later, their homology with sequences deposited in GenBank was confirmed with a BLASTn analysis (25).

A multiple sequence alignment of both genes was performed using the ClustalW65 Multiple Sequence Alignment tool (26). The orthologous gene of *Dirofilaria immitis* was used as the outgroup sequence for 12S rRNA and 5.8S-ITS2-28S in each analysis. Subsequently, two phylogenetic trees were constructed using the Maximum Likelihood Tree (ML) method with the MEGA-X program: Molecular Evolutionary Genetics Analysis v10.2.679 (27). The following options were configured: (i) Phylogeny test: Bootstrap method, (ii) Number of Bootstrap replications: 1000, (iii) Evolutionary model of the method: Tamura-Nei, and (iv) ML heuristic method: Nearest-Neighbor-Interchange (NNI).

2.4 Statistical data analysis

For data analysis, summary measures were considered to be quantitative and frequency measures were used. The Mann–Whitney U test assessed differences in quantitative variables, while Fisher's exact test was used for categorical variables. Data were considered statistically significant with a value of p <0.05. STATA statistical software (version 17; StataCorp, College Station, TX, United States) was used for all these analyses.

The Minimum Infection Rate (MIR) was calculated only for *Aedes* (*Ochlerotatus*) *albifasciatus* due to the limited number of mosquitoes collected from other species. It was assumed that a mosquito pool had at least one infected mosquito if *A. reconditum* DNA was found. As a result, MIR was calculated using the formula (number of positive pools/total number of mosquitoes studied) x100 (28).The MIR was estimated using the Wilson confidence interval method for binomial proportions with a 95% confidence interval (CI).

2.5 Ethics statement

The study was approved by the Comité de Cuidado y Uso de Animales de Laboratorio (CICUAL) of the Universidad Católica del Maule under the number 09–2021. Blood samples from canines were taken by trained personnel. The informed consent document was obtained from all the dogs' owners, and data like the age, breed, address, and the dog's sleeping location were recorded.

3 Results

3.1 Detecting filarial nematodes in canine blood

A total of 200 blood samples were collected from dogs. The average age of the 200 dogs was 5.9 ± 3.8 years, with 101 females accounting for 50.5%. One hundred and sixty-six dogs slept outside, of which 31 (18.7%) exhibited dermatological symptoms or signs compatible with filarial infections, and 3 (9.1%) showed signs but slept indoors. Regarding gender and the presence of symptoms, there were no differences between the positive (microfilaremic dogs) and negative (amicrofilaremic dogs) groups.

A difference in the average age was observed between microfilaremic dogs ($n = 15$) and those amicrofilaremic ($n = 184$). The negative dogs had an average age of 5.7 ± 3.8 (years), whereas the positive dogs had an average age of 7.4 ± 4.6 (years). The difference between the two groups in this sample did not reach statistical significance ($p = 0.056$). Additionally, when considering sleeping locations, 82.1% of amicrofilaremic dogs slept outside, whereas 100% of microfilaremic dogs slept outside without any statistical significance ($p = 0.059$).

It is important to note that several dogs from the same household were infected (Table 1: sample IDs 63, 64, 65; IDs 139, 145, 147; IDs 177, 183, 185, 186).

3.1.1 Microscopy identification by modified Knott's test

8% (16/200: 95% C.I.: 4.9–12.68) of the blood samples tested positive for the modified Knott's Test. The average length of 10 microfilariae was 260.07 µm (260.07 ± 6.59), and the average width was 5.01 µm (5.01 ± 0.51) (Supplementary Table 2). The measurements of microfilariae found in this study agree with the description of *A. reconditum* (Figure 2) according to Magnis et al. (29) who reported a length of 265 µm (264.83 ± 5.47) and a width of 5 µm (4.63 ± 0.52). However, it is important to note that one of the positive samples presented a single larva 244.14 µm length and 8.1 µm wide. This larva exhibited a pronounced buccal cavity and clearly observable esophagus and intestine. However, due to the divergence in these morphological features compared to known filariae, it was not possible to identify a precise species. (Supplementary Table 2 and Figure 2). This larva was excluded from the statistical analysis.

3.1.2 Molecular identification of filarial nematodes

At least one of the two PCRs targeting nematodes and filaria (12 s rRNA; 5.8S-ITS2-28S) was successfully amplified in 15 out of 16 blood samples from dogs that tested positive using the modified Knott's test. Ten of the 16 positive blood samples amplified a 650 bp PCR product of the 5.8S-ITS2-28S. However, two of these samples exhibited several non-specific amplification bands in addition to the 650 bp product (Supplementary Figure 1). Concurrently, a PCR product amplifying the 12S rRNA region was obtained for 9 out of the 16 samples. Combining both PCRs allowed the successful amplification of all samples (Supplementary Table 2). Moreover, all samples tested negative for the COI gene of *D. immitis*.

The fragments obtained through sequencing, targeting both genes, in 13 out of the 16 amplified samples corresponded to *A. reconditum*, showing 98–100% homology to GenBank entries (Supplementary Table 2). For samples positive to both PCRs, the best sequences were selected based on sequencing specificity using chromatogram analysis for subsequent BLASTn. However, the sequencing results of three samples were inconsistent with both PCR and morphometric findings.

A phylogenetic tree was constructed based on the best sequences in terms of quality and length: five for 5.8-ITS2-28S and six for 12S rRNA. The 5.8S-ITS2-28S sequences obtained formed a distinct cluster closely related to *Acanthocheilonema reconditum* (accession numbers KX932116.1, KX932124.1, and KX932127.1). Notably, this cluster was separated from its sister genus *Dirofilaria* (accession number LN626267.1) (Figure 3A). Similarly, the phylogenetic tree generated for the 12S rRNA region demonstrated the grouping of *Acanthocheilonema* sequences with those of *Acanthocheilonema reconditum* (accession numbers OR778872.1 and MZ678927.1), and *Acanthocheilonema vitae* (KP760315.1). To root the tree, the 12S rRNA gene sequence of *Dirofilaria immitis* (accession numbers AP017707.1:9539–10301) was clustered in a separate taxon, serving as the outgroup (Figure 3B).

3.2 Identification through morphological analysis of species of mosquitoes collected

A total of 724 adult female mosquitoes were collected between December and March. Based on morphological characteristics, they were assigned to two genera and three species: 91% were identified as *Ae.* (*Och.*) *albifasciatus*, 4.4% *Culex pipiens*, and 2.3% *Cx. apicinus*. However, 2.2% of the specimens could not be identified due to missing or damaged morphological features crucial for their classification.

3.2.1 Identification of filarial nematodes In mosquitoes.

Seven hundred and twenty-four samples of female mosquitoes were studied using PCR for 12S rRNA for nematodes and *D. immitis* COI gene. Whole mosquito bodies were grouped into pools of 2–11 samples or kept in individual tubes, depending on the species and geographic collection point. This process yielded 79 pools and 62 specimens for DNA extraction. The number of pools was as follows: *Ae.* (*Och.*) *albifasciatus* ($n = 659$, 65 pools, 43 individuals), *Cx. pipiens* (n = 32, six pools, 17 individuals), *Cx. apicinus* ($n = 17$, four pools, two individuals), and unidentified species ($n = 16$, 16 individuals).

PCR screening targeting nematodes' 12S rRNA (Supplementary Table 3) revealed positivity in 15 pools (all from *Ae.* (*Och.*) *albifasciatus*) and nine individuals (Five from *Cx. pipiens* and four from *Ae.* (*Och.*) *albifasciatus*). The minimum infection rate (MIR) was calculated only for *Ae.* (*Och.*) *albifasciatus* at 23% (95% CI 14.41–34.75) due to the low number of specimens collected in the other species. All positive pools and individuals were subjected to sequencing more than once; however, only six sequences of satisfactory quality were obtained. BLASTn similarity searches using 12S rRNA sequences obtained from three pools of *Ae.* (*Och.*) *albifasciatus* revealed 99.54, 98.85, and 98.13% similarity to *Acanthocheilonema reconditum* (accession number MZ678927.1). Additionally, two pools of *Ae.* (*Och.*) *albifasciatus* showed 97.87 and 93.3% similarity to *Setaria equina* (accession number AJ544835.1),

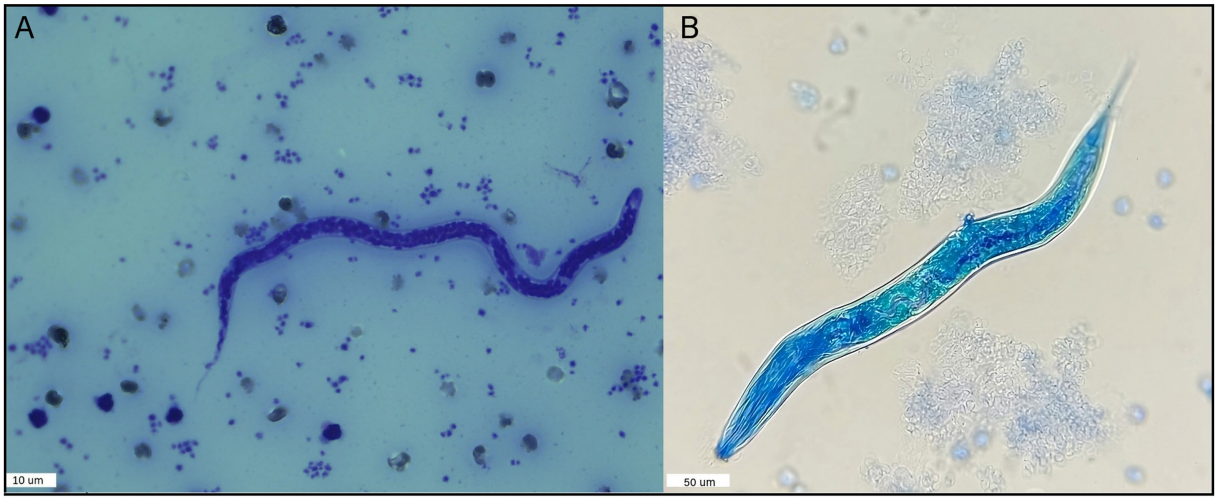

FIGURE 2
Nematode specimens found in the blood of dogs in this study using the modified Knott's test. **(A)** Microfilariae example found in 15 dog samples identified as *A. reconditum* following measurement according to Magnis et al. (29), using the modified Knott's test and Giemsa stain. **(B)** Larvae found in one dog blood sample using the modified Knott's Test.

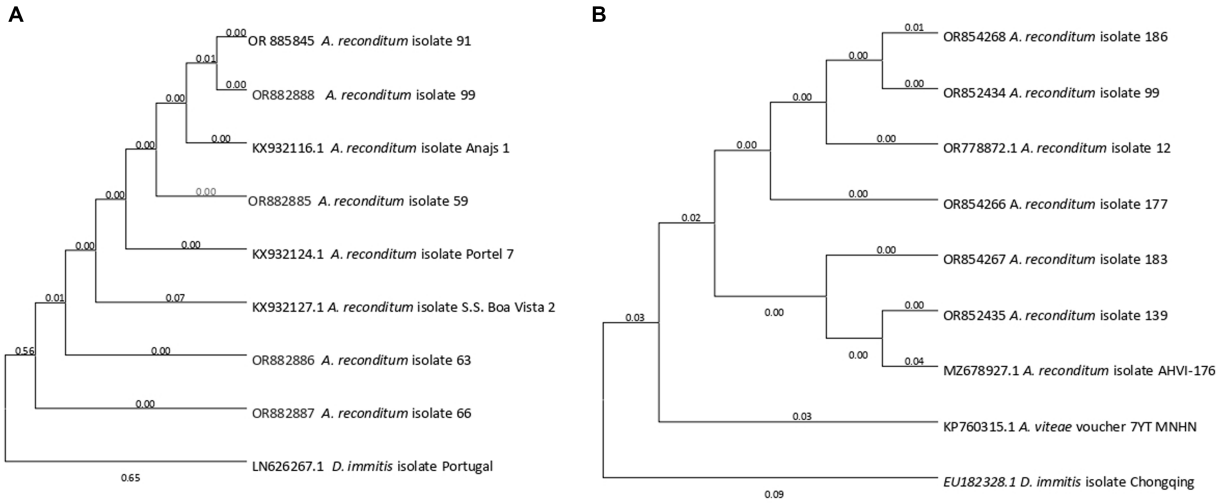

FIGURE 3
The phylogenetic placement of the *Acanthocheilonema reconditum* sequence obtained in this study, as inferred through Maximum Likelihood analysis. **(A)** Partial 5.8-ITS2-28S gene was used. **(B)** Partial 12S rRNA gene was used. All sequences were rooted with *Dirofilaria immitis*.

and one individual of *Cx. pipiens* displayed 99.10% similarity to *Acanthocheilonema reconditum* (accession number MZ678927.1).

PCR screening targeting *D. immitis* COI gene yielded negative results for all mosquito samples.

Please refer to the Supplementary material for detailed information on the identification of filarial nematodes in mosquitoes (Supplementary Table 3).

4 Discussion

One of the aims of this study was to investigate the presence of microfilariae in dogs from two semi-rural locations in Región del Maule, a zone in central Chile characterized by a temperate Mediterranean climate with wet winters and hot, dry summers. Agriculture, forestry, livestock, and fishing are the most prevalent industries in this region, accounting for 28 and 60% of the workforce in Villa Alegre and San Clemente, respectively. Approximately 17.1% of the population in Villa Alegre and 20.1% in San Clemente lack basic services such as drinking water and sewage treatment (30).

No previous studies have examined the prevalence of filariae in dogs from this region, and there is no official epidemiological data on vector-borne disease surveillance in humans or animals. Few scientific publications have reported the occurrence of vector-borne diseases in Chile. Regarding filarial nematodes, Alcaíno et al. (16) reported a prevalence of 29.9% in dogs from the north, center, and south of Chile

(excluding Región del Maule), with 99.4% of infections attributed to the genus *Dipetalonema* (currently *Acanthocheilonema*). A recent study by Cevidanes et al. (13) reported a 1% prevalence of *A. reconditum* in the blood of dogs from the Metropolitan Region of Chile. In contrast, our study revealed a significantly higher prevalence of 7.5% for *A. reconditum*. *Acanthocheilonema* parasites are primarily transmitted by fleas (*Ctenocephalides*, *Pulex*, and *Echidnophaga* spp.) or lice (*Heterodoxus* and *Linognathus* spp.) (6, 31, 32). Research suggests that transmission of this nematode depends on the proximity between infected and non-infected dogs (32). This is likely due to the limited mobility of adult fleas and lice away from their hosts, making vector transmission more probable when animals are housed together (33). Consistent with our findings, several dogs from the same household were infected with *A. reconditum*. Additionally, the habits and characteristics of the environment in which dogs live predispose them to infection. Previous studies conducted in diverse regions worldwide have demonstrated that rural dogs often face exposure to or infection by various vector-borne pathogens (34–36).

Age has been reported as a risk factor for filarial infection in dogs (37), most likely related to the accumulation of transmission periods and, subsequently, opportunities for an infection to occur in hosts not under preventive treatment. However, in this study, age could not be identified as a risk factor for filarial infection, likely because all the animals were older than two years in age (38) compared to other studies where age ranges typically started before one year of age.

Regarding gender and the presence of clinical signs, there were no differences between the positive and negative groups. This is in contrast to the study of Lopez et al. (12), where the authors examined 50 dogs with and 50 without clinical signs, finding a notably higher number of dogs with microfilaremia among those symptomatic dogs, and this difference reached statistical significance.

Infections caused by *A. reconditum* exhibit distinct epidemiological features compared with those caused by *Dirofilaria* species. The distinctive attributes of factors influencing the transmission and establishment of *Dirofilaria* spp. in different regions, including the presence of reservoir hosts and the abundance and stability of vectors, ultimately shape their epidemiology (38).

Acanthocheilonema reconditum is an enzootic species in South America. For example, in a recent study in Colombia, 3.4% positivity of microfilariae by microscopic examination was reported (102/2971); out of 102 samples, 82 were analyzed, and 49 were identified as *A. reconditum* by PCR-RFLP ((39). In Brazil, a higher distribution of *A. reconditum* than *D. immitis* (7.2% versus 2.2% in 418 tested samples) was reported (40). In a case reported in Brazil, scientists found a slightly smaller *A. reconditum* species (41). A previous study carried out in semi-rural areas in Santiago, the Chilean capital (12), reported microfilariae measuring between 260 µm and 283 µm, falling in the range for *A. reconditum*. However, data on microfilariae length and width reported in the literature vary considerably (29). Therefore, amplification and DNA sequencing from canine microfilariae are required to correctly identify the species causing the infection. In our study, we did not detect any *Dirofilaria* species. Interestingly, two neighboring countries of Chile, namely Argentina (42) and Bolivia (43), are enzootic for *Dirofilaria* species, with a high prevalence observed in dogs from specific territories.

In our study, we encountered several inconsistencies in the results of both PCRs, which were addressed using touchdown PCR. Nevertheless, some of the obtained sequences exhibited low quality and were incongruent with the PCR results and the observed morphological characteristics of the microfilariae. These results are likely related, among other reasons, to the low microfilaraemia in the samples studied and the PCR detection limit (44). In this regard, Latrofa et al. (45) reported a PCR detection limit for *A. reconditum* of 8 mfs/mL, a high value if we consider a PCR and the low microfilaraemia found, and Espinosa et al. (39) reported a sensitivity of 68% using the same primers used in this study. It is also necessary to design primers specific for species of filarial nematodes that infect canines since the expected band size ranges, according to the literature, cannot differentiate between species. It is important to highlight that the application of Touchdown PCR made it possible to reduce the non-specific bands in the PCR of the dog and mosquito samples, as described in the purpose of this technique (22). The touchdown PCR has been used with good results for other insect samples (46). Undoubtedly, using more sensitive molecular methods for filarial detection could reveal an increasing number of previously unidentified or unreported filarial genera and species in a wide range of invertebrate hosts.

Another objective of this study was to investigate the presence of nematodes DNA in mosquitoes. *Ae.* (*Och.*) *albifasciatus* and *Cx. pipiens* are among the mosquito species found in this study and are known to be competent vectors for *Dirofilaria*. These species (of mosquito) have been reported to serve as vectors for *Dirofilaria* in countries neighboring Chile (47, 48). Although we did not detect *D. immitis* or *D. repens* in the studied mosquitoes, we found *Acanthocheilonema* sp. DNA in 15 pools of *Ae.* (*Och.*) *albifasciatus,* and in 5 individuals of *Cx. pipiens* from the same location as the positive dogs. Other studies have assessed filaroid nematodes in mosquitoes using the same primers (49), but only Manoj et al. (44) have found 3% positivity of *A. reconditum* in *Cx. pipiens.* We agree that this filaroid helminth could have been acquired by mosquitoes while feeding on infected dogs, and positive results for parasite DNA do not necessarily imply that they are competent vectors for these parasites. Additionally, we found two positive pools for *S. equina* DNA within the 15 positive pools for *Ae.* (*Och.*) *albifasciatus* specimens, consistent with the presence of horses in the area where the mosquitoes were collected. To our knowledge, this is the first report of *Setaria* parasite circulation in mosquitoes from Chile. *Setaria* parasites are a genus of filarial nematodes that infect swine, camels, cattle, equines, and other domestic mammals. In particular, *S. equina* is a common vector-borne pathogen in equines worldwide and is particularly prevalent in tropical zones. *S. equina* has been associated with transmission by *Ae. aegypti* and *Cx. pipiens,* where the first larval stage (L1) develops into the third stage (L3) within two weeks in their thoracic muscles. Adult worms are mainly found in the peritoneal cavities of horses and donkeys. Although usually considered non-pathogenic, they may induce various degrees of peritonitis and migrate to the eye, brain, lung, and scrotum, causing lacrimation, blindness, paraplegia, and neurological disturbances in the equines (50–52). Although *S. equina* has been documented in horses in Chile (53), no molecular characterization has been performed for this parasite.

These findings highlight the importance of pathogen surveillance in mosquitoes and reservoirs, such as dogs in Chile. This proactive approach is essential due to potential human and animal health implications. Several challenges remain to be addressed, such as identifying other vectors in Chile, evaluating

host species in different geographic distribution areas, and investigating the biological cycles and developmental stages.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: GenBank accession number: OR885845, OR882885,OR882886, OR882887,OR854268, OR852434, OR778872, OR854266, OR854267,OR852435, OR852433.1, OR852432.1.

Ethics statement

The animal studies were approved by Comité de Cuidado y uso de Animales de Laboratorio (CICUAL) de la Universidad Católica del Maule. 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

BC-F: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. CRG: Investigation, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. APG: Formal analysis, Investigation, Methodology, Resources, Writing – original draft. SP: Methodology, Resources, Writing – review & editing. SB: Resources, Supervision, Validation, Writing – review & editing. APC: Investigation, Resources, Writing – review & editing. IQ: Investigation, Resources, Writing – review & editing. MS: Investigation, Resources, Writing – review & editing. RA: Investigation, Resources, Writing – review & editing. CB: Investigation, Resources, Writing – review & editing. CS: Methodology, Writing – review & editing. RL-Y: Methodology, Writing – review & editing. CAAR: Methodology, Writing – review & editing, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as 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.2023.1334832/full#supplementary-material

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Intestinal parasites infecting captive non-human primates in Italy

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Non-human primates (NHPs) living in captive conditions are susceptible to intestinal parasites that can contribute to mortality and morbidity, and cause zoonotic infections. Thus, parasite surveys on NHP populations under human care are relevant as part of the evaluation of NHPs welfare and in the zoonotic disease risk assessment, as well as in the exploration of parasite transmission pathways, according to the One-Health concept. This study aimed to identify intestinal parasites infecting NHPs living in two wildlife recovery centers and in a zoological garden, in Italy. Ninety-three fecal samples from *Macaca tonkeana, Macaca fascicularis, Sapajus apella, Chlorocebus aethiops, Macaca fuscata, Macaca sylvanus,* and *Cebus capucinus* were collected at Piano dell'Abatino Park (Lazio), and fecal smears and flotation were performed in order to identify parasites according to morphological keys. Additionally, one carcass of *M. fuscata* from the Bioparco Zoological Garden of Rome (Lazio) and one of *M. fascicularis* from the Center for the Recovery of Exotic and Maremma Wild Animals (Tuscany) were necropsied and intestinal adult nematodes were collected and characterized at morphological and molecular level, using the mitochondrial cox1 and rrnL markers. Protozoans (*Entamoeba coli, Iodamoeba bütschlii, Dientamoeba fragilis*-like, *Giardia* sp.), chromists (*Balantidium/Buxtonella* sp.) and nematodes (*Capillaria* sp., *Trichuris* sp., strongyliform larvae and *Oesophagostomum* sp.) were found through fecal smears and flotation. The collected adult nematodes from dead NHPs were morphologically identified as whipworms (genus *Trichuris*). Phylogenetic analyses grouped *Trichuris* specimens into the *Trichuris trichiura* complex of species, with specimens from *M. fuscata* clustering into a host-specific branch, and whipworms from *M. fascicularis* clustering within a clade formed by *Trichuris* infecting several primate species, including humans. The results here collected revealed the presence of potentially zoonotic parasites circulating in captive primates in Italy, providing useful information for the formulation of management and care plans for captive NHPs, and for the elaboration of safety measures for visitors and animal keepers.

KEYWORDS

captive primates, intestinal parasites, Italy, molecular characterization, zoonosis

1 Introduction

Intestinal parasites are often responsible for diseases in animals living in confined environments such as sanctuaries, zoological gardens and wildlife rescue centers (1). Captive animals may be more susceptible to protozoan and helminth parasites with direct life cycles, which are more prevalent and prone to disseminate in confined conditions where the animals might be more stressed due to overpopulation and malnutrition, showing clinical signs as diarrhea and dehydration, and requiring veterinary care (2, 3). Parasite transmission mainly occurs through the fecal-oral route via direct contact with infected hosts (or their fecal material), or indirectly through the ingestion of contaminated water or food (4). In a confined environment, the low hygienic measures may lead to high levels of environmental contamination, and the handlers' movements among different premises without safe and hygiene measures may contribute to the dissemination of such parasites inside and outside the workplace. Captive non-human primates (NHPs) may act as reservoirs for zoonotic parasites and the frequent use of pharmacological treatments may lead to the selection of resistance traits (1, 5). Therefore, confined environments are of great interest for parasitological studies, involving the One-Health concept.

Parasitological investigations have been carried out worldwide in zoological parks housing NHPs. For instance, *Giardia duodenalis* infections were reported in several NHPs hosted in 12 zoological gardens in China (6), while in a study carried out in Malaysia in three zoos hosting 69 specimens of NHPs, there were reported 21 species of intestinal parasites with a high prevalence of nematodes like *Ascaris* spp. and *Oesophagostomum* spp., only one animal positive to *Blastocystis* and no observation of *Giardia* spp. (7). Moreover, a large survey on intestinal parasites infecting NHPs hosted in two research centers in Brazil reported a large occurrence of *Balantidium coli* and *Entamoeba* sp. among protozoans, and a general low frequency of helminths, with predominance of *Trichuris trichiura* (8).

In Europe, parasitological surveys on NHPs have been performed in zoological enclosures such as the Dublin Zoological Garden (Ireland) (9), the Belgrade Zoo (Serbia) (10), the Kiev Zoo (Ukraine) (11), the Brno Zoological Garden (Czech Republic) (12), the Sofia Zoo (Bulgaria) (13), the Wroclaw Zoo (Poland) (14), the Košice Zoological Garden (Slovakia) (15), among others (13, 16). Nematodes (e.g., *Ascaris* sp., *Trichuris* sp., *Strongyloides* sp.) are the most common parasites detected, followed by cestodes and trematodes (13). Furthermore, *G. duodenalis*, *Cryptosporidium hominis, Blastocystis* sp., and *Entamoeba dispar* circulation between NHPs and their zookeepers has been identified in European zoological gardens, with the confirmation of zoonotic transmission events involving *Blastocystis* sp. and a highly suspected zoonotic transmission of *C. hominis* (4). Additionally, subcutaneous *Taenia crassiceps* cysticercosis in a ring-tailed lemur in a Serbian zoo has been reported (17).

In Italy, some surveys on intestinal parasites infecting NHPs living in zoological gardens have been conducted so far. In central Italy, *Cryptosporidium* sp. and *Trichuris* sp. have been found infecting *Lemur catta* at the Giardino Zoologico of Pistoia (18), while, at the Bioparco Zoological Garden of Rome, *G. duodenalis* has been reported infecting *L. catta,* and *Entamoeba* spp. was diagnosed in *Cercocebus torquatus*, *Chlorocebus aethiops*, *Macaca fuscata*, *Mandrillus sphinx*, *Pan troglodytes*, *L. catta*, and *Pongo pygmaeus* (19). In southern Italy, *Trichuris* sp., *Strongyloides fuelleborni*, and *Cryptosporidium* sp. infected *Papio cynocephalus* at the Fasano Zoo Safari, while *G. duodenalis* was found infecting *L. catta*, *Cercopithecus mona*, *Alouatta caraya*, *Nomascus concolor*, *Colobus guereza*, and *Semnopithecus entellus* in a zoological garden in the Benevento province (20). Moreover, *Cyclospora* was detected in *P. troglodytes* from a wildlife animal rescue center, and in *Macaca fascicularis* from an experimental primate research center (21). Eight taxa of intestinal parasites (*Trichuris* sp., *Oesophagostomum* sp., *Entamoeba coli*, *Endolimax nana*, *Iodamoeba bütschlii*, *Chilomastix mesnili*, *B. coli*, and *Blastocystis* sp.) were recorded infecting *M. fascicularis* in a biomedical research center (22).

Concerning necropsies carried out on dead captive NHPs, *Trichuris* sp. from *Eulemur albifrons*, and *Strongyloides* sp. from *Macaca sylvanus* have been found at the Natura Viva zoo (23), and *Echinococcus granulosus* from *L. catta* at a zoo in northern Italy (24). At the Bioparco Zoological Garden of Rome, larval forms of *Taenia martis* from *L. catta* (25), and adult *Trichuris* sp. from *L. catta*, *M. fuscata* and *C. aethiops* have been reported (26, 27). Larval forms of *Mesocestoides* sp. from *Saguinus midas* were collected at a wildlife recovery center (28).

Despite their importance in public health and NHPs welfare, the currently available information on intestinal parasites infecting captive NHPs in Italy is still limited to fragmented data. Thus, here we provide a survey on intestinal parasites circulating in NHPs hosted in two wildlife rescue centers and in one zoological garden in central Italy.

2 Materials and methods

Fecal samples and adult nematodes were collected during 2020–2022, from NHPs living in confined environments in Italy.

2.1 Fecal samples

Ninety-three fecal samples from *Macaca tonkeana* (Tonkean black macaque) ($n = 23$), *Macaca fascicularis* (long-tailed macaque) ($n = 16$), *Sapajus apella* (tufted capuchin) ($n = 43$), *Macaca fuscata* (Japanese macaque) ($n = 2$), *Macaca sylvanus* (Barbary macaque) ($n = 4$), *Chlorocebus aethiops* (grivet) ($n = 2$) and *Cebus capucinus* (capuchin monkey) ($n = 3$) were collected at the Piano dell'Abatino Park (Lazio), in the framework of a routine parasitological survey. In this habitat, the animals are hosted in different premises, as detailed below. One premise hosts two individuals of *C. aethiops* and two individuals of *C. capucinus*; one premise is dedicated to *M. fascicularis*, with seven individuals; two not-separated premises for *M. sylvanus* with 11 individuals; one premise for only one individual of *M. fuscata*; three premises for *S. apella*, with 11, 12, and 14 individuals; and four premises for *M. tonkeana* with eight, six, thirteen, and fourteen individuals. Fresh samples were collected directly from the soil inside the premises and were not attributed to a specific individual. For each sample, one aliquot was stored in 10% formalin solution, and one aliquot in 70% ethanol solution. Samples were examined both macroscopically, to verify the presence of nematodes or cestodes, and microscopically. Morphological identification of protozoan and helminth parasites was performed after direct fecal smears (29) and

flotation with a salt-sugar solution (SG: 1.28) (30) useful for general purposes. Slides from direct fecal smears and flotation were examined with a microscope, and at least 10 fields were screened at objective magnification ×100, ×200, ×400, and ×1,000, successively. This protocol was used to qualitatively identify parasite eggs, cysts and oocysts. Photos of parasites were taken for morphological identification. For some parasite taxa the identification was possible only to the genus level.

2.2 Adult nematodes

Two dead macaques were inspected during necropsies carried out at the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "Mariano Aleandri" to identify the cause of death. Ten entire adult nematodes (three males and seven females) and few disrupted nematode body portions were collected from the caecum of one *M. fascicularis* hosted at the Center for the Recovery of Exotic and Maremma Wild Animals (CREMWA) (Tuscany). From one *M. fuscata* hosted at the Bioparco Zoological Garden of Rome (Lazio), eight adult nematodes (all females - not well preserved) were collected from the caecum. Nematodes were repeatedly washed with saline solution, and then used for morphological observation after clarification in lactophenol. A body portion was used for molecular characterization based on sequence analyses of the two partial mitochondrial regions *cox*1 and *rrn*L, informative for phylogenetic assignment (31, 32). The obtained sequences were compared to homologous GenBank retrieved data, and used for phylogenetic inferences with the maximum likelihood (ML) method by MEGA7 (33), after testing for the best evolutionary models explaining the data (33). The only available homologous sequences of *Trichuris* sp. from the same host species *M. fascicularis* (JF690967) was not reliably attributable to this genus, thus it was excluded from the analysis. Sequences of *Trichinella spiralis* and *Trichinella britovi* were used as outgroups (AF293969, KM357413). Additional file 1 and file 2 show the material used for comparative analyses.

3 Results

3.1 Fecal samples

Four taxa of protozoans (*Entamoeba coli, Iodamoeba bütschlii, Dientamoeba fragilis*-like, and *Giardia* sp.), one taxon of chromist (*Balantidium/Buxtonella* sp.), and four taxa of helminths (*Capillaria* sp., *Trichuris* sp., strongyliform larvae and *Oesophagostomum* sp.) were identified in the fecal samples from NHPs living at the Piano dell'Abatino Park (Table 1). None infected animals showed gastrointestinal symptoms. Representative images from microscopic analyses are available in the Figure 1.

The capuchin monkeys were the only primate species in which no gastrointestinal parasites were observed. The following potentially zoonotic parasites were detected: *Giardia* sp. was found infecting the grivet, *Trichuris* sp. infecting the long-tailed macaque, *Oesophagostomum* sp. was observed in the Tonkean black macaque and *Capillaria* sp. in the tufted capuchin monkey. *Trichuris* sp. and *Capillaria* sp. were not identified at species level due to negative results of molecular identification assays.

3.2 Adult nematodes

The general gross morphology of *Trichuris* adult specimens collected from *M. fascicularis* and *M. fuscata* intestinal caeca was congruent with a filiform long anterior part and a broad and handle-like posterior part, typical of whipworms. The cuticle presented transversal striation and the anterior portion of the body showed bacillary bands. Males (Figure 2) and females (Figure 3) showed similar morphological features described for *Trichuris trichiura* from *Papio papio* and *M. sylvanus* (31), *Trichuris* sp. from *M. sylvanus* (34, 35) and *T. ursinus* from *Papio ursinus* (36). The eggs measurements ranged from 25.50–27.90 × 54.30–56.80 µm in *Trichuris* from *M. fascicularis* and from 30–35 × 53–61.6 µm in *Trichuris* from *M. fuscata*.

Regarding the molecular characterization, ten high quality *rrn*L sequences (nine from *M. fascicularis* and one from *M. fuscata*) and four *cox*1 sequences (all from *M. fascicularis*) were obtained from the collected nematodes and used for phylogenetic inferences in comparison to GenBank retrieved data, with final datasets of 43 input and 460 bp and of 32 input and 341 bp, respectively. Both phylogenetic trees identified the presence of two main clades, namely "Clade 1" and "Clade 2" (31). The *rrn*L ML consensus tree in Figure 4 described Clade 1 named as the *T. suis* clade, including *Trichuris colobae* as a sister clade of *T. suis* + *Trichuris* sp. from *Chlorocebus*. The *Trichuris* specimens from *M. fascicularis* here analyzed clustered into the "subclade c" of the "Clade 2" or *T. trichiura* clade branch (indicated in red color) (31), with high statistical support (99–100%). The "subclade c" branch included *T. trichiura* individuals collected in a broad host range for primates, such as the Japanese macaque, the Barbary macaque, the green monkey, the baboon, and humans from Africa and Europe. The specimen from *M. fuscata* here collected grouped in the subclade defined as MF in previous reports (branch indicated in blue color) from the same host species living in the Bioparco Zoological Garden of Rome (26).

A similar topology was obtained for the *cox*1 ML consensus tree (Additional file 3), in which specimens of *Trichuris* from *M. fascicularis* were included in the "Clade 2 subclade c" together with *Trichuris* from other macaques and baboons. Such evidences confirmed that specimens infecting *M. fascicularis* here analyzed can be identified as *T. trichiura,* given the similarity with this taxon reported also in other primates, including humans. No good quality sequences were obtained at this marker for *Trichuris* infecting *M. fuscata*.

4 Discussion

The present study investigated the presence of intestinal parasites infecting NHP species living in captivity in Italy. Based on morphological analyses from fecal samples of NHPs living at Piano dell'Abatino Park, nine parasite taxa were identified, all of them presenting direct life cycles. However, due to the sampling from premises with multiple hosts, without tracing primate individuals during defecation, the precise estimation of epizootiological parameters such as prevalence, intensity and abundance was not possible.

In this study, *Balantidium/Buxtonella* sp., *E. coli,* and *I. bütschlii* were the most frequently detected parasites. Most parasite taxa identified in this study have been previously reported in captive NHPs

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TABLE 1 List of parasites identified according to host species.

Parasite taxa	*Cebus capucinus* (*n* = 3 *N* = 2)	*Chlorocebus aethiops* (*n* = 2 *N* = 2)	*Macaca fascicularis* (*n* = 16 *N* = 7)	*Macaca fuscata* (*n* = 2 *N* = 1)	*Macaca sylvanus* (*n* = 4 *N* = 11)	*Macaca tonkeana* (*n* = 23 *N* = 41)	*Sapajus apella* (*n* = 43 *N* = 37)
Entamoeba coli	0	0	10	2	4	23	0
Iodamoeba bütschlii	0	2	8	1	1	11	4
Giardia sp.	0	1	0	0	0	0	0
Dientamoeba fragilis-like*	0	0	6	0	0	0	0
Balantidium/Buxtonella sp.	0	0	7	2	1	17	1
Capillaria sp.	0	0	0	0	0	0	1
Trichuris sp.	0	0	4	0	0	0	0
Strongyliform larvae*	0	0	0	0	1	0	5
Oesophagostomum sp.	0	0	0	0	0	1	0

Number of positive samples for each parasite taxa, per primate species (*n*, number of samples analyzed; N, number of primates hosted in the premises). *Based on morphology it was not possible to identify the genus/species.

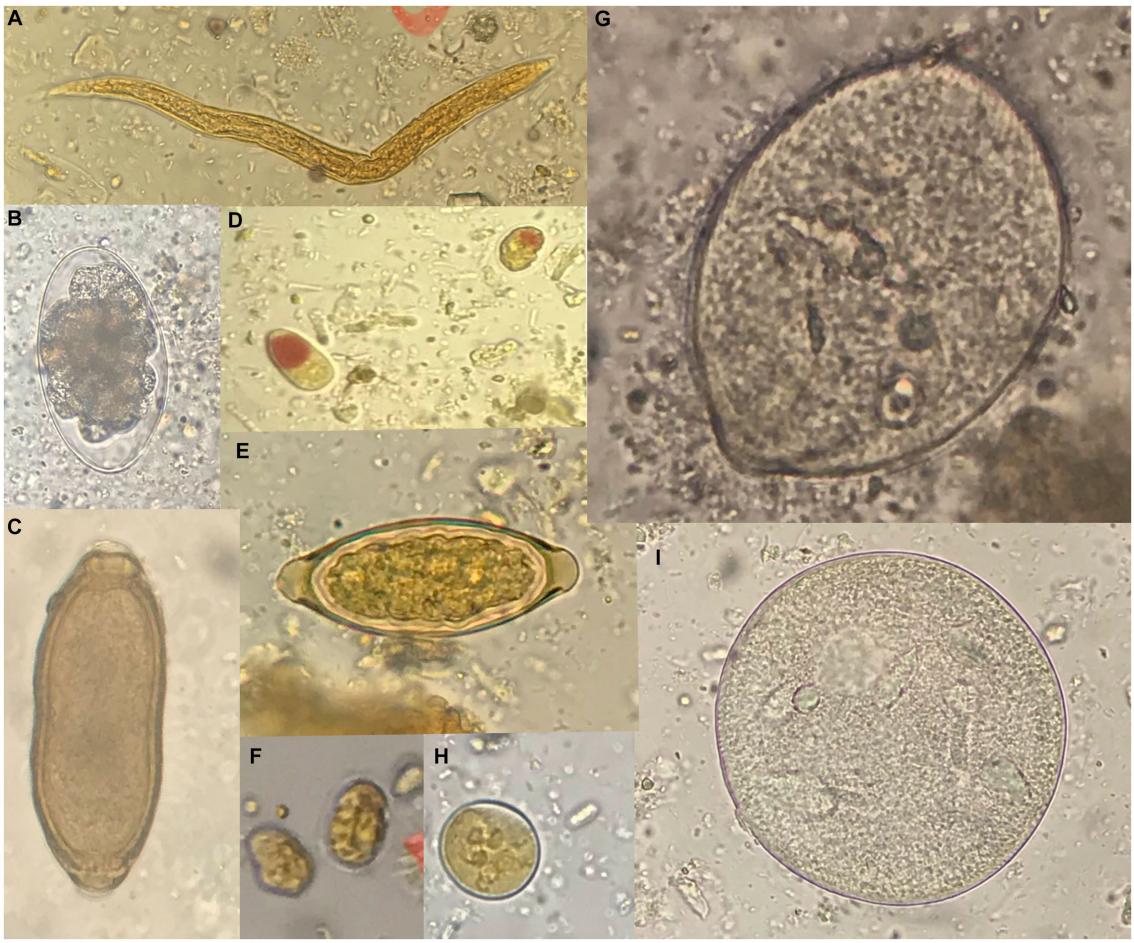

FIGURE 1
Representative images of parasites detected by microscopy. **(A)** Strongyliform larva (40x). **(B)** *Oesophagostomum* sp. (50 × 85 µm). **(C)** *Capillaria* sp. (40 × 25 µm). **(D)** *Iodamoeba bütschlii* (10 × 12 µm). **(E)** *Trichuris* sp. (25 × 55 µm). **(F)** *Giardia* sp. (10 × 8 µm). **(G)** *Balantidium/Buxtonella* sp. trophozoite (90 µm). **(H)** *Entamoeba coli* (20 µm). **(I)** *Balantidium/Buxtonella* sp. cyst. Measures refer to the samples shown in the figure.

in Europe, as is the case of *Trichuris* sp., *Oesophagostomum* sp., *Balantidium* sp., *Giardia* sp., *E. coli* and *I. bütschlii* (19, 22). *Giardia* sp. was found in only one individual, and taking into account that this parasite is usually more frequently found in studies on NHPs in zoos, we should consider that in this case it may not be a true infection but cysts accidentally ingested from the environment. Additionally, due to

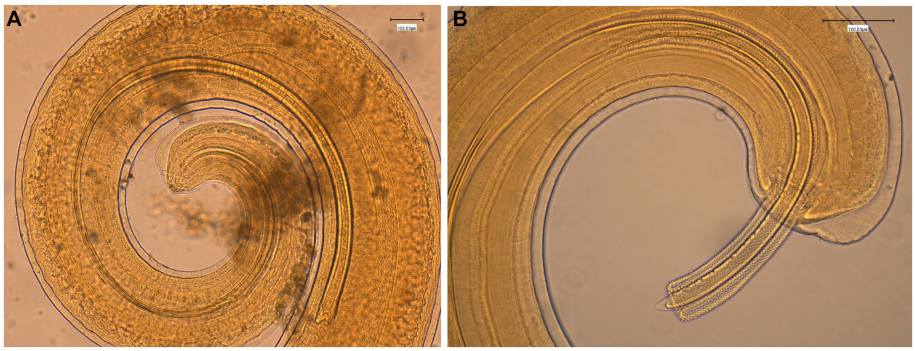

FIGURE 2
Morphology of male *Trichuris* sp. from *Macaca fascicularis*. **(A)** Posterior end showing the arrowed and invaginated spicule, with distal and proximal cloacal tube and ejaculatory duct. **(B)** Posterior end with evaginated spicule and spicule sheath with spines.

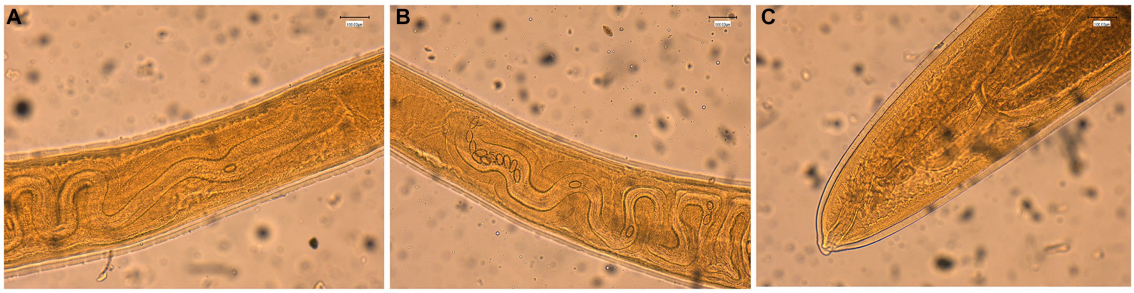

FIGURE 3
Morphology of female *Trichuris* sp. from *Macaca fascicularis*. **(A)** Vulva region with visible tegument covered by spines. **(B)** Circumvoluted vagina with eggs. **(C)** Posterior end showing the end of uterus and cloaca.

the intermittent shedding of cysts, in some cases it is necessary the examination of fecal samples on consecutive days (47), and in this study no sampling on consecutive days was performed.

Balantidium/Buxtonella sp. was found infecting five of the seven NHP species sampled. Pigs are the main reservoir host of *Balantidium*, while rodents and NHPs may function as alternative reservoir hosts (37). Wild boars are also present at the study site, but in a small number and located in a separate facility from the NHPs. Thus, in this case swine are unlikely to participate in the transmission cycle (even if it cannot be definitively ruled out due to handler's movements, or by the rain/wind that can easily transport the cysts from one facility to another), while wild rodents are very common within the primate enclosures. For future studies it is highly recommended the use of integrative taxonomy accounting for morphological characteristics combined with molecular approach for species identification, as it has been demonstrated how misleading the cyst morphology-based diagnostics of *Balantidium* sp. and *Buxtonella* sp. can be, leading to ambiguity in the epidemiology of these infections (38). In Italy, both *Buxtonella* sp. and *Balantidium* sp. have been reported, for instance *Buxtonella sulcata* infecting cattle in central Italy (39), and *Balantidium coli* infecting swine in the south of the country (40). Given the uncertainty in the taxonomic assignment, we have chosen to indicate this finding as *Balantidium/Buxtonella* sp.

Molecular testing should be also recommended for the optimal identification of *D. fragilis* (41). In our survey, *D. fragilis*-like was found in samples from *M. fascicularis*, and this parasite was recently reported infecting free-ranging *M. fascicularis* in Indonesia (42). Additionally, future molecular studies to determine the species of the strongyliform larvae found infecting *S. apella* and *M. sylvanus* are required, in particular to confirm or exclude the presence of *Strongyloides* sp., a zoonotic parasite of paramount relevance, reported in Italy both in dogs and humans (43). *Capillaria* sp. was found in one sample of a tufted capuchin monkey, however, the molecular approach for species identification gave negative results, probably due to difficulties in the genomic DNA isolation and/or PCR inhibitors. *Capillaria* sp. has been reported infecting different NHP species (44), including capuchin monkeys: *C. capucinus* in Panama (45) and *C. albifrons* in Ecuador (46). However, these reports were based on microscopy, thus, the use of molecular testing is also here suggested for the identification at species level to elucidate the zoonotic potential.

Trematodes, cestodes and acanthocephalans have been previously reported in free-ranging primates (48). Considering that the methods used in this study allow the detection of these parasite taxa, the lack of findings could be related to the different diets and habits of captive individuals compared to free-ranging NHPs.

Given the close phylogenetic relationship between human and NHPs, continuous parasitological surveys on captive primates should be encouraged for the monitoring of zoonotically transmitted parasites, for instance within conservation and management of threatened primate species, and in the recovery of traded NHPs. In the present study, four out of the seven NHP species under investigation are considered endangered (EN) or vulnerable (VU) by the IUCN, and while no animals hosted at Piano dell'Abatino Park showed clinical signs or symptoms of

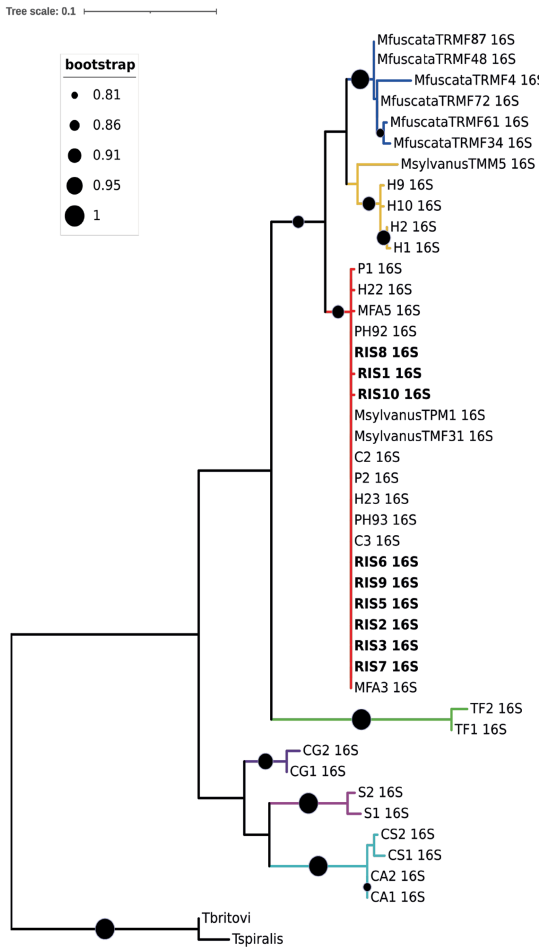

FIGURE 4
Phylogenetic tree based on *rrn*L of *Trichuris* spp. Maximum likelihood consensus tree of partial mitochondrial *rrn*L of *Trichuris* spp. analyzed in the present study (for specimen codes information see Supplementary File S1). Circles at nodes indicate the bootstrap statistical support, according to the legend on the left. Colors represent clades and sub-clades and correspond to those reported by Rivero et al. (31).

gastrointestinal origin, two animals died at the Bioparco Zoological Garden of Rome and in the CREMWA, probably because of *Trichuris* infection. So far, *Trichuris* spp. have been reported by morphological and/or molecular characterization in the following *Macaca* species: the Japanese macaque (26, 27, 32), the Barbary macaque (34, 35) and the long tailed macaque (22), the latter investigated only in terms of eggs presence in stool samples without any molecular characterization. Such studies revealed the presence of two separated taxonomic entities able to infect Japanese macaques living in confined environments, one specific to this host, and one shared also with other primates (26, 27, 32). Analogous molecular results were obtained also regarding the Barbary macaque hosted in the Castellar Zoo (Spain), infected by two genotypes within the *T. trichiura* lineage, supported also by morphological data (35).

Here we provide for the first time morphological and molecular data of *T. trichiura* infecting *M. fascicularis,* to share with the scientific community for comparative purposes. We obtained reliable data from the analyses of adult *Trichuris* infecting the dead long tailed macaques hosted at the CREMWA, and despite no molecular data were obtained from fecal samples from the animals hosted at Piano dell'Abatino Park, the eggs size observed in the two sample sites were overlapping, suggesting *T. trichiura* circulation. The long-tailed macaque from the CREMWA analyzed in the present study lived in a colony of around 30 individuals (49), thus the finding of *Trichuris* infection may represent a high risk for the other macaques belonging to the colony. It is also a concern, taking into account that *M. fascicularis* has been recently listed as an endangered species with a decreasing population trend, according to the International Union for Conservation of Nature (IUCN) (50), mainly due to the high demand in the national and international trade, and the hunting for subsistence. Moreover, there is a risk for handlers and visitors in terms of zoonotic transmission. Therefore, it is necessary the constant monitoring to trace the presence of eventual parasitic species of zoonotic interest, in both confined environments and in native areas where NHPs live near or in close contact with humans.

In conclusion, this parasitological survey revealed the presence of potentially zoonotic parasites circulating in NHPs in Italy, providing useful information for the formulation of their management and care plans, and for the elaboration of safety measures for visitors and animal keepers. Regular parasitological surveys in captive NHPs using both microscopy and molecular analyses should be recommended, in order to monitor the impact of parasitosis on the health status of captive NHPs and to properly assess the potential zoonotic transmission risk.

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 Biological material taken from alive animals was not invasively collected, while material taken during necropsies was authorized by the ethical approval of the Istituto Zooprofilattico Sperimentale Lazio e Toscana.

Author contributions

SR: Conceptualization, Funding acquisition, Methodology, Writing – original draft. SC: Conceptualization, Formal analysis, Methodology, Writing – original draft. MMDF: Formal analysis, Writing – review & editing. CD: Resources, Writing – review & editing. FB: Supervision, Writing – review & editing. NC: Resources, Writing – review & editing. SD'A: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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SUPPLEMENTARY MATERIAL 1
Trichuris sp. material used for phylogenetic inference based on the partial mitochondrial ribosomal *rrn*L region. Information on specimen codes, host species, GenBank accession number and literature references are provided.

SUPPLEMENTARY MATERIAL 2
Trichuris sp. material used for phylogenetic inference based on the partial mitochondrial *cox*1 region. Information on specimen codes, host species, GenBank accession number and literature references are provided.

SUPPLEMENTARY MATERIAL 3
Maximum likelihood consensus tree of the *Trichuris* spp. partial mitochondrial *cox*1 sequences analyzed in the present study. Numbers at nodes indicate the bootstrap statistical support (for specimen codes information see Additional file 2).

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Moniezia benedeni drives CD3⁺ T cells residence in the sheep intestinal mucosal effector sites

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Introduction: T cells are the core of the cellular immunity and play a key role in the regulation of intestinal immune homeostasis. In order to explore the impact *Moniezia benedeni* (*M. benedeni*) infection on distributions of CD3⁺ T cells in the small intestine of the sheep.

Methods: In this study, sheep pET-28a-CD3 recombinant plasmid were constructed and expressed in *BL21* receptor cells, then the rabbit anti-sheep CD3 polyclonal antibody was prepared through recombinant protein inducing. The *M. benedeni*-infected sheep (infection group, $n = 6$) and healthy sheep (control group, $n = 6$) were selected, and the distributions of CD3⁺ T cells in intestinal *laminae propria* (LP) and mucous epitheliums were observed and analyzed systematically.

Results: The results showed that the rabbit anti-sheep CD3 polyclonal antibody had good potency and specificity. In the effector area of small intestine, a large number of CD3⁺ T cells were mainly diffusely distributed in the intestinal LP as well as in the mucous epitheliums, and the densities of intestinal LP from duodenum to jejunum to ileum were 6.01 cells/$10^4\,\mu m^2$, 7.01 cells/$10^4\,\mu m^2$ and 6.43 cells/$10^4\,\mu m^2$, respectively. Their distribution densities in mucous epitheliums were 6.71 cells/$10^4\,\mu m^2$, 7.93 cells/$10^4\,\mu m^2$ and 7.21 cells/$10^4\,\mu m^2$, respectively; in the infected group, the distributions of CD3⁺ T cells were similar to that of the control group, and the densities in each intestinal segment were all significantly increased ($p<0.05$), meanwhile, the total densities of CD3⁺ T cells in duodenum, jejunum and ileum were increased by 33.43%, 14.50%, and 34.19%. In LP and mucous epitheliums, it was increased by 33.57% and 27.92% in duodenum; by 25.82% and 7.07% in jejunum, and by 27.07% and 19.23% in ileum, respectively.

Discussion: It was suggested that *M. benedeni* infection did not change the spatial distributions of CD3⁺ T cells in the small intestine of sheep, but significantly increased their densities, which lays a foundation for further research on the regulatory mechanism of sheep intestinal mucosal immune system against *M. benedeni* infection.

KEYWORDS

***Moniezia benedeni*, sheep small intestine, CD3⁺ T cell, *laminae propria*, mucous epitheliums**

1 Introduction

The small intestine has digestive, absorptive, secretory, and immunological functions (1, 2). Meanwhile, it is usually exposed to a variety of microorganisms [e.g., bacteria (3), viruses (4)] and parasites (5, 6), etc. The intestine can rely on multilayered defense barriers, such as mechanical-physical barriers (7), chemical barriers (8, 9) and immune barriers (9),

which can prevent the invasion of pathogenic microorganisms and antigens. T cells, as the main component of lymphocytes, have biological functions such as direct killing of target cells, assisting B cells to produce antibodies, response reactions to specific antigens, and cytokine production (10). Many studies have confirmed that the toxicity of antibody dependent cell mediated cytotoxicity (ADCC) can be induced by specific antibodies that bind to the parasite. It is the key to the host's immune response against parasitic infections (11). CD3 (cluster of differentiation 3) acts as a T cell receptor (TCR) that transduces the activation signals generated by the recognition of antigens by the TCR into T cells, resulting in T cell activation (12, 13), and is also an important surface marker molecule of T cells (14).

In intestinal mucosal immunity, *lamina propria* (LP) is an important effector site of mucosal immune responses (15). The intestinal lamina propria T cells (LPL) mainly assist B cells in synthesizing and secreting IgA (16). It can not only prevent the contact between mucosa and pathogenic microorganisms (17), neutralize and regulate the distribution of body flora (18, 19), but also play a role with complement and lysozyme, resulting in the dissolution of pathogens (20, 21), and maintain intestinal homeostasis. Intestinal intraepithelial lymphocytes (IELs) are the first immune cells in the intestinal mucosal immune system to contact foreign antigens, microorganisms, and parasites (22). It has a variety of functions, such as inhibiting mucosal hypersensitivity (23), neutralizing the cytotoxic effects of exogenous cytotoxicity (24), and secreting lymphokines (25). Most IELs contain numerous cytoplasmic granules that facilitate cytotoxic activity. Additionally, they can express effector cytokines, including interferon-gamma (IFNγ) and interleukins (IL)-2, IL-4 or IL-17. Obviously, the host can strengthen local immunity to resist infection by pathogenic microorganisms through mucosal immune-related cell proliferation (26, 27).

Parasites are a major cause of disease in livestock. According to statistics, there are 2,169 species of livestock and poultry parasites identified in China, including 404 species of nematodes, 203 species of protozoa, 373 species of trematodes, 150 species of tapeworms, 10 species of acanthocephalans and 1,030 species of arthropods (28). *Moniezia benedeni* (*M. benedeni*) is usually parasitizing the small intestines of cattle and sheep, and its main pathogenic effects are mechanical blockage, nutrient seizure, and toxicity (29). Animals exhibit weight loss as a clinical symptom, anemia, localized gastrointestinal distension, dysentery or severe constipation (30), and common psychiatric symptoms such as spasms, gyratory movements, head tilting and empty chewing, causing death in some severe cases (31). When the parasite invades the host, it can trigger the host's immune response (32), mainly type II immune responses, which involves the production of specific immunoglobulins and cytokines, promoting the proliferation of intestinal epithelial cells and the increase of intestinal mucus to promote the excretion of the parasite (33). The results showed that in *cysticular echinococcus* infection, the expression of host's CD69, CD44 and CD40L of $CD4^+$ and $CD8^+$ T cells was up-regulated and the expression of CD62L was down-regulated. The number of regulatory T cells expressing $CD4^+$ $CD25^+$ $FoxP3^+$ increased significantly (34). Our previous studies have confirmed that *M. benedeni* infection significantly reduces the density of small intestinal IgA^+, IgG^+, and IgM^+ cell distributions (35). However, the effect of *M. benedeni* infection on the distribution and expression of T-lymphocytes in the effector sites in the small intestine in sheep and the resulting anti-parasitic immune response are not clear. The aim of this study is to analyse the distribution characteristics and distribution density of $CD3^+$ T cells in the effector zone of the small intestine of sheep infected with the cestode *M. benedeni* through bioinformatic analysis of CD3, preparation of polyclonal antibodies, immunohistochemistry and immunofluorescence. On this basis, the study of the effect of *M. benedeni* infection on the pattern of changes in T lymphocytes in sheep's small intestine lays the foundation for further elucidating the regulatory mechanism of the sheep intestinal mucosal immune system in response to *M. benedeni* infection.

2 Materials and methods

2.1 Experimental animals and experimental design

Uninfected (control group, $n = 6$) and *M. benedeni* infected sheep (infected group, $n = 6$) were selected, respectively. They were anaesthetised intravenously with sodium pentobarbital (20 mg/kg) and then exsanguinated to death. Secondly, the abdominal cavity of the executed sheep was opened, and the duodenum, jejunum and ileum tissues were quickly cut out, and the tissue samples taken were fixed in 4% formaldehyde solution, embedded and sliced according to the conventional methods to make paraffin sections (4 μm). All tissue samples of the duodenum, jejunum and ileum were collected in sterile tubes for ELISA and western blotting detection. The histological samples of them were fixed in a 4% neutral paraformaldehyde solution for more than 15 days. Purchase of healthy male New Zealand White rabbits from the Laboratory Animal Center of Lanzhou Institute of Veterinary Medicine, Chinese Academy of Agricultural Sciences, China, weight about 2.2 kg.

2.2 Preparation and western blotting analysis of polyclonal antibody against CD3 in sheep

Referring to the coding region (CDS) of the sheep CD3 gene sequence (GenBank: S53077.1), the mRNA has 1,343 bp in length, with a coding region from position 134 to 713, and it translates into a protein consisting of 193 amino acids. The prediction of transmembrane structure revealed a total of 115 amino acids for CD3 in the extramembrane region, and this extramembrane portion (1–114) was intercepted using Editseq (DNAStar 7.0). The signal peptide was predicted and truncated (1–21), leaving 95 amino acids, which correspond to a base sequence of 285. Then the enzyme cutting site was determined, and finally sent to Genewiz Biotechnology Co., Ltd. for sequence synthesis. The CD3 was connected with pET-28a (+) vector, and transformed into DH5α receptor cells. Finally, the correctly sequenced positive recombinant plasmid was obtained as pET-28a-CD3.

The constructed pET-28a-CD3 recombinant plasmid was transfected into 50 μL of *BL21*(DE3) competent cells under aseptic conditions. Single colony were picked in 5 mL of LB liquid medium containing Kan^+, then cultured at 37°C and 220 rpm on a shaker overnight. The overnight bacteria were transferred into 5 mL of LB liquid medium containing Kan^+ according to 1:100, cultured at 37°C and 220 rpm until the OD_{600} value reached 0.6–0.8. One milliliter fluid was taken in a 1.5 mL centrifuge tube as the preinduction control, and

the remaining solution was added with 1 mol/L IPTG according to 1:1000. The cultured was induced at 37°C and 220 rpm for 6 h. One milliliter fluid was taken in a 1.5 mL centrifuge tube for the post-induction control. The precipitate was collected by ultrasonically crushing in an ice bath, and the supernatant and precipitate were separately collected and sampled for SDS-PAGE. The collected precipitates were combined with affinity column (containing HIS-tagged proteins), and the proteins were purified. The concentration of the proteins was determined spectrophotometrically. Purified sheep CD3 recombinant protein was emulsified and injected at multiple points into rabbits, targeting the popliteal lymph nodes and subcutaneously on the back. After four immunizations, blood was collected from the heart and centrifuged to obtain rabbit anti-sheep CD3 polyclonal antibodies.

The purified recombinant protein was electrophoresed on a 15% SDS-PAGE and transferred to PVDF membrane, which was sealed by adding skimmed milk powder at 37°C. The PVDF membrane was conjugated with rabbit antiserum (diluted 1:500) and incubated overnight at 4°C. After being washed 3 times with TBS-T, the HRP-labeled secondary antibody (diluted 1:8000) was added and incubated for 2 h at room temperature. Finally, after being washed 3 times with TBS-T, the ECL luminescent solution was added for color development.

2.3 Immunohistochemical staining procedures

The paraffin sections were dewaxed with water, placed in citrate buffer (power 900 W, action 10 min), cooled naturally, and washed with distilled water for 2 min × 3 times. Then, they were treated with 3% hydrogen peroxide at room temperature for 15 min and washed again with distilled water for 2 min × 3 times; the distilled water surrounding the tissues was drained off using filter paper. Then 5% BSA (from a ready-to-use immunohistochemical staining kit) blocking solution was added dropwise and allowed to act at 37°C for 40 min. The excess liquid was shaken off from the tissues, and diluted primary antibody was added dropwise. The tissues were incubated at 4°C overnight. After washing with PBS (0.01 mol/L, pH 7.2) for 2 min × 3 times, the secondary antibody (goat anti-mouse/rabbit IgG of HRP) was added dropwise and incubated at 37°C for 30 min. After washed with PBS for 5 min × 4 times, the appropriate amount of SABC was added dropwise, incubated at 37°C for 30 min, and washed with PBS for 5 min × 4 times; DAB color development kit (20×, Goods number: ZLI-9018, Beijing Zhongsui Jinqiao Biotechnology Co., Beijing, China) was used at room temperature, and the reaction was terminated by washing with water. Then the nucleus were stained with hematoxylin for 50 s, washed with water for 10 min, differentiated for 5 times. Dehydrated, and mounted with neutral balsam. Serial sections were made, and the antibody stock solution was diluted to 1:200, 1:400, 1:600, 1:800, 1:1000, 1:1200, respectively, to observe the staining effect, and the concentration was finally determined to be 1:600.

2.4 Statistical analysis

Using a digital scanner (3DHISTECH Pathology Slice Scanner, Shandong Spirit Medical Technology Co.) to observe the location and characteristics of $CD3^+$ T cell distribution. For each segment of intestine, 5 slices were selected and 10 visual field of mucous epitheliums were randomly selected for each slice; the number of positive cells in each mucous epithelium was counted, and the density of positive cells was calculated. Statistical analysis was performed using Origin 2022 and SPSS 23.0 software, using one-way ANOVA (LSD method was used for *post hoc* analysis) to analyze the differences between the distribution densities of positive cells between the groups; and the *t*-test of independence was used to analyze the significance of the differences between the groups infected with the same site and the control group, the significant difference was considered at $p < 0.05$.

2.5 Immunofluorescence staining

Paraffin sections were dewaxed to water, placed in citrate buffer (power 900 W, action 10 min), cooled naturally, and washed in distilled water for 2 min × 3 times. Shake off the liquid around the section, draw a circle around the tissue with a histochemical pen (to prevent the antibody from flowing out), and incubate the circle with a drop of BSA for 30 min. Gently shake off the sealing solution, dilute the sections with primary antibody (1:600), and incubate the sections in a wet box at 4°C overnight. The slides were washed in PBS on a shaker for 5 min × 4 times, and the secondary antibody (Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) ab150077, Abcam) was added dropwise for 50 min at room temperature and protected from light. The slides were placed in PBS and washed on a shaking table for 5 min × 4 times, and DAPI staining solution was added dropwise in the circle, and incubated at room temperature and protected from light for 10 min. The slides were placed in PBS and washed on a shaking table for 5 min × 4 times. Spontaneous fluorescence quencher was added to the circle for 5 min, and rinsed under running water for 10 min. The sections were shaken dry and sealed with an anti-fluorescence quenching sealer. Finally, the distribution of $CD3^+$ T cells in sheep small intestine was observed under a fluorescence microscope, and the images were acquired (The DV Elite™ Imaging System, GE, United States; DAPI UV excitation wavelength 330–380 nm, emission wavelength 420 nm, blue light; FITC excitation wavelength 465–495 nm, emission wavelength 515–555 nm, green light).

3 Results

3.1 Similarity comparison and phylogenetic tree construction of CD3 in sheep

Phylogenetic homology comparisons and phylogenetic tree construction were performed using MAGA11.0 software based on the CDS region of the CD3 gene sequences obtained from the NCBI database for pig, rabbit, human, sheep, horse, domestic cat, chicken, tiger, chimpanzee and dog. As shown in Figure 1A, the similarity between pig and rabbit, human, sheep, horse, domestic cat, chicken, tiger, chimpanzee and dog CDS regions was 70.3%, 76.5%, 79.6%, 78.5%, 69.8%, 50.6%, 70.9%, 75.9%, and 73.8%, respectively. Sheep and pigs were found to be the most closely related species (Figure 1B). They were followed by humans, chimpanzees, domestic cats and tigers. In contrast, chicken were found to be the most distantly related species (Figure 1).

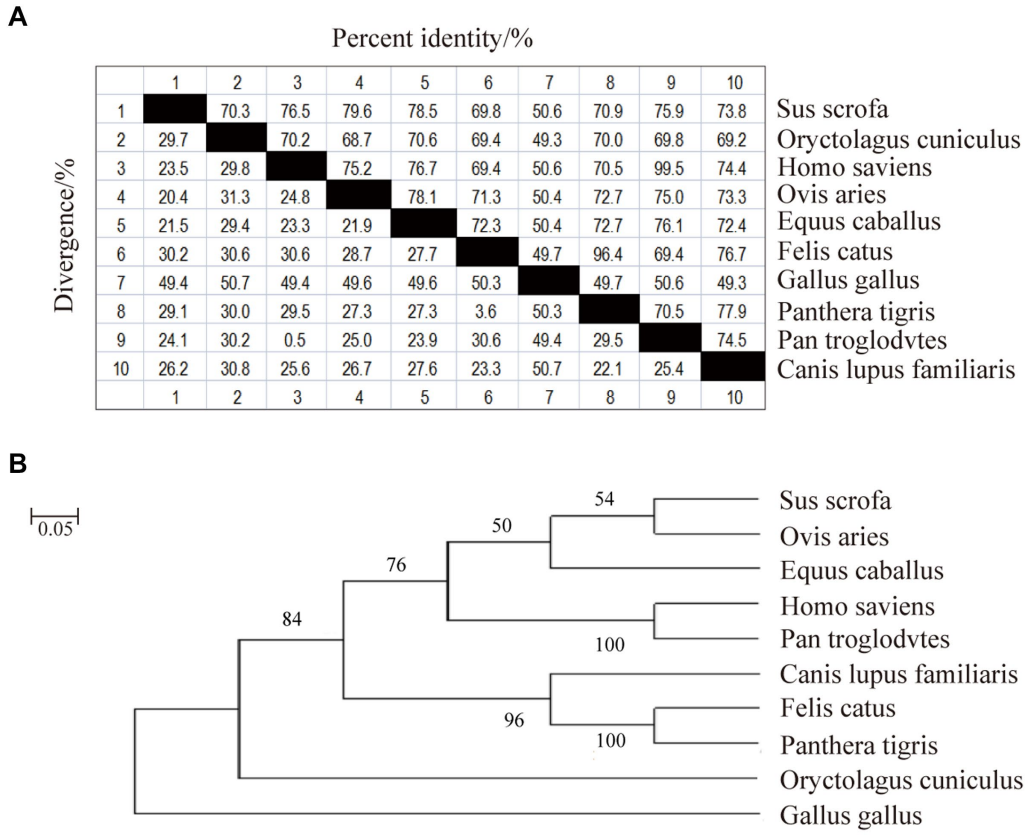

FIGURE 1
Nucleotide sequence similarity analysis and phylogenetic tree of sheep CD3 gene. **(A)** CD3 nucleotide sequence similarity analysis. **(B)** CD3 nucleotide sequence evolutionary tree analysis.

3.2 Bioinformatics analysis of CD3 in sheep

3.2.1 Physical and chemical properties

CD3 encodes 192 amino acids with a molecular weight of 21555.57U; the theoretical isoelectric point (PI) is 6.73, indicating that CD3 is an acidic protein; and the predicted instability index (PI) is 19.83, proving that CD3 is a stable protein. According to Table 1, leucine was the most abundant among the 20 amino acids encoding CD3, accounting for 22%, or 11.5%, while phenylalanine was the least abundant with only 2%, or 1.0% (Table 1). The predicted theoretical half-lives were 30 h in mammalian reticulocytes cultured *in vitro*, > 20 h in yeast, and >10 h in *E. coli*.

3.2.2 Prediction of hydrophilic/hydrophobicity, transmembrane regions and signaling peptides

The prediction of hydrophobicity results for the amino acid sequence of sheep CD3 protein (Figure 2A), revealed that the lowest value was at amino acid position 50/240/399, which was −0.322 and the most hydrophilic. The highest value is at amino acid position 508/509, which was 2.267 and the most hydrophobic. Amino acids in the hydrophilic region accounted for more amino acids than those in the hydrophobic region, so the sheep CD3 protein was a hydrophilic protein. The prediction analysis of hydrophilicity (Figure 2Ba) and antigenic epitope (Figure 2Bb) indicated that it was a hydrophilic protein with high antigenic index. The transmembrane structure predicted that all the amino acids were absent from the transmembrane region (Figure 2C). The signal peptide predicted that the protein did not have a signal peptide structure (Figure 2D).

3.2.3 Secondary structure projections and three-tier structural projections

As shown in Figure 3A, the CD3 protein consists of α-helix (16.15%), extended strand (26.56%), β-turn (4.69%), and irregular curl (52.60%) regions, with the highest percentage being accounted for by the CD3 protein's irregular curl region. This suggests that there are larger binding sites within CD3, indicating its classification as a mixed-type protein with a more complex biological function. The tertiary structure of sheep CD3 protein was predicted using the online sequencing software SWISS-MODEL,[1] and the coverage of the prediction model was 98%, which indicated that the model was reasonably constructed. The results of the tertiary structure model prediction showed the consistency with the results of the secondary structure prediction (Figure 3B).

1 http://www.expasy.ch/swissmod/SWISS-MODEL.html

TABLE 1 Amino acid composition of CD3 in sheep.

Amino acids	Quantity	Proportion	Amino acids	Quantity	Proportion
Ala(A)	11	5.7%	Thr(T)	10	5.2%
Cys(C)	6	3.1%	Gly(G)	17	8.9%
Arg(R)	11	5.7%	Asn(N)	13	6.8%
Asp(D)	7	3.6%	Gln(Q)	11	5.7%
Glu(E)	14	7.3%	His(H)	2	1.0%
Ile(I)	6	3.1%	Leu(L)	22	11.5%
Lys(K)	10	5.2%	Met(M)	5	2.6%
Phe(F)	2	1.0%	Pro(P)	10	5.2%
Ser(S)	9	4.7%	Trp(W)	4	2.1%
Tyr(Y)	9	4.7%	Val(V)	13	6.8%

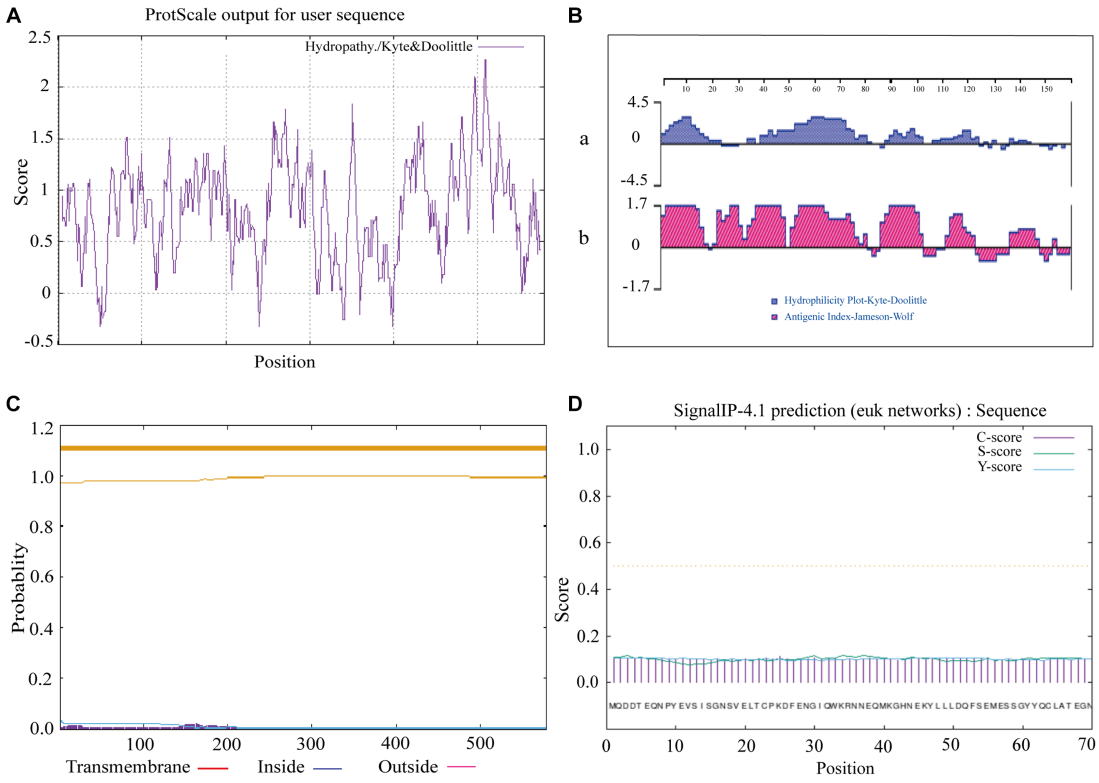

FIGURE 2
Ovine CD3 hydrophilicity, antigenic epitopes, protein transmembrane regions and signal peptide prediction **(A)**. Prediction of hydrophobicity of sheep CD3 protein. **(B)** Prediction of hydrophilicity and antigenic epitope of sheep CD3 [**(a)** hydrophilicity prediction; **(b)** antigenic epitope prediction]. **(C)** Prediction of transmembrane region of sheep CD3 protein (red line, transmembrane region; blue line, intramembrane region; purple line, extra-membrane region). **(D)** Sheep CD3 signal peptide prediction (C-score, shear site value; S-score, signal peptide region value; Y-score, parameter combining S- and C-values).

3.2.4 Analysis of phosphorylation and glycosylation sites and protein interactions of CD3 in sheep

Online software for its phosphorylation[2] and glycosylation[3] prediction analysis revealed 19 specific phosphorylation sites and no glycosylation sites (Figures 4A,B). STRING analysis showed an average local clustering coefficient of 0.941. As shown in Figure 4C, there are interactions between sheep CD3 and proteins such as CD28, ITK, CD3G, CD3D, SYK, CD4, CD247, ZAP70, LCK, CD19, and CD8A; these proteins exhibit strong interconnections among themselves.

3.3 Preparation for anti-sheep CD3 polyclonal antibodies

The supernatants and precipitates of the sonicated proteins were collected separately for SDS-PAGE. The results showed that

2 https://services.healthtech.dtu.dk/service.php?NetPhos-3.1
3 https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0

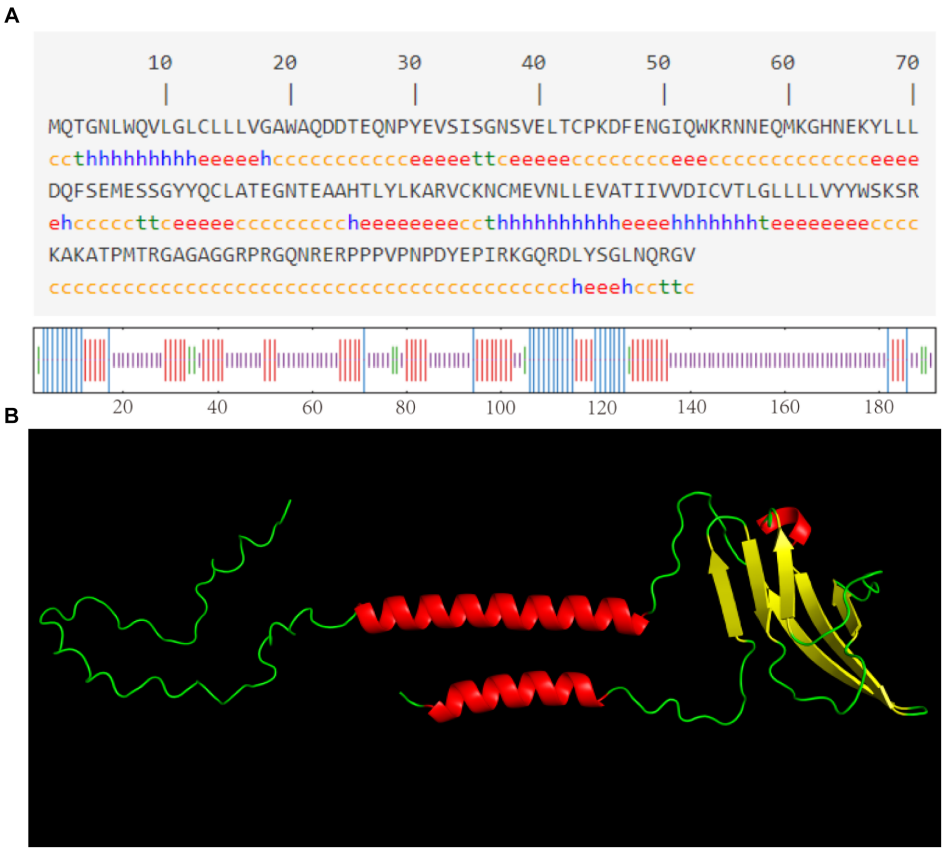

FIGURE 3
(A) Predicted secondary structure of sheep CD3 protein h, α-helix; e, extended chain; t, β-turn; c, irregularly coiled. **(B)** Predicted tertiary structure of CD3 protein.

compared with the products without induction by recombinant bacteria, the post-induction products appeared obvious expression bands (Figure 5A). Additionally, target bands appeared in the precipitates of the recombinant bacteria-induced products after sonication and centrifugation. It indicates that the recombinant protein CD3 is successfully expressed in *BL21* and exists as an inclusion body. The standard curve was obtained by plotting the relative mobility against the logarithm of the molecular weight of standard proteins (Figure 6). After elution, the purified recombinant protein was found to be free of heterogeneous proteins as detected by SDS-PAGE (Figure 5B), which indicated that the protein was of high purity. The results of western blotting showed that there was a clear protein blot appearing at about 13.7 kDa on the PVDF membrane (Figure 5C), which indicated that the rabbit anti-sheep CD3 antibody could specifically bind to the recombinant protein.

Based on the relative mobility of the protein to be measured, its relative molecular mass was determined from the standard curve (Table 2). The correlation coefficient $R^2 > 0.99$ indicates that the established standard curve can be used to determine the relative molecular mass of the protein. The mobility of CD3 in electrophoresis was found to be 3.69, and the calculated relative molecular mass of CD3 is 13.7 kDa.

3.4 The pattern of the effect of *Moniezia benedeni* infection on the distribution of CD3⁺ T cells

Immunofluorescence results showed that sheep CD3⁺ T cells were mainly diffusely distributed around the intestinal LP and within the mucous epitheliums of the duodenum (Figures 7A,B), jejunum (Figures 8A,B) and ileum (Figures 9A,B). Immunohistochemical results showed that the distribution densities of CD3⁺ T cells in each intestinal segment, from the duodenum to the jejunum and ileum were 6.64 cells/10^4 μm^2, 7.62 cells/10^4 μm^2 and 6.15 cells/10^4 μm^2, respectively. The highest distribution density was found in the jejunum, followed by the duodenum and ileum. After *M. benedeni* infection, the distribution densities of total CD3⁺ T cell were significantly increased (Table 3 and Figure 10), with densities of 8.86 cells/10^4 μm^2 (duodenum), 8.73 cells/10^4 μm^2 (jejunum) and 8.93 cells/10^4 μm^2 (ileum), respectively. Each density increased by 33.43% (duodenum), 14.50% (jejunum) and 34.19% (ileum).

Statistical analysis of the distribution density of CD3⁺ T cells within mucous epitheliums and intestinal LP in each intestinal segment showed that the distribution densities of CD3⁺ T cells on the mucous epitheliums were 6.71 cells/10^4 μm^2 (duodenum), 7.93 cells/10^4 μm^2 (jejunum) and 7.21 cells/10^4 μm^2 (ileum), respectively.

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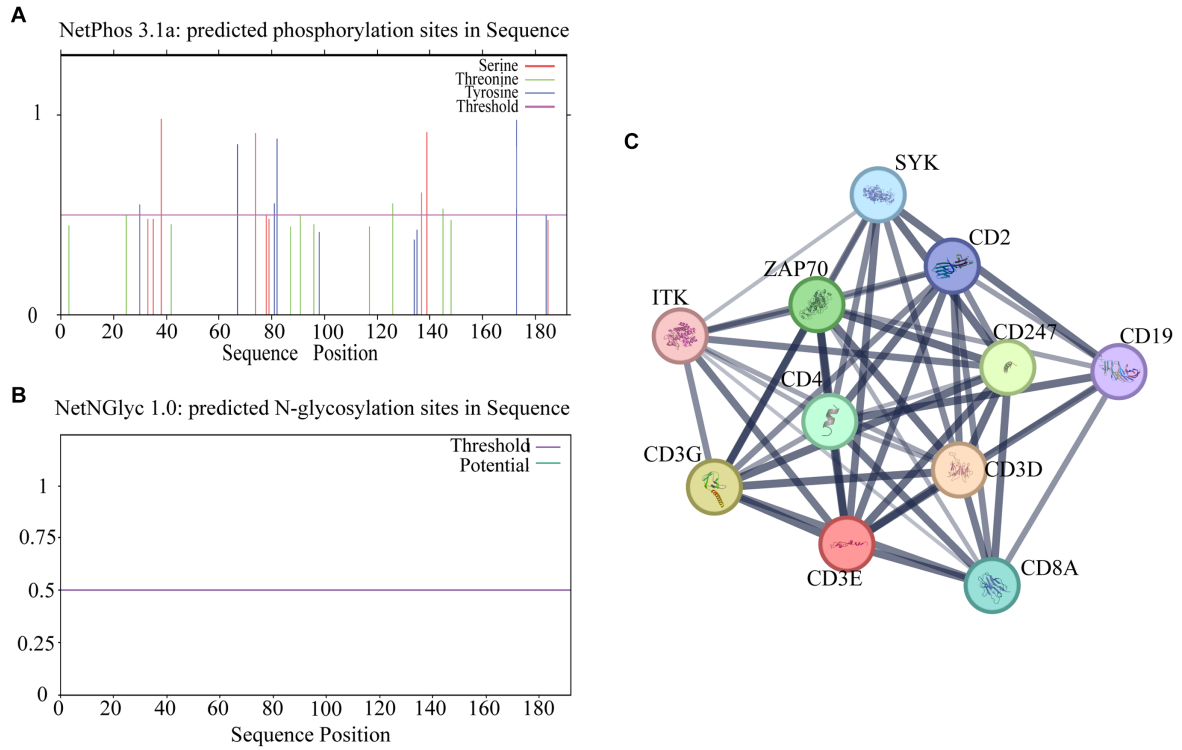

FIGURE 4
Analysis of potential phosphorylation and glycosylation sites of CD3 in sheep and other protein interactions. **(A)** Phosphorylation site prediction. **(B)** Glycosylation site prediction. **(C)** Protein interaction analysis. The thickness of the lines indicates the intensity of the interaction between proteins, and the thicker the lines indicate the greater the interaction between proteins.

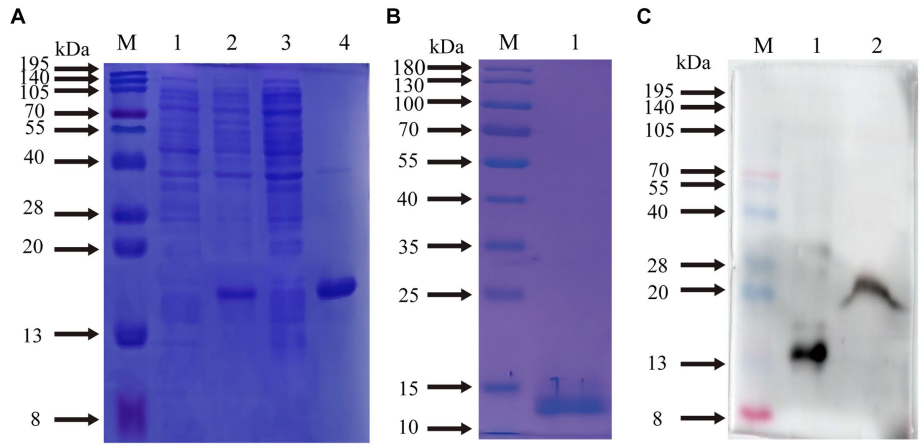

FIGURE 5
Prediction of CD3 expression form in sheep and WB results. **(A)** M. protein molecular quality standard; (1) recombinant bacterial pre-induction product; (2) recombinant bacterial post-induction product; (3) supernatant of recombinant bacterial induction product; (4) precipitation of recombinant bacterial induction product. **(B)** M. protein molecular quality standard; (1) purified proteins. **(C)** M. protein molecular quality standard; (1) purified proteins; (2) natural total proteins.

The distribution densities of $CD3^+$ T cells on the intestinal LP were 6.01 cells/$10^4\,\mu m^2$ (duodenum), 7.01 cells/$10^4\,\mu m^2$ (jejunum) and 6.43 cells/$10^4\,\mu m^2$ (ileum), respectively. The distribution densities of $CD3^+$ T cells in the mucous epitheliums of each intestinal segment after *M. benedeni* infection were 8.59 cells/$10^4\,\mu m^2$ (duodenum), 8.49 cells/$10^4\,\mu m^2$ (jejunum) and 8.60 cells/$10^4\,\mu m^2$ (ileum), respectively, with an increase of 27.92% (duodenum), 7.07% (jejunum) and 19.23% (ileum) in the intestinal LP of each intestinal segment. The distribution densities of $CD3^+$ T cells were 8.03 cells/$10^4\,\mu m^2$ (duodenum), 8.82 cells/$10^4\,\mu m^2$ (jejunum) and 8.17 cells/$10^4\,\mu m^2$ (ileum), which

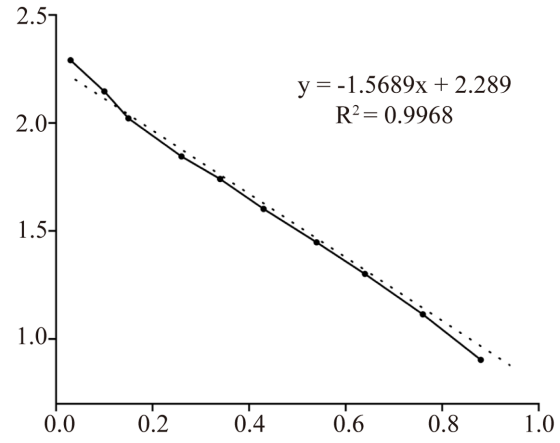

FIGURE 6
Protein relative molecular mass standard curve.

increased by 33.57% (duodenum), 25.82% (jejunum) and 27.07% (ileum), respectively (Table 4 and Figure 11). The most dramatic increase in the distribution density of CD3⁺ T cells in jejunal mucous epitheliums and intestinal LP was observed ($p < 0.05$).

4 Discussion

The results showed that sheep CD3 consisted of 192 amino acids, which had a high affinity with pigs (about 79.6%) and the lowest affinity with chickens (about 50.6%). Protein interactions analysis showed that there was interaction between sheep CD3 and CD4, CD247 and other proteins. It has been demonstrated that two ζ chains of CD3 molecule were encoded by CD247 gene, which form a TCR/CD3 complex with T cell antigen receptor αβ (TCRαβ) or γδ (Tcrγδ) and CD3γ, ε, δ chains in a non-covalent bond (36). TCR mainly recognizes and binds MHC antigenic peptide complexes, and CD3 transduces the signals recognized by TCR and induce activation of T

TABLE 2 Determination of protein band mobility in CD3-SDS-PAGE.

Enterprise	Measured value									
Relative molecular mass M/kDa	195	140	105	70	55	40	28	20	13	8
Logarithm of relative molecular mass LgM	2.29	2.14	2.02	1.84	1.74	1.60	1.44	1.30	1.11	0.90
Sample migration distance L/cm	0.15	0.50	0.75	1.30	1.70	2.15	2.70	3.20	3.80	4.40
Relative mobility Mr	0.03	0.1	0.15	0.26	0.34	0.43	0.54	0.64	0.76	0.88

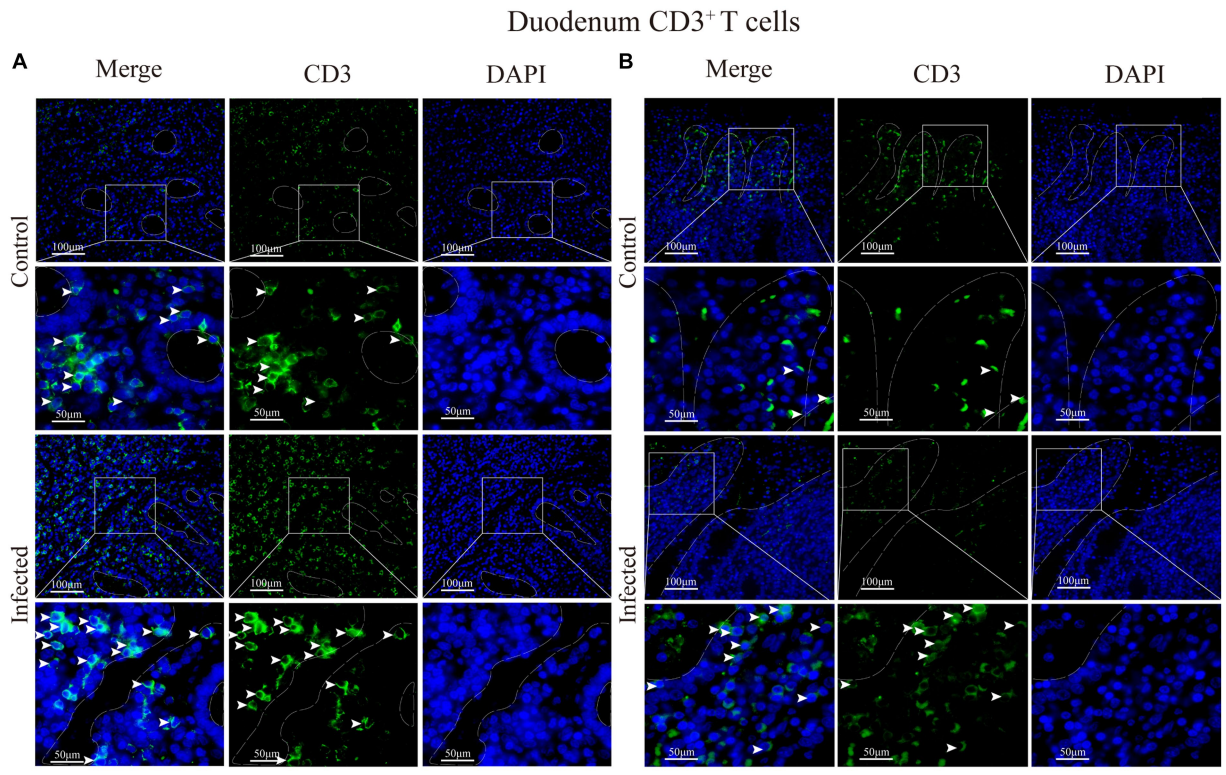

FIGURE 7
(A) Distribution of CD3⁺ T cells in the intestinal LP of the duodenum. **(B)** Distribution of CD3⁺ T cells in the duodenal mucous epitheliums. The white arrows represent T cells.

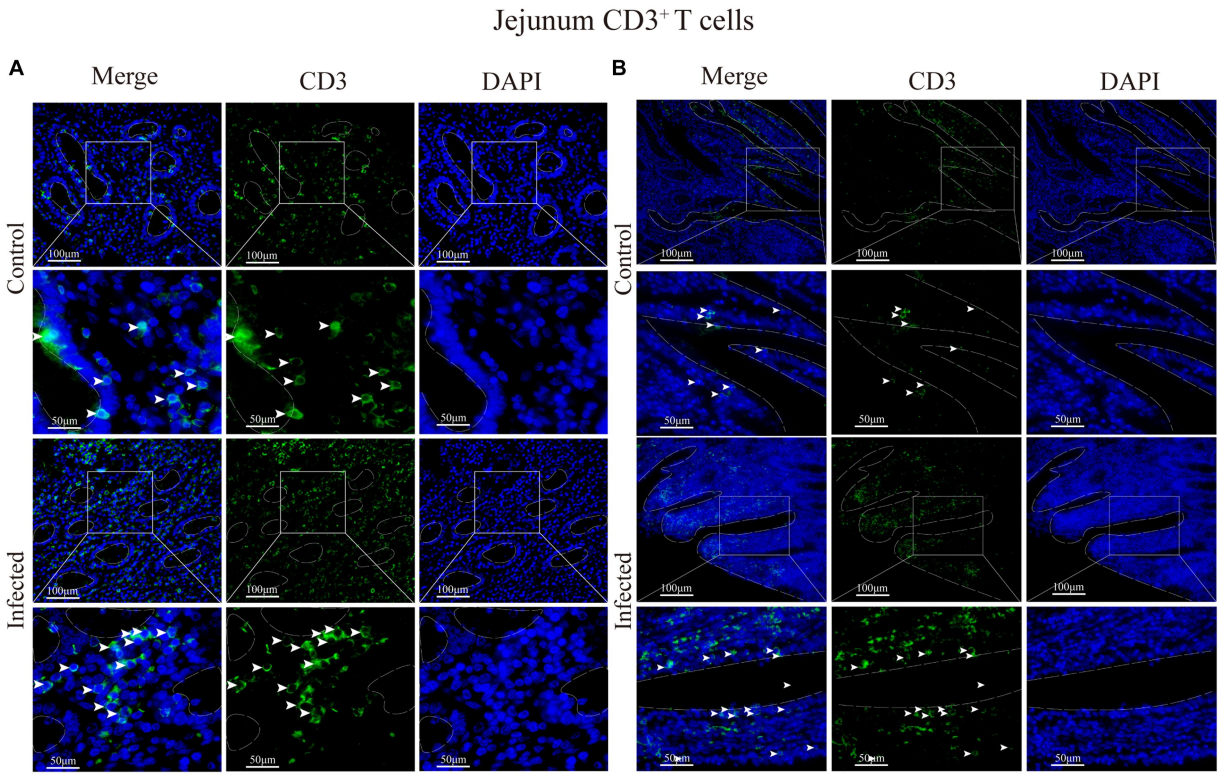

FIGURE 8
(A) Distribution of CD3$^+$ T cells in the intestinal LP of the jejunum. **(B)** Distribution of CD3$^+$ T cells in the duodenal mucous epitheliums. The white arrows represent T cells.

lymphocyte. The initiation of T lymphocyte activation is determined by the level of TCR/CD3 membrane expression levels (37). Our predictive analysis shows that the molecular weight of sheep CD3 was 21555.57 U, the theoretical isoelectric point (PI) was 6.73, the predicted instability index (PI) was 19.83, and there were more amino acids in the hydrophilic region than in the hydrophobic region. It was indicated that CD3 was an acidic, hydrophilic, and stabile protein without extramembrane region and signal peptide. It mainly plays biological roles in the cell membrane, and the analysis of amino acid fractions reveals that leucine is the most predominant. It also suggests that the sheep CD3 recombinant protein would have a good immunogenicity. Polyclonal antibody prepared in this study had good specificity. The construction of recombinant plasmid. The experimental results also confirmed that through the recombinant plasmid was constructed, prokaryotic expression and preparation, the rabbit polyclonal antibody against sheep CD3 recombinant protein had good specificity. These results will lay a foundation for further investigation of the effects of *M. benedeni* infection on T cells in sheep small intestine.

The intestine maintains the normal digestive, absorptive, and secretory function, it also paly an important mucosal defense functions (38). The intestinal mucosal immune system can be divided into induction sites and effector sites (39). The former mainly contains M cells, dendritic cells (40), macrophages (41) and intestinal epithelial cells (42), which are mainly responsible for the uptake and transport of antigens. The latter includes IELs and LP lymphocytes, where transmitted antigens are activated to produce antibodies and various immune factors (43). IEL, as the first immune cell to interact with foreign antigens, microorganisms and parasites in the body's immune system (44), can induce local and systemic immune responses to clear antigens by secreting related cytokines (45). The CD3 molecule transduces activation signals generated by T cell receptors to recognize antigens (46). So the determination of the change characteristics of CD3$^+$ T lymphocytes is very important to evaluate the intestinal immune response to parasitic infection.

The results of this study showed that in the control group, CD3$^+$ T cells were distributed in the mucosal epithelium and the lamina propria around the intestinal gland, and their densities were different, among which the distribution density in LP of the jejunum was higher than that in LP of the duodenum and ileum. Our previous studies have confirmed that under physiological conditions, the distribution of IgA$^+$, IgG$^+$, and IgM$^+$ cells in the small intestine of sheep presents obvious local specificity (47). Therefore, under normal conditions, the distribution characteristics of CD3$^+$ T cells in the small intestine of sheep are similar to those of antibody secreting cells, both of which have significant local specificity.

M. benedeni infection did not change the spatial distribution of CD3$^+$ T cells, but led to increased distribution density of CD3$^+$ T cells in each intestinal segment. CD3$^+$ T cells were significantly increased in LP and IEL in duodenum and ileum compared with in jejunum. This characteristic change is exactly the opposite of the castration effect of *M. benedeni* infection on intestinal antibody secreting cells (48). The function of IEL can directly affect the integrity of mucosal immune barrier (49). Studies have shown that

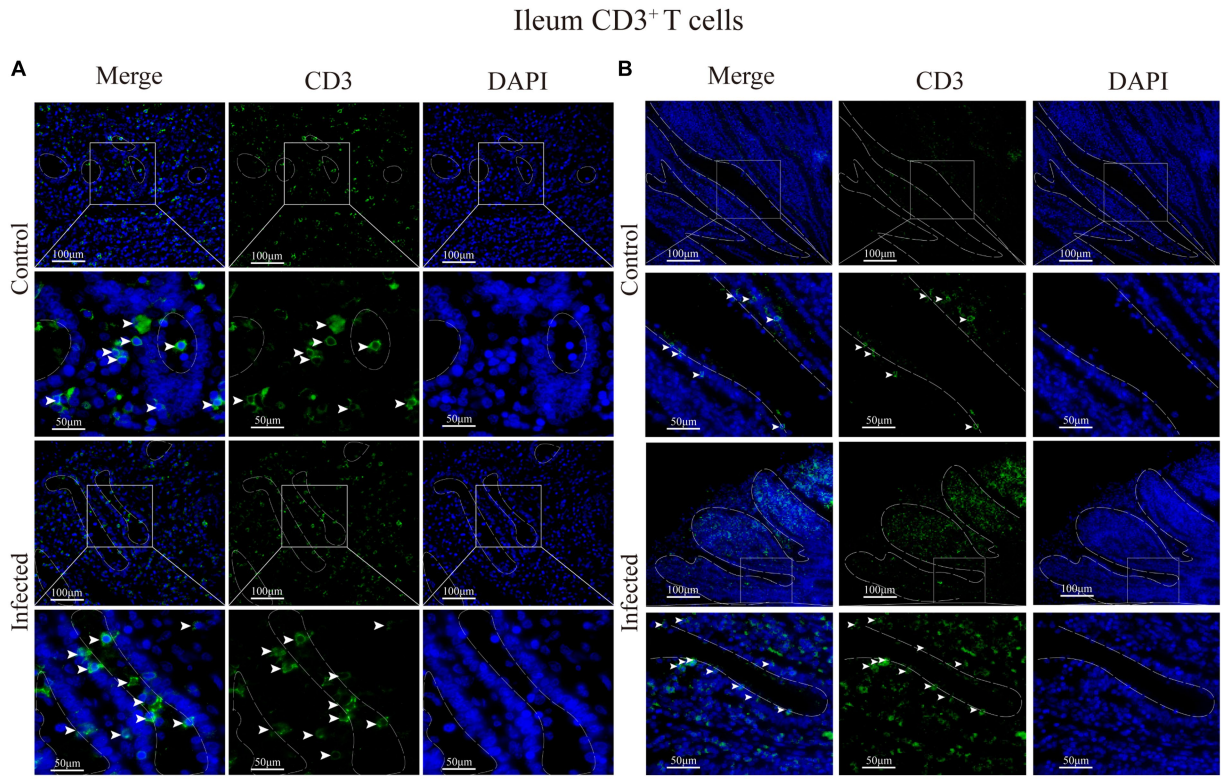

FIGURE 9
(A) Distribution of CD3⁺ T cells in the intestinal LP of the ileum. **(B)** Distribution of CD3⁺ T cells in the ileal mucous epitheliums. The white arrows represent T cells.

TABLE 3 Changes in the density of CD3⁺ T cells in the small intestine of sheep after *M. benedeni* infection.

	Duodenum	Jejunum	Ileum
Control (cells/10^4 μm^2)	6.64 ± 0.83^{Aa}	7.62 ± 1.68^{Ba}	6.15 ± 0.92^{Ca}
Infected (cells/10^4 μm^2)	8.86 ± 0.88^{Ab}	8.73 ± 1.17^{Ab}	8.93 ± 1.02^{Bb}
Rate of increase	33.43%	14.50%	34.19%

Rise rate = (infected group − control group)/control group×100%. Differences in data in the same row are indicated by capital letters, and different letters indicate significant differences ($p < 0.05$); differences in data in the same column are indicated by lowercase letters, and different letters indicate significant differences ($p < 0.05$).

in a mouse model of *Eimeria vermicularis* infection, the number of IEL increases sharply when the number of coccidium oocysts increases (50). Therefore, the increase in the number of CD3⁺ T cells in IEL can be considered to play an important immunomodulatory and immunoprotective role in *M. benedeni* infection. The LP is the main effect site of mucosal immune response. A large amount of IgA secreted by plasma cells can enter the mucosal surface through the mediation of secretory segment to neutralize antigens (51). After infection, the distribution density of CD3+ T cells in LP increased by 33.57% (duodenum), 25.82% (jejunum) and 27.07% (ileum) respectively. Studies have shown that helminth infection can induce immune regulation of autoimmune-mediated inflammatory diseases, promote Th2 cells balance, facilitate IgE class conversion or activation of polyclonal B cells, and significantly increase IgE expression levels (52). This anti-inflammatory state is thought to be driven by T and B regulatory cells and parasite secretions that have the ability to promote immune regulation. Therefore, the results of this study suggest that after infection with *M. benedeni*, the number of CD3⁺ T cells in each intestinal segment of the host increases, and the cellular immune response is significantly enhanced, which is conducive to mediating the host mucosal immune response by CD3⁺ T cells, maintaining the integrity of the mucosal epithelium, and inhibiting intestinal mucosal hypersensitivity. It plays an important regulatory role in anti-bacterial (53), anti-viral (54), anti-infection and anti-local cell carcinogenesis (55, 56), reducing mechanical damage (57), diluting metabolites (58), and neutralizing toxins (59) produced by worms. This study provides an important basis for understanding the molecular mechanism of parasite infection and revealing the interaction between parasite and host, and lays a foundation for studying the changes of different subtypes of T cells. However, the effect of *M. benedeni* infection on the increase of T lymphocytes in

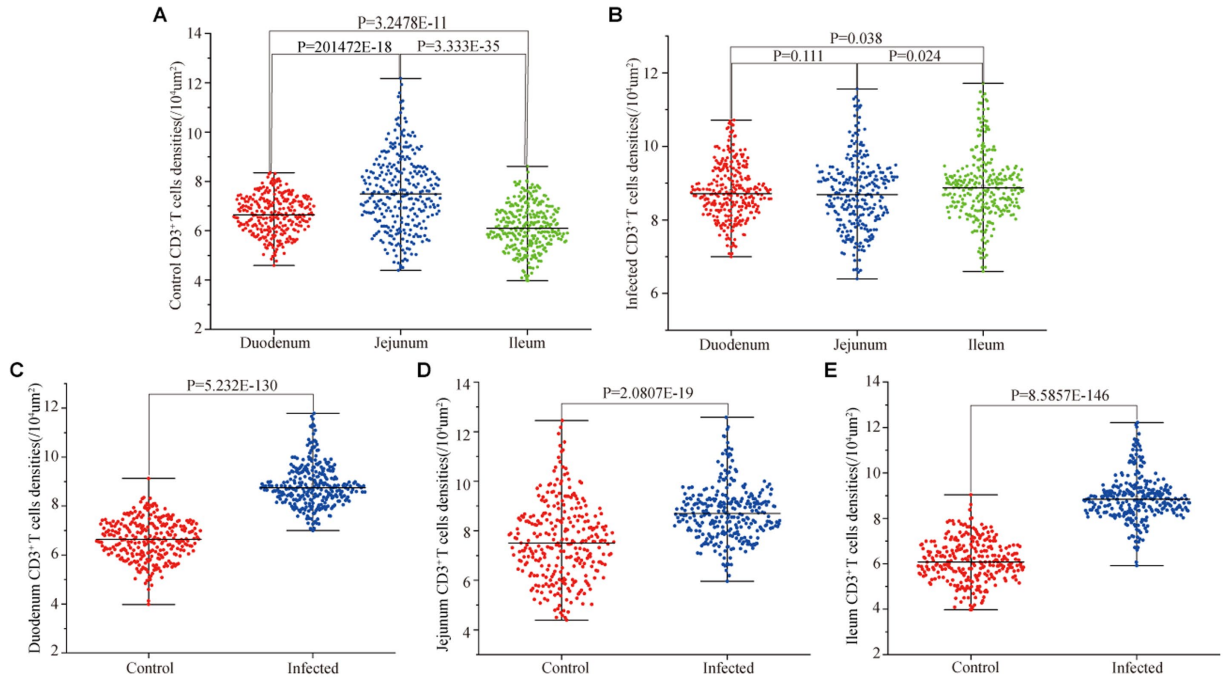

FIGURE 10
Effect of *M. benedeni* infection on the distribution density of small intestinal CD3⁺ T cells in sheep. **(A)** Distribution density of small intestinal CD3⁺ T cells in control sheep. **(B)** Distribution density of small intestinal CD3⁺ T cells in infected sheep. **(C)** Distribution density of duodenal CD3⁺ T cells by *M. benedeni* infection. **(D)** Distribution density of jejunum CD3⁺ T cells by *M. benedeni* infection. **(E)** Distribution density of small intestinal CD3⁺ T cells by *M. benedeni* infection on ileal CD3⁺ T cell distribution density; $p < 0.05$ indicates significant difference.

TABLE 4 Changes in the density of CD3⁺ T cells in the mucous epitheliums and the intestinal LP of sheep small intestine after *M. benedeni* infection.

		Duodenum	Jejunum	Ileum
Mucous epitheliums	Control (cells/10^4 μm^2)	6.71 ± 0.88^{Aa}	7.93 ± 1.01^{Ba}	7.21 ± 1.01^{Ca}
	Infected (cells/10^4 μm^2)	8.59 ± 0.99^{Ab}	8.49 ± 0.88^{Ab}	8.60 ± 0.80^{Bb}
	Rate of increase	27.92%	7.07%	19.23%
Intestinal LP	Control (cells/10^4 μm^2)	6.01 ± 0.82^{Aa}	7.01 ± 0.73^{Ba}	6.43 ± 1.01^{Ca}
	Infected (cells/10^4 μm^2)	8.03 ± 1.01^{Ab}	8.82 ± 0.83^{Bb}	8.17 ± 0.77^{Cb}
	Rate of increase	33.57%	25.82%	27.07%

Rise rate = (infected group − control group)/control group × 100%. Differences in data in the same row are indicated by capital letters, and different letters indicate significant differences ($p < 0.05$); differences in data in the same column are indicated by lowercase letters, and different letters indicate significant differences ($p < 0.05$).

sheep small intestine was significantly different in different intestinal segments, which may be related to the metabolites secreted by *M. benedeni* infection or the cell differentiation of the host mucosal epithelium, which needs to be confirmed by further studies.

5 Conclusion

In this study, specific rabbit anti-sheep CD3 polyclonal antibody was successfully prepared. CD3⁺ T cells were dispersed in the LP surrounding intestinal glands and intestinal epithelium of sheep small intestine, and their distribution density was relatively high, especially in IEL, the distribution density in jejunum was higher than that of in duodenum and ileum. The spatial distribution of CD3⁺ T cells in the small intestine of sheep was not changed after infection by *M. benedeni*, but the distribution density of CD3⁺ T cells in each intestinal segment was increased. It is suggested that *M. benedeni* infection leads to a significant increase of CD3⁺ T cells in the small intestine, thereby enhancing cellular immunity or strengthening mucosal immunity against *M. benedeni* infection. In addition, the high distribution density of CD3⁺ T cells in the IEL and LP in all intestinal segments provides the basis for studying mucosal immunity and maintenance epithelial integrity, It also plays a role in inhibiting intestinal mucosal hypersensitivity and the recogniting whether epithelial cells are infected by bacteria and viruses or not. This lays the foundation for further studies on the regulatory mechanisms of the intestinal mucosal immune system against *M. benedeni* infection in sheep.

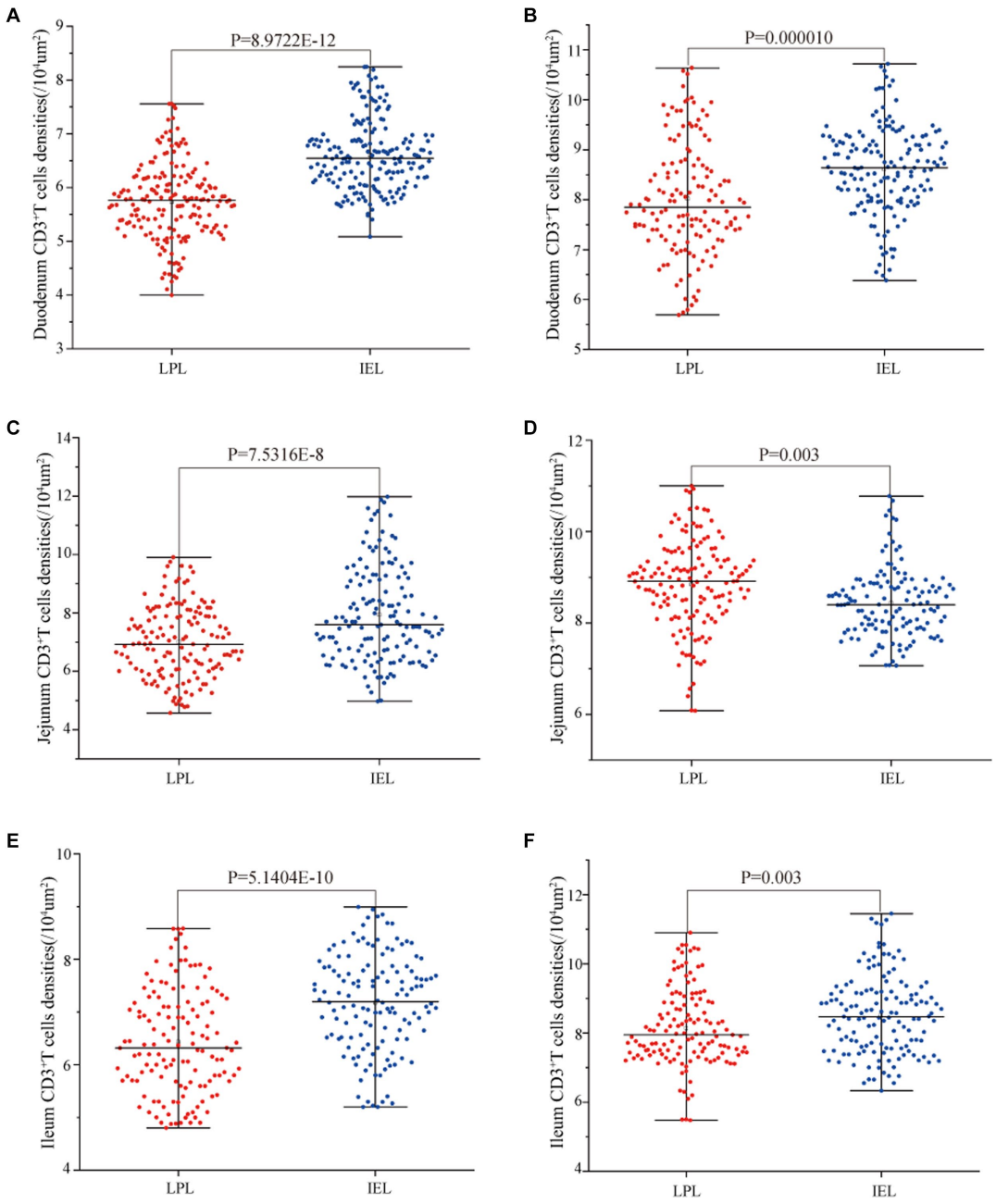

FIGURE 11
Effect of *M. benedeni* infection on the distribution of CD3⁺ T cells in the intestinal LP and mucous epitheliums in the small intestine of sheep **(A,B)** are the distribution densities of CD3⁺ T cells in the intestinal LP and mucous epitheliums of the duodenum by *M. benedeni* infection; **(C,D)** are the distribution densities of CD3⁺ T cells in the intestinal LP and mucous epitheliums of the jejunum by *M. benedeni* infection; **(E,F)** are the distribution densities of CD3⁺ T cells in the intestinal *LPxaq* and mucous epitheliums of the ileum by *M. benedeni* infection. Distribution density of CD3⁺ T cells (**A,C,E** are control groups, and **B,D,F** are infection groups). $p < 0.05$ indicates significant difference.

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 study was approved by Animal Care and Use Committee (IACUC) of College of Veterinary Medicine of Gansu Agricultural University (Approval No.: GSAU-Eth-VMC-2021-021). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WC: Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing. WY: Supervision, Writing – review & editing, Methodology. JP: Methodology, Writing – review & editing. ZH: Writing – review & editing, Validation. BW: Writing – review & editing,

Supervision. BX: Writing – review & editing, Investigation. XF: Investigation, Writing – review & editing. WH: Investigation, Writing – review & editing. WW: Writing – review & editing, Project administration. WZ: Project administration, Writing – review & editing, Conceptualization, Funding acquisition, Supervision, Validation.

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Conflict of interest

BX was employed by Lanzhou Safari Park Management Co.

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Microfilaremic infection in canine filariosis in Colombia: a challenge in morphological and molecular diagnostics

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Canine filariosis is caused by filiform nematodes and affects several species of animals as well as humans. The disease produces a wide range of symptoms that can often be confused with other diseases, which increases the complexity of its diagnosis. The search for methodologies to facilitate its diagnosis is a challenge, and specific and differential identification of the parasite species causing the disease holds key to a successful diagnosis. In Colombia, there is a problem of underdiagnosis of filariosis in microfilaremic dogs infected by *Dirofilaria immitis* and *Acanthocheilonema reconditum*, and of microfilaremias not related to heartworm disease. The highest prevalences have been reported for *D. immitis* infections, although new cases of *A. reconditum* infections are beginning to appear. The aim of this study was to differentiate the microfilariae infections caused by *D. immitis* and *A. reconditum* by a morphological and molecular characterization of microfilariae so as to facilitate an accurate diagnosis of canine filariosis in the metropolitan area of Bucaramanga (Colombia). For this purpose, 400 blood samples with anticoagulants were collected from the dogs and analyzed with the help of a commercial immunochromatography kit for the detection of *D. immitis* circulating antigen. The Woo, Knott, and polymerase chain reaction (PCR) techniques were employed for determining the parasite count, morphological observation, and molecular identification of microfilariae present in the dogs respectively. The prevalence of microfilaremic dogs in Bucaramanga metropolitan area was 18.75% (75/400). The prevalence of dogs that tested positive for *D. immitis* in the antigen and in PCR tests was 1.25% (5/400) and 1% (4/400), respectively. Furthermore, the PCR test revealed that 17.75% of the microfilaremic dogs tested positive for *A. reconditum* (71/400) (first report in the metropolitan area of Bucaramanga), with one animal co-infected by both species, and 0% for *D. repens* (0/400). However, by morphological characterization, 4% of the microfilariae (3/75) corresponded to *D. immitis*, 20% (15/75) to *D. repens*, and 76% (57/75) to *A. reconditum*. The use of molecular diagnostic methods such as PCR aids in the specific identification of the parasite, thus making it a more accurate method than the morphological characterization of microfilariae. The identification of the parasites by PCR helps improve the veterinary diagnosis of canine filariosis in Colombia, which would

lead to the establishment of an appropriate treatment protocol for each species of filaria and also to the generation of reliable data to be used at the clinical and epidemiological levels.

KEYWORDS

filariosis, zoonosis, Colombia, dogs, *Dirofilaria immitis*, *Acanthocheilonema reconditum*

1 Introduction

Canine filariosis is a parasitic disease caused by filiform nematodes at different stages of their life cycle (1). These parasites are transmitted by vectors that are widely distributed, with the main host being dogs, both domestic and wild, as well as humans, who act as accidental hosts (2). In veterinary medicine, there are families of filarials that hold importance for their impact on public health due to their zoonotic potential, although filarials such as *Onchocerca lupi, Acanthocheilonema dracunculoides (sin: Dipetalonema dracunculoides), A. reconditum (sin: Dipetalonema reconditum),* and *Cercopithifilaria (sin: Acanthocheilonema grassi)* (1, 3–7) cause low pathogenicity and other pathogens such as *Dirofilaria immitis* and *D. repens* are responsible for heartworm disease and subcutaneous dirofilariosis, respectively (8, 9).

These diseases produce different clinical manifestations. On the one hand, they may lead to ataxia, incoordination, marked leukocytosis, and hemoglobinuria, caused by an infection by *A. dracunculoidess*, which are lodged in the peritoneal cavity (8, 10, 11). Granulomas formed at the cutaneous level due to the presence of the parasite in muscle fascia, subcutaneous tissue, peritoneal cavity, and kidney are associated with an infection by *A. reconditum* (12, 13). On the other hand, *D. immitis* can cause chronic cough, dyspnea, lipothymia, weakness, anorexia, weight loss, and dehydration, depending on the parasite load or variation in the physical exercise performed by the animal. The greater the physical activity, the greater the arterial damage (9). In addition, the symptoms caused by *D. repens* depend on the location of the nodules it infects and are generally limited to local inflammation, mainly in subcutaneous and ocular tissues, erythema, and pruritus. Occasionally, much more severe systemic immune reactions may develop, manifesting as fever or lymphadenopathy (14, 15).

Different vector species are involved in the transmission of these parasites. *Rhipicephalus sanguineus* (tick), *Ctenocephalides* spp., and *Pulex irritans* (fleas) are the vectors known to transmit *A. dracunculoidess* and *A. reconditum* (7), whereas *Culex* spp., *Aedes* spp., and *Anopheles* spp. (culicid mosquitoes) are implicated in the transmission of *D. immitis* and *D. repens* (16).

A wide variety of studies have reported the prevalence of *D. immitis* worldwide in dogs, mainly due to the availability of commercial diagnostic techniques for the detection of circulating antigens (9, 16–18). However, for other species, cases are reported accidentally, epidemiological studies are rare, and there are no commercial diagnostic techniques available for their detection (13, 19–21). In Colombia, the prevalence of *D. immitis* in dogs is 0.91–53.2%, depending on the sampling site (22–27), and it is 10.82% in Bucaramanga (28). However, there is only one study that has reported the presence of *D. repens* using molecular techniques in three blood samples of dogs that are found in shelters in Bucaramanga (29). There are two reports on the presence of *A. reconditum* in microfilaremic dogs with prevalences varying between 4.81 and 61.3% (13, 30).

This complex diagnostic condition and the fact that it is common to observe dogs with a myriad of symptoms that can be associated with various diseases makes the identification, differentiation, and diagnosis of filarial species at the veterinary clinic level very important. Therefore, the aim of this study was to provide techniques for the morphological and molecular characterization of the filariae present in dogs in the metropolitan area of Bucaramanga, Colombia, in order to improve the identification of filarial species and also contribute to their detection in other Colombian regions.

2 Materials and methods

2.1 Sampling area

Bucaramanga Metropolitan Area, the capital city of the Department of Santander, consists of three municipalities: Floridablanca, Piedecuesta, and Girón in central Colombia (Figure 1). This metropolitan area is spread over 1,479 km^2, and its municipal area occupies 165 km^2. It is located 959 m above the mean sea level. Its climate is tropical with an average temperature of 23°C and a maximum of 30°C, and the area experiences rainy and dry seasons throughout the year. The region receives significant annual rainfall at an average of 1,159 mm (31, 32). The estimated human population of the metropolitan area of Bucaramanga is 1.1 million, out of which 95% live in urban areas (33). The approximate domestic canine population is 7,906, according to the latest canine census of 2018–2019, of which a large number are stray dogs (34, 35).

2.2 Samples used

The samples collected from 400 owned domestic dogs (non-roaming) from January to September 2023 were used for this study. The veterinary staff members of the Biovet Veterinary Clinical Laboratory collected the samples. The written informed consent of the owners was considered as an inclusion criterion, and confidentiality of patient information was always maintained. All dogs included in the study were over 1 year of age. The variables considered for the analysis were age, sex, municipality of residence, socioeconomic status, whether dogs lived inside or outside of the house, and use or non-use of ectoparasite medication. The socioeconomic classification of the dogs was made based on the six socioeconomic hierarchical strata: (1) misery; (2) poverty; (3) poverty with some economic

FIGURE 1
Sampling area: metropolitan area of Bucaramanga (Colombia) by satellite image.

resources (4) middle class; (5) upper middle class; and (6) upper class. They were recategorized into vulnerable strata 1 and 2; middle class 3 and 4; and upper class 5 and 6 (36). All data are shown in Table 1.

2.3 Parasitological microscopic and immunocromatographic techniques

The blood samples from the dogs were collected in 1 mL K2 EDTA plastic microtubes and were maintained at 4°C. The Woo technique was used as a screening method to detect microfilaremic dogs (37). One-third of blue-line microhematocrit tubes were filled with whole blood, then sealed with plasticine, and centrifuged for 5 min at 11,000 rpm. Finally, they were observed under an optical microscope with a 10× objective, and their movement, which was either progressive rectilinear or non-progressive undulating, was recorded.

Microfilaremic samples were analyzed by the modified Knott technique (38). In brief, 1 mL of blood with K2 EDTA was mixed with 9 mL of 2% formalin and centrifuged at 1500 rpm for 5 min. After discarding the supernatant, 3 drops of methylene blue were added to the sediment. Afterward, 10 µL of this treated sample was spread on a slide and observed under a light microscope with a 40× objective. Microfilariae were identified by the following morphological criteria without sheath: a sharp cephalic end and a straight and sharp tail (*D. immitis*); a blunt cephalic end and a sharp and filiform tail, often ending in an umbrella hook (*D. repens*); and a blunt cephalic end with a prominent hook, and a flat and curved hooked tail (*A. reconditum*) (38–40). The microfilariae load in the positive samples was quantified in 20 µL of the sample that was diluted to 1:100.

Dog serum samples were tested for the presence of *D. immitis* antigens using a commercial immunochromatographic test kit (Uranotest Dirofilaria®, Urano Vet SL, Barcelona, Spain; sensitivity: 94.4%, specificity: 100%) according to the manufacturer's instructions.

2.4 Molecular PCR endpoint detection

2.4.1 DNA extraction

The commercial kit Corpogen® DNA 2000 was used for the extraction of the genetic material following the manufacturer's instructions. Briefly, 350 µL of each blood sample from microfilaremic dogs was processed. The samples were washed with a washing solution and columns and centrifuged at 12,000 rpm for 1 min four times. Then, 500 µL of lysis buffer and 50 µL of proteinase K were added. After 12 h of incubation at 56°C, they were vortexed and 500 µL of saline solution was added. They were then incubated on ice for 5 min and centrifuged at 12,000 rpm for 10 min. Then 600 µL of isopropanol was added, and the sample was shaken gently by inversion, centrifuged at 12,000 rpm for 5 min, and the supernatant was removed by inversion. The pellet was washed twice with 250 µL of 70% ethanol and centrifuged between washes at 12,000 rpm for 1 min. It was allowed to air dry for 20 min and finally the DNA was reconstituted with 100 µL of reconstitution solution for 1 h at 65°C. All solutions were provided by the manufacturer.

2.4.2 *Dirofilaria immitis* and *Dirofilaria repens* multiplex PCR assay

The processed samples were subjected to multiplex PCR reactions for the detection of *D. immitis* and *D. repens* as per Gioia et al. (41). In brief, multiplex PCR reactions were performed in a SimpliAmp™ (Applied Biosystems™) using two sets of primers in the same mixture reaction. We used two general primer pair 12SF (5-GTTCCA GAATAATCGGCTA-3) and 12SRdeg (5-ATTGACGGATG(AG) TTTGTACC-3) and specific forward primer for *D. immitis* (12SF2B 5-TTTTTTTACTTTTTTTTTTGGTAATG-3) and a specific reverse primer for *D. repens* (12SR2 5-AAAAGCAACACACACAAATAA(CA) A-3) with an equimolar combination of general and specific primers in a single tube. The hybridization of these

TABLE 1 Identified sequences and GenBank accession number.

Sample ID	Species filaria	Gene	Query coverage (%)	Sequence	GenBank number
4	*Acanthocheilonema reconditum*	12S ribosomal RNA	94	yATTCGGGAGTAAAGTTGTATTTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCC TTATTTGTTTAATTTTTTTTGGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAA CAGATATATATTTGGCTTATAGATTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTGAAATT GGAAAAGAAAGTAATTTATTTTTTAGTGTTTTAGTGAATTTAATAAATAGAGTGGTACAAACCTCCCCGTCAATT	PP214446
6	*Acanthocheilonema reconditum*	12S ribosomal RNA	89	CGATAATACYTRCCATAATATCATGATMTGWGTATTYTATTTTTYWATWTWATWTWTGTAAATATTTTAATTTTTTATTTTTAATT GAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTTAAA CCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTGGCAC ATGTATGATTGTTTTGTTATTATGTTATYTGTAATGCTTTAAACTTTTTTTGTTTTAAACAGATATATATTTGGCTTATAGATTTTT TTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTATTTTTTTTTGAAWTGSAAAAGAAGWATTTWTTTTTAGTGTTTA GWGATTTATAATAGAGYGYACMACCMTCCGTCATA	PP214447
9	*Acanthocheilonema reconditum*	12S ribosomal RNA	93	GCGTATACTCATCCGTACATACGTTATTTTTGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTTTAATT GAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTTAAA CCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTGGCACA TGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTTGGCTTATAGATTTTT TTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTTGAAATTGGAAAAGAAAGTAATTTATTTTTTAGT GTTTTAGTGAATTTAATAAATAGAGTGGTACAAAATTC	PP214448
11	*Dirofilaria immitis*	12S ribosomal RNA	95	TGGATTACTCTCTTTCGTGTACATTCTTACGATTTTTTTTGTTTTTTTGTTTTATGTTTTTTTTTGTAAAATATTTAAATTTATTTAT GTTTTTTTGTAATATTGAAAATTTGGTGTTTGAACTGGATTAGTACCCAGGTAATCAAAATTTATTAATTCGGGAGTAAAGTTTTGT TTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTTTTTTGGAGAGCCCTCTTTTATAGTGAATTTTGTT GGCGCATGTATGATTGTTTAGTTTTTACTTTTTTGGTAATGCTTTGTGTTTTATACATTTAAAACAGATATATATTTGGCTTATGGA TTTATTTTTCATGTGTTACTATTGTTAATTTTCTTTGGATTAATTTTTAATTTTTTTTGTTGAAATTGGAAAAGAAAGTAATTTTTTC TTAATGTAATAATGAATTTAATAAATAAAGTGGTACAAATCCCACCCTAAAAG	PP158631:32
12	*Acanthocheilonema reconditum*	12S ribosomal RNA	80	CTCCATATMCTGCCAGCACATSAYTAYATMTGTSTGTTYTAYTTATTCTAYYKTATWTATGTAATATTTTAATTTTTTATTTTTAAT TGAATAATGTTTAAATTTGTTTTGTGACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAGTTGTATTTAAACCG AAAATATTGACTGACTTTAGATTTTTCTTTGGATATGTGTTAGGAGAGCCCTCCTTATTTGTTAATTTTTTTTGGCACATGTATGATG TTTTGTTATTATGTTATTTGTAATGCTTTAACTTTTTTTGTTTAAACAGATATATATTTGGCTTATAGATTTTTTTGCTGTATTACTA TWGWTAATTTTCTTTGGTATTTTTTTATTTTTTTGAAATRSAAARAAGWATTYATTTTTAGTGTTTTAGWGAATTAKAATMSMSC GCCCCACCMTCCGTCATA	PP214449
13	*Acanthocheilonema reconditum*	12S ribosomal RNA	93	AGGCAGCTCTCCTTCTTACAAGCGATAATTTTAGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTTT AATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTAT TTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTG GCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTTGGCTTATAGA TTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTTGAAATTGGAAAAGAAAGTAATTTATTTTT TAGTGTTTTAGTGAATTTAATAAATAGAGTGGTACAAACCCCCCCCTCAAAAAA	PP214450

(Continued)

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TABLE 1 (Continued)

Sample ID	Species filaria	Gene	Query coverage (%)	Sequence	GenBank number
14	*Acanthocheilonema reconditum*	12S ribosomal RNA	92	AAACGTACTATCATTCGCTGCAATATTTATTTTTAGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTA TTTTTAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAG TTGTATTTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATT TTTTTTTGGCCCATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAAATATATATTT GGCTTATAAATTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTGAAATTGAAAAAAAAA GTAATTTATTTTTTAGGGTTTTAGGGAATTTAATAAATAAAGGGGTCCAAACCCCCCCCCCAATAA	PP214451
15	*Acanthocheilonema reconditum*	12S ribosomal RNA	91	GGGAGACGATATTTAGTTTCTACGTTATTTTTGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTTT AATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTAT TTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTT TGGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTTGGCTTA TAGATTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTGAAATTGGAAAAAAAAGTAATTT ATTTTTTAGGGTTTTAGTGAATTTAATAAATAGAGGGGTACAAACTCCCCCCCCCAATATTAA	PP214452
16	*Acanthocheilonema reconditum*	12S ribosomal RNA	100	AGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATG TGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTGGCACATGTATGATTGTTTTGTTATTATGTTA	PP214453
18	*Acanthocheilonema reconditum*	12S ribosomal RNA	89	TKKTCMTATWTTWATWWTATWTWGTAAATATTTTAATTTTTTATTTTTATTGRATAAATGTTTAAAATTTGTTTTGTGAACTGG ATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTGTATTTAAACCGAAAAATATTGACTGACTTTAGATTTTTC TTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTAATTTTTTTTGGCACATGTATGATGTTTTGTATTATGTTA	PP214454
19	*Acanthocheilonema reconditum*	12S ribosomal RNA	100	ATATTTTAATTTTTTATTTTTAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTAT TAATTCGGGAGTAAAGTTGTATTTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTC CTTATTTGTTTAATTTTTTTTTGGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAAC AGATATATATTTGGCTTATAGATTTTTTTTTGCATGTATTACTATTGKTAA	PP214456
26	*Acanthocheilonema reconditum*	12S ribosomal RNA	99	AATATTTTAATTTTTTATTTTTAATYGAATAAAAGKTTTAAAATTTGTTTTGKGAACTGGGATTAGTACCCCRGTAATCAAAGTTTW TTAATTCGGGAGTAAAGTTGTATTTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGRATATGTGTTAGGAGAGCCCYC CTTAWTTGTTTAATTTTTTTTTGGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAAC AGATATATATTTGGCTTATAGATTTTTTTTTGCATGTATTACTATTGTTATT	PP214457
27	*Acanthocheilonema reconditum*	12S ribosomal RNA	94	GCCATGACTATCCTTCCATACTTGTTTATTTTTAGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTT TAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTAT TTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTTG GCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTTGGCTTATAGA TTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTTATTTTTTTTTTGAAATTGGAAAAAAAAGTAATTTATTTT TTAGTGTTTTAGTGAATTTAATAAATAGAGTGGTACAAACCCCCCCCGTCAATAA	PP214458

(Continued)

TABLE 1 (Continued)

Sample ID	Species filaria	Gene	Query coverage (%)	Sequence	GenBank number
28	*Acanthocheilonema reconditum*	12S ribosomal RNA	100	TTATTTTTGTGTTTTTTATTTTTTATTTATTTTWGKAAAATATTTTAATTTTTTATTTTTAATTGAATAAATGTTTAAAATTTGT TTTGTGAACTGGATTAGKACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTTAAACCGAAAAAATATTGACTGA CTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTGGCACATGTATGATTGTTTTGTT ATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTTGGCTTATAGATTTTTTTTGCATGTATTWCT ATTGTTAATTTTCTTTG	PP214459
30	*Acanthocheilonema reconditum*	12S ribosomal RNA	91	GGCAGGACKGAAACCTAAGAYARTTACTTTCTTTTCCAATTTCAAAAAAAAATAAAAAAAATATCCAAAGAAAATTAACAATAG TAATACATGCAAAAAAAATCTATAAGCCAAATATATATCTGTTTTAAAACAAAAAAAGTTTAAAGCATTACAAATAACATAATA ACAAAACAATCATACATGTGCCAAAAAAAATTAAACAAATAAGGAGGGCTCTCCTAACACATATTCCAAAGAAAAATCTAAAGT CAGTCAATATTTTTTCGGTTTAAATACAACTTTACTCCCGAATTAATAAACTTTGATTACCTGGGTACWAATCCAGTTCACAAAA CAAATTTTAAACTTTATTCAATTAAAATAAAAAATTAAAATATTTTACWAAATAAAATAAAAAATAAAAACACAAAAWTAAATTT ACAACAWTTAGAGTTAAAAATTAAAATGGTTGCCGAYCTTCYGAACA	PP214459
33	*Acanthocheilonema reconditum*	12S ribosomal RNA	92	AGTCAACTCAATTGTTGTATATTTATTTTTGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTTT AATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTG TATTTAAACCGAAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTT TTTTTGGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTT GGCTTATAGATTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTGAAATTGGAAAAAA AAGTAATTTATTTTTTAGTGTTTTAGTGAATTTAATAAATAGAGTGGTACAAACACCCCCTCATAAGG	PP214460
39	*Acanthocheilonema reconditum*	12S ribosomal RNA	92	ACCGGTACTCTATTGTTATATTATTTAATTTTGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATT TTTAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGT TGTATTTAAACCGAAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATT TTTTTTGGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTT GGCTTATAGATTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTGAAATTGGAAAAAAAA GTAATTTATTTTTTAGTGTTTTAGTGAATTTAATAAATAGAGTGGTACAAACACCCCCTCTA	PP214461
41	*Acanthocheilonema reconditum*	12S ribosomal RNA	92	AAAAAACTCTACTTGTTATATTCGTTATTTTTAGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTT TAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTAT TTAAACCGAAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTT GGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAAGGCTTTAAACTTTTTTTGTTTTAAAACAAATATATATTTGGCTTATA AATTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTGAAATTGAAAAAAAAAGTAATTT ATTTTTTAGGGTTTTAGGGAATTTAATAAATAAAGGGGTAAAAACCCCCCCCCAATAAAAA	PP214462
42	*Acanthocheilonema reconditum*	12S ribosomal RNA	100	TTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTTTAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTG GATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTTAAACCGAAAAAAATATTGACTGACTTTAGATTT TTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTTGGCACATGTATGATTGTTTTGTTATTATGTTA TTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAAATATATATTTGGCTTATAAATTTTTTTTGCATGTATTACTATTGTTAA TTTTCTTTGGATATTTTTTTTATTTTTTTTTGAAATTGAAAAAAAAAGTAATTTATTTTTTAGGGTTTTAGGGAATTTAA	PP214463

(Continued)

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TABLE 1 (Continued)

Sample ID	Species filaria	Gene	Query coverage (%)	Sequence	GenBank number
45	*Acanthocheilonema reconditum*	12S ribosomal RNA	100	TTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTTTAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTTAACTTTTTTTGTTTTAAAACAAATATATATTTGGCTTATAAATTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTTATTTTTTTTTTGAAATTGAAAAAAAAA	PP214464
49	*Acanthocheilonema reconditum*	12S ribosomal RNA	92	AACCTCTTCTATTGTTGTATATTTATTTTTGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTTTAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTGGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTTGGCTTATAGATTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTTGAAATTGGAAAAAAAAGTAATTTATTTTTTAGTGTTTTAGTGAATTTAATAAATAAAGTGGTACAAACCCCCCCCCCCAAAAAAA	PP214465
53	*Acanthocheilonema reconditum*	12S ribosomal RNA	92	TTTGGACTCTATTGTTGTAATATTTATTTTTGTGTTTTTTATTTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTTTAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTGGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTTGGCTTATAGATTTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTTGAAATTGGAAAAAAAAGTAATTTATTTTTTAGTGTTTTAGTGAATTTAATAAATAAAGGGGTACAAACACCCCCCCTCAAAAAAA	PP214466
55	*Acanthocheilonema reconditum*	12S ribosomal RNA	92	CCCTACGTCTCCTTGTTATAATATATTTTTGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTTTAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTGGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTTGGCTTATAGATTTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTTGAAATTGGAAAAGAAAGTAATTTATTTTTTAGTGTTTTAGTGAATTTAATAAATAGAGTGGTACAAACACCCCCTCATAAGG	PP214467
67	*Acanthocheilonema reconditum*	12S ribosomal RNA	90	ATCCTCTTGGTTCCGTCAGCCGTCGAGCTTCGTGTAGTACATTATTTTTACTTTATTTGTGCAAAATATTTTACTTTTTAACCTTCAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTTTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTTGGCCCCTGTATGAATGGTTTGTTATTATGTTATTTGGAATGCTTTAAACTTTTTTTGGTTTAAAACAAAAAAATATTTGGCTTAAAAATTTTTTTTGCCTGGATTACTATTGGTAATTTTCTTTGGAAATTTTTTTTTATTTTTTTTTTGAAATTGGAAAAAAAAGTAATTTATTTTTTTAGGGGTTTAAGGAATTTAATAAATAGAGGGGGACAAACCCACCCCTCAAATAA	PP214468

(Continued)

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TABLE 1 (Continued)

Sample ID	Species filaria	Gene	Query coverage (%)	Sequence	GenBank number
74	*Acanthocheilonema reconditum*	12S ribosomal RNA	92	CGTTCGTTGCGCTGTAGCCGTATATCTTCTCTATAGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTA TTTTTAATTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAG TTGTATTTAAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATT TTTTTTGGCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTTG GCTTATAGATTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTTATTTTTTTTTTGAAATTGGAAAAGAAAG TAATTTATTTTTTAGTGTTTTAGTGAATTTAATAAATAGAGTGGTACAAACCTCCCCGTCAAATA	PP214469
78	*Dirofilaria immitis*	12S ribosomal RNA	95	ATACACTCATTTGTTGTATATTACGATTTTTTTTGTTTTTTTGTTTTATGTTTTTTTTTGTAAAATATTTAAATTTATTTATGTTTT TTTGTAATATTGAAAATTTGGTGTTTGAACTGGATTAGTACCCAGGTAATCAAAATTTATTAATTCGGGAGTAAAGTTTTGTTTAAA CCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTTTTTTGGAGAGCCCTCTTTTATAGTGAATTTTGTTGGCGC ATGTATGATTGTTTAGTTTTTACTTTTTTGGTAATGCTTTGTGTTTTATACATTTAAAACAGATATATATTTGGCTTATGGATTTAT TTTTCATGTGTTACTATTGTTAATTTTCTTTGGATTAATTTTTAATTTTTTTGTTGAAATTGGAAAAGAAAGTAATTTTTTCTTAAT GTAATAATGAATTTAATAAATAAAGTGGTACAACACTCCTTCATAAGGT	PP214470
83	*Acanthocheilonema reconditum*	12S ribosomal RNA	93	ATCGTCTCTATTGTTGTATATTTATTTTTGTGTTTTTTATTTTTTTATTTTATTTTTGTAAAATATTTTAATTTTTTATTTTTAA TTGAATAAATGTTTAAAATTTGTTTTGTGAACTGGATTAGTACCCAGGTAATCAAAGTTTATTAATTCGGGAGTAAAGTTGTATTT AAACCGAAAAAATATTGACTGACTTTAGATTTTTCTTTGGAATATGTGTTAGGAGAGCCCTCCTTATTTGTTTAATTTTTTTTTG GCACATGTATGATTGTTTTGTTATTATGTTATTTGTAATGCTTTAAACTTTTTTTGTTTTAAAACAGATATATATTTGGCTTATA GATTTTTTTTGCATGTATTACTATTGTTAATTTTCTTTGGATATTTTTTTTATTTTTTTTTTGAAATTGGAAAAAAAAGTAATTTA TTTTTTAGGGTTTTAGTGAATTTAATAAATAAAGGGGTACAAACCCCCCCCCAAATAA	PP214470

oligonucleotides amplifies the approximately 500 bp conserved region (12SF/12SRdeg) of filarials with a simultaneous amplification of the 204 bp *D. immitis* (12SF2B/12SRdeg) and/or 327 bp *D. repens* (12SF/12SR2) specific fragment. The final volume per reaction was 20 µL (1 µL genomic DNA, $MgCl_2$, 1.5 mM, 0.2 mM dNTP, 0.5U Tucan Taq DNA polymerase (Corpogen), and 1 µM of each of the four primers) and the reaction had a thermal profile of 92°C for 1 min. Furthermore, 40 cycles at 92°C for 30 s, at 49°C for 45 s, at 72°C for 1 min, and final elongation step at 72°C for 10 min were performed. The amplification products were run on 2.5% ethidium bromide agarose gel at 95 V for 40 min followed by UV visualization. The specificity of the multiplex PCR assay for the two species was assessed by a control amplification of the DNA extracted from adult *D. immitis* and *D. repens* worms from the worm repository of the Zoonotic Diseases and One Health group of the University of Salamanca.

2.4.3 *Acanthocheilonema reconditum* PCR assay

The PCRs were performed in a SimpliAmp™ (Applied Biosystems™) using a set of primers in the same mixture reaction. We used the general primer pair CxFrec (5′-GTGTTGA GGGACAGCCAGAATT-3′) and CXRrec (5′-GAACGTATATTCT GGATAGTGACCA-3′) previously designed on the COX1region. The sequences of the gene coding for COX1 of *A. reconditum* were obtained from GenBank (accession number MZ540221.1; MW656249.1; MW246127.1; MW138007.1; MT230063.1; MT193075.1; JF461456.1) and aligned using the online version of ClustalW2 (42).

The PCR was performed using an equimolar volume of primers (CxFrec/ CXRrec) in a single-tube reaction. The hybridization of these oligonucleotides amplifies the conserved region of approximately 118 bp (CxFrec/CXRrec). The final volume per reaction was 30 µL (3 µL genomic DNA, $MgCl_2$, 1.5 mM, 0.2 mM dNTP, 0.5U Tucan Taq DNA polymerase (Corpogen), and 1 µM of each of the four primers). The thermal profile of the reaction was: at 95°C for 5 min; 40 cycles at 95°C for 60 s, at 50°C for 60 s, at 72°C for 30 s, and the final elongation step at 72°C for 5 min. The amplification products were run on 2.5% ethidium bromide agarose gel at 95 V for 40 min followed by UV visualization.

2.4.4 Amplicon purification and DNA sequencing

The purification of the amplicon and sequencing was carried out following Gioia et al. (41) with some modifications. In brief, the specificity of the PCR amplification corresponding to *D. immitis* and *A. reconditum* on representative positive blood samples and to *D. immitis* and *D. repens* adult worms was assessed by amplicon purification followed by DNA sequencing. The species-specific amplicons were run on 2.5% ethidium bromide agarose gel followed by UV visualization. The concentration of the purified amplicons was spectrophotometrically measured using a ND-100 Spectrophotometer. The purified amplification products were then sequenced by SANGER sequencing (Macrogen Korea). The obtained sequences were aligned to the expected target sequences using the basic local alignment search tool (BLAST) (42).

The purified amplification products were then sequenced by SANGER sequencing (Macrogen Korea). The obtained sequences were aligned to the expected target sequences using BLAST (42) and should have close to 100% coverage (GenBank accession number KF707482.1, OR852434.1, MZ678927.1, OR854266.1, OR854267.1).

2.5 Statistical analysis

Data were analyzed using the SPCC 20.0 statistical program for Windows (SPSS Inc./IBM, Chicago, IL, United States). A descriptive analysis was carried out employing univariate analysis to determine the frequencies and bivariate analysis using chi-square test, from which a statistical analysis was carried out to determine the association between the variables. In all the cases, the significance level was set at $p < 0.05$.

Cohen's Kappa coefficient, sensitivity and specificity were calculated to evaluate the efficacy of the diagnostic tests for the diagnosis of filariosis. The values obtained were classified as: coefficient < 0 (no agreement), between 0 and 0.19 (slight agreement), between 0.20 and 0.39 (fair agreement), between 0.40 and 0.59 (moderate agreement), 0.60 and 0.79 (substantial agreement), and 0.80 and 1.00 (almost perfect agreement).

3 Results

Of the 400 dog samples tested, 75 had the presence of circulating microfilariae (18.75%) and 1.25% tested positive for the *D. immitis* antigen (5/400). In the 75 microfilaremics, progressive rectilinear movement was observed in 97.3% (73/75) of the samples and non-progressive undulant in 2.66% (2/75) of them. By morphology (head and tail), compatibility with *D. immitis* was seen in 4% of the microfilariae (3/75) that had a sharp cephalic end and a straight and sharp tail (Figure 2). Compatibility with *D. repens* was observed in 20% (15/75) of the samples characterized by a blunt cephalic end and a sharp and filiform tail, often ending in an umbrella hook (Figure 3). Compatibility with *A. reconditum* was noted in 76% (57/75) of the samples that featured a blunt cephalic end with a prominent hook and a flat and curved hooked tail (Figure 4).

Microfilaremic dogs samples were tested with D. immitis antigen test and 5/400 (1,25%) were positive. By PCR, 4/75 (5.3%) were positive for *D. immitis* and 71/75 (94.6%) for *A. reconditum*, with microfilaremic prevalences in relation to the total samples included in the study of 1% for *D. immitis* (4/400) and 17.25% for *A. reconditum* (71/400). Randomly, 27 fragments corresponding to 2 microfilaremic dogs with PCR product positive for *D. immitis* (2/4) and 25 for *A. reconditum* (25/71) were sequenced were sequenced by the Sanger technique (Macrogen, Korea) using primers 12SF and 12Rdeg for filarial generic, identifying the sequences as *D. immitis* with close to 100% query coverage and *A. reconditum* (Table 1). All microfilariae samples were negative for *D. repens*. One *D. immitis* antigen-positive dog showed microfilaremia and PCR-positive to *A. reconditum*. The Kappa index concordance for the results of the comparison of antigen testing for *D. immitis* with Woo test, morphological identification of *D. immitis* (Knott) with PCR *D. immitis* and morphological identification of *A. reconditum* (Knott) with *A. reconditum* PCR for the diagnosis of canine filariosis is shown in Table 2.

All prevalences obtained by sex, age, municipality (Bucaramanga, Floridablanca, Girón and Piedecuesta), race, socioeconomic status and place of residence are shown in Table 3. Significant differences were found between the variables sex ($\chi^2 = 6.57$, df = 1, $p < 0.01$), municipalities ($\chi^2 = 26{,}37$, df = 7, $p < 0.000071$) and use of ectoparasiticides ($\chi^2 = 11.93$, df = 1, $p < 0.002$) for microfilaremic filariosis; the variables sex ($\chi^2 = 6.32$, df = 1, $p < 0.012$), municipalities ($\chi^2 = 19.60$, df = 3, $p < 0.001$) and use of

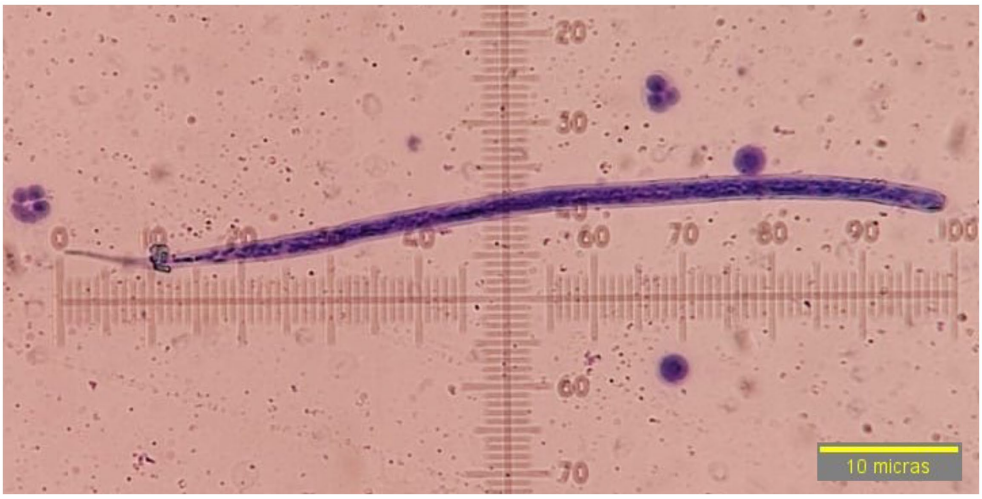

FIGURE 2
Microfilariae of *Dirofilaria immitis*, observed at 40× by modified Knott's technique without sheath, sharp cephalic end, straight and sharp tail.

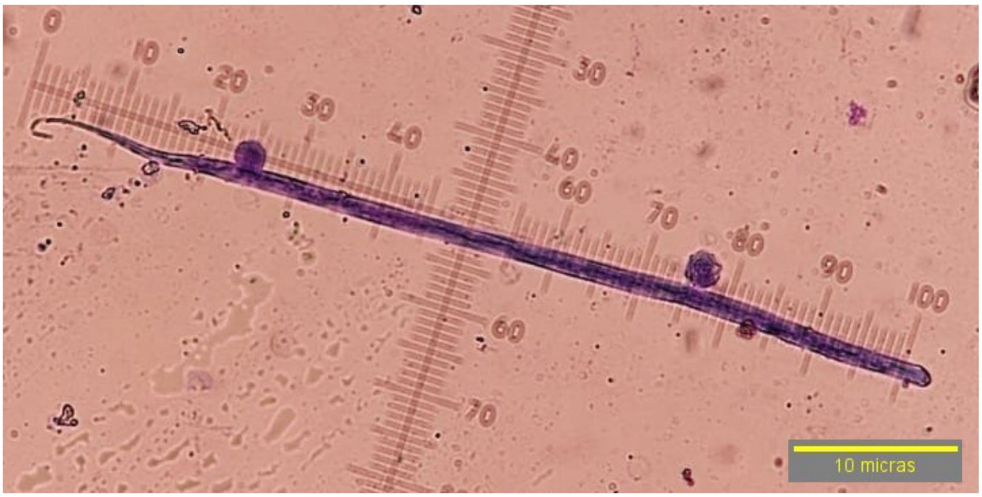

FIGURE 3
Microfilariae suggestive of *Dirofilaria repens*, observed at 40× by modified Knott's technique without sheath, blunt cephalic end, sharp filiform tail, ending in an umbrella handle.

ectoparasiticides (χ^2=8.75, df=1, p<0.003) for *A. reconditum* and for dirofilariosis the only association was observed with use of ectoparasiticides (χ^2=84.80, df=1, p<0.028) (Table 4).

4 Discussion

Filariae exhibit complex life cycles involving different hosts, as they develop through several larval stages. Considering that different specific vectors (mosquitoes, flies, ticks, fleas, and lice) are involved in their transmission to complete their biological cycle, the varying rates of infection, coinfection, and symptomatology, and the different treatments required to treat the infections caused by them, it is necessary to differentiate the various filariae species present in the dog for an accurate diagnosis of canine filariosis (2, 5, 43, 44). In our study, firstly, dogs with circulating microfilariae in the blood were identified by the Woo test, which was used as a screening test (45). Secondly, for the morphological identification of the species to determine the corresponding microfilariae, the Knott technique was used (10, 46–48). And, thirdly, to verify or elucidate the species of filaria that were visualized based on their morphological characteristics and when the result is not congruent and exact, as sometimes happens, due to the similarity between species and the strength of the smear, their molecular identification was carried out by PCR and the product was subsequently sequenced to identify the parasitic species (30, 49, 50).

The Woo and Knott techniques could generate false-negatives because of low parasitemia, interactions of a single sex of parasites, immaturity of the parasites, ectopic location of the adult worms, and

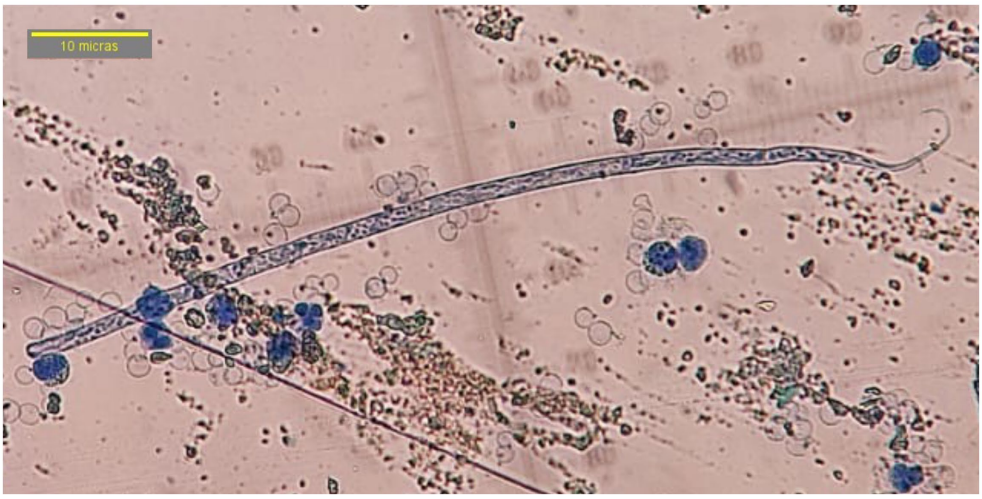

FIGURE 4
Microfilariae of *Acanthocheilonema reconditum*, observed in 40× by modified Knott's technique without sheath, blunt cephalic end with prominent hook, flat and curved hooked tail.

TABLE 2 **Comparative assessment of the performance of the methods used in diagnostics in microfilaremic dogs according to Kappa index, confidence interval, sensitivity and specificity of the tests analyzed.**

Test	Samples n/n total (%)	Kappa index	IC 95%	Sensitivity	Specificity
Ag Test Di × WOO	4/400 (1%)	0.078	0.038–0.118	80	82
Morfology Knott × PCR Di	4/75 (5.3%)	0.000	0.0–0.0	75	100
Morfology Knott × PCR Ar	57/75 (76%)	0.000	0.0–0.0	77	100

Ag, antigen; Ar: *Acanthocheilonema reconditum*; IC: confidence interval; Di: *Dirofilaria immitis*; PCR: Polymerase Chain Reaction.

application of microfilaricides, leading to a decrease in the sensitivity of the tests to identify the presence of filariae (46, 48–55). This is where the identification of antibodies and circulating filarial antigens in blood becomes important (9, 16, 40, 54, 56–58). Commercial tests for the detection of only circulating antigens of *D. immitis* in dogs and cats are available in Colombia, with sensitivities and specificities versus necropsy of over 94 and 100% respectively, allowing identification of dogs without microfilaremia and in which even a single adult worm is present (10, 28, 40). Even with high sensitivities and specificities, certain factors can affect the results, such as the age and sex of the parasite, the species of parasite other than *D. immitis*, not following the manufacturer's instructions, or the use of lactones (8, 40, 49, 59). It is, therefore, necessary to develop and apply highly sensitive molecular methods based on single or multiple reactions for the identification of the genetic material of the different species present in the dogs at the time of veterinary consultation in order to establish an appropriate treatment protocol to treat the infections caused by each species of filaria (11, 40, 51, 60).

In our study, we observed microfilariae to be morphologically compatible with *D. immitis*, *D. repens*, and *A. reconditum*. However, PCR test and the subsequent sequencing of the specific amplified fragment ruled out the presence of *D. repens* and showed at least one dog with morphologically compatible microfilariae and testing positive for *D. immitis* antigen (gold standard for dirofilariosis), in which the sequencing after PCR also confirmed the presence of *A. reconditum*, thus pointing to the presence of a co-infected animal in this area. Similar findings have been reported by other authors (13, 41, 48, 50), recommending the use of PCR in veterinary clinics as a routine diagnostic method and in epidemiological studies wherever possible.

In our study, the integration of the methods used in the study such as WOO microscopy for the detection of microfilariae and species differentiation using PCR allowed the identification of the species for filariae present, which provide reliable data for clinical and epidemiological use.

In Colombia, the prevalence of *D. immitis* in dogs ranges from 0.91 to 53.2%, (22–27). The prevalence is 10.82% in Bucaramanga and the seroprevalence in humans is 6.71% (28). The prevalence varies between 4.81 and 61.3% for *A. reconditum* (13, 30) and the presence of *D. repens* is detected by morphological techniques in dogs that are put in shelters in Bucaramanga (29). In our study, the prevalence of *D. immitis* was 1% and that of *A. reconditum* was 17.75%, with one animal co-infected by both species, decreasing significantly from previously reported prevalences. This decrease could be due to the sampling performed. In the other studies, the samples used were mostly from uncontrolled stray dogs and dogs with owners with a poor socio-economic status, whereas in this study the dog samples came from veterinary clinics where the dogs underwent an annual check-up. The results observed for *A. reconditum* in dogs in this study represents the first report of the detection of this species in northeastern Colombia. In addition, we also found significant

TABLE 3 Filariosis prevalence in dogs in the metropolitan area of Bucaramanga by variables.

	Sample (n)	+ Microfilaremic	Prevalence mf	+ Ag Di test	Prevalence Ag Di	+ PCR Di	Prevalence PCR Di	+ PCR Ar	Prevalence PCR Ar
Sex									
Male	161 (40.3%)	40	24.84%	3	1.86%	2	1.24%	38	26.60%
Female	239 (59.8%)	35	14.64%	2	0.83%	2	0.83%	33	13.80%
Age									
1–2 years	113 (28.2%)	20	17.69%	1	0.88%	1	0.88%	19	16.81%
3–6 years	145 (36.3%)	26	17.93%	3	2.06%	2	1.37%	24	16.55%
>7 años	142 (35.5%)	29	20.42%	1	0.70%	1	0.70%	28	19.71%
Breed									
Mestize	313 (78.3%)	59	18.84%	4	1.27%	3	0.95%	56	17.89%
Pure	87 (21.8%)	16	18.39	1	1.14%	1	1.14%	15	17.24%
Municipalities									
Bucaramanga	145 (36.3%)	22	15.17%	2	1.37%	1	0.68%	21	14.48%
Floridablanca	74 (18.5%)	9	12.16%	1	1.37%	1	1.35%	8	10.18%
Girón	86 (21.5%)	31	36.04%	2	2.35%	2	2.35%	29	33.72%
Piedecuesta	95 (23.8%)	13	13.68%	0	0%	0	0%	13	13.68%
Residential zone									
Urban	206 (51.5%)	44	21.35%	4	1.94%	3	1.12%	41	19.90%
Rural	194 (48.5%)	31	15.97%	1	0.52%	1	0.51%	30	15.40%
Socioeconomic level									
Stratum 1	194 (48.5%)	33	17.01%	1	0.52%	1	0.51%	32	16.40%
Stratum 2	16 (4%)	2	12.50%	1	6.25%	1	6.25%	1	6.25%
Stratum 3	120 (30%)	28	23.33%	2	1.67%	2	1.66%	26	21.60%
Stratum 4	66 (16.5%)	12	18.18%	1	1.52%	0	0%	12	18.18%
Stratum 5	2 (0.5%)	0	0%	0	0%	0	0%	0	0%
Stratum 6	2 (0.5%)	0	0%	0	0%	0	0%	0	0%
Place of permanence									
Indoors	315 (78.8%)	62	19.68%	5	1.58%	4	1.26%	58	18.41%
Outdoors	85 (21.3%)	13	15.29%	0	0%	0	0%	13	15.29%
Type of dwelling									
House	363 (90.8%)	65	17.90%	4	1.10%	3	0.82%	62	17.07%
Apartment	37 (9.3%)	10	27.02%	1	2.70%	1	2.70%	9	24.32%
Living with other animals									
Dogs	185 (46.3%)	26	14.05%	1	0.54%	0	0%	26	14.04%
Cats	35 (8.8%)	9	25.17%	1	2.85%	1	2.85%	8	22.85%
Various species	180 (45%)	40	22.22%	3	1.66%	3	1.66%	37	20.50%
Ectoparasiticides									
Yes	293 (73.25%)	43	14.67	2	0.68%	1	0.34%	42	14.33%
No	107 (26.75%)	32	29.90%	3	2.83%	3	2.80%	29	27.10%
Which?									
Benzoylureas	1 (0.3%)	0	0%	0	0%	0	0%	0	0%
Fenilprazoles	7 (1.8%)	0	0%	0	0%	0	0%	0	0%
Isoxazolinas	135 (33.8%)	23	17.03%	1	0.74%	1	0.74%	22	16.26%
Various	150 (37.5%)	20	13.33%	1	0.66%	3	0.02	29	19.33%
None	107 (26.8%)	32	29.90%	3	2.80%	0	0%	20	19.69%
Skin problems such as alopecia, nodules and scoriation.									
Yes	100 (25%)	17	17%	0	0%	0	0%	17	17%
No	300 (75%)	58	19.33%	5	1.66%	4	1.33%	54	18%
Total	400	75	18.75%	5	1.25%	4	1.0%	71	17.75%

Ag: antigen; Ar: *Acanthocheilonema reconditum*; Di: *Dirofilaria immitis*; PCR: Polymerase Chain Reaction.

10.3389/fvets.2024.1368307

TABLE 4 Analysis of association of variables in dogs exposure to microfilaremic filariosis: *D. immitis* and *A. reconditum*.

Variables	microfilaremic filariosis			Exposure to *D. immitis*			*A. reconditum*		
	χ^2	df	$p<0.05$	χ^2	df	$p<0.05$	χ^2	df	$p<0.05$
Sex	6.57	1	0.01*	0.16	1	0.689	6.32	1	0.012*
Age	0.40	2	0.816	0.35	2	0.83	0.58	2	0.746
Municipalities	26.37	7	0.000071*	2.71	3	0.437	19.60	3	0.001*
Socioeconomic level	3.38	5	0.64	6.16	5	0.291	3.79	5	0.580
Residential zone	1.89	1	0.168	0.89	1	0.345	1.34	1	0.246
Type of dwelling	1.83	1	0.176	1.19	1	0.275	1.20	1	0.272
Place of residence	0.84	1	0.358	1.09	1	0.296	0.44	1	0.504
Ectoparasiticides	11.93	1	0.002*	4.80	1	0.028*	8.57	1	0.003*
Skin problems	0.26	1	0.605	1.34	1	0.24	0.05	1	0.821
Living with other animals	5.21	2	0.074	3.89	2	0.143	3.32	2	0.190

*$p<0.05$.

differences by socioeconomic level, which may be because of a non-uniform number of samples at all levels and variation in the administration of ectoparasiticides. A higher prevalence rate of parasitic infection was reported in animals in which ectoparasiticides were not administered, as other authors have reported (22, 27, 28, 46, 56, 57).

5 Conclusion

The use of highly sensitive diagnostic methods allows the identification of filarial species, leading to their classification and establishment of the appropriate treatment protocol to treat infections caused by each filarial species. Employing these methods also leads to generation of reliable data to be used at the clinical and epidemiological levels. The search for innovative diagnostic methodologies is fundamental to the development of veterinary care. In our study, we found, for the first time, the presence of a co-infected animal that tested positive for *D. immitis* antigen as well as for *A. reconditum* in the PCR microfilaremia in northeastern Colombia. It is therefore necessary to carry out a differential diagnosis of filariosis in dogs in this region and other nearby areas to improve the diagnosis and avoid clinical errors after treatment. Furthermore, taking into account that they are zoonotic diseases and that humans can be affected with a variety of symptoms and also become asymptomatic (silent infections), it is necessary to conduct epidemiological studies widely and improve the diagnosis of filariosis in order to control the disease more efficiently.

Data availability statement

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

Ethics statement

The animal studies were approved by the Ethics Committee of the Cooperative University of Colombia (No. 006-2020). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners of the animals for the participation of animals in this study.

Author contributions

MVE-M: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Investigation, Resources, Validation. VHA-Q: Conceptualization, Funding acquisition, Methodology, Writing – review & editing, Writing – original draft, Resources, Validation. CRC: Methodology, Writing – review & editing, Data curation. JEJD: Data curation, Methodology, Writing – review & editing. MTO: Data curation, Methodology, Writing – review & editing. ADB: Data curation, Methodology, Writing – review & editing. MFC: Data curation, Methodology, Writing – review & editing. RM: Conceptualization, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Prediction and validation of potential transmission risk of *Dirofilaria* spp. infection in Serbia and its projection to 2080

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Animal and human dirofilariosis is a vector-borne zoonotic disease, being one of the most important diseases in Europe. In Serbia, there are extensive studies reporting the presence of *Dirofilaria immitis* and *D. repens*, mainly in the north of the country, where the human population is concentrated and where there is a presence of culicid mosquitoes that transmit the disease. Ecological niche modeling (ENM) has proven to be a very good tool to predict the appearance of parasitosis in very diverse areas, with distant orography and climatologies at a local, continental, and global level. Taking these factors into account, the objective of this study was to develop an environmental model for Serbia that reflects the suitability of the ecological niche for the risk of infection with *Dirofilaria* spp. with which the predictive power of existing studies is improved. A wide set of variables related to the transmission of the parasite were used. The potential number of generations of *D. immitis* and the ecological niche modeling method (ENM) were used to estimate the potential distribution of suitable habitats for *Culex pipiens*. The highest probability of infection risk was located in the north of the country, and the lowest in the southern regions, where there is more orographic relief and less human activity. The model was corroborated with the location of *D. immitis*-infected dogs, with 89.28% of the country having a high probability of infection. In addition, it was observed that the percentage of territory with optimal habitat for *Culex* spp. will increase significantly between now and 2080. This new model can be used as a tool in the control and prevention of heartworm disease in Serbia, due to its high predictive power, and will serve to alert veterinary and health personnel of the presence of the disease in the animal and human population, respectively.

KEYWORDS

***Dirofilaria* spp., infection risk, ecological niche modeling, *Culex pipiens*, projection, Serbia, Europe**

1 Introduction

Vector-borne diseases have a significant negative impact on both animals and humans worldwide (1). One of the most important factors to consider is anthropogenic global warming, which has led to changes in the composition of terrestrial and coastal ecosystems, one of the main causes being the increase in temperature and the consequent spread of new vector species to previously vector-free areas (2–4). In the case of Europe, moreover, the increase in the intensity of human activity, as well as new agricultural methods and the expansion of irrigated cultivation, has led to a substantial increase in countries close to traditional endemic countries such as Portugal, Spain, France, Italy, Greece, and Turkey (5–7).

Dirofilariosis is a worldwide vector-borne zoonotic disease and one of the most important animal diseases in Europe. *Dirofilaria immitis* and *D. repens* are the most important causative agents of the disease in its definitive hosts, which are domestic and wild canids and felids. The domestic dog is the main reservoir or the one for which most data are known, and its vectors belong to the genera *Culex* spp. and *Aedes* spp. and are widely represented throughout the European continent (7–12). Humans act as accidental hosts, coming into contact with the parasite more frequently in places where microfilaremic reservoirs exist, which can lead to human dirofilariosis (10).

In Europe, changes in its distribution pattern have been documented, with most countries being endemic with a broad change in the last 20 years (7, 10, 13). The distribution of the disease is favored by the presence of vectors, as well as with the presence of fresh water, high humidity, and average temperatures. When the environmental temperature increases, the period in which the larvae mutate inside the vector is shortened (14, 15).

In Serbia there are several studies that report the presence of cardiopulmonary dirofilariosis in dogs, being 3.17–16.1% in the north, in the capital (Belgrade) 22.01%, even with coinfections with *D. repens* in 3.97% of the dogs, and in Kosovo 9% (16–21). In recent years, prevalences in dogs have increased in the north of the country, with ranges between 12.7 and 33.3%, together with the presence of some microfilaremic dogs and in the south (Kosovo) with prevalences due to *D. immitis* of 14.8% (20, 22–25). In addition, studies of the presence of *D. immitis* in wild animals such as gray wolf and red fox, golden jackals, and wolves have been reported with prevalences between 1.55–7.32 and 7.79% in wild cats (26–28) and for the first time, the presence of *Dirofilaria* spp. in three species of culicid mosquitoes: *Cq. richiardii*, *Cx. pipens*, and *Och. caspius* (29).

Ecological niche modeling (ENM) has proven to be a very good tool in predicting the occurrence of parasitosis in very diverse area, with distant orographies and climatologies at local, continental and global levels (30–38). These models are based on the processing of robust environmental and bioclimatic variables, as well as others directly related to vector, and thus assess the probability of transmission of vector diseases (5, 39–43). One of the most important models for this situation and one of the most widely used is the maximum entropy algorithm (Max-Ent), which uses presence data and produces robust and very accurate statistical models (42, 44–46).

In Serbia there are no specific investigations that have allowed predicting the risk of *Dirofilaria* spp. infection, but there are studies (5, 47) for the European continent that incorporate cartographic information in their spatial analysis with GIS temperature records. However, there are no studies for Serbia that take into account orography, climate, environment, human activities or population centers, among others. Considering that with ENMs it is possible to relate the presence of a zoonosis to biotic variables, extrapolate it to other areas without vector presence data and know its dynamics over time at high resolution, as well as take preventive control measures to avoid the expansion or eradication of a zoonosis, the arm of this study was to develop an environmental model for Serbia that reflects the suitability of the ecological niche for the risk of infection by *Dirofilaria* spp., taking into account, in addition to the average annual temperature, other bioclimatic and environmental variables, and the number of generations of *Dirofilaria* spp. that can be developed in the vector, as a novel contribution that improves the predictive models carried out at the European level, improving their resolution and significance.

2 Methods

2.1 Description of the study area

Serbia (44°0′59.5″ N 21°0.352′ E) is a country in southeastern Europe located on the landlocked Balkan Peninsula, bordered by Hungary to the north, Romania and Bulgaria to the east, North Macedonia and Albania to the south, and Bosnia and Herzegovina, Croatia, and Montenegro to the west. The province of Vojvodina, in the northern third of the country, is part of the Central European Pannonian Plain. The rest of the country is mountainous, with the Dinaric Alps in the center, west, and southeast. The easternmost part of the country is the Wallachian Plain, while the western border is determined by the Carpathian Mountains. The Southern Carpathians meet the Balkan Mountains in the southeast of the country, following the course of the Great Morava River. Most of Serbia's territory (92%) belongs to the Danube River basin, which dominates the north of the country. Besides the Danube, the main rivers are its tributaries the Sava (coming from the west), the Tisza (coming from the north), the Drina (coming from the south) and the Morava, the latter flowing almost entirely through Serbia in the mountainous southern regions. Due to the geography of the terrain, natural lakes are few and far between, but there are numerous bodies of water of artificial origin. The country's climate is continental, alternating between a Mediterranean climate influenced by the Adriatic Sea in the south with warm, dry summers and autumns, and relatively cold winters with heavy snowfall in the interior; and in the north there is a continental climate with cold winters and warm, humid summers (48) (Figure 1).

2.2 *Culex pipiens* habitat suitability modeling and *Dirofilaria* spp. generations

Culex pipiens georeferenced points from Serbia were used from data previously obtained by Kurucz et al. (29), Kemenesi et al. (49) and Južnič-Zonta et al. (50). This mosquito species was selected for modeling as it is one of the most abundant species in Europe and has been reported as a vector of dirofilariosis in Serbia (7) and processed at a spatial resolution of 1 km^2.

Environmental and bioclimatic variables were obtained in the same way as Rodríguez-Escolar et al. (42). In fact, 19 bioclimatic

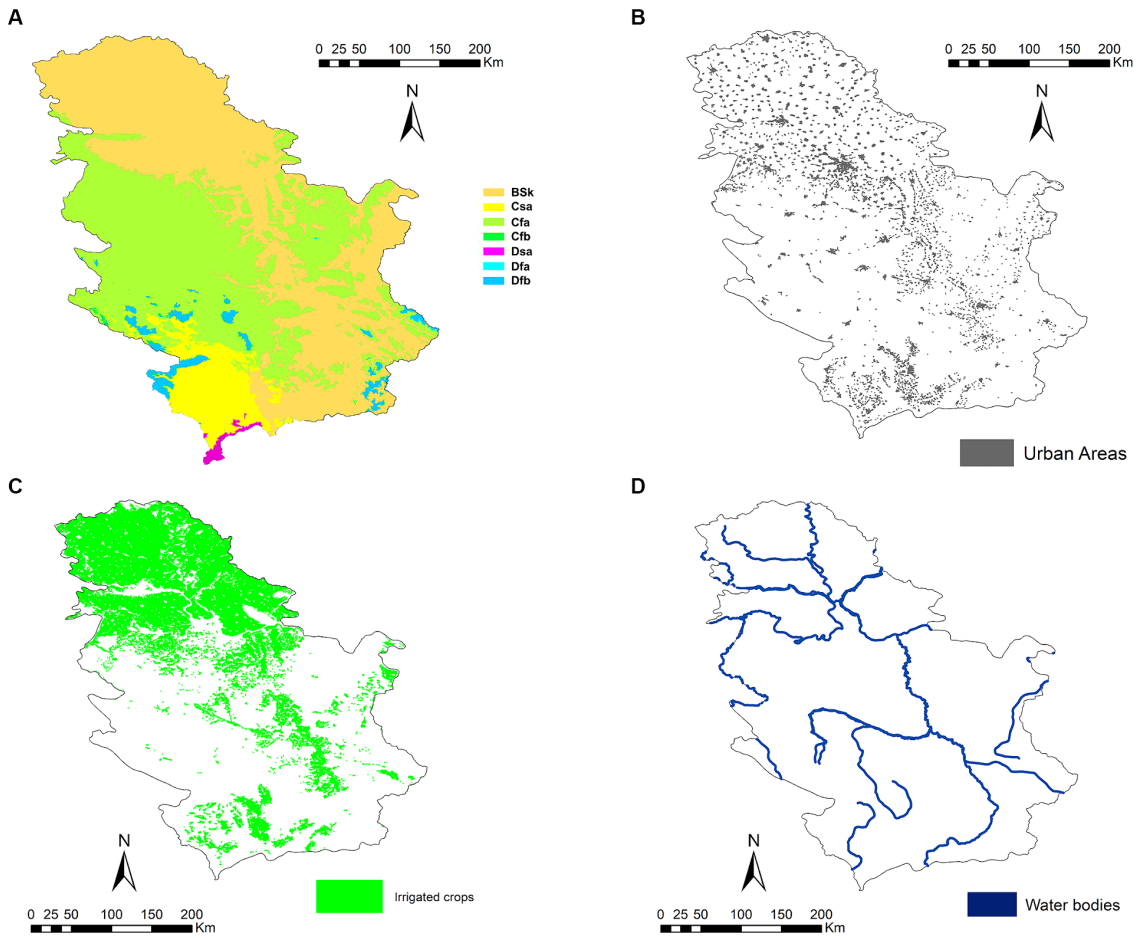

FIGURE 1
(A) Climates according to the Köppen Climate Classification System (BSh: hot semi-arid climate; BSk: cold semi-arid climate; Csa: hot-summer Mediterranean climate; Csb: warm-summer Mediterranean climate; Cfa: humid subtropical climate; Cfb: temperate oceanic climate; Dsb: humid continental climate; Dsc: subarctic climate; Dfa: hot-summer humid continental climate; Dfb: humid continental climate; Dfc: subarctic climate; and ET: Tundra), **(B)** human populations, **(C)** irrigated crops, and **(D)** water bodies in Serbia.

variables were downloaded from the World Clim website (51, 52) at a spatial resolution of 1 km^2 for the years between 1970 and 2000 (current data), plus projected data for 2040, 2060, and 2080 (53). All variables were related to temperature and precipitation. Of the 19 bioclimatic variables, seven were selected taking into account a multicollinearity test performed in R based on Pearson's correlation coefficient, in the same way as. In this study, variables with a cross-correlation coefficient $r > \pm 0.75$ were discarded and, according to vector biology, the following variables were selected: mean annual temperature (°C) (BIO_1), isothermality (BIO_3), seasonality of temperature (DE × 100) (BIO_4), mean temperature of the wettest quarter (°C) (BIO_8), mean temperature of the driest quarter (°C) (BIO_9), annual precipitation (mm) (BIO_{12}), *y* and seasonality of precipitation (coefficient of variation) (BIO_{15}). In addition, five environmental variables (human footprint: built environment, population density, electric power infrastructure, cropland, grazing land, roads, railways and waterways (53), the presence of irrigated crop areas, the location of rivers and water bodies (54), and the density of shrubs and herbaceous plants (55) due to their effect on vector distribution) were selected.

To model the habitat suitability and geographic distribution of *Cx. pipiens* in the study area, the methodology of Morchón et al. (43) were used. In fact, we used the Maxent program (56) to calculate the habitat suitability of a species across environmental constraints (57). With the Kuenm package in R (58), the 119 best models generated in Maxent were chosen by combining a set of variables, 17 values of the regularization multiplier (0.1–1.0 at intervals of 0.1, 2–6 at intervals of 1, and 8 and 10), and the seven possible combinations of three feature classes (linear, quadratic, and product). The model performance was assessed in terms of statistical significance (Partial_ROC < 0.05), omission rates (OR = 5%), and model complexity using the Akaike information criterion corrected for small sample sizes (AICc). Significant models with an omission rate ≤ 5% were selected. Then, from this set of models, those with an AICc delta value of ≤2 were selected as the final candidate models. The candidate models were built using the "kuenm_cal" function, and the evaluation and selection of the best model were carried out using the "kuenm_ceval" function. Finally, the final ENM (best-fit model) was generated using the variables and the same parameters as previously selected. Ten bootstrap replications with logistic outputs were performed. The

evaluation of these final models was based on the ROC_partial, OR, and AICc calculations using an independent dataset. The creation of the final models was carried out by using the "Kuenm_mod" function.

The number of annual *Dirofilaria* spp. generations was calculated using the model described by Genchi et al. (5, 39, 47), Rodríguez-Escolar et al. (42), and Morchón et al. (43) and in the R-software (v.4.3.0) with daily average temperature data between 1990 and 2016 in Serbia (59, 60). With this model, it is possible to quantify the complete development of microfilariae of *Dirofilaria* spp. up to larvae 3 within the culicid vectors (extrinsic incubation) where it is necessary to accumulate 130 growth degree days (GDD), in 30 days, at most, this number being the life expectancy of the culicid mosquito.

2.3 *Dirofilaria* spp. risk map and its validation

To obtain a risk map of *Dirofilaria* spp. in Serbia, we multiplied (weighting approach) the final ENM of *Cx. pipiens* and *Dirofilaria* spp. generations from the raster calculator in ArcMap 10.8. To validate the resulting *Dirofilaria* spp. risk map, points of presence of *D. immitis* and *D. repens* infected dogs were obtained from all over the country (17, 19–22, 24–26, 61–69) and overlaid on the risk map to see in which area they were living.

2.4 Forward projection and rank change analysis

To assess the potential effects of climate change on heartworm transmission risk dynamics, we employ the best performing *Cx. pipiens* model to extrapolate the bioclimatic variables analyzed for three different time periods: the 2040s (2021–2040), the 2060s (2041–2060), and the 2080s (2061–2080). Additionally, three different RCPs 8.5 scenarios were used with the HadGEM3-GC21-LL model (70). This model is one of the most widely used today to simulate the climate response to increasing greenhouse gas concentrations in Europe (71).

Once the estimates were made, it was necessary to determine the percentage of increase or decrease in suitable habitat for *Cx. pipiens* for Serbia. In fact, we convert the NEM and future projections into a binary map of presence and absence using the 10th percentile of the current model as a threshold. With the biomod2 script of the R program, a range shift analysis was performed to determine in which territories the greatest changes in *Cx. pipiens* distribution occur, as result of climate change, for the 2040, 2060, and 2080 scenarios compared to today (72).

3 Results

3.1 Habitat suitability model for *Culex pipiens*

The curve value (AUC) of the *Cx. pipiens* ecological niche model for Serbia was 0.975, indicating very good predictive power. Habitat suitability for *Cx. pipiens* ranged from 0 to 0.93 (Figure 2), with the variables contributing most to the ENM Human footprint and BIO_{15} (Table 1). Of the 13 variables used, those with the highest contribution were the human footprint and BIO_{15} (Precipitation Seasonality) with a percentage contribution of 53 and 32.8%, respectively. The rest of the variables had lower values of 6.6%. Considering the map obtained, the area of highest habitat suitability for *Cx. pipiens* in Serbia is in the northern part of the country, an area that is part of the Pannonian plain with a larger human footprint and less mountainous than the south, where there is generally low suitability.

3.2 Number *Dirofilaria* spp. generations

The highest value (>2.8) of the number of generations of *Dirofilaria* spp. was found in the Pannonian plain area (north of the country), where the number of generations is high due to the lower altitude (Figure 3). In the south, due to a more rugged orography, generations decrease with altitude (down to 0.09) except for the areas close to the main river basins.

3.3 Potential risk of transmission of *Dirofilaria* spp.

The result of the *Dirofilaria* spp. transmission risk map in Serbia is shown in Figure 4. Generally speaking, the highest risk is found in the northern part of the country, decreasing as one moves toward the southern areas, with a more rugged relief and less human presence. In terms of territory, five ranges of values have been established (very high, high, medium, low, and very low), with 6.3 and 17.2% corresponding to very high and high risk areas respectively; 19.3% of the territory has a medium risk, 20.7% a low risk, and 36.5% a very low risk. The places where the risk of transmission is high or very high coincide with areas of low altitude, high human footprint and irrigated crops. In the south, the risk is generally low due to a more mountainous orography, with the exception of the basins of the main rivers as they are at a lower altitude.

To test our transmission risk map and validate it, geo-referenced points of *D. immitis* and *D. repens* infected dogs were superimposed. Of the *Dirofilaria* spp. positive dogs, 89.28% were found in very high-risk areas, 9.57% in high-risk areas, and 1.16% in moderate risk areas. In both low and very low risk areas, the percentage of positive dogs was 0% (Figure 5).

3.4 Future projection for the years 2040, 2060, and 2080 according to the climate change scenario RCP 8.5

The range change analysis shows a remarkable increase in the extent of suitable habitats for *Cx. pipiens* in 2040 and 2060, with the exception of 2080 where the change is very little appreciable (Figure 6). The percentage gain of territory for *Cx. pipiens* was 44.8% for 2040, 104.1% for 2060, and 2.9% for 2080. Notably, in 2080, there is a 65.7% percentage loss of suitable territory for the vector. Increases in areas suitable for the mosquito vector occur toward higher altitude areas in the south.

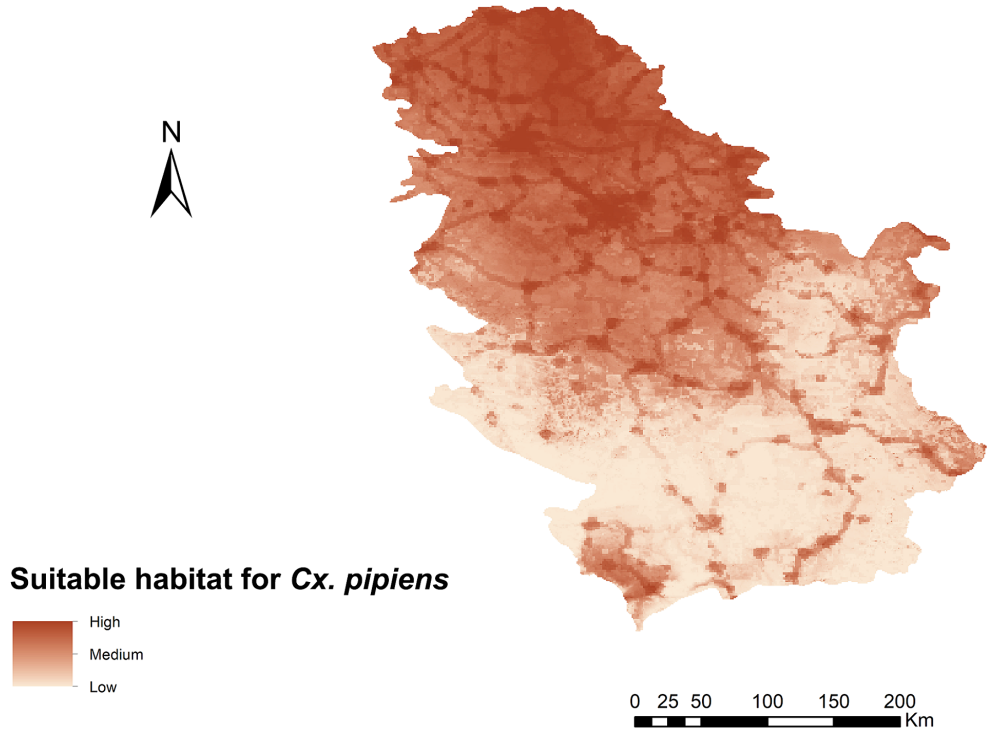

FIGURE 2
Ecological niche model for *Cx. pipens* in the geographical area of Serbia representing suitable habitat.

TABLE 1 Analysis of the contribution of the 13 environmental and bioclimatic variables to the ecological niche model for *Cx. pipens*.

Variable	Percent contribution
Human footprint	53%
BIO_{15} (Precipitation seasonality)	32.8%
BIO_{12} (Annual precipitation)	6.6%
BIO_3 (Isothermality)	4.7%
Rivers	1.4%
Herbaceous density	0.9%
Irrigated crops	0.3%
Water bodies	0.2%
BIO_1 (Annual mean temperature)	0.1%
BIO_4 (Temperature seasonality)	0%
Shrub density	0%
BIO_8 (Mean temperature of wettest quarter)	0%
BIO_9 (Mean temperature of driest quarter)	0%

4 Discussion

Serbia in one of the countries in southeastern Europe where prevalences in infected dogs have continued to increase in recent years with ranges between 12.7 and 33.3%, mainly in the north of the country (20–25, 73) and where, for the first time, *Cq. richiardii*, *Cx. pipens*, and *Och. caspius* have been identified as vector species of the disease (29). This study is the first to map the risk of *Dirofilaria* spp. infection in Serbia using the distribution of the territory suitable for the survival of *Cx. pipiens*, one of the main and most abundant vectors of the disease in Europe (7), as well as including new predictor variables, and which has been validated using the presence of *Dirofilaria* spp. infected dogs as a reference. Within the biased spectrum of predictor variables that have been taken into account to date in most predictive models for Northeastern Europe (annual temperature records) (5, 39, 47, 74–77), in this study, we have incorporated several variables directly linked to the vector's life cycle (humidity, rainfall, areas of naturally and/or artificially stagnant freshwater, rivers, density of herbaceous plants, irrigated agricultural areas, location of human populations, communications, agricultural activities, exchange of goods, and travel), as well as weighting with the number of generations of *Dirofilaria* spp. in the vector, with a robust and highly predictive result.

With the utilization of ecological niche modeling tools, it is possible to create risk models for zoonotic diseases that take into account a variety of abiotic variables regarding the development of a species, these tools predict the most likely habitats for the mosquitoes that carry the disease and have a high degree of resolution, even in areas where surveillance data are lacking (78). In South of Europe, a previous study has been utilized to validate the risk map associated with *Dirofilaria* spp. with the addition of the geolocation of infected animals, obtaining a higher resolution projection (1 km^2) with a high significant and consistent (42, 43).

Genchi et al. (5) produced a map of the potential number of *Dirofilaria* spp. generations, where Serbia was located with average values, similar to those of the rest of central European countries, being higher in the north of the country. In our study, we have observed that the risk of infection by *Dirofilaria* spp. predominates in the north, which corroborates previous data, and centralizes the risk in places

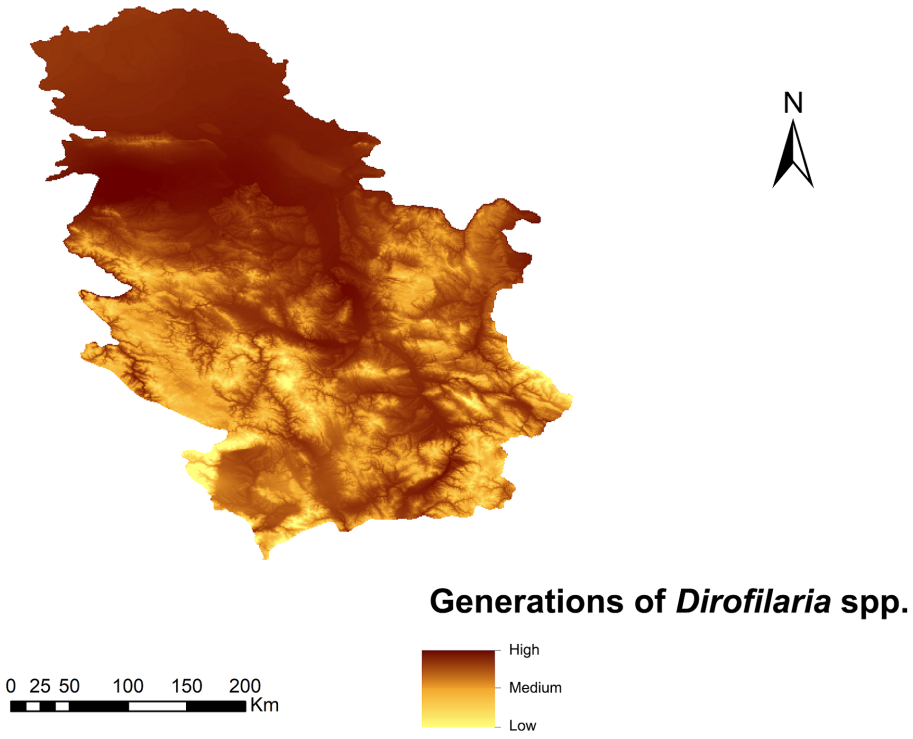

FIGURE 3
Prediction of the number of generations of *Dirofilaria* spp. in Serbia.

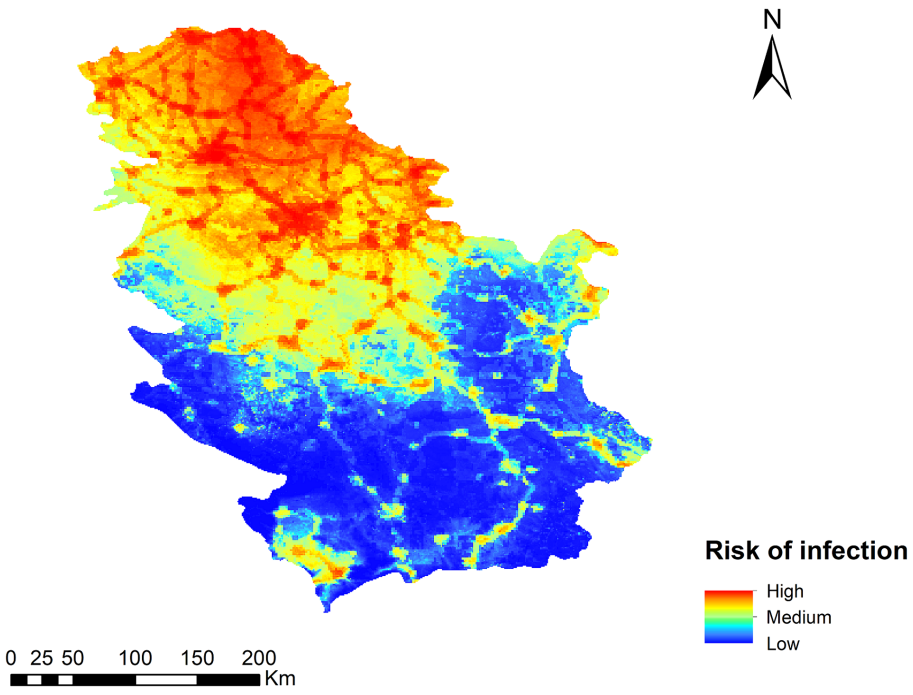

FIGURE 4
Ecological niche model of the risk of *Dirofilaria* spp. infection in Serbia.

where human population, agricultural activity, and average rainfall are concentrated, these being the variables that contributed most to the model, suggesting the presence of *Cx. pipiens* is related to the presence of irrigated areas, a high density of human population and animals infected by *D. immitis* and/or *D. repens* and an increase in humidity. Moreover, if we take into account the wild carnivore population (7, 13,

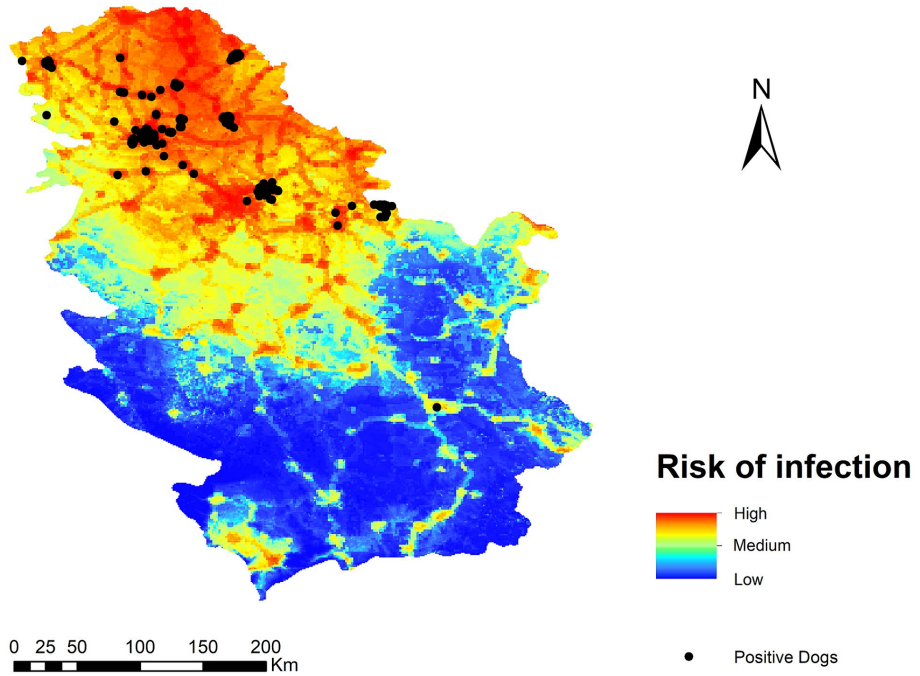

FIGURE 5
Ecological niche model of the risk of *Dirofilaria* spp. infection in Serbia with the locations of infected dogs according to Kurucz et al. (29), Kemenesi et al. (49), and Južnič-Zonta et al. (50).

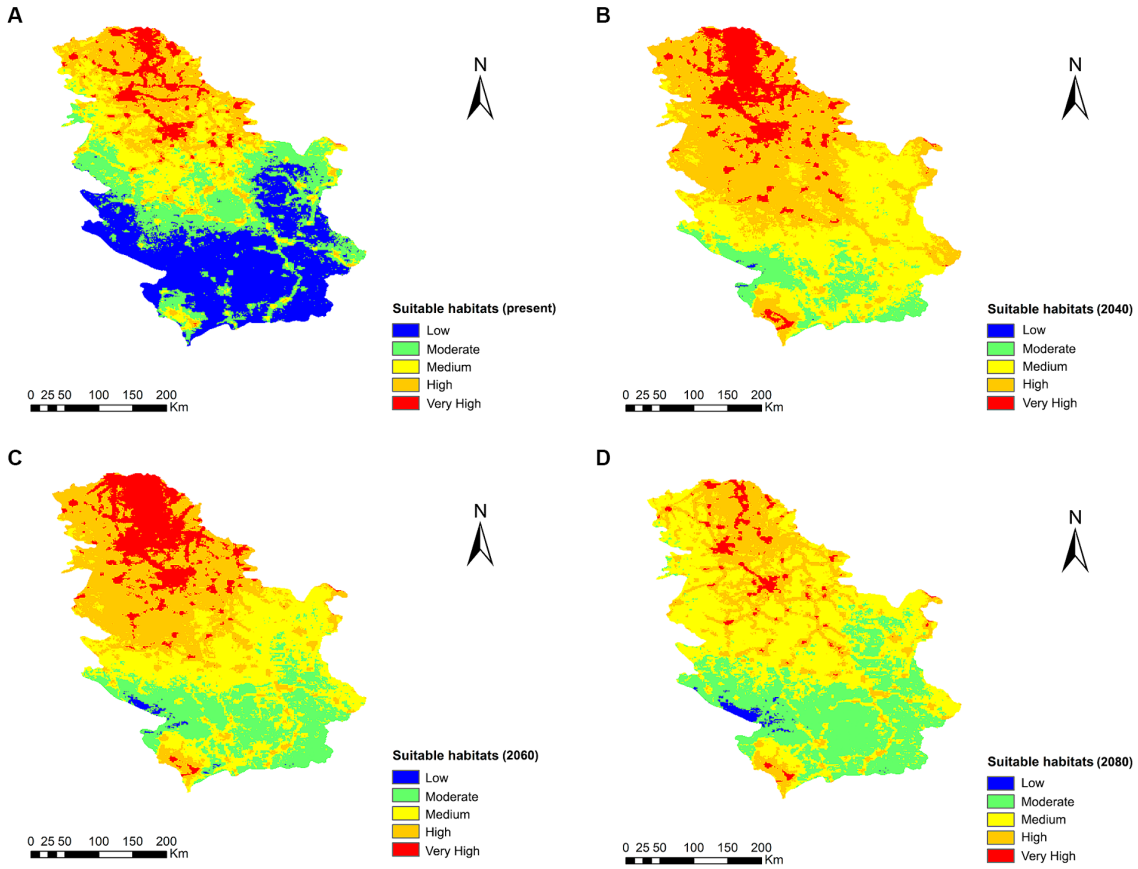

FIGURE 6
Suitable habitats for *Cx. pipens* at present **(A)** and their projections into the future, 2040 **(B)**, 2060 **(C)**, and 2080 **(D)**, in Serbia under the climate change scenario RCP 8.5.

24, 25, 27, 79–82) and others (82), our model increases in reliability as studies of *Dirofilaria* spp. infected animal populations show concentrated positivity, as well as infected domestic dogs, in the north of the country. There are also data from neighboring countries with high rates of *Dirofilaria* spp. infection such as Hungary, Romania, Bulgaria, Croatia, Bosnia, and Herzegoniva (7, 12, 15, 77, 83–90), which may increase the risk of infection.

The results of the 2040, 2060, and 2080 projections under climate change scenario RCP 8.5 revealed a displacement of the current distribution area of *Cx. pipiens* toward new territories, mainly in the south of the country, in where there is a significant potential increase in *Cx. pipiens* habitat, and therefore risk of infection, throughout the country and mainly in the south, with a 104.1% gain of ideal habitat for culicid vectors in 2060, although in 2080, there is a 65.7% percentage loss of suitable vector territory, decreasing in the north but remaining similar in the south. This is in line with other studies where there is an increase in temperatures, which is consolidated in areas with previously colder and in the future temperate climates, due to climate change and the transmission dynamics of certain vector-borne diseases (34, 42, 74, 90), therefore, from the point of view of One Health, measures should be taken by the Serbian government administration to take appropriate control measures and to interrupt the expansion and establishment of the vectors transmitting the disease.

In conclusion, this model will allow both health and veterinary scientists to diagnose the disease in previously unsuspected/clean areas, take more effective control measures, and further investigate the epidemiology of dirofilariosis in animals and humans. Consequently, disease alerts will be increased, considering each population's specific situation. Further studies should be carried out to investigate the infection risk at a local level in order to take the necessary and optimal preventive measures to interrupt the spread of dirofilariosis in southern Europe in the coming years. Similar situations are already occurring in countries bordering Serbia, such as Croatia, Romania, Bulgaria, Hungary, and Greece. Thanks to this type of ecological niche model for *Cx. pipiens* and the prediction of the risk of infection for *Dirofilaria* spp., it will be possible to help health and veterinary personnel to carry out control measures both in areas where the disease is already diagnosed and in others where the health alert is lower. All of this will facilitate the action of veterinarians and doctors and the monitoring of the disease in specific locations in the country.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. All figures are originals created by the authors with Maxent and ArcMap 10.8 software.

Author contributions

IR-E: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. RH-L: Investigation, Software, Supervision, Validation, Writing – review & editing, Methodology. JS-A: Investigation, Software, Supervision, Writing – review & editing, Methodology, Validation. MC-C: Data curation, Formal analysis, Writing – review & editing. SS: Concep1tualization, Data curation, Investigation, Supervision, Validation, Writing – review & editing, Visualization. MŽ: Data curation, Investigation, Writing – review & editing. DM: Data curation, Investigation, Visualization, Writing – review & editing. RM: Conceptualization, Data curation, Funding acquisition, Investigation, Resources, Supervision, Writing – original draft, Writing – review & editing, Methodology, Validation, Visualization.

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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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Analysis of the current risk of *Leishmania infantum* transmission for domestic dogs in Spain and Portugal and its future projection in climate change scenarios

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Canine leishmaniosis, caused by the protozoan parasite *Leishmania infantum*, is a cosmopolitan vector-borne zoonosis, transmitted principally by *Phlebotomus perniciosus* in Spain and Portugal, where it is considered an endemic disease. Ecoinformatics tools such as ecological niche models (ENM) have been successfully tested to model the distribution of the risk of infection of different parasitosis as they take into account environmental variables vital for their survival. The risk map proposed in this study combines the potential distribution of *Ph. perniciosus* in the Iberian Peninsula and the calculation of the infection rate of the parasite in the vector to model the risk of contracting the disease in a more realistic way. In fact, this weighting strategy improves the predictive power of the resulting model (R^2=0.42, p=<0.01) compared to the *Ph. perniciosus* ENM model alone (R^2=0.13, p>0.05). The places with the highest risk of transmission are the southwest and central peninsular area, as well as the Mediterranean coast, the Balearic Islands and the Ebro basin, places where the ideal habitat of *Ph. perniciosus* and the infection rate is also high. In the case of future projections under climate change scenarios, an increase in the risk of infection by *L. infantum* can be observed in most of the territory (4.5% in 2040, 71.6% in 2060 and 63% in 2080), mainly in the northern part of the peninsula. The use of ENMs and their weighting with the infection rate in *Ph. perniciosus* is a useful tool in predicting the risk of infection for *L. infantum* in dogs for a given area. In this way, a more complete model can be obtained to facilitate prevention and control.

KEYWORDS

***Leishmania infantum*, leishmaniosis, *Phlebotomus perniciosus*, Spain, Portugal, dogs, ecological niche model, infection risk**

1 Introduction

Vector-borne zoonotic diseases pose significant health challenges for both animals and humans, accounting for 61% of human diseases of zoonotic origin (1–4). These diseases are increasingly prevalent across the European continent due to globalization and climate change. Factors such as rising temperatures, vector movement, increased migration and tourism involving infected people and animals, and inadequate management diseases control measure among other factors, contribute to this trend (5–8).

Canine leishmaniosis stands as a vector-borne zoonotic disease caused by *Leishmania infantum*, a protozoan parasite that affecting both animals and humans alike, with dogs being the main domestic reservoir. Its primary vectors in Iberian Peninsula are *Phlebotomus perniciosus* and *Phlebotomus ariasi* species (9, 10). These, when feeding on blood from the definitive host, ingest amastigotes (tissue form), which then develop to promastigotes (infective form) in the intestine of the vector and subsequently migrate to the proboscis (11). This process is temperature-dependent, increasing logarithmically the percentage of sandflies infected by *L. infantum* between 10 and 30°C, the survival range of the vector (12).

Its distribution is cosmopolitan and dynamic, both spatially and temporally, subject to multiple social and environmental factors. In Europe, the countries located in the Mediterranean basin (France, Greece, Italy, Spain, and Portugal) are endemic, with a much higher incidence of canine leishmaniosis than human leishmaniosis (13). Within the entire peninsular and insular territory of Spain and Portugal, most of its surface is considered endemic. In Spain, reported seroprevalences range from 0.86 to 24.66%, with the highest reports in the south and on the Mediterranean coast (14–16). In Portugal, canine leishmaniosis is found throughout the territory with a heterogeneous distribution, with the highest seroprevalence observed in the center of the country, with values close to 30% (13).

In the context of prevention and control of animal and human leishmaniosis, it is essential to emphasize the various tools used for the prevention of infection (17). One of these is mapping to visualize areas where there is a risk of disease infection, as it allows early identification of risk areas, facilitates planning of interventions, optimizes resource allocation, supports epidemiological surveillance and improves risk communication to the population. Ecoinformatics tools, such as Geographic Information Systems (GIS) and ecological niche models (ENM), can be employed to manage zoonotis parasitosis. These tools facilitate modeling the distribution of the disease by considering the bioclimatic and environmental variables necessary for their maintenance (18). ENMs assign suitability values to the environmental habitats where an organism lives, achieved through the correlation between the known distribution records of the species and the environmental variables that influence it (19). These models have already been used to assess the potential risk of zoonotic disease transmission utilizing records of parasite presence, infected hosts (20, 21) and potential transmitting vectors (22, 23).

For leishmaniosis, these tools have been specifically applied specifically to the Mediterranean basin and other parts of the world to model the risk of infection concerning environmental variables such as precipitation, temperature, and vegetation (17, 24–32). Local studies in the Iberian Peninsula have assessed the risk of L. *infantum* infection using GIS tools. The initial risk map was constructed in the community of Madrid based on the distribution of vectors (*Ph. perniciosus* and *Ph. ariasi*), indicating high risk nuclei in individualized foci in the Center and South of the region (33). The second study, in East-Central Portugal, was focused solely on the presence of infected hosts, and suggests that irrigated crops and olive groves, open forests, and watercourses influence infection distribution (34). However, these studies did not integrate ecological niche models of the vectors with parasite development within them, extracting the full potential of these techniques and being much more realistic. The ability to model the development of the parasite inside the vectors together with the distribution of the latter through ENMs, has made infection risk mapping a supplementary tool in control plans for other vector-borne diseases, such as dirofilariosis on a larger scale (35–37).

The aim of this study was to develop an infection risk map for *L. infantum* in the Iberian Peninsula (Spain and Portugal) and the Balearic Islands as well as its projection to 2080 through the use of ENM, taking into account the habitat suitability of *Ph. perniciosus,* its main vector in the study area, and the calculation of the infection rate of *L. infantum* in the vector.

2 Materials and methods

2.1 Area of study

The Iberian Peninsula (40°14′24" N 4°14′21" W), formed by the countries of Spain and Portugal, and the Balearic Islands (Spain), were established as a study area. This territory is located in the southeast of the European continent, close to Morocco (Africa) and only separated from it by the Strait of Gibraltar (Figure 1). Both Spain and Portugal have overseas territories such as the Canary Islands, the Azores or Madeira, but these have not been taken into account in this study due to the particularity of their biogeographical characteristics, which are very different from those of the continent. The Iberian Peninsula covers a territory of approximately 590,000 km^2 and the Balearic Islands 4,992 km^2. Most of the peninsular territory is surrounded by coastline, surrounded to the east and south by the Mediterranean Sea, to the north by the Cantabrian Sea and to the west by the Atlantic Ocean. Regarding the continental territory, it is mostly made up of a large plateau with an average altitude of 600 meters crossed by both large hydrographic basins and a multitude of mountain ranges, providing the peninsula with a great diversity of environments. The mountain ranges that divide the peninsula are the Pyrenees, the Cantabrian Mountains, the Iberian System, the Central System and the Penibaetic System. The basins of the Ebro River (northeast) and the Guadalquivir River (south) are the main river basins of the peninsula, followed by other smaller basins such as the Guadiana (southwest), Júcar and Segura (east), Duero and Tajo (west) and Miño (northwest).

The Iberian Peninsula has a wide variety of climates, which give it a great biological importance. The northwest of the peninsula is an area of cool summers, mild winters and high humidity and precipitation throughout the year. The Levantine coast is an area with a Mediterranean climate with hot, dry summers and mild winters. The south is characterized by a warm and dry African-influenced climate with summer drought; while, in the central plateau, whipped by strong winds, the climate is very hot in summer and very cold in winter with rainfall normally restricted to spring and autumn. It is worth noting the notable difference in the climate with respect to the altitude, with

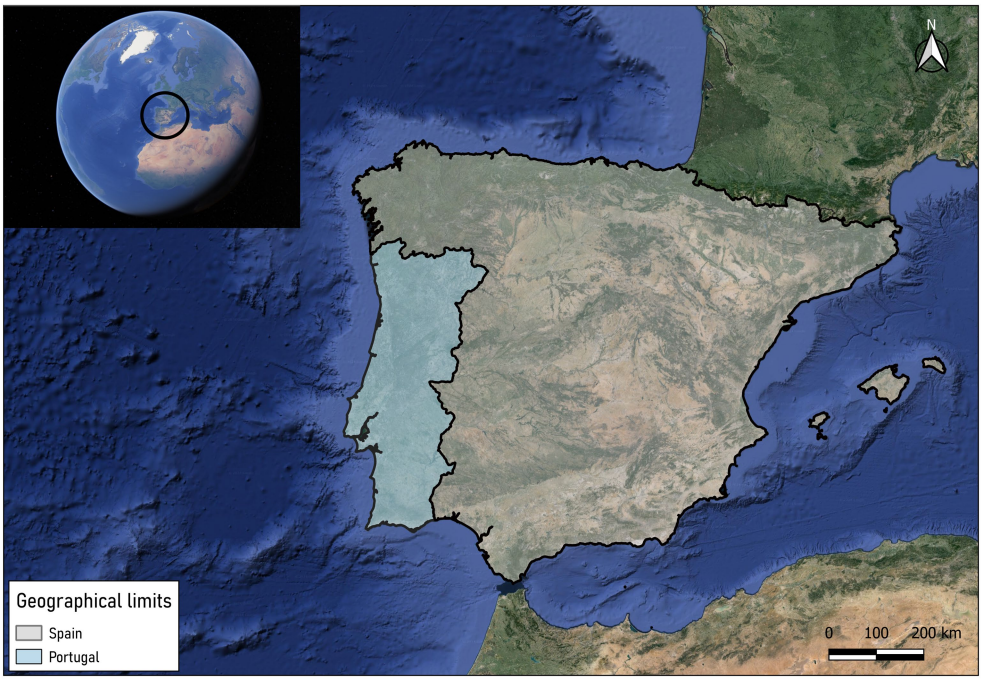

FIGURE 1
Location of Portugal and Spain and Balearic Islands (Spain).

the high mountain areas having mild summers and being covered with snow in winter (38).

2.2 *Phlebotomus perniciosus* habitat suitability modeling

2.2.1 Distribution data

To model habitat suitability for *Ph. perniciosus*, the main vector of canine leishmaniosis in the Iberian Peninsula and Europe (14, 39–41), we collected records of presence of this species from published studies (9, 42–44) and Global Biodiversity information Facility (GBIF) (45) to obtain the most representative face-to-face attendance possible. In order to obtain only presence points and eliminate sampling biases, the data were processed at a resolution of 1 km^2. Finally, 3,032 vector presence data were obtained for use in the model.

2.2.2 Bioclimatic and environmental data

At a spatial resolution of 1 km^2, 19 bioclimatic variables related to temperature and precipitation were downloaded from World Clim (46), for present-day conditions and projected scenarios for 2040, 2060, and 2080 (47). Subsequently, a multicollinearity analysis was performed on these variables in the R software using Pearson's correlation coefficient (48). To avoid cross-correlation between the 19 bioclimatic variables, those with a value of $r > \pm 0.75$ were eliminated and taking into account the biological needs of the vector, the variables chosen were BIO_1 (Annual Mean Temperature), BIO_2 (Mean Diurnal Range: The mean of the monthly temperature ranges), BIO_3 (Isothermality: Mean Diurnal Range (BIO_2) / Temperature Annual Range (BIO_7) × 100), BIO_8 (Mean Temperature of Wettest Quarter), BIO_{12} (Annual Precipitation) and BIO_{15} (Precipitation Seasonality: Standard deviation of weekly or monthly precipitation values as a percentage of the mean of those values). Next, the environmental variables were downloaded: density of shrubs and herbaceous plants (49) and the human footprint (50) which includes 8 variables (built environment, population density, electric power infrastructure, farmland, grazing land, roads, railways and waterways) reflecting the impact of human activities on the ecosystem. All downloaded data layers were processed in ArcMap 10.8 to ensure uniform extent, resolution (1 km^2 per pixel) and coordinates system (GCS_WGS_1984).

2.2.3 Modeling approaches

The maximum entropy algorithm MaxEnt was used (51) to model the vector's ecological niche from the Kuenm package of the R software (version 4.3.0) (48), automating the process (52). MaxEnt employs points of presence and environmental variables to estimate habitat suitability, which can be defined as the area in where specific environmental conditions necessary for the survival or reproduction of a species exist (53). To model *Ph. perniciosus*, 119 models were created with Kuenm for a set of variables, 17 regularization multiplier values (0.1–1.0 at 0.1 intervals, 2–6 at intervals of 1, 8, and 10) and the seven possible combinations of three feature classes (linear, quadratic, and product). The performance of the models created was assessed considering the significance of the partial receiver operating characteristic (partial ROC), with 100 iterations and 50% data for bootstrapping, skip rates (OR = 5%) and model complexity (Akaike information criterion - AIC). From the models that met the evaluation criteria, the final model was chosen based on the mean ratio of the area under the curve (AUC) obtained with points of occurrence independent of the calibration. The best-fit model (final model) was generated using the same parameters selected in the previous step. Ten

replicates were developed per bootstrap with logistic outputs, and re-evaluated based on criteria ROC_parcial, OR and AICc.

2.3 *Leishmania infantum* infection rate in phlebotomine

The infection rate (% of *Ph. perniciosus* infected by *L. infantum*) was calculated as Rioux et al. (12), applied to our vector, by using the formula $y = 0.718[1 - e^{-0.237(x-8)}]$ (y is % of *Ph. perniciosus* infected by *L. infantum* and x is the Annual Mean Temperature). The frequency distribution was adjusted to a theoretical ascending logarithmic curve, which allowed estimating the infection rate between 10 and 30°C, which is the temperature range in which the parasite can survive and replicate in the *Ph. ariasi* vector, as reported by Rioux et al. (12). The infection rate was carried out using the program R-4.3.0.

2.4 *Leishmania infantum* risk map and its validation

An infection risk map for *L. infantum* is a visual representation that identifies the geographical areas, within a given environment, where a certain risk of parasite transmission (high, medium or low) may exist. The risk of *L. infantum* infection refers to the probability that a host may become infected taking into account different factors such as the presence of the vector, the prevalence of the disease in a given population, the presence of natural reservoirs of the pathogen, environmental conditions favorable for vector reproduction, the availability of standing water for vector reproduction, and the proximity between vectors and hosts, among other factors. To generate the risk map of *L. infantum* infection in the study area, once the final ENM for *Ph. perniciosus* was generated, it was multiplied using a weighting approach with the map of the infection rate of *L. infantum* in *Ph. perniciosus*.

Our risk map was validated employing a regression analysis between the mean risk of infection and the seroprevalence of canine disease in all the autonomous communities of Spain and regions of Portugal, reported by Almeida et al. (13) and Montoya-Alonso et al. (16). In addition, geolocations of dogs infected by *L. infantum* were superimposed on the risk map. The geolocation of these infected animals is also derived from the same studies previously employed (13, 16). Simultaneously, seroprevalence data were compared using the unweighted vector ENM with the same approximation.

2.5 Forward projection and rank change analysis

Three suitable habitats for *Ph. perniciosus* were generated with the previously selected parameters, incorporating projections of the bioclimatic variables analyzed for the time periods 2021–2040 (2040), 2041–2060 (2060) and 2061–2080 (2080). The RCP 8.5 scenario, which represents high CO_2 emissions, was utilized using the HadGEM3-GC21-LL model (54), to study the effect of climate change in the future, because of high greenhouse gas emissions in Europe (55). The infection rate of *L. infantum* corresponding to each of the three future scenarios was also calculated using the BIO_1 (Annual Mean Temperature) of each time period.

The suitability habitats were weighted with the rate of infection of *L. infantum* and risk maps corresponding to each of the three periods analyzed were generated. Subsequently, the current risk map and the three projected future risk maps were transformed into presence/absence binary maps using the logistic threshold of training presence of the 10th percentile of the current map. This process is essential to perform a range-change analysis in order to establish alterations in the risk of *L. infantum* infection in the future. Finally, the percentage of cells that gained or lost risk of infection as a result of climate change was calculated for the maps projected to 2040, 2060, and 2080 compared to the present map using the biomod2 package of the R software (56).

3 Results

3.1 Habitat suitability model for *Phlebotomus perniciosus*

Figure 2 shows the developed ENM, indicating the suitability of *Ph. perniciosus* habitat across the Iberian Peninsula and the Balearic Islands. The maximum suitability value recorded was 0.83 (high suitability), while the minimum value was 0.0004 (low suitability). Table 1 shows the contribution degree of each of the variables in the vector model, with the variables with the highest percentage of contribution attributions to the human footprint (40.14%) and the BIO_1 (20.83%). The remaining variables contribute between 9.37 and 2.53%, with the latter being the lowest percentage. Those areas with a high suitability value correspond to the southwest of the peninsula, followed by others such as the center, the Levantine coast and the Balearic Islands, the Ebro basin and some areas of the north coast of the peninsula. Conversely, regions in the interior northwest and the central east, as well as the mountainous areas characterized by lower population density and cooler temperatures, exhibits less suitable habitat.

3.2 Map of *Leishmania infantum* infection rate in *Phlebotomus perniciosus*

Figure 3 illustrates the potential resulting map depicting the rate of *L. infantum* infection in *Ph. perniciosus* across the Iberian Peninsula and the Balearic Islands. Areas with the highest infection rate are concentrated to those of the southwest and south of the peninsula, the Mediterranean coast, the Balearic Islands and the Ebro basin, and the north and northwest coasts of the territory. The northern plateau had a medium infection rate, while the mountain areas, at higher altitudes display percentages close to 0.

3.3 Map of potential risk of transmission of *Leishmania infantum*

Figure 4 presents the map with the potential risk of transmission by *L. infantum* in the mainland and the Balearic Islands. Different infection risk values are represented using a color palette from red to

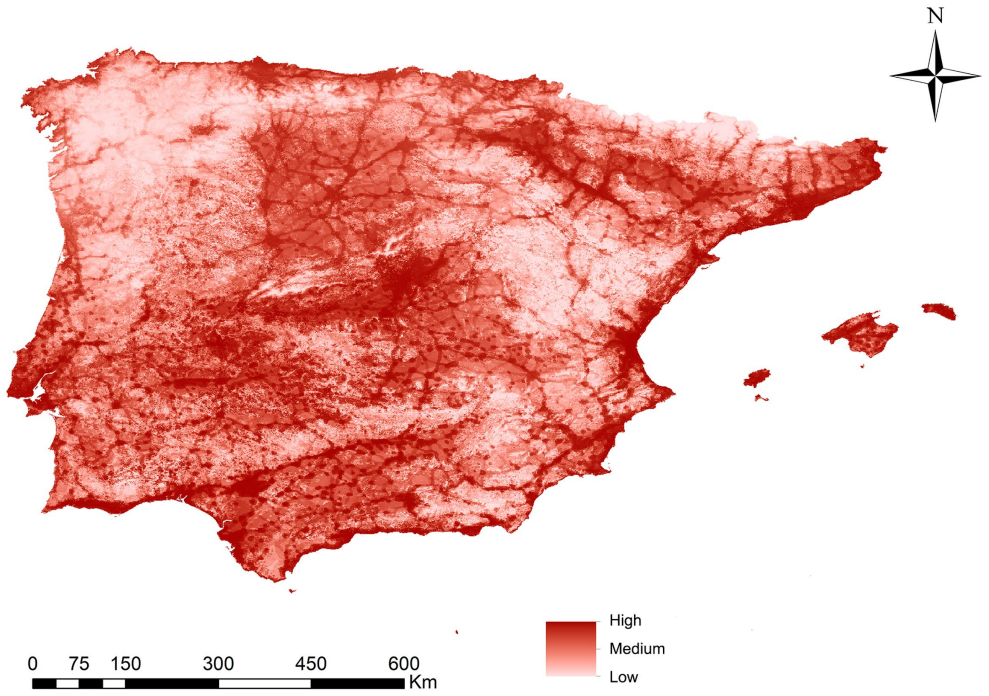

FIGURE 2
Ecological niche model (suitable habitat) for *Phlebotomus perniciosus* in Iberian Peninsula (Portugal and Spain) and Balearic Islands (Spain).

TABLE 1 Analysis of the contribution of the 9 environmental and bioclimatic variables to the ecological niche model for *Phlebotomus perniciosus*.

Variable	Percent contribution
Humanfootprint	40.14%
BIO_1 (annual mean temperatura)	20.83%
BIO_2 (Mean Diurnal Range)	9.37%
BIO_{12} (anual precipitation)	8.93%
Shrubs density	6.63%
BIO_3 (isothermality)	4.48%
Herbaceous density	4.36%
BIO_8 (mean temperature of wettest quarter)	2.73%
BIO_{15} (precipitation seasonality)	2.53%

blue, with the highest value being 0.56 and the lowest 0. The territory is divided into five risk ranges established by natural jenks (Very High, High, Medium, Low and Very Low), with 23.8% of the study area identified as very high/high risk areas, 23% as medium risk areas, and 53.2% as low/very low risk areas. There is a risk of infection throughout the study area except for high-altitude areas. The places with the highest risk of transmission correspond to the southwest and center of the peninsula, as well as the coast near the Mediterranean Sea, the Balearic Islands and the Ebro basin, places which coincide with areas characterized by ideal *Ph. perniciosus* habitats and high infection rate. Areas such as the northern plateau and the north and northwest coast have intermediate risk values. Areas of the interior of the peninsula, mountainous areas and higher altitudes with cooler temperatures exhibit risk values close to zero.

3.4 Validation of the *Leishmania infantum* potential transmission risk map

The result of the regression calculation was a positive and significant relationship between the infection risk map for *L. infantum* and the seroprevalence in infected dogs by each autonomous community in Spain and regions in Portugal ($\beta \pm SE = 61.15 \pm 16.39$, $R^2 = 0.42$, $p < 0.01$) (Figure 5). The results obtained from the unweighted vector ENM did not fit significantly with the seroprevalence data ($\beta \pm SE = 20.79 \pm 12.38$, $R^2 = 0.13$, $p > 0.05$), highlighting the importance of combining it with the infection rate.

Regarding the dogs infected with *L. infantum* and geolocated, 82.6% were in areas estimated to be at very high/high risk areas, 13.2% in medium risk areas and 4.2% in low/very low risk areas (Figure 6).

3.5 Forward projection of potential risk of transmission de *Leishmania infantum*

In the projection of potential transmission risk maps to the three future scenarios (2040, 2060 and 2080) of *L. infantum* through *Ph. perniciosus*, a latitudinal shift of the risk of infection toward the north of the peninsula is observed in both 2060 and 2080 (Figure 7). When the range-change analysis was carried out, the percentage of the territory where the risk of infection for *L. infantum* increases was in the North of the peninsula with 4.5% by 2040, 71.6% in 2060 and 63% in 2080. However, there is also a loss in the percentage of territory where there is a risk of infection, mainly in the south of the peninsula, being 9.6, 14.4 and 27.9% for 2040, 2060 and 2080, respectively.

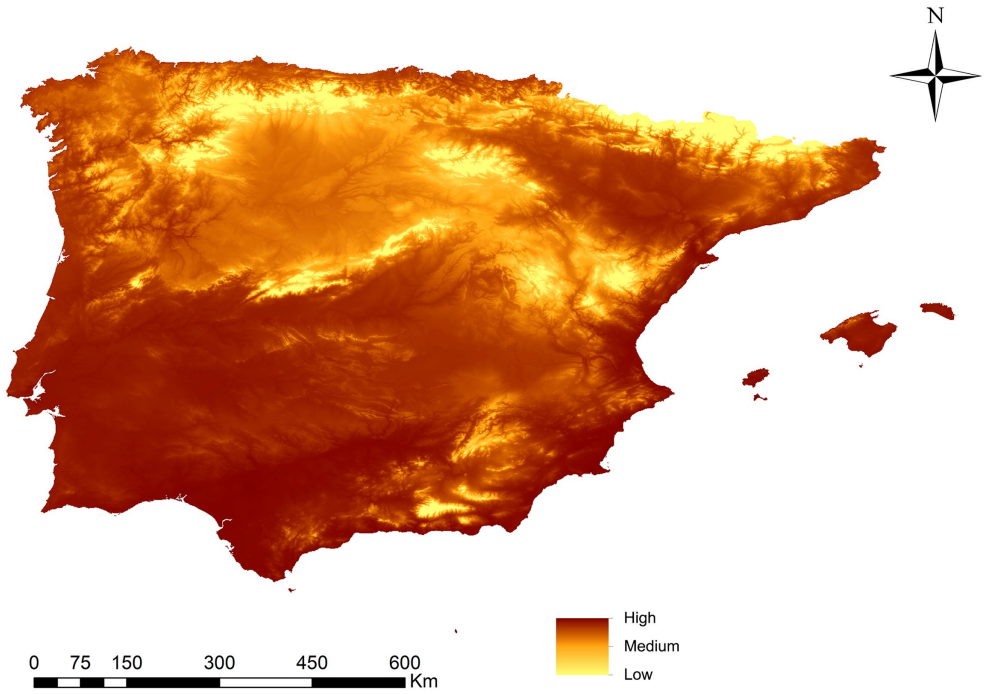

FIGURE 3
Prediction of *Phlebotomus perniciosus* infection rate in Iberian Peninsula (Portugal and Spain) and Balearic Islands (Spain).

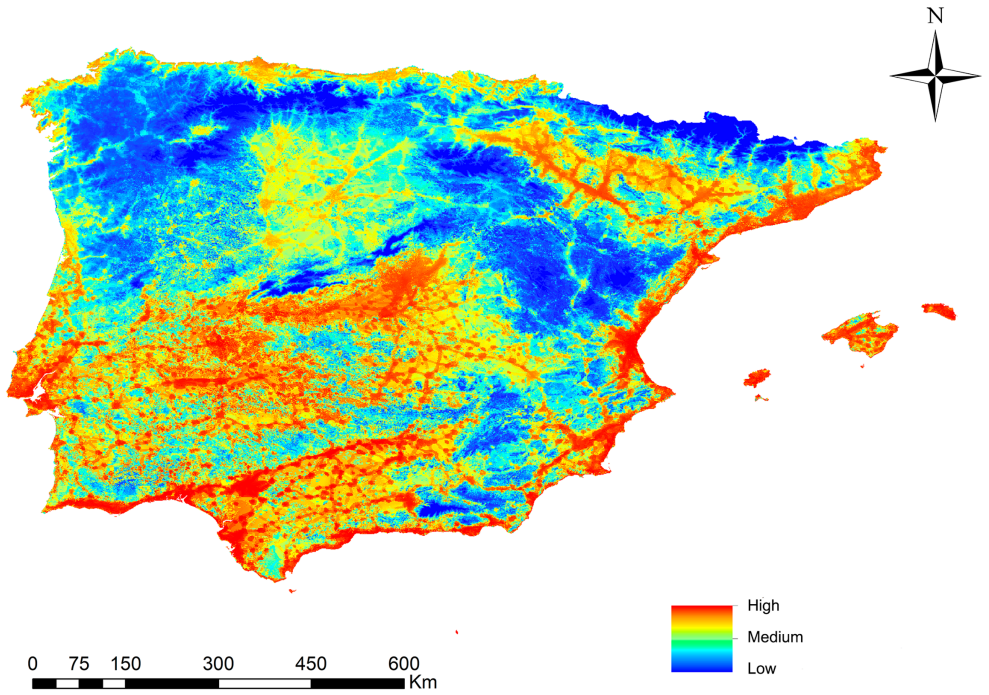

FIGURE 4
Ecological niche model of the risk of *Leishmania infantum* infection in Iberian Peninsula (Portugal and Spain) and Balearic Islands (Spain).

4 Discussion

This study provides quantitative data on the risk of *L. infantum* infection in Iberian Peninsula (Spain and Portugal) and the Balearic Islands (Spain). The novelty of this study lies in the weighted use of both the calculation of habitat suitability by ENM of *Ph. perniciosus*, and the infection rate of *L. infantum* in the sandfly to predict the presence of the vector and the infectivity of the disease more accurately.

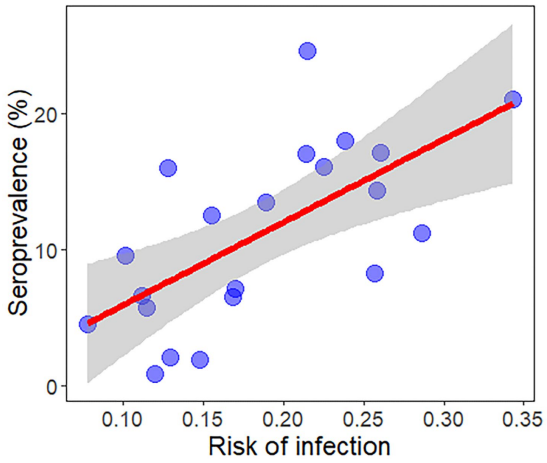

FIGURE 5
Regression plot for the validation of the ecological niche model between the mean risk of infection and disease prevalence in dogs most recently to date in all Spanish autonomous communities and Portuguese regions of the Iberian Peninsula and in the Balearic Islands (Spain) reported by Almeida et al. (13) and Montoya-Alonso et al. (16).

Prior to our study, the methodology of the ENMs has already been applied to try to model the distribution of leishmaniosis both in Europe and in other continents, using data on either the presence of vectors or infected hosts (17, 24–32). The same is applied to the Iberian Peninsula, where there are only two GIS studies have individually use the records of infected hosts or the distribution of their vectors, respectively (33, 34).

The risk map proposed in this work combines the potential distribution of the main vector of *L. infantum* in the Iberian Peninsula and the calculation of the parasite infection rate in the vector to model the risk of contracting the disease in a more realistic way. In fact, this weighting strategy improves the predictive power of the resulting model ($R^2 = 0.42$, $p = < 0.01$) compared to the *Ph. perniciosus* suitability model alone ($R^2 = 0.13$, $p = > 0.05$).

The variables that contribute most to explaining the potential distribution of *Ph. perniciosus* are the human footprint (built environment, population density, electric power infrastructure, cropland, grazing land, roads, railways, and waterways) and BIO_1 (Mean Annual Temperature). Areas where human pressure is high are an ideal habitat for the maintenance of *Ph. perniciosus* populations. These areas with high anthropic presence, such as parks and agricultural land, also have important reservoirs of *L. infantum* (rabbits, rats, cats) associated with them, making it possible to efficiently maintain the biological cycle of canine leishmaniosis with high loads of infected sandflies (57–63). In addition, high prevalences of *L. infantum* infection in urban lagomorph populations have been linked to recent outbreaks of human leishmaniosis in Spain (57, 62), where annual incidences in humans (0.4–3.18 cases/100,000 inhabitants) and different prevalences in animals [29% in foxes (*Vulpes vulpes*), 13% in beech martens (*Martes foina*), 33% in wolves, 33.3% in rats, 15.6% in stray cats, 100% in rabbits, 8% in badgers and 1/3 of infected Egyptian mongooses] with special presence in southeastern Spain (11, 60–63) have been reported. On the other hand, the average annual temperature has a positive influence on the biology and ecology of the sandfly (rate of egg production, development of juvenile stages, annual number of generations, feeding behavior, period of activity and survival of adults) (64, 65). Other variables with a minor influence on the suitability models obtained include the diurnal mean range and the seasonality of rainfall. This last variable is also associated with the habitat types identified as influencing the distribution of hares and other wild reservoirs of *L. infantum* (natural grasslands, coniferous forests, lands occupied mainly by agriculture, lands with significant areas of natural vegetation and non-irrigated farmlands) that are characterized by moderate to high annual rainfall (66). Currently, areas with a higher seroprevalence of *L. infantum* in Spain suffer from drought, which may negatively influence sandfly populations and affect the transmission of the disease.

Regarding the variables associated with the rate of infection of *L. infantum*, the average annual temperature also influences its development, with the percentage of infected sandflies increasing logarithmically as the temperature rises within their survival ranges (12).

Our combined risk model indicates that actually, areas of the interior of the peninsula, mountainous and higher altitude areas with low temperatures, (which decrease both the habitat suitability of the vector and the rate of infection of these by the parasite) have risk values close to 0. On the other hand, the areas with a higher risk of infection (the south-west and center of the Peninsula, as well as the coast near the Mediterranean Sea, the Balearic Islands and the Ebro basin) coincide with areas with a high human presence, high average annual temperatures and with the basins of large rivers such as the Tajo, the Ebro and the Guadalquivir.

In the case of future projections under climate change scenarios, an increase in the risk of infection by *L. infantum* can be observed in most of the territory (4.5% in 2040, 71.6% in 2060 and 63% in 2080), mainly in the northern part of the peninsula. However, in some areas of the south of the territory, there would be a decrease in risk over time (9.6% in 2040, 14.4% in 2060 and 27.9% in 2080), which may be due to the foreseeable decrease in water resources, and the reduction of wetlands and vegetation in these areas (67, 68). This work predicts that canine leishmaniosis, in line with other vector-borne diseases, will shift latitudinally and toward higher altitude areas, altering its dynamics both spatially and temporally, colonizing areas where it was previously absent (22, 32, 35, 36, 69, 70). The effect of climate change on the seasonality and distribution of these types of vector-borne diseases will be more pronounced within the temperature ranges conducive to transmission occurs (71, 72).

As future approaches to applying of ENMs in vector-borne zoonotic diseases, it is possible to use the weighting tool not only with the niche model of one of its vectors, but also with more than one that inhabit the same territory, each with different ecological niches, provides sufficient data are available to model their distribution. In this way, a more comprehensive model could be obtained to facilitate the prevention and control of these diseases by veterinary and other specialist personnel.

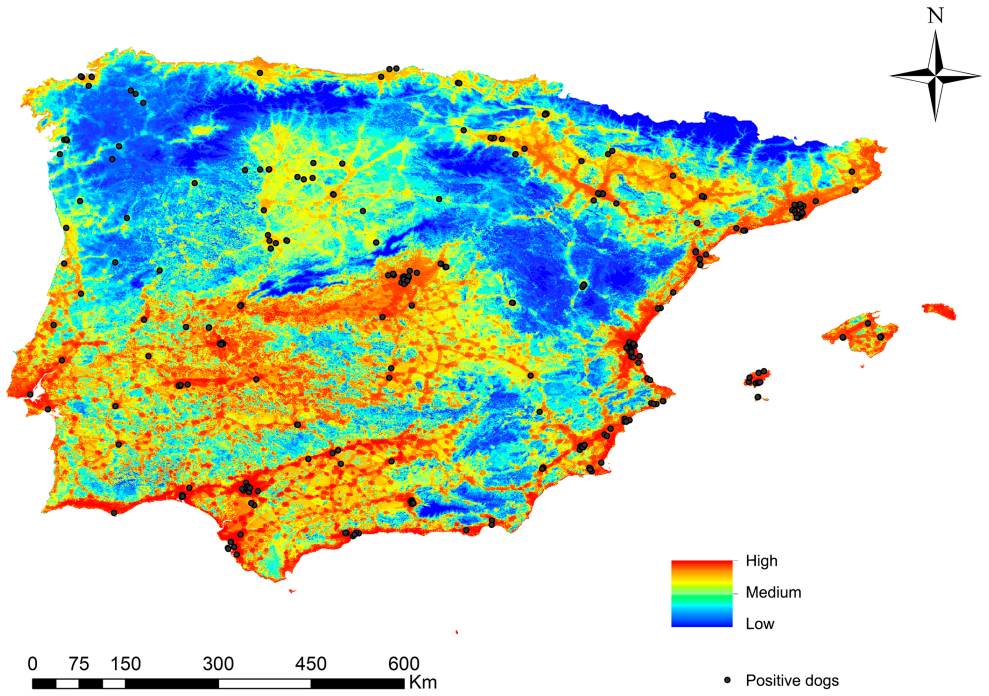

FIGURE 6
Ecological niche model of the risk of *Leishmania infantum* infection in Iberian Peninsula (Portugal and Spain) and Balearic Islands (Spain) and the geolocations of infected dogs in Iberian Peninsula and Balearic Islands (Spain).

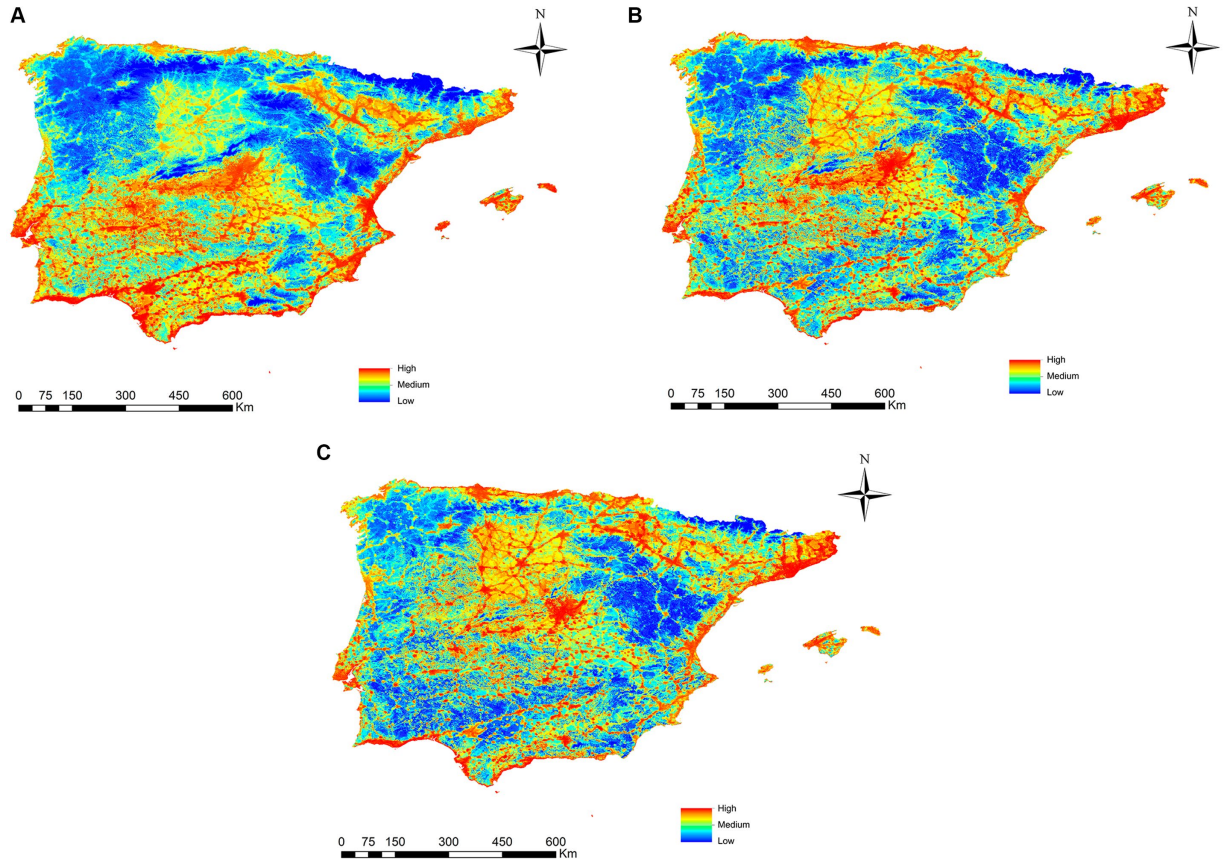

FIGURE 7
Projections of the risk of *Phlebotomus perniciosus* infection for 2040 **(A)**, 2060 **(B)** and 2080 **(C)** in Iberian Peninsula (Portugal and Spain) and Balearic Islands (Spain) under the climate change scenario RCP 8.5.

Data availability statement

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

Author contributions

IR-E: Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing, Data curation, Software. AB-d: Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Software. MC-C: Formal analysis, Investigation, Methodology, Writing – review & editing. DB-B: Investigation, Writing – review & editing. SD-E: Investigation, Writing – review & editing. REH-L: Investigation, Supervision, Validation, Writing – review & editing. JÁS-A: Conceptualization, Investigation, Methodology, Supervision, Validation, Writing – review & editing. RM: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, 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

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Erratum: Analysis of the current risk of *Leishmania infantum* transmission for domestic dogs in Spain and Portugal and its future projection in climate change scenarios

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An Erratum on

Analysis of the current risk of *Leishmania infantum* transmission for domestic dogs in Spain and Portugal and its future projection in climate change scenarios

by Rodríguez-Escolar, I., Balmori-de la Puente, A., Collado-Cuadrado, M., Bravo-Barriga, D., Delacour-Estrella, S., Hernández-Lambraño, R. E., Sánchez Agudo, J. Á., and Morchón, R. (2024). *Front. Vet. Sci.* 11:1399772. doi: 10.3389/fvets.2024.1399772

Due to a production error, there was a mistake in the order of Figures 5, 6, which were swapped incorrectly while the order of their captions remained unchanged.

Figures 5, 6 and their correct legends appear below:

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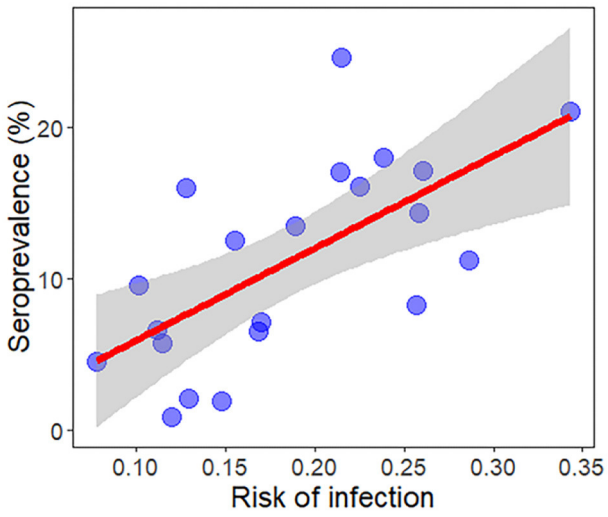

FIGURE 5
Regression plot for the validation of the ecological niche model between the mean risk of infection and disease prevalence in dogs most recently to date in all Spanish autonomous communities and Portuguese regions of the Iberian Peninsula and in the Balearic Islands (Spain) reported by Almeida et al. (13) and Montoya-Alonso et al. (16).

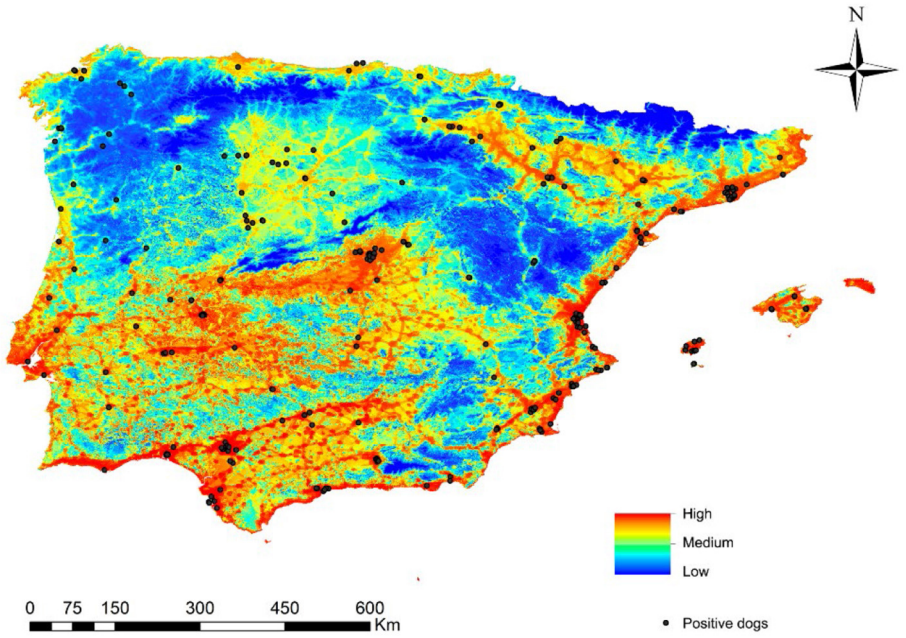

FIGURE 6
Ecological niche model of the risk of *Leishmania infantum* infection in the Iberian Peninsula (Portugal and Spain) and Balearic Islands (Spain) and the geolocations of infected dogs in the Iberian Peninsula and the Balearic Islands (Spain).

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Epidemiological analysis of *Dirofilaria immitis* (Spirurida: Onchocercidae) infecting pet dogs (*Canis lupus familiaris*, Linnaeus, 1758) in Baixada Fluminense, Rio de Janeiro

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Dirofilaria immitis infection is routinely detected in dogs during veterinary care in tropical and subtropical regions worldwide. Parasitological tests for the detection of this infection are routinely performed only in areas with a high prevalence. Baixada Fluminense, a region in Rio de Janeiro, was considered heartworm-free until local veterinarians began to receive blood exams results indicating the presence of microfilariae (MF). A laboratory database was hence used to collect data from 2017 to 2020 to understand the extent of spread of the parasite in this area. The results of complete blood count analysis and MF or heartworm antigen detection tests conducted on canine samples sent from veterinary clinics in Baixada Fluminense (Magé, Duque de Caxias, Guapimirim, Nova Iguaçu, and São João de Meriti municipalities) were included. In total, the results of 16,314 hematological tests were considered. The overall prevalence of *D. immitis* was 3.4% (554/16,314), considering that only one test result was obtained per animal on the same day. This study is highly relevant because it indicates the spreading geographic distribution of the worms, heightens awareness among local health professionals and the general population, and encourages compliance with prophylactic measures to prevent further spread of parasite.

KEYWORDS

***Dirofilaria immitis*, heartworm, zoonosis, epidemiology, pet dog**

1 Introduction

Some filarioids, such as *Dirofilaria immitis*, *Dirofilaria repens*, *Acanthocheilonema reconditum*, *Oncocherca lupi*, and *Cercopithifilaria grassii*, belonging to the family Onchocercidae (Spirurida), are transmitted by arthropod vectors and cause canine filariasis (1). Worldwide, more than seventy species of the Culicidae family participate in the

transmission of *D. immitis* and the main mosquito-vectors are: *Aedes*, *Ochlerotatus*, *Culex*, *Anopheles* (2). Competent vectors ingest microfilariae (MF) when they take a blood meal. In about 10 to 14 days, depending on the environmental temperature, the larvae develop into third stage (L3) and migrate to the head of the mosquito. When the mosquito takes the next blood meal the L3 migrates to the new definitive host. Once the new host is infected the L3 molts to L4 and in approximately 120 days young adults can be found in the pulmonary arteries and right chambers of the heart. Adult males and females' mate and produce MFs that can be found in the peripheral blood stream approximately in 7 to 9 months (3).

Canine clinical signs are multifactorial. Most dogs are asymptomatic and when they become sick, coughing, weight loss and exercise intolerance are frequent. Severe disease includes signs of congestive right heart failure (4). An update on the South American seroprevalence showed that no infected dog has been reported in Chile and that in the other countries were the infection has been detected, prevalence rates range from 14.41% in Argentina to 1.6% in Colombia, 8.9% in Mexico, 5.5% in Peru and 15.2% in Venezuela (5). The overall prevalence of canine *D. immitis* infection in Brazil was 13.03% (6). *Dirofilaria immitis* canine infection is common in the coastal regions of Brazil, with a high prevalence of 23.1% (7).

In the State of Rio de Janeiro, during the active search for cases of canine heartworm disease, seroprevalence was recorded in some locations in the metropolitan region where no survey had been carried out. In the west zone, a study showed that 21.6% of canines were infected (8); another research showed that laboratories that received samples from different neighborhoods in the city of Rio de Janeiro reported only 7% of nematode infections in dogs (9) and occurrences were reported during veterinary care on Ilha do Governador showing that 14.5% of dogs were infected by *D. immitis* (10).

The Baixada Fluminense region was considered to be indene, until 2004 when a record of a case with a frequency rate of 0.9% in the municipality of Nova Iguaçu (11). After 2017, *D. immitis* has been detected at a higher frequency with autochthonous cases reported in this area (12, 13). The Baixada Fluminense region has recently been recognized as a new focus area for onchocercid infections (13). Undoubtedly, global climate change and anthropogenic actions favor increased human, canine, and mosquito population densities and, thus, the spread of the infection (14).

Traveling with dogs is increasing owing to the easiness. Some families travel with multi-species pets. Although this practice may be incentivized, the associated health issues must not be ignored. One way to counteract these health issues is through good pet care, including preventive measures that undoubtedly impose chemoprophylaxis on *D. immitis*. Therefore, infections monitoring and spreading awareness, particularly in areas without parasite circulation, must be prioritized locally. This study aimed to analyze the epizootiological factors including the prevalence of infections *of D. immitis* in domestic dogs in Baixada Fluminense, Rio de Janeiro, Brazil.

2 Materials and methods

2.1 Ethical aspects

The study was approved by the Animal Use Ethics Committee of the Oswaldo Cruz Institute/Oswaldo Cruz Foundation (CEUA-IOC-L009/2020) and the Oswaldo Cruz Institute/Oswaldo Cruz Foundation Human Research Ethics Committee (CEP CAAE: 30759620.1.0000.5248).

2.2 Study location

The study was performed as a retrospective analysis of the Laborlife Clinical Analysis Laboratory[1] database from January 2017 through December 2020, including dogs that lived in Baixada Fluminense (total area of 43,696 km^2; below 200 meters altitude), metropolitan region of the State of Rio de Janeiro. The Atlantic Forest Biome touches the border areas of Baixada Fluminense compromising a vast area of environmental conservation with ecological stations and parks, a semi-humid tropical climate, and the average annual temperature of 24°C.[2] The municipalities included in this study were Nova Iguaçu (22° 45′33″S, 43° 27′04″W), Magé (22° 39′10″S, 43° 02′26″W), Guapimirim (22° 32′14″S, 42° 58′55″W), Duque de Caxias (22° 47′ 08″S, 43° 18′42″W) and São João de Meriti (22° 48′14″S, 43° 22′22″W).

2.3 Data collection

The data was limited to that of blood samples obtained from dogs over 12 months of age to avoid bias due to the long prepatent period of the infection and that collected by attending veterinarians of private clinics or hospitals located in one of the five municipalities of Baixada Fluminense (Metropolitan Rio de Janeiro). The data included: (i) *D. immitis* antigen detection test results (lateral flow immunochromatographic assay – Alere™ Dirofilariasis Ag Test Kit; BioNote, Inc., Republic of Korea, or enzyme immunoassay – SNAP® 4Dx® Plus; IDEXX Laboratories, Westbrook, MN, United States); (ii) results of modified Knott's test to detect microfilariae (15); and (iii) unexpected findings obtained during blood smear for CBC or hemoparasite investigation. When an infection was detected in a dog using one technique, results from other methods were excluded to avoid duplication. When antigen detection test result was available, it was considered first. Knott's test results were considered when the antigen test result was unavailable, and blood smear results were considered only when none of the other were available. In these cases, the presence of microfilariae was recorded.

2.4 Statistical analysis

Was evaluated the following characteristics were evaluated: the municipality of residence, age (>12 months), sex (male or female), and tests for the detection of adult worms and microfilariae. Pearson's chi-squared test was used to determine the association between these characteristics and the test results. As some variables had more than two categories, a post-hoc analysis of the adjusted standardized residuals was performed to identify each variable's specific pairs of associated categories. The *p*-values were adjusted using the Bonferroni

1 https://www.laborlife.com.br/portal/

2 http://www.ceperj.rj.gov.br/

TABLE 1 Epizootiological data associated with the prevalence of *D. immitis* in canines in 2017–2020 in Baixada Fluminense, RJ.

Characteristics	*D. immitis n* (%)		*p*-value	Total *n* (%)
	No	Yes		
Municipalities			0.000*	
Magé	2,892 (17.7)[a]	270 (1.7)[b]		3,162 (19.4)
Duque de Caxias	9,547 (58.5)[a]	241 (1.5)[b]		9,788 (60.0)
São João de Meriti	971 (6.0)[a]	5 (0.0)[b]		976 (6.0)
Nova Iguaçu	465 (2.9)[a]	6 (0.0)[b]		471 (2.9)
Guapimirim	1,885 (11.6)[a]	32 (0.2)[b]		1,917 (11.7)
Age (years)			0.020*	
1–7	7,591 (46.5)[a]	244 (1.5)[b]		7,835 (48.0)
8–14	3,223 (19.8)[a]	135 (0.8)[b]		3,358 (20.6)
15 or more	337 (2.1)[a]	7 (0.0)[a]		344 (2.1)
Uninformed	4,609 (28.3)	168 (1.0)		4,777 (29.3)
Sex			0.000*	
Female	8,165 (50.0)[a]	242 (1.5)[b]		8,407 (51.5)
Male	7,595 (46.6)[a]	312 (1.9)[b]		7,907 (48.5)
Tests			0.000*	
Unexpected findings	14,837 (90.9)[a]	335 (2.0)[b]		15,172 (93.0)
D. immitis antigen	183 (1.1)[a]	62 (0.4)[b]		245 (1.50)
Modified Knott's test	740 (4.5)[a]	157 (1.0)[b]		897 (5.50)
Total	15,760 (96.6)	554 (3.4)		16,314 (100)

*p-value < 0.05; [a,b,c] each letter indicates categories of variables that do not differ at a significance level of 0.05.

method to account for multiple comparisons. All analyses were performed using SPSS Statistics software version 24 (16) with an α significance level of 5%.

3 Results

The analysis included 16,314 test results, of which 3.4% were positive for *D. immitis* (Table 1). The highest overall prevalence was observed in Magé, where 8.5% (270/3,162) of the dogs tested positive, followed by Duque de Caxias, where 2.5% tested positive (241/9,788) (Table 1). The space–time distribution shown in Figure 1 indicates that these municipalities have remained the same over the years, with a greater number of cases than those in the others. According to antigen tests, 25.3% (62/245) of the dogs were positive for the *D. immitis*, 17.5% (157/897) were positive for the modified Knott's test, and 2.2% (335/15,172) were positive for unexpected findings of microfilariae (Table 1; Figure 2).

The results indicated a significant association between infection and all canine characteristics (Tables 1, 2). Compared to dogs treated at veterinary clinics in Magé, those treated in Duque de Caxias, São João de Meriti, Nova Iguaçu, and Guapimirim were 73% (OR = 0.270; CI95% = 0.226–0.323), 94.5% (OR = 0.055; CI95% = 0.023–0.134), 86.2% (OR = 0.138; CI95% = 0.061–0.312), and 81.8% (OR = 0.182; CI95% = 0.125–0.263) less likely, respectively, to have positive results (Tables 1, 2). Furthermore, dogs treated in São João de Meriti were 79.6% less likely (OR = 0.204; CI95% = 0.084–0.496) to be infected than those treated in Duque de Caxias (Tables 1, 2). Age was also a significant factor, with dogs aged 8–14 years being 30.3% more likely (OR = 1.303; CI95% = 1.052–1.614) to be infected than those aged 1–7 years. Male dogs were 38.6% more likely (OR = 1.386; CI95% = 1.168–1.644) to be infected than female dogs (Tables 1, 2). Moreover, the *D. immitis* antigen test showed 59.6% (OR = 1.596; CI95% = 1.141–2.233) more positive results than the modified Knott's test (Tables 1, 2).

4 Discussion

According to a previous report using multiplex PCR, at least 93.5% of dogs in the study area were infected with *D. immitis* (13). Hence, in this study MF detected by conventional tests displaying the morphology of the anterior and of the posterior ends in agreement with *D. immitis* description (17, 18) were assumed to be *D. immitis* by the laboratory. This incomplete identification of the larvae morphology can be considered a limitation in this study.

Male dogs were infected more frequently than female dogs. This has been previously reported (19) however, no hypothesis has been proposed to explain this difference (19, 20). Empirical observations have shown that spaying and neutering dogs in the study area are rare, suggesting that females need to be better cared for to avoid unwanted litter and that male dogs are mainly restricted to backyards or sometimes allowed to roam free, as observed elsewhere (19, 20). This human behavioral manner, along with the predisposition of male dogs, suggests that the difference may be attributed to the exposure to infected mosquitoes instead of the sex.

The frequency of infections among older dogs (8–14 years) may have been higher than that among younger dogs by chance. When

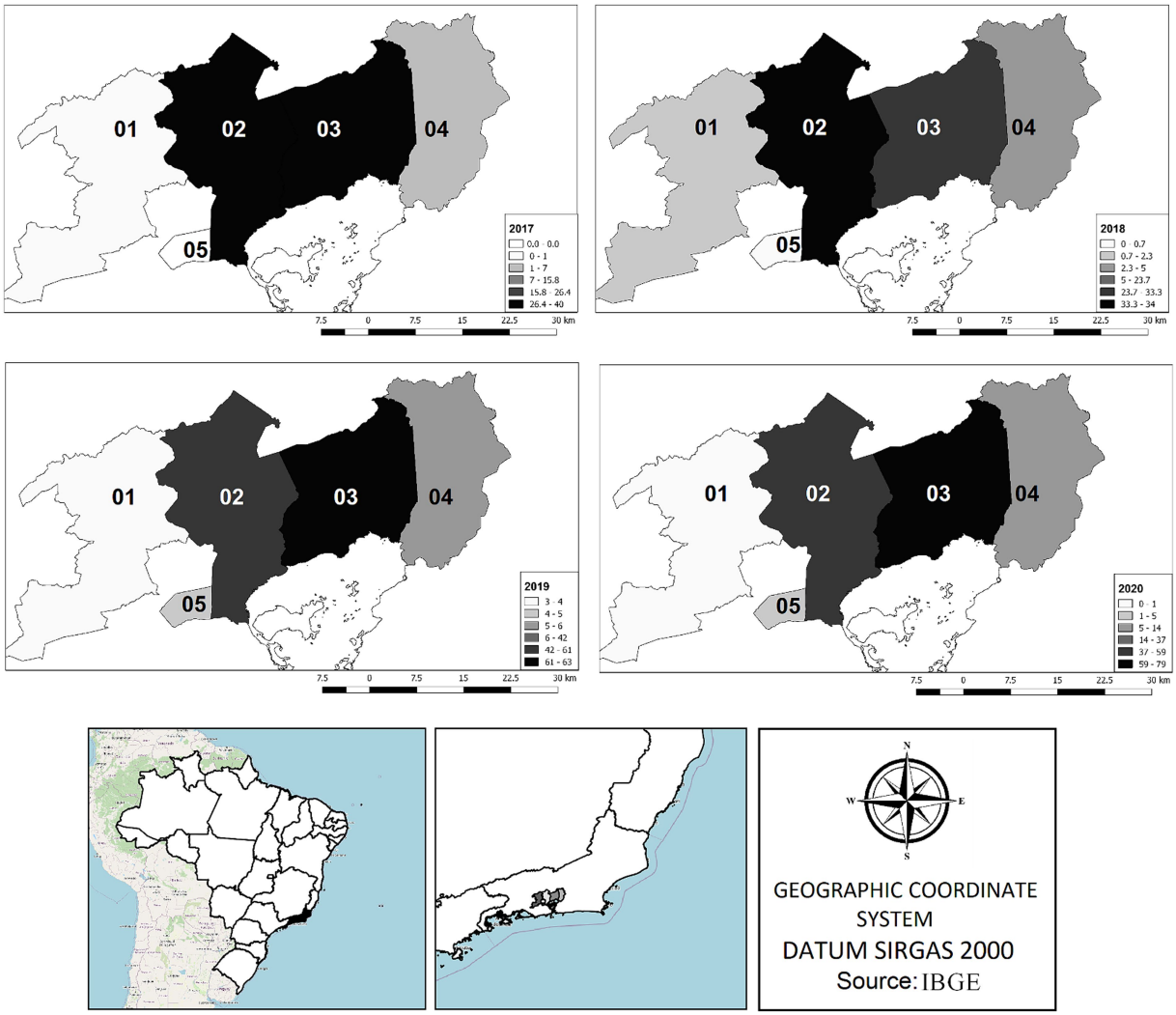

FIGURE 1
Positive cases of *Dirofilaria immitis* in dogs (aged >12 months) in Baixada Fluminense, Rio de Janeiro State, Southeast Brazil (QGis software version 2.18). 1- Nova Iguaçu, 2- Duque de Caxias, 3- Magé, 4- Guapimirim, 5- São João de Meriti.

moderately challenged as observed (frequency of 3.4%), the longer the exposure to the vectors, the higher will be the risk. This contrasts with the results of a previous study conducted in long-known focus areas for *D. immitis* high-challenge transmission (frequency > 20%). In those areas, the length of time the dogs lived in the focus did not increase the infection frequency, perhaps because the focus was established, and transmission was quick (7). Therefore, it may be inferred that Baixada Fluminense is an area where *D. immitis* transmission is a recent event as a possible result of global environmental changes that demand extended periods of transmission (14).

In addition to the human population density, the presence of microfilaremic dogs (21) conditions the establishment of an enzootic cycle and the emergence of cases of human pulmonary dirofilariasis in areas of socio-environmental vulnerability, making it a worrying factor according to the One Health concept (22, 23). Therefore, implementing public policies for the management of environmental sanitation, control of vector mosquitoes by the endemic sector, and educational planning for health professionals by the local authorities is of paramount importance (24, 25). The study area once considered free of heartworm transmission, currently presents data suggesting the existence of this parasite (13). Therefore, once transmission in the area has been established, veterinarians must be prepared to guide pet owners to adhere to prevention and treatment measures.

With the occurrence of infected dogs in Baixada Fluminense documented herein, factors related to anthropogenic and climate, in addition to the presence of infected dogs (26, 27), may facilitate the establishment of competent mosquito populations and enhance the transmission of *D. immitis* in the region. Considering that Baixada Fluminense is a section of the state's lowlands tangential to the oceanic coast and is a permanent conservation area, wild animals may also be affected (28). Most of the *D. immitis* infections documented in Brazil are in coastal areas (7, 29, 30), although infections are not restricted to these environments (31). The geographical dispersion of the parasite *D. immitis* in Brazilian previously indene regions are scarce (12, 13, 32, 33), however in Europe this spreading receives attention and is seen as a possible consequence of global climate changes (14, 34).

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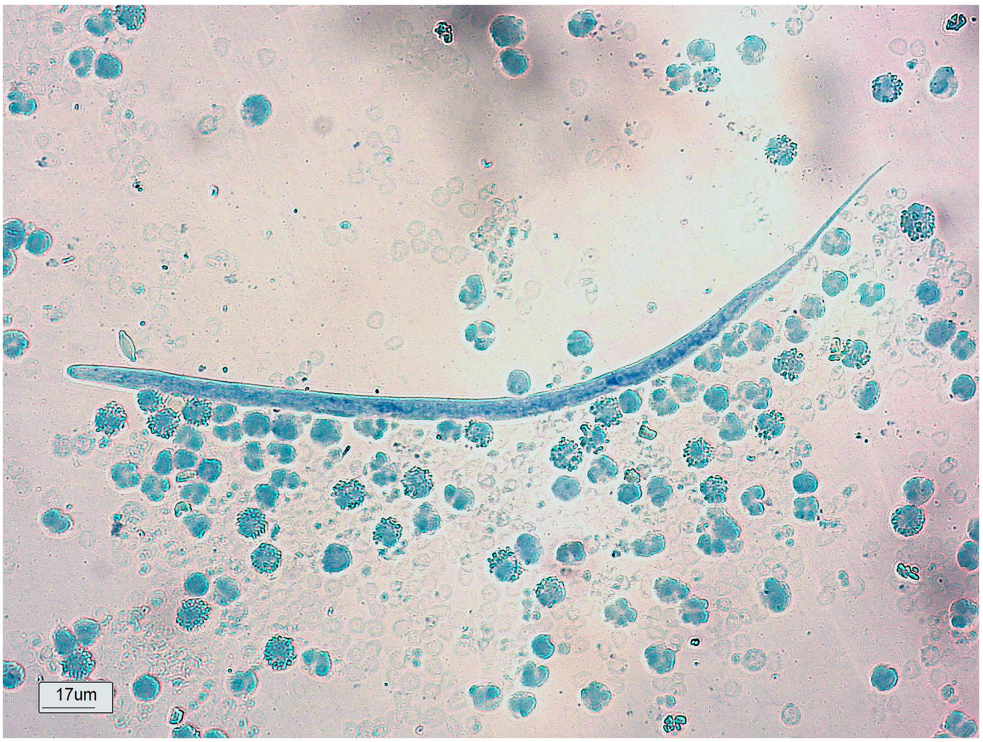

FIGURE 2
Microfilariae of *Dirofilaria immitis*, detected by the modified Knott's technique.

TABLE 2 The Odds ratio between each variable's categories differs, referring to the chi-square posthoc test.

Characteristics	OR	CI95%
Municipalities		
Magé vs. Duque de Caxias	0.270	0.226–0.323
Magé vs. São João de Meriti	0.055	0.023–0.134
Magé vs. Nova Iguaçu	0.138	0.061–0.312
Magé vs. Guapimirim	0.182	0.125–0.263
Duque de Caxias vs. São João de Meriti	0.204	0.084–0.496
Age (years)		
1–7 vs. 8–14	1.303	1.052–1.614
Sex		
Female vs. Male	1.386	1.168–1.644
Tests		
Unexpected findings vs. *D. immitis* antigen	15.005	11.030–20.411
Unexpected findings vs. Modified Knott's test	9.396	7.666–11.516
Modified Knott's test vs. *D. immitis* antigen	1.596	1.141–2.233

OR, odds ratio; CI95%, confidence interval 95%.

Thus, a broad epidemiological investigation must be conducted to monitor the prevalence of *D. immitis* in local dog populations by performing specific routine laboratory tests for detection and this filarioid identification. The rapid tests for antigen research are readily available and have greater specificity and sensitivity for the detection of *D. immitis* and to be recommended for clinical and epidemiological research (7, 30, 31).

The general recommendation is to request the modified Knott test (15) associated with antigen test (3) to detect and confirm parasitism in case of "occult infection" once 30% of the canine population will never be microfilaremic and because the predictive value of the antigen tests may provide false-positive results in a low-frequency area (30, 35).

5 Conclusion

The recently detected *D. immitis* infection in dogs in the lowland Baixada Fluminense region makes the area a candidate for canine heartworm transmission. This reinforces the need for an integrative approach among health professionals with a broad one-health perspective to implement public policies that promote health.

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 Animal Use Ethics Committee of the Oswaldo Cruz Institute/ Oswaldo Cruz Foundation. 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

VA: Conceptualization, Data curation, Project administration, Investigation, Methodology, Writing – original draft. PS: Writing – original draft, Methodology. ÉP: Formal analysis, Methodology, Writing – original draft. PA: Methodology, Writing – original draft. NL: Methodology, Formal analysis, Writing – review & editing. GG: Methodology, Writing – review & editing, Conceptualization, Project administration, Supervision. AM: Conceptualization, Data curation, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

NL is a consultant for Boehringer Ingelheim and Zoetis in Brazil.

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

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Molecular characterization of *Cryptosporidium* in wild rodents from the Inner Mongolian Autonomous Region and Liaoning Province, China: assessing host specificity and the potential for zoonotic transmission

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Introduction: Wild rodents are key hosts for *Cryptosporidium* transmission, yet there is a dearth of information regarding their infection status in the Inner Mongolian Autonomous Region and Liaoning Province of China. Therefore, the present study was conducted to determine the prevalence and genetic characteristics of *Cryptosporidium* among wild rodents residing in these two provinces.

Methods: A total of 486 rodents were captured, and fresh feces were collected from each rodent's intestine for DNA extraction. Species identification of rodents was performed through PCR amplification of the vertebrate cytochrome b (*cytb*) gene. To detect the presence of *Cryptosporidium* in all fecal samples, PCR analysis and sequencing of the partial small subunit of the ribosomal RNA (*rRNA*) gene were performed.

Results: Four species of rodents were identified: *Rattus norvegicus*, *Mus musculus*, *Apodemus agrarius*, and *Cricetulus barabensis*. Positive results for *Cryptosporidium* were obtained for 9.2% (18/195), 6.6% (7/106), 5.6% (5/89), and 6.3% (6/96) of these rodents, respectively, with an average infection rate of 7.4% (36/486). The identification revealed the presence of five *Cryptosporidium* species, *C. ubiquitum* ($n = 8$), *C. occultus* ($n = 5$), *C. muris* ($n = 2$), *C. viatorum* ($n = 1$), and *C. ratti* ($n = 1$), along with two *Cryptosporidium* genotypes: Rat genotype III ($n = 10$) and Rat genotype IV ($n = 9$).

Discussion: Based on the molecular evidence presented, the wild rodents investigated were concurrently infected with zoonotic (*C. muris*, *C. occultus*, *C. ubiquitum* and *C. viatorum*) as well as rodent-adapted (*C. ratti* and Rat genotype III and IV) species/genotypes, actively participating in the transmission of cryptosporidiosis.

KEYWORDS

***Cryptosporidium*, prevalence, wild rodent, genotyping, public health, China**

1 Introduction

Cryptosporidium, a parasitic apicomplexan organism, infiltrates the epithelial cells of the small intestine, leading to infections that are the second most prevalent cause of severe diarrhea among young children residing in regions with limited resources (1). Additionally, *Cryptosporidium* is a significant opportunistic pathogen among immunocompromised individuals, such as those living with Human Immunodeficiency Virus (HIV), transplant recipients, cancer patients undergoing chemotherapy, and those undergoing hemodialysis treatment (2). Moreover, water-borne and food-borne outbreaks of *Cryptosporidium* are common among the general population. Globally, more than 1,200 outbreaks have been attributed to the transmission of *Cryptosporidium* through waterborne sources (3). Additionally, over 8 million cases of cryptosporidiosis were reported annually due to foodborne outbreaks (4). Therefore, cryptosporidiosis holds immense significance in public health, necessitating proactive measures to prevent and control its occurrence.

By using genotyping technology, over 170 species and genotypes of *Cryptosporidium* have been identified, existing across a diverse range of hosts (5). Human cryptosporidiosis is primarily attributed to either the anthroponotic *C. hominis* or zoonotic *C. parvum*. Additionally, humans can become infected with another 20 species/genotypes of *Cryptosporidium* (6). Although these infections occur at a lower frequency, recently, there has been a noticeable increase in reports of human infections caused by species other than *C. hominis* and *C. parvum*, such as *C. meleagridis*, *C. ubiquitum*, *C. cuniculus*, *C. andersoni* and *C. viatorum* (5, 6). These species of *Cryptosporidium* possess the ability to infect a diverse array of animals, and the majority of human infections caused by them may occur through animals, either via direct contact or ingestion of feces-contaminated oocysts in water or food (6). To effectively contain the transmission of *Cryptosporidium*, it is crucial to embrace a "One Health" approach that recognizes the intricate interdependence between humans, animals, and the environment (7). Rodents, which are widely distributed globally with a vast array of activities, maintain close ties to humans, animals, and the environment. Consequently, they exert significant influence on their ecosphere, particularly due to their ability to transmit *Cryptosporidium* oocysts into the environment, thereby affecting both humans and animals (8).

Extensive research has been conducted on rodents infected with *Cryptosporidium*, revealing an average prevalence of 19.8% when molecular detection methods are employed (8). Molecular confirmation has identified more than 26 species and 59 genotypes of *Cryptosporidium* across more than 54 rodent species (5, 8). Although most species and genotypes are host-specific or exhibit a limited host range, virtually all known *Cryptosporidium* species and genotypes capable of infecting humans have been detected in rodents (5, 8). Consequently, rodents pose a significant public health risk as reservoirs of zoonotic *Cryptosporidium* species. To effectively evaluate the prevalence of *Cryptosporidium* in rodents and support the development of policies aimed at preventing its transmission to humans and other animals, continuous monitoring of *Cryptosporidium* in rodents, particularly wild rats, is imperative, especially in regions where no sampling conducted before.

In China, the Inner Mongolian Autonomous Region and Liaoning Province are mainly dependent on agriculture and animal husbandry as their economic sources. Rodents are widely distributed in these regions and are active on farms and livestock farms. However, currently, the prevalence of *Cryptosporidium* in rodents, especially wild ones, in these two provinces is still unclear. Therefore, this study aimed to conduct a molecular diagnosis of *Cryptosporidium* in wild rodents in the Inner Mongolian Autonomous Region and Liaoning Province of China, determine *Cryptosporidium* infection rates and evaluate the risk of zoonotic transmission of *Cryptosporidium* at the species level.

2 Materials and methods

2.1 Ethical concerns

The protocols used in the present study underwent a meticulous review process and were ultimately approved by the Research Ethics Committee of Wenzhou Medical University (approval number SCILLSC-2021-01).

2.2 Sample collection

Between November 2023 and February 2024, a cumulative total of 486 wild rodents were collected, with 229 rodents originating from Harqin Banner in Inner Mongolia and 257 rodents originating from Jianping County in Liaoning Province, China (Figure 1 and Table 1). Rodents were captured by utilizing cage traps baited with a mixture of peanut and sunflower seeds. For each designated capture location, approximately 50 cage traps were methodically placed in a straight line, ensuring a uniform spacing of 5 meters between each trap and effectively establishing transects. At 4:00 PM, the transects were positioned and retrieved the following morning at 8:00 AM. Each rodent captured was euthanized humanely via CO_2 asphyxiation and promptly transported to the laboratory within 48 h, ensuring its safety in sealed containers containing ice. A fecal sample weighing 0.5 grams was collected from the rectum of each rodent.

2.3 DNA extraction

Exclusively designated for DNA extraction, 0.2 grams of each fecal sample was processed, while the remaining portion was preserved as a backup and stored at a chilled temperature of −80°C. Using the QIAamp DNA Mini Stool Kit (Qiagen, Germany), genomic DNA was extracted from each processed sample. During the extraction process, the lysis temperature was increased to 95°C, while all other steps were performed strictly according to the manufacturer's guidelines. Subsequently, the DNA was reconstituted in 200 µL of AE elution buffer, provided with the kit, and was subsequently stored at −20°C prior to PCR analysis.

2.4 Identification of rodent species

To identify the rodent species, the vertebrate cytochrome b (*cytb*) gene (421 bp) was amplified via PCR from the fecal DNA. The primer sequences were 5'-TACCATGAGGACAAATATCATTCTG-3′ and 5'-CCTCCTAGTTTGTTAGGGATTGATCG-3′, and PCR conditions were as follows: 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 30 s. Initial denaturation

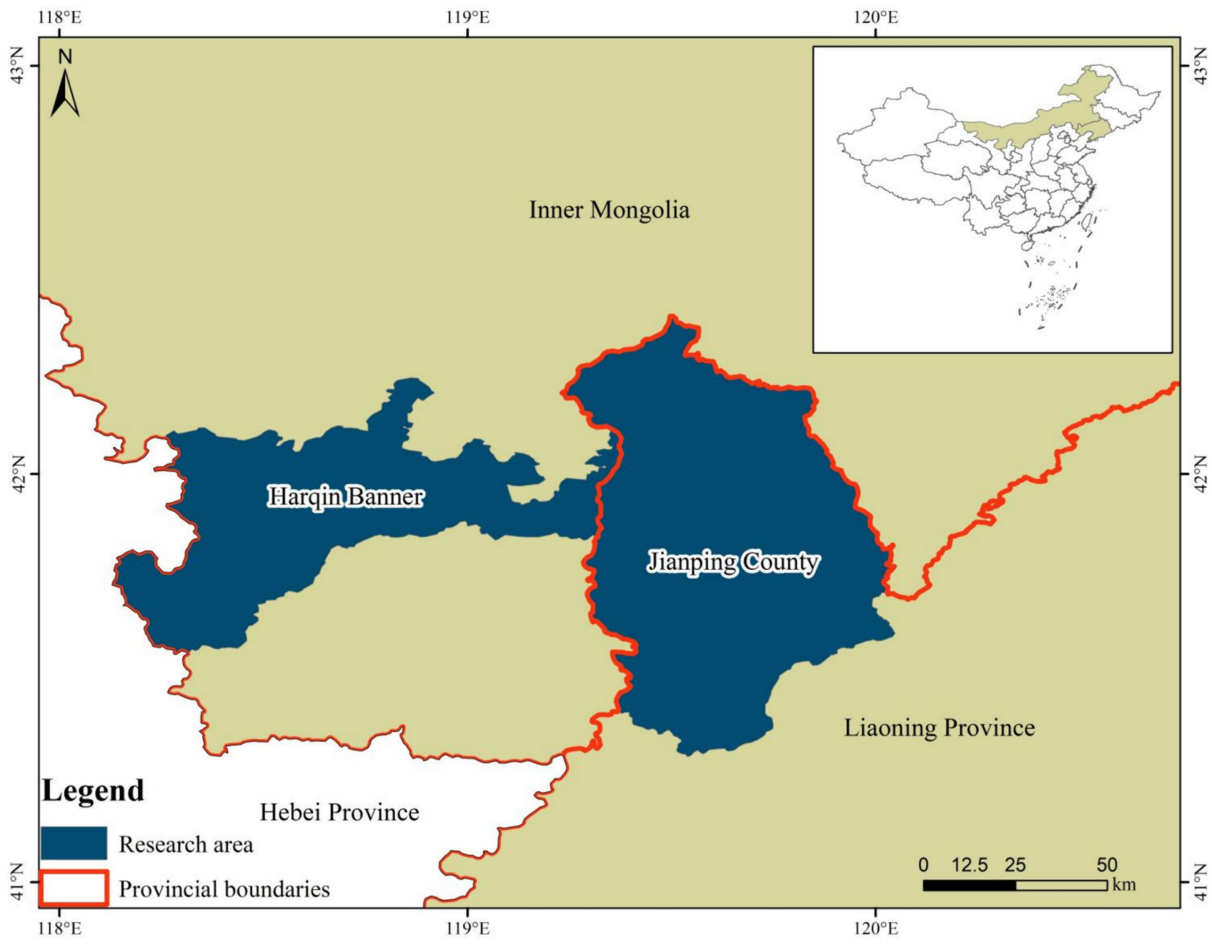

FIGURE 1
Map of the wild rodent sampling location in the Inner Mongolian Autonomous Region and Liaoning Province, China.

was performed at 94°C for 5 min, followed by a final extension at 72°C for 5 min. These conditions followed the previously described protocol by Verma and Singh (10).

2.5 *Cryptosporidium* genotyping

All DNA samples were subjected to nested PCR utilizing primers previously developed by Xiao et al. in 1999 to amplify an 830 bp fragment of the partial small subunit ribosomal RNA (*SSU rRNA*) gene of *Cryptosporidium* (9). The primers used for the primary PCR were 5'-TTCTAGAGCTAATACATGCG-3′ and 5'-CCCTAATCC TTCGAAACAGGA-3′, while the primers used for the secondary PCR were 5′-GGAAGGGTTGTATTTATTAGATAAAG-3′ and 5'-AAGGA GTAAGGAACAACCTCCA-3′. Both PCR amplification steps were conducted under identical conditions, commencing with initial denaturation at 94°C for 3 min. This was followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. TaKaRa Taq DNA Polymerase was used for PCR amplification, with positive controls using DNA from chickens infected with *C. baileyi* and negative controls using deionized water without DNA templates. PCR products were analyzed via gel electrophoresis on a 1.5% agarose gel in TAE buffer, with GelRed (Biotium Inc., Fremont, California, United States) serving as the staining agent.

2.6 Sequencing analysis

The PCR products of the expected size were purified using a DNA gel purification kit from Sangon Biotech (Shanghai, China). These purified products were then sequenced using the Sanger sequencing method by Sangon Biotech (Shanghai) Co., Ltd., on an ABI Prism 3,730 XL DNA analyzer. Sequencing was performed with the same primers used for the secondary PCR and was facilitated by a BigDyeTerminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, United States). To guarantee the precision of the nucleotide sequence, sequencing was carried out from both ends of the product, and further PCR products were sequenced whenever mutations were identified. After acquiring the sequences, they were carefully edited using DNASTAR Lasergene version 7.1.0 and aligned with reference sequences retrieved from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) using the basic local alignment search tool (BLAST) and ClustalX 2.0 software (http://www.clustal.org/) to accurately identify the *Cryptosporidium* species.

TABLE 1 Prevalence and distribution of *Cryptosporidium* species/genotypes in the investigated rodents from the Inner Mongolian Autonomous Region and Liaoning Province of China.

Regions	Rodent species	Positive/examined (%)	Species/genotype of *Cryptosporidium* (*n*)
Liaoning (Jianping)	*Apodemus agrarius*	3/62 (4.3)	*C. ubiquitum* (1), *C. viatorum* (1), Rat genotype III (1)
	Cricetulus barabensis	1/36 (2.8)	*C. occultus* (1)
	Mus musculus	1/28 (3.6)	*C. muris* (1)
	Rattus norvegicus	11/103 (10.7)	Rat genotype III (4), Rat genotype IV (4), *C. occultus* (3)
	Subtotal	16/229 (7.0)	Rat genotype III (5), *C. occultus* (4), Rat genotype IV (4), *C. muris* (1), *C. ubiquitum* (1), *C. viatorum* (1)
Inner Mongolia (Harqin Banner)	*Apodemus agrarius*	2/27 (7.4)	Rat genotype III (2)
	Cricetulus barabensis	5/60 (8.3)	Rat genotype IV (4), *C. occultus* (1)
	Mus musculus	6/78 (7.7)	Rat genotype III (3), *C. ubiquitum* (2), *C. muris* (1)
	Rattus norvegicus	7/92 (7.6)	*C. ubiquitum* (5), *C. ratti* (1), Rat genotype IV (1)
	Subtotal	20/257 (7.8)	*C. ubiquitum* (7), Rat genotype IV (5), Rat genotype III (5), *C. muris* (1), *C. occultus* (1), *C. ratti* (1)
Total		36/486 (7.4)	Rat genotype III (10), Rat genotype IV (9), *C. ubiquitum* (8), *C. occultus* (5), *C. muris* (2), *C. viatorum* (1), *C. ratti* (1)

2.7 Phylogenetic analyses

The SSU rRNA sequences of *Cryptosporidium* spp. obtained in this study were combined with reference sequences to construct a phylogenetic tree using Mega 7.0 software. The Tamura–Nei model-based Maximum Likelihood method was chosen to analyze the phylogenetic relationships. To ensure the reliability of the evolutionary tree, a bootstrap analysis was conducted with 1,000 replicates. The reference sequences necessary for tree construction were retrieved from GenBank and previous research studies.

2.8 Statistical analyses

Statistical analysis was performed utilizing SPSS version 22.0 (SPSS Inc., United States). The chi-square test was utilized to determine the disparities in the occurrence of *Cryptosporidium* spp. across diverse regions and rodent species. A p value less than 0.05 was considered to indicate statistical significance.

2.9 Nucleotide sequence accession numbers

The nucleotide sequences of *Cryptosporidium* obtained in this study have been deposited in the GenBank database under accession numbers PP527771 to PP527783.

3 Results

3.1 Rat species identification

In this study, PCR and sequencing analysis of the *cytb* gene revealed the presence of four rodent species: *Apodemus agrarius* ($n = 89$), *Cricetulus barabensis* ($n = 96$), *Mus musculus* ($n = 106$) and *Rattus norvegicus* ($n = 195$). No additional data were gathered for these wild rodents (Table 1).

3.2 Prevalence of *Cryptosporidium* infection

Nested PCR was performed on 486 fecal samples to assess the presence of *Cryptosporidium* species by analyzing the *SSU rRNA* gene. The results revealed that 36 samples were positive for this parasite, yielding an average infection rate of 7.4%, with 7.0% (16/229) in Liaoning (Jianping) and 7.8% (20/257) in Inner Mongolia (Harqin Banner) (Table 1). Statistical analysis did not indicate any significant differences in *Cryptosporidium* prevalence between the two regions ($\chi^2 = 0.11$, df = 1, $p = 0.74$). Regarding rodent species variation, the highest infection rate of *Cryptosporidium* was observed for *R. norvegicus* (9.2%; 18/195), followed by *M. musculus* (6.6%; 7/106), *A. agrarius* (5.6%; 5/89), and *C. barabensis* (6.3%; 6/96). The difference in the infection rate of *Cryptosporidium* among the rodent species groups was not statistically significant ($\chi^2 = 1.65$, df = 3, $p = 0.65$).

3.3 Distribution of *Cryptosporidium* species/genotypes

Five species of *Cryptosporidium*, namely, *C. ubiquitum* ($n = 8$), *C. occultus* ($n = 5$), *C. muris* ($n = 2$), *C. viatorum* ($n = 1$), and *C. ratti* ($n = 1$), as well as two genotypes—*Cryptosporidium* Rat genotype III ($n = 10$) and *Cryptosporidium* Rat genotype IV ($n = 9$)—have been identified through sequencing the PCR products of 36 *Cryptosporidium*-positive samples (Table 1).

In Liaoning, *Cryptosporidium* Rat genotype III emerged as the dominant species, accounted for 31.3% (5/16) of the positive samples,

followed by *C. occultus* and *Cryptosporidium* Rat genotype IV each comprising 25.0% (4/16). The remaining three species, *C. muris*, *C. ubiquitum*, and *C. viatorum*, contributed equally with a share of 3.3% (1/16) each. On the other hand, in Inner Mongolia, *C. ubiquitum* emerged as the predominant species, accounting for 35.0% (7/20) of the positive samples. *Cryptosporidium* Rat genotype III and *Cryptosporidium* Rat genotype IV followed closely, each comprising 25.0% (5/20) of the positive samples. The remaining species, *C. muris*, *C. occultus*, and *C. ratti*, contributed 5.0% (1/20) each (Table 1).

Among the different rodent species, *R. norvegicus* carried a diverse range of species/genotypes of *Cryptosporidium*, including *C. ubiquitum*, *C. ratti*, *C. occultus*, *Cryptosporidium* Rat genotype III and *Cryptosporidium* Rat genotype IV. In contrast, *C. barabensis* was limited to carrying only *C. occultus* and *Cryptosporidium* Rat genotype IV. The remaining two rodent species each harbored three species/genotypes: *C. ubiquitum*, *C. viatorum* and *Cryptosporidium* Rat genotype III in *A. agrarius* and *C. ubiquitum*, *C. muris* and *Cryptosporidium* Rat genotype III in *M. musculus* (Table 1).

3.4 Genetic identification of *Cryptosporidium* species/genotypes

Among the nine SSU rRNA sequences belonging to *Cryptosporidium* Rat genotype IV, six sequences were found to be identical to each other, sharing perfect 100% similarity with the *Cryptosporidium* genotype W19 variant sequence (AY737581) previously isolated from water samples in the United States (US). The three remaining sequences of *Cryptosporidium* rat genotype IV were identical to each other and had not been previously described. They exhibited a remarkable similarity of 99.87% to the *Cryptosporidium* genotype W19 variant sequence (AY737582), which was also detected in US waters. The sole difference among them was a single nucleotide substitution, specifically from A to G, at position 441 (Table 2).

Ten SSU rRNA sequences of *Cryptosporidium* Rat genotype III revealed five types, with one type represented in five samples sharing an identical sequence (JX294367) with *Cryptosporidium* Rat genotype III from wild black rats in northern Australia. The second type represented two samples were novel, exhibiting 99.01% similarity (12 nucleotide differences, including 9 substitutions and 3 insertions) with JX294363, which was found in wild black rats from Australia. The remaining three types were each found in a single sample and were previously undescribed, differing from the *Cryptosporidium* Rat genotype III sequence (JX294367) of wild black rats from northern Australia by three (T to C at position 482 and T delete at positions 439 and 440), seven (T to G at position 93, G to A at position 398, T to C at position 438, T delete at positions 439 and 440, T to C at position 481, G to A at position 599), and six (T to C at position 481, G to A at position 559, T to C at position 593, T to A at position 683, G to A at position 739, and G to T at position 755) nucleotides (Table 2).

The present study identified eight sequences of *C. ubiquitum* that were consistent with each other and exhibited 100% similarity to the *C. ubiquitum* sequence (MW043441) isolated from cattle in Bangladesh. The two *C. muris* sequences were also identical and exhibited 100% similarity with MW090931, which was isolated from wastewater and sewage in Guangzhou, China (Table 2).

Among the five sequences of *C. occultus* obtained in this study, two exhibited 100% homology with MG699179, which was identified in *Meriones unguiculatus* from the Czech Republic. The remaining three sequences of *C. occultus* were homologous to each other and were novel, sharing 99.51% similarity with MG699179, differing by four nucleotides (Table 2).

The sequences of *C. ratti* and *C. viatorum* identified here were novel and differed by one nucleotide from MT504541 in *R. norvegicus* in the Czech Republic and from MK522269, which was found in *Leopoldamys edwardsi* from China (Table 2).

The phylogenetic analysis of the ssu rRNA sequences has confirmed that the sequences obtained in the present study, corresponding to *C. viatorum*, *C. ubiquitum*, *C. occultus*, *Cryptosporidium* Rat genotype IV, *C. ratti*, *Cryptosporidium* Rat genotype III, and *C. muris*, have clustered together with their respective reference sequences, forming distinct and clearly identifiable groups within the phylogenetic tree (Figure 2).

4 Discussion

Cryptosporidium infections among rodents have been reported in 19 countries, with global prevalence rates ranging from 0.7 to 100%. The overall average infection rate for rodents is 19.8%, indicating a widespread distribution of this parasite in rodent populations worldwide (8, 11). The present study revealed an average positive rate of 7.4% (36/486) for *Cryptosporidium* among the surveyed wild rodents. Explaining the disparities in prevalence rates among studies is challenging due to the numerous influencing factors. Although the four wild rodent species (*R. norvegicus*, *M. musculus*, *A. agrarius*, and *C. barabensis*) investigated in this study did not show significant differences in infection rates, rodent species may still have an impact on *Cryptosporidium* infection rates. For instance, Zhang et al. recently summarized the occurrences of *Cryptosporidium* infections across 54 rodent species, encompassing wild, domestic pet, farm, and laboratory animals. Specifically, the prevalence rates among these rodent categories are as follows: 20.5% for wild animals, 27.0% for domestic pets, 14.5% for farm animals, and 2.7% for laboratory animals (8). Additionally, geographical location is another crucial factor, with overall infection rates varying across Asia, Europe, South America, North America, and Africa at 18.6, 28.0, 15.2, 7.3, and 2.2%, respectively (11). However, it is worth noting that these rates could be influenced by the limited number of studies conducted in each region. Specifically, Africa, South America, and North America have only one or two studies, thus limiting their representativeness (8, 11). Therefore, to gain a comprehensive understanding of the epidemiology of *Cryptosporidium* in rodents, it is imperative to conduct broader geographical surveys that encompass a more diverse range of species and individuals.

The present study identified five species and two genotypes of *Cryptosporidium* among the surveyed wild rodents. Among these, *C. ratti* and *Cryptosporidium* Rat genotypes III, and IV are predominantly found in rodents, exhibiting a narrow host range that is typically rodent-specific (5). Although sporadically reported in other animals, such as camels, goats, black bears and cats, these species and genotypes have not been documented in humans and are rarely encountered in other hosts, rendering their potential pathogenicity uncertain (5, 12–15). Nevertheless, their ability to be detected in streams in the US and in raw sewage water in various countries

TABLE 2 Sequence similarity analysis of *Cryptosporidium* species/genotypes in this study with reference sequences from GenBank.

Cryptosporidium species/genotypes (n)	Accession number	Identities (Nucleotide difference at position)	Ref accession numbers in host from country
C. muris (2)	PP527778	100% (/)	MW090931 in wastewater and sewage from China
C. occultus (2)	PP527779	100% (/)	MG699179 in *M. unguiculatus* from the Czech Republic
C. occultus (3)	PP527780	99.51% (T to A at position 444, a T insertion at position 446, A to T at positions 482 and 488)	MG699179 in *M. unguiculatus* from the Czech Republic
C. ratti (1)	PP527782	99.87% (G to T, at position 81)	MT504541 in *R. norvegicus* from the Czech Republic
C. ubiquitum (8)	PP527777	100% (/)	MW043441 in cattle from Bangladesh
C. viatorum (1)	PP527783	99.87% (G to A, at position 502)	MK522269 in *Leopoldamys edwardsi* from China
Rat genotype III (2)	PP527781	99.01% (12 nucleotide differences, including 9 substitutions and 3 insertions)	JX294363 in wild black rats from Australia
Rat genotype III (5)	PP527773	100% (/)	JX294367 in wild black rats from northern Australia
Rat genotype III (1)	PP527774	99.51% (T to C at position 482 and T delete at positions 439 and 440)	JX294367 in wild black rats from northern Australia
Rat genotype III (1)	PP527775	99.15% (T to G at position 93, G to A at position 398, T to C at position 438, T delete at positions 439 and 440, T to C at position 481, G to A at position 599)	JX294367 in wild black rats from northern Australia
Rat genotype III (1)	PP527776	99.27% (T to C at position 481, G to A at position 559, T to C at position 593, T to A at position 683, G to A at position 739, and G to T at position 755)	JX294367 in wild black rats from northern Australia
Rat genotype IV (6)	PP527771	100% (/)	AY737581 in water from the US
Rat genotype IV (3)	PP527772	99.87% (A to G, at position 441)	AY737582 in water from the US

underscores the need for further investigations to delineate their actual host range and assess their impact on public health (16–19).

Cryptosporidium muris, a dominant parasite in rodents, has been identified in more than 20 rodent species as well as in pigs, pigeons, camels, black-boned goats, sheep, horses, and captive zoo animals (5, 6). Multiple reports exist of *C. muris* infections in humans, primarily in low-income countries and HIV$^+$ patients, with limited reports in high-income nations (20). Although our study identified *C. muris* in only two specimens of *Mus musculus*, this finding not only confirms that *Mus musculus* is the primary host of *C. muris* but also suggests that *C. muris* infection may serve as a significant link in disease transmission to humans and other animals. Additionally, *C. occultus* primarily infects rats and has also been reported in ruminants such as cattle, water buffaloes, yaks, deer, alpacas and bactrian camels (5, 21). Limited human cases have also been reported (22). This study is the first to identify *C. occultus* in *R. norvegicus* and *C. barabensis*, further expanding its host range and indicating that these two rodent species play active roles in the transmission of this parasite.

Cryptosporidium ubiquitum and *C. viatorum* are two commonly encountered zoonotic species that infect humans (6). Among these, *C. ubiquitum* often occurs in rodent species, encompassing 21 distinct genotypes, exhibiting an exceptional capacity to infect a diverse array of hosts, such as primates, carnivores, and ruminants (5, 23). In the present study, *C. ubiquitum* was identified in *R. norvegicus*, *A. agrarius*, and *M. musculus*, with a preponderance in *R. norvegicus* from Inner Mongolia. This observation might suggest the potential for cross-species transmission of *C. ubiquitum* between rodents and goats/sheep, considering its widespread detection in these animals in Inner Mongolia (24). Although no human cases of *C. ubiquitum* infection have been documented in this region thus far, the known pathogenicity of this species toward humans cannot be discounted, given its numerous reported cases in the United States, Canada, and the United Kingdom (23). Therefore, individuals, particularly those residing in Inner Mongolia, should exercise caution and refrain from contact with brown rats and other wild rodents to mitigate the risk of cryptosporidiosis transmission from rodent sources. Moreover, it has been confirmed here that *A. agrarius* has been infected with *C. viatorum*, which initially identified in humans in 2012 (25). Cases of *C. viatorum* infection have been documented in 13 countries, including China (22). Currently, *C. viatorum* has only been described in rodent species such as *R. rattus*, *R. lutreolus*, *Leopoldamys edwardsi*, and *Berylms bowersi* (26–28). These findings suggest that rodents serve as the primary hosts for this parasite, further emphasizing their crucial role in its transmission.

FIGURE 2
Phylogenetic tree of *Cryptosporidium* species based on partial *SSU rRNA* gene sequences (~800 bp). The tree was generated using the Maximum Likelihood method based on the Tamura-Nei model. Bootstrap values (> 50%) derived from 1,000 replicates are displayed to the left of the nodes for reliability assessment. The sequences generated in the present study are indicated with solid circles.

5 Conclusion

This study revealed a 7.4% infection rate of wild rodents with *Cryptosporidium* spp. in the Inner Mongolian Autonomous Region and Liaoning Province, China. Molecular analysis revealed the presence of nonhuman infectious *C. ratti*, *Cryptosporidium* rat genotypes III and IV, and zoonotic species such as *C. ubiquitum*, *C. occultus*, *C. muris*, and *C. viatorum*. These findings indicate that rodents may play a crucial role in maintaining and disseminating these infections, posing a potential risk to public

health. Therefore, a comprehensive multidisciplinary "One Health" approach is imperative to gain a thorough understanding of rodent-related *Cryptosporidium* and potential transmission routes.

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 protocols of the present study underwent a rigorous review process and were ultimately approved by the Research Ethics Committee of Wenzhou Medical University, with the approval number SCILLSC-2021-01. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LL: Writing – review & editing, Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. Q-X: Methodology, Writing – original draft, Writing – review & editing, Funding acquisition, Resources. AJ: Writing – original draft, Writing – review & editing, Investigation, Methodology. FZ: Investigation, Methodology, Writing – review & editing, Writing – original draft. WZ: Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition, Resources. FT: Conceptualization, Data curation, 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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Morphology, morphometry, and phylogeny of the protozoan parasite, *Eimeria labbeana*-like (Apicomplexa, Eimeriidae), infecting *Columba livia domestica*

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Introduction: *Eimeria* spp. are intracellular protozoan parasites of the phylum Apicomplexa causing economic losses to various wild and domestic animals. An eimerian species infecting *Columba livia domestica* was identified in this study.

Methods: A total of 15 faecal samples were examined by floatation technique, a prevalence rate of 60% was reported. Eimerian oocysts were sporulated in 2.5% potassium dichromate solution then identified using morphological and molecular (DNA amplification of the *18S rRNA* and *ITS-1* genes) diagnostic techniques.

Results: Sporulated oocysts were identified as *Eimeria labbeana*-like, after morphometry with typical bi-layered wall with spherical to subspherical oocysts morphology. A polar granule is present, but no micropyle or oocyst residuum. Sporocysts are elongated ovoidal with stieda body. Sporocyst residuum with many granules and sporozoites with refractile bodies and nucleus. Both *18S rRNA* and *ITS-1* sequences have been deposited in GenBank database. DNA sequences from the partial *18S rRNA* generated from the oocysts were found to be related to eimerian and isosporan parasites found in domestic pigeons. For the first time, *ITS-1* sequences for *E. labbeana*-like were provided.

Conclusion: The necessity of using molecular techniques to describe pigeon intestinal coccidian parasites in conjunction with traditional morphology-based tools was emphasized in this work in order to understand the biology of such parasites.

KEYWORDS

pigeons, coccidia, molecular technique, phylogeny, Saudi Arabia

Introduction

Coccidiosis is a parasitic disease of all bird's intestinal tract caused by protistan parasites the genera of *Eimeria*, *Isospora*, *Caryospora*, and *Tyzzeria* (1, 2). Because of the walls of oocysts, these coccidian organisms may survive in the environment. Infected birds discharge microscopic oocysts in their feces, causing other birds to become infected via ingesting sporulated oocysts. The discharged oocysts require a time, in the surrounding environment outside the host, to sporulate to produce sporulated oocyst containing sporozoites within sporocysts (infective stage) that can infect another host, hence completing the life cycle (3). Disease may have a negative impact on farm animals

by costs for treatment, prevention, eradication, decontamination, and restocking. In birds, life cycle of members of the genus *Eimeria* begins when sporulated oocysts are ingested by susceptible birds. Coccidia infiltrates the intestinal lining after being ingested, undergo both sexual and asexual reproduction, and cause tissue damage (4). Post-mortem examination of the host and fecal examination can confirm the existence of this disease (5–7).

Several species of the genus *Eimeria* have been described infecting pigeons employing the traditional morphological description, parasite biology, and typical macroscopic lesions, including *E. chalcoptereae* (8), *E. choudari* (9), *E. columbae* (10), *E. columbapalumbi* (11), *E. columbarum* (12), *E. columbinae* (13), *E. curvata* (14), *E. duculai* (15), *E. gourai* (15), *E. janovyi* (16), *E. kapotei* (17), *E. labbeana* (18), *E. labbeana*-like (19), *E. livialis* (20), *E. mauritiensis* (21), *E. palumbi* (22), *E. sphenocerae* (23), *E. tropicalis* (24), *E. turturi* (25), *E. waiganiensis* (26), and *E. zenaidae* (27). *E. labbeana* is the most pathogenic and often reported species, located in small intestine of pigeons and causing diarrhea, enteritis, and even mortality (19).

However, due to inadequate description and lack of measurements for several eimerian species from the Columbidae in the past, it has been difficult to assign and confirm identities of existing species. Duszynski et al. (28) stated that just two species (*E. labbeana* and *E. columbarum*) are likely to occur in pigeons and considered as valid species. Due to these challenges, molecular methods are required to reliably delimit taxa and infer phylogenetic relationships among members of the genus *Eimeria* (29). Several approaches based on the polymerase chain reaction (PCR) have been developed to characterize avian eimerian species, including the amplification of the nuclear genes such as small subunit (8, 13, 19, 30), large subunit (8, 19) rRNA; and the internal transcribed spacer region 1 (ITS-1) (5), as well as the mitochondrial cytochrome c oxidase subunit I (COI) (8, 13, 19, 31).

This study was carried out to describe and characterize the eimerian oocysts recovered from domestic pigeons using morphological and molecular tools.

Materials and methods

Sample collection

A commercial poultry farm in Riyadh (Saudi Arabia) yielded 15 specimens of domestic pigeon, (*C. livia domestica*). Pigeons were housed indoors in well-ventilated cages with free access to food and water *ad libitum* and were raised following the institution's criteria for animal care and use in research (approval number KSU-SU-23-45).

Fecal examination

Fecal samples, from each bird, weighing around 1 g were collected in separate screw-capped plastic containers labeled properly and delivered to the Parasitology Laboratory Research at the Department of Zoology, College of Science. The samples were initially analyzed to determine their consistency and color, as well as the presence of mucus, blood, and other contaminants. Standard microscopical procedures were used to examine the presence or absence of coccidia oocysts. Flotation technique with Sheather's sucrose solution (specific gravity 1.27) was employed in order to concentrate the oocysts in positive samples (32).

Sporulation of oocysts

According to Levine (33), the oocysts were placed in a 2.5% (w/v) potassium dichromate solution, left at room temperature, and checked to track the sporulation process. For further investigation, the sporulated oocysts were washed three times in phosphate-buffered saline and stored at 4°C.

Morphology and morphometry

Following the standards of Silva et al. (2) and Saikia et al. (5), eimerian species were identified based on oocyst morphology and sporulation time. Photographs were taken with a Leica DM 2500 microscope (NIS ELEMENTS software, version 3.8). The size (including length and width) and shape index (length/width ratio) of 50 oocysts from each fecal sample were measured using ocular micrometer. All measurements are given in microns (μm) and a range (mean in parentheses) using ImageJ 1.53e software (Wayne Rasband and contributors, National Institute of Health, United States).

Molecular techniques

DNA extraction

Purified oocysts were suspended in 100 μL sodium hypochlorite at 65°C for 45 min. For 1 h at 65°C, the samples were combined with 350 μL of CTAB extraction buffer (2% cetyltrimethylammonium bromide, 1% polyvinylpyrrolidone, 100 mM Tris–HCl, 1.4 M NaCl, 20 mM EDTA) (34). An ultrasonicator (Thermo Fischer Scientific, United States) was used to disrupt the rigid wall of sporulated oocysts. The genomic DNA was extracted from excysted sporozoites using Isolate II fecal DNA extraction kit (Meridian Bioscience, London, United Kingdom). DNA samples were kept at −20°C until further processing.

Polymerase chain reaction

The methods described by Al-Quraishy et al. (35) to amplify the *18S rRNA* and *ITS-1* regions were used for PCR. The PCR reaction was carried out in accordance with the suggested PCR conditions and the genus-specific primers published by Orlandi et al. (36) for the *18S rRNA* and Kawahara et al. (37) for the *ITS-1* regions. Gel electrophoresis of amplified DNA was run on 1.5% (w/v) agarose gel (Sigma-Aldrich, United States) stained with SYBR Safe DNA gel dye (Thermo Fischer Scientific, Canada) was used to visualize PCR results. The gel was loaded with a DNA ladder (100 bp DNA, Fermentas) and the expected product size was visualized using a gel documentation system (BioRad, United States).

Sequencing and phylogenetic analysis

Positive PCR products were sequenced in the forward direction using Macrogen® sequencing facility (Seoul, South Korea). The identity of the generated sequences was checked using a BLAST search and aligned with relevant sequences using the CLUSTAL-X method

(38). The phylogenetic trees were generated using Bayesian Inference (BI) and maximum likelihood (ML) methods using Mr. Bayes and MEGA 11 software, respectively (39, 40). Distances were estimated using the Kimura 2-parameter model, and the numbers at the branch of the tree demonstrate bootstrap support from 1,000 replications. Markov Chain Monte Carlo chains were run for 2,000,000 generations, the log-likelihood scores were plotted, and the final 75% of trees were used to produce consensus trees. The *18S rRNA* gene sequence of *Toxoplasma gondii* (L24381) was included in the tree as an outgroup.

Results

Gross examination revealed color and consistency variations in the fecal samples, including greenish feces and watery diarrhea, in 9 of 15 samples. Microscopic examination recorded that that 60% ($n = 9$) of 15 fecal samples contained unsporulated coccidian oocysts, and the affected pigeons expressed weakness and reduced appetite. Unsporulated oocysts reached full sporulation after 1–2 days when left at 2.5% $K_2Cr_2O_7$ at room temperature (25± 2°C). Sporulated oocysts recovered in the present study correspond with the description criteria of the genus *Eimeria*, with close similarity to *Eimeria labbeana*-like as described below.

Morphology and morphometry

The sporulated oocysts were spherical to subspherical in shape (Figure 1A). The oocyst wall was bilayered (Figures 1A,C), the outer layer was thinner than the inner layer measuring 1.4–1.7 (1.5). Fifty oocysts were measured, with sizes ranging from 18.8 to 21.9 in length and 15.9–16.7 in width (Table 1). The average size was 20.4 × 16.4 µm without a micropyle or oocyst residuum (Table 1). Their length-width ratio (shape index) ranged from 1.2 to 1.3 (1.2) (Table 1). The oocyst possessed an ovoid polar granule. Oocysts sporulation within 24–36 h. The sporocysts were elongated ovoidal with a single-layered (Figures 1A,B), ranging in size from 11.9 to 13.8 in length and 5.1–6.5 in width (Table 1). Sporocysts had an average size of 12.7 × 5.9 µm (Table 1). Their shape index ranged from 1.9 to 2.1 with a mean of 2.1. Stieda body was present, 0.7–1.0 (0.8) × 1.2–0.9 (1.1) µm, however, substieda body is not present. A sporocyst residuum is a spherical mass made up of several granules (Figures 1A,B). Sporozoites were elongated, lying lengthwise head to tail inside the sporocyst, with two refractile bodies (Figures 1A–C), one of which is spherical and 3.1–3.8 (3.5) × 1.5–2.2 (1.9) µm. A nucleus was seen directly in the posterior refractile body (Figures 1A,B).

Molecular analysis

Partial *18S rRNA* and *ITS-1* gene regions were successfully amplified and yielded ~613 and ~600 bp, respectively. Two sequences of were obtained from the partial *18S rRNA* and were deposited in GenBank database with the accession numbers OR264478 and OR264479. The two sequences were identical with only one mutation at position 182 of the alignment (with a transversion C/G). Phylogenetic analysis revealed that the two sequences generated from the *E. labbeana*-like in the present study shared a common ancestor with *E. labbeana*-like from the GenBank database (KT305927 from *C. livia domestica* from Australia) with high ML bootstrap values and high BI posterior probability as

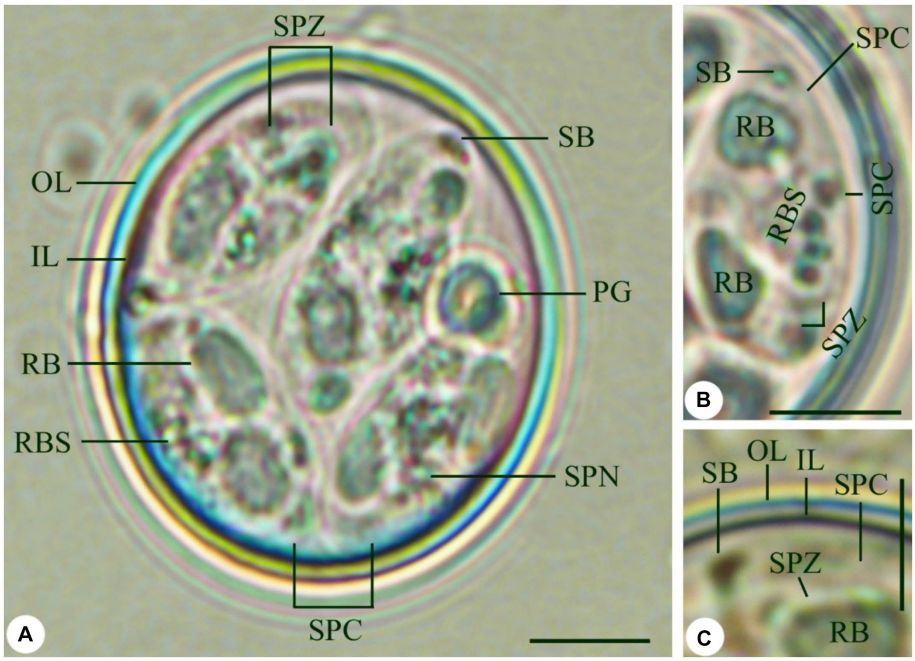

FIGURE 1
Eimeria labbeana-like infecting pigeons. **(A)** Sporulated oocyst. **(B,C)** High magnifications of sporocyst with sporozoites and refractile body (OL, Outer layer; IL, Inner layer; RF, Refractile body; SB, Stieda body; PG, Polar granule; SPC, Sporocyst; RBS, Residuum of sporocyst; SPZ, Sporozoite; SPN, Sporozoite nucleus) Scale = 5 µm.

TABLE 1 Morphological characteristics of sporulated oocysts for the recovered *Eimeria labbeana* and *E. labbeana*-like species from Columbidae.

Eimeria species	Host species	Oocysts			Micropyle	Residuum	Sporocyst size		Locality
		Shape	Length	Width			Length	Width	
Eimeria labbeana Pinto (18)	*C. livia*	Subspherical to ovoidal	17–21	16–18	+	–	11–14 (12.4)	5–7 (6.4)	Asia, India
Eimeria labbeana Nieschulz (12)	*C. livia*	Subspherical to ellipsoidal	15–18 (16.7)	14–16 (15.3)	–	–	12.4	6.4	Asia, India
Eimeria labbeana-like Yang et al. (19)	*C. livia*	Subspherical	18.9–22 (20.2)	15.7–18.9 (16.1)	–	+	12.5–14.5 (13)	5.5–7 (6.1)	Australia
Eimeria labbeana Elseify et al. (45)	*Coturnix ypsilophora*	Subspherical to spherical	21.5–22.6	16.9–19.8	–	– –	10.54–16.68	6.2–10.6	Egypt
Eimeria labbeana Saikia et al. (5)	*C. livia domestica*	Subspherical to spherical	19.50–23.43 (21.02)	16.41–19.03 (17.98)	–	–	– –	– –	India
Eimeria labbeana Joseph et al. (46)	*C. livia domestica*	Subspherical	16.5	15	–	–	– –	– –	Nigeria
Eimeria labbeana Aboelhadid et al. (52)	*C. livia domestica*	Subspherical to ovoidal	15–18.9	14–17.5	–	–	– –	– –	Egypt
Eimeria labbeana Al-Agouri et al. (53)	*C. livia domestica*	Subspherical to spherical	16.5	15	–	+	– –	– –	Libya
Eimeria labbeana Oliveira et al. (30)	*Streptopelia decaocto*	Subspherical to ellipsoidal	16–21 (18.7)	14–18 (15.7)	+	–	10–14 (12.2)	5–7 (6.4)	Portugal
	C. palumbus	Subspherical to ellipsoidal	16–21 (19)	14–18 (15.9)	+	–	10–14 (12.3)	5–7 (6.0)	Portugal
Eimeria labbeana-like (Present study)	*C. livia domestica*	Subspherical to spherical	18.8–21.9 (20.4)	15.9–16.7 (16.4)	–	–	11.9–13.8 (12.7)	5.1–6.5 (5.9)	Saudi Arabia

+ present, – absent, – – not detected.

shown in Figure 2. Furthermore, they clustered with DNA sequences of the same region obtained from *Eimeria* spp. from Columbidae. They were distinct from those *Eimeria* spp. from Phasianidae and Turdidae. Three Sequences were obtained from the *ITS-1* region and were deposited at GenBank database with the accession numbers OR270024-OR270026. The obtained sequences were different from all *ITS-1* sequences deposited in GenBank database with an identity of less than 75%. However, the last part of the sequences (80 bp) which constitutes the *5.8S rRNA* region was highly similar to several eimerian species with 100% identity to *E. subspherica* of bovines.

Discussion

The fecal examination is the most commonly used laboratory technique in veterinary practice for diagnosis of the parasitic infections (41). According to the current data, multiple methods for identification of *Eimeria* species are utilized in field diagnosis. In the current study, 9 of 15 samples tested positive for coccidian oocysts, yielding an overall prevalence of 60% which is in agreement with different reports from various countries (75% by Ramesh et al. (42) from Chennai (India), 67.58% by Gül et al. (43) from Van City (Turkey), 61.36% by El-Sayed (44) from Sharkia Governorate (Egypt), 59.6% by Aleksandra and Pilarczyk (6) from Pomerania province (German), 58.2% by Elseify et al. (45) from Qena province (Egypt), 56.2% by Joseph et al. (46) from Maiduguri Metropolis Borno State (Nigeria), and 52% by Hui et al. (47) from Shanghai (China)). It has been reported that young and growing pigeons lack acquired immunity to coccidian infections and outbreaks can occur under conditions of poor hygiene. Clinical manifestation of pigeon intestinal coccidiosis appeared in the form of greenish watery diarrhea, a decrease in food intake, and body weakness. These findings are consistent with those published by Bandyopadhyay et al. (16), Dalloul and Lillehoj (48), Bhrami et al. (49), Quiroz-Castañeda et al. (50), and Gadelhaq and Abdelaty (51), who all found that coccidiosis had pathological effects on domestic pigeons, resulting in significant losses.

Researchers used different criteria to identify eimerian species excreted in the droppings of pigeons including the morphology and morphometry of oocysts, pre- and patent periods, and sporulation time (5, 11, 13, 19, 29, 51, 52). Based on morphology, it has been confirmed that *E. labbeana*-like is infecting pigeons in a commercial poultry farm in Riyadh area (Saudi Arabia). Yang et al. (19) found oocysts with similar morphological features from coccidian infection in *C. livia* in Australia, however, they have reported oocysts with oocystic residuum, which is not visible in their photomicrographs and may corresponded to some debris stuck

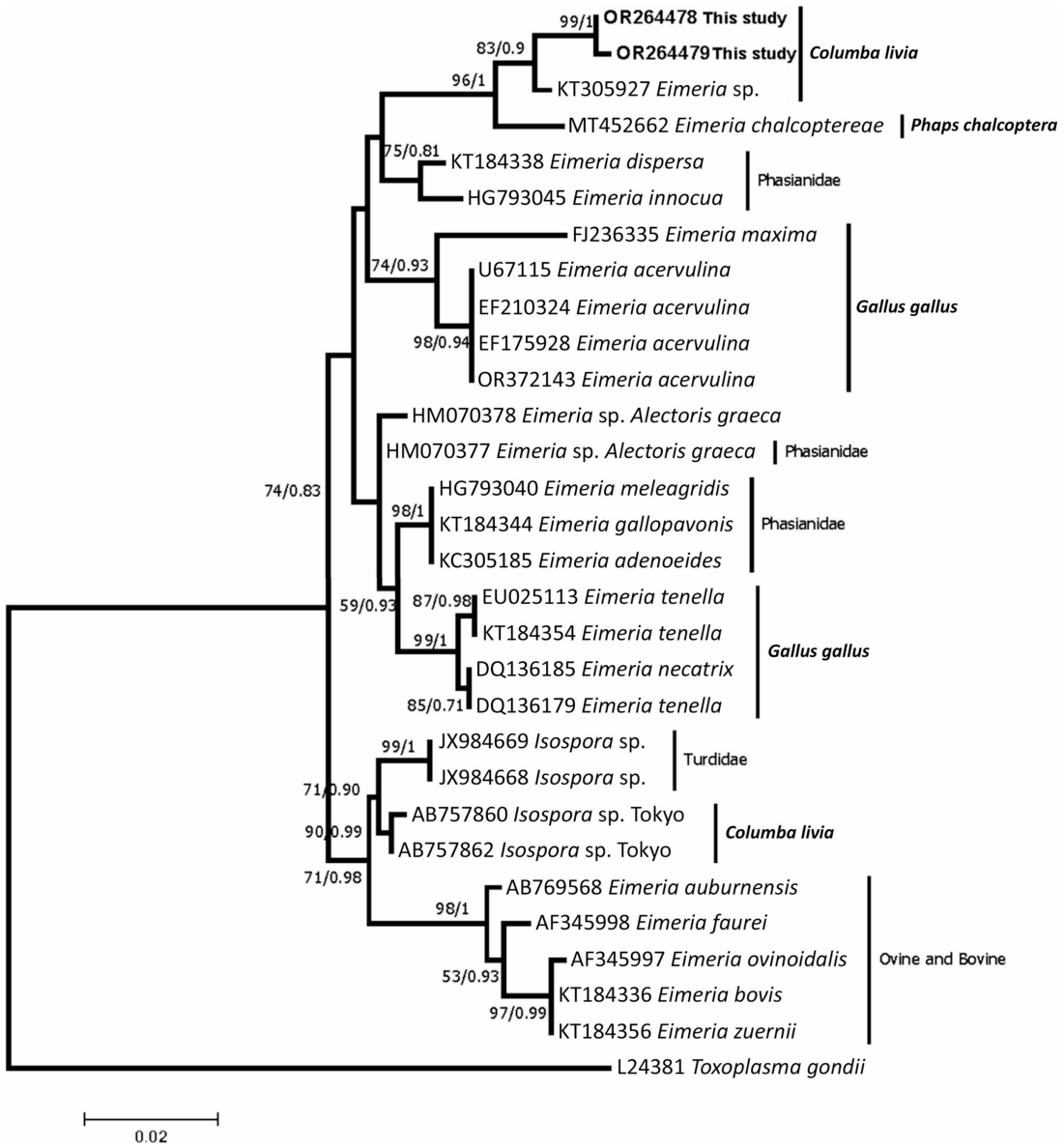

FIGURE 2
A consensus phylogenetic tree constructed with maximum likelihood (ML) and Bayesian Inference (BI) methods, showing phylogenetic relationships between *Eimeria labbeana*-like and related taxa in NCBI GenBank database with *Toxoplasma gondii* as an outgroup. The ML and BI trees are inferred from the partial *18S rRNA* sequences data generated from the *E. labbeana*-like detected from *C. livia domestica* (OR264478 and OR264479 shown in bold) and related taxa from GenBank database. Numbers indicated at branch nodes are bootstrap values and posterior probability (ML/BI). Only bootstraps >50% are shown.

externally to the oocyst wall. When comparing the oocysts detected in the present study with the group of *E. labbeana* species previously described from the Columbidae, the following findings can be made: (i) The oocyst studied in this study, or those from Australia, was far from the type locality of *E. labbeana*. (ii) The morphometric data of the oocysts showed variation in the size of the oocysts which were larger than that described by Nieschulz (12), Joseph et al. (46), Aboelhadid et al. (52), Al-Agouri et al. (53), and Oliveira et al. (30). (iii) The oocyst shape of *E. labbeana* was spherical to subspherical except for those described by Pinto (18), Nieschulz (12), Aboelhadid et al. (52), and Oliveira et al. (30) who highlighted the polymorphic nature of the oocysts, which could be sub-spherical and/or ellipsoidal. (iv) There was no micropyle except for those identified by Pinto (18) and Oliveira et al. (30). (v) There was no oocyst residuum except for those described by Al-Agouri et al. (53).

Partial *18S rRNA* sequences of the eimerian oocysts from the present study indicated that the sequences are related to the 18S rDNA sequences obtained from eimerian parasites from the Columbidae. One of the sequences (KT305927) obtained from *Eimeria* sp. which regarded by Yang et al. (8) as *E. labbeana*-like from *C. l. domestica* in Australia. However, three sequences from *Isospora* sp. (AB757861, AB757863, AB757864) obtained from *C. l. domestica* from Japan and a sequence from *E. chalcoptereae* from a bronzewing pigeon (*Phaps chalcoptera*) in Australia (8).

The *18S rRNA* sequences obtained in the present study differed from those from *Isospora* sp. and *E. chalcoptereae*, However, they showed high similarity to sequences from *E. labbeana*-like reported by Yang et al. (19) with 98.5% similarity. Morphological description of *E. labbeana* or *E. labbeana*-like oocysts showed remarkable variation. Since molecular data for *E. labbeana*-like were only available from Yang et al. (19) and the present study. We, therefore, suggest that the sequences reported in the present study and that reported by Yang et al. (19), since they have a high similarity of 98.5%, may probably be for the same species which was *E. labbeana*-like. Even though they were from two different and distant localities and they were similar in morphology and morphometry except for the presence of oocyst residuum in the oocysts of Yang et al. (19). All other descriptions of *E. labbeana* did not show oocyst residuum except for those descriptions from Yang et al. (19) and Al-Agouri et al. (53). Both Yang et al. (19) and Al-Agouri et al. (53) in their description of *E. labbeana*-like or *E. labbeana* mentioned the presence of oocyst residuum, however, the oocyst residuum was inconspicuous in their photographs which may probably be an artifact. During the present study, we have reported sequences for the *ITS-1* and the 5.8S rRNA regions and there were no sequences for *E. labbeana* or related *Eimeria* spp. which found in GenBank database. Yang et al. (19) studied the cytochrome c oxidase I sequence variation in *E. labbeana*-like and they found it related to *E. dispersa* from the wild turkey (*Meleagris gallopavo*). This probably resulted from the unavailability of related sequences in GenBank database. Despite repeated attempts, it was not possible to obtain sequences from cytochrome c oxidase I in the present study.

Conclusion

This study provides additional knowledge about the oocysts of *Eimeria labbeana*-like in *C. livia domestica* (its type host) from Riyadh (Saudi Arabia). Moreover, unique genetic sequences were added in GenBank database for *18S rRNA* and *ITS-1* regions that recovered for this eimerian species. More research is needed to incorporate preventative and control approaches to reduce the economic impact of *E. labbeana*-like infection.

Data availability statement

The data presented in the study are deposited in the parasitological collection of the museum, College of Science, King Saud University, Riyadh, Saudi Arabia. Two DNA sequences of partial 18S rRNA gene were deposited at GenBank and were given the accession numbers OR264478 and OR264479. In addition to three additional sequence of partial *ITS-1* gene region with the accession numbers OR270024-OR270026.

Ethics statement

The animal study was approved by the Research Ethical Committee (REC) at King Saud University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SA: Methodology, Resources, Software, Writing – review & editing, Conceptualization, Data curation, Investigation, Project administration, Supervision, Validation, Visualization, Writing – original draft, Formal analysis. RA-G: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. SAQ: Project administration, Resources, Software, Writing – original draft, Data curation, Investigation, Supervision, Validation, Visualization, Writing – review & editing, Conceptualization, Formal analysis, Methodology. EA-S: Formal analysis, Methodology, Resources, Software, Visualization, Writing – review & editing, Conceptualization, Data curation, Investigation, Project administration, Supervision, Validation, Writing – original draft. OM: Conceptualization, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing, Data curation, Investigation, Project administration, Resources, Software, Supervision, Validation.

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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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Case report: First report of potentially zoonotic *Gongylonema pulchrum* in a free-living roe deer (*Capreolus capreolus*) in Slovenia

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Adult female and male *Gongylonema* nematodes were found in the oesophagus of a free-living roe deer (*Capreolus capreolus*) in Slovenia during passive health surveillance of wildlife. The genus *Gongylonema* was determined by light microscopy based on the genus-specific cuticular bosses in the anterior part of the parasite. Molecular methods were used to confirm the species *Gongylonema pulchrum*, which has zoonotic potential. Although *Gongylonema* species are considered common and distributed worldwide, this is the first report of *G. pulchrum* in an animal on the territory of Slovenia and the first molecular report in a roe deer worldwide. The parasite is likely to be underdiagnosed, misdiagnosed or goes unnoticed as the animals show little or no clinical signs and minor pathological lesions. Slaughterhouse workers, hunters and veterinarians should be aware of this elusive parasite. Examination and evisceration of the upper digestive tract of animals should therefore be carried out more carefully.

KEYWORDS

***Gongylonema pulchrum*, nematode, zoonosis, roe deer (*Capreolus capreolus*), oesophagus, PCR, sequencing**

1 Introduction

Gongylonema sp. is a spirurid nematode that forms zigzag patterns in the submucosa of the upper digestive tract of domestic and wild mammals, birds and sometimes humans (1–6). Its main definitive hosts are ruminants, and its global prevalence has been described as common (7–10). In free-living wild herbivores, it has been found in roe deer (*Capreolus capreolus*) (11), European fallow deer (*Dama dama*) (12), bison (*Bison bison*) (13), white-tailed deer (*Odocoileus virginianus*) (14), spotted deer (*Axis axis*), sambar (*Rusa unicolor*), mouse deer (*Tragulus meminna*), nilgai (*Boselaphus tragocamelus*), serow (*Capricornis sumatraensis*), giraffe (*Giraffa camelopardalis*) (15), wild mouflon (*Ovis aries musimon*), sika deer (*Cervus nippon*), feral alien Reeves's muntjacs (*Muntiacus reevesi*) and water buffalo (*Bubalus bubalis*) (6, 9, 16, 17). The parasite was also found in other game species, such as red fox (*Vulpes vulpes*) and wild boar (*Sus scrofa*) (17). With an estimated population of 10 million animals, the roe deer is the most common and widely distributed deer species in Europe (18). In Slovenia, about 80% of the country's territory serves as permanent habitat for roe deer, which emphasizes

their large presence (19). It is therefore not surprising that the roe deer is one of the most important game species in the country (20).

Gongylonema species have an indirect life cycle, in which the intermediate hosts are coprophagous beetles (families *Scarabaeidae*, *Tenebrionidae*, *Hydrophilidae* and *Histeridae*) and some cockroaches (*Blattella* spp.) (21–23). Definitive hosts can become infested by ingesting infested insects or through contaminated food or water (24). Infestation with *Gongylonema* sp. in ruminants usually has no effect on animal health, apart from rare reports of mild to moderate local inflammation with signs of discomfort and irritation in the oesophagus (7, 10). Humans act as accidental hosts, with patients most commonly reporting an intermittent, migratory, worm-like sensations in the upper oesophagus and oral cavity (22, 25–27). An association between *Gongylonema pulchrum* infestation and squamous cell carcinoma was hypothesized in a 17-year-old female ruffed lemur (*Lemur macaco* subsp. *variegatus*) and a 59-year-old man (28, 29). In Slovenia, only one case of autochthonous infestation with *G. pulchrum* in a human was documented in 2019 (22). Although *Gongylonema* sp. is recognized as a parasite of ruminants in Slovenia (30), there are no studies or reports of infestation of animals with this parasite to support this statement.

The aim of this paper is to report the presence of *Gongylonema* sp. in the oesophagus of a free-living roe deer (*C. capreolus*) in Slovenia and its molecular identification as *G. pulchrum* using PCR and Sanger sequencing of the obtained PCR amplicons.

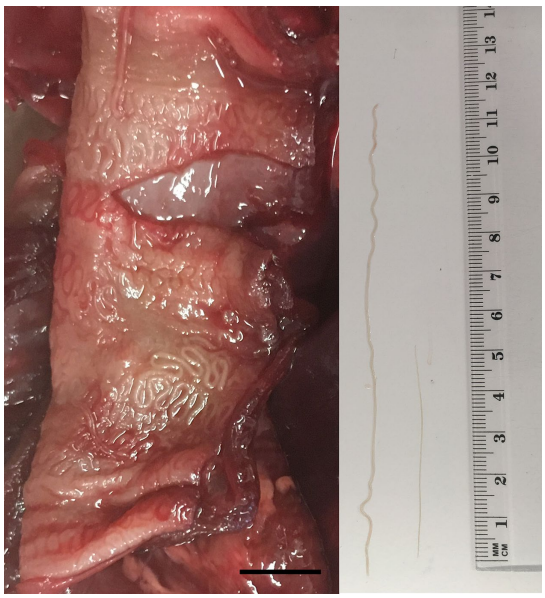

FIGURE 1
Serpentine-like structures in the subserosa of the oesophagus of a juvenile female roe deer (left); the scale bar represents 1 cm. From the subserosa, white thread-like worms were collected (right); the male (shorter nematode) was approximately 5 cm and the female (longer nematode) 12 cm long.

2 Case description

In March 2023, the necropsy of a juvenile female roe deer from a hunting ground near the town of Gornji Grad (Lower Styria, Slovenia) was performed at the Veterinary Faculty (Ljubljana, Slovenia) as part of a national passive surveillance programme. The death of the animal followed extensive tissue and organ damage caused by a predator. A detailed parasitological examination of the lungs (for lungworms) and surrounding tissues (heart, oesophagus) unexpectedly revealed serpentine-shape changes in the subserosa of the oesophagus (Figure 1, left). On extraction, the white thread-like worms were 5–12 cm long (Figure 1, right). Under the light microscope, cuticular bosses typical of the genus *Gongylonema* were observed in the anterior part of the parasites (Figure 2, left). A total of ten females and one male were collected. The male was 5 cm long and had asymmetrical caudal wings with two short, differently sized spicules (Figure 2, right), indicating a juvenile male (8). The females were about 12 cm long and had a pronounced uterus filled with embryonated oval eggs.

After morphological examination of the nematodes, molecular methods were used to determine the species; one female nematode was stored in sterile physiological saline solution at −20°C for subsequent molecular analysis. DNA was extracted from the mid-body section of the parasite using the iHelix kit (Institute of Metagenomic and Microbial Technologies, Slovenia; https://www.ihelix.eu/) according to the manufacturer's instructions. The extraction protocol included bead-beating (45 s at 6400 rpm) three times using a tissue homogenizer (MagNA Lyser Instrument; Roche, Switzerland), combined with enzymatic and heat-induced lysis between mechanical shearings. DNA was eluted to a final volume of 100 µL and stored at −20°C until further analysis. For species determination, PCR and Sanger sequencing were employed, targeting the overlapping segments of the ribosomal RNA (rRNA) genes (rDNA). Twelve universal eukaryotic primer pairs (Supplementary Table S1) were used for PCR amplification as previously described (1, 9, 31); each primer pair was used in a separate PCR reaction. In brief, 25-µl reaction mixtures contained 2.5 µL of DNA, 0.5 U of Platinum Taq DNA Polymerase (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA), 2.5 mM $MgCl_2$ and 1× PCR buffer supplied by the manufacturer, 1 µM of each primer, and 0.25 mM of each dNTP (Applied Biosystems by Thermo Fisher Scientific). Amplification was performed in the VeritiPro Thermal Cycler (Applied Biosystems by Thermo Fisher Scientific) according to the following protocol (applied for all PCR reactions/primer pairs): initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1 min, and final extension at 72°C for 10 min. The obtained PCR amplicons were analyzed with the QIAxcel capillary electrophoresis system (Qiagen, Germany) using the QIAxcel DNA High Resolution Kit, QX Alignment Marker 15–3,000 bp, QX Size Marker 100–2,500 bp, OM500 separation method and a sample injection time of 10 s according to the manufacturer's instructions.

Ribosomal PCR amplicons ($n = 12$) were sequenced in both directions (Eurofins Genomics Europe, Germany). The retrieved sequence fragments were imported into Geneious Prime v2022.1.1 (Biomatters, New Zealand) and mapped to a 6,091-bp reference *G. pulchrum* (GeneBank accession no. AB495389.2) to enable reconstruction of a nearly complete *Gongylonema* rDNA region containing also the internal transcribed spacers (ITS) 1 and 2; *G. pulchrum* was selected as suspected according to the origin of the isolate (16). A 6010-bp consensus 18S rDNA - ITS1-5.8S rDNA - ITS2 - 28S rDNA sequence was obtained, which was aligned to three (of 22 available >6,000-bp long *G. pulchrum* sequences of rDNA in GenBank; accessed on 24 April 2024) selected *G. pulchrum*

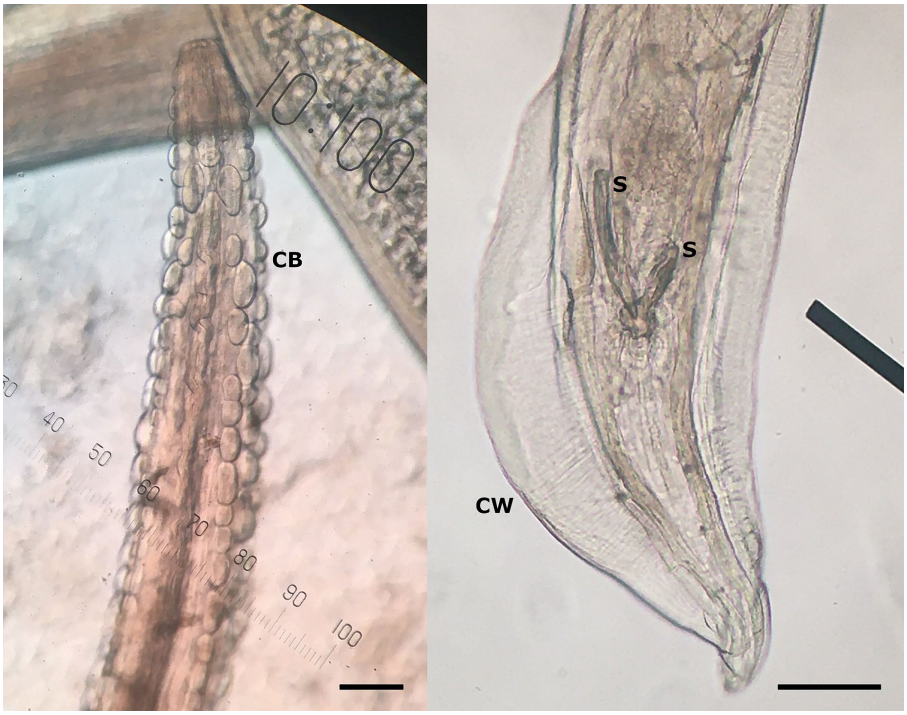

FIGURE 2
Cuticular bosses (CB) on the anterior part of the nematode, typical for genus *Gongylonema* sp. (left), and posterior part of the male *Gongylonema pulchrum* with caudal wings (CW) and two short spicules (S) of different sizes (right) at 100× magnification. The scale bar represents 100 µm.

(AB495389.2, AB495394.1, AB495397.1) and one *Gongylonema nepalensis* (LC278392.1) sequence of rDNA. Of note, the retrieved sequence fragments were also mapped to a 6,114-bp reference *G. nepalensis* (LC278392.1) and the consensus sequence obtained was identical to the 6,010-bp consensus after mapping to *G. pulchrum*. The constructed consensus shared most similar single nucleotide polymorphisms (SNPs) and insertions/deletions (indels) to *G. pulchrum* sequences and much less similar to *G. nepalensis*. After the blast search (https://blast.ncbi.nlm.nih.gov/; accessed on April 24, 2024), the consensus sequence was most similar to *G. pulchrum* (100–99.64% identity where query cover was 100%) and less to *G. nepalensis* (97.22–97.07%); lower than 100% query cover (<93%) was obtained for the *Gongylonema* species *G. aegypti* and *G. neoplasticum*. The results of molecular identification showed that the nematode belonged to *G. pulchrum*. The obtained *G. pulchrum* genomic rDNA sequence, comprising 18S rDNA, ITS1, 5.8S rDNA, ITS2 and 28S rDNA, was submitted to GenBank under the accession number PP594418.

To confirm the results of molecular identification, all >6,000-bp long rDNA sequences of the genus *Gongylonema* were retrieved from GenBank (accessed on 1 July 2024); a total of 31 rDNA sequences of *G. pulchrum* ($n = 22$ from Japan, China, Iran and Slovenia; the *Gongylonema* isolate from Slovenia was the only one of human origin), *G. nepalensis* ($n = 4$ from Nepal and Italy), *G. neoplasticum* ($n = 2$ from Japan) and *G. aegypti* ($n = 3$ from Egypt) were obtained. The sequences were complemented with the rDNA of *G. pulchrum* obtained in the present study and the phylogenetic tree was constructed in MEGA11 (32) (Figure 3); the maximum likelihood method and Tamura-Nei model were used with default parameters (33). The 6,705-bp long rDNA sequence of *Stegophorus macronectes* (HE793715.1), belonging to the same order (*Rhabditida*) and suborder (*Spirurina*) as *Gongylonema* spp., was used as an outgroup to root the tree. A clear clustering according to *Gongylonema* species was observed, but no sub-species clustering, indicating a high genetic similarity of *G. pulchrum* and the correct identification of the roe deer isolate as *G. pulchrum*.

3 Discussion

This is the first molecular report of *G. pulchrum* in a roe deer and the first report of a *Gongylonema* nematode found in an animal in Slovenia. The report also complements the recent human case of *G. pulchrum* reported from Slovenia, which was thought to be an autochthonous infestation (22). The molecular protocols and analyses are presented in detail to facilitate further use in diagnostic laboratories, as many warm-blooded animals are infested with *Gongylonema* nematodes, which are also potential zoonotic agents (1–6).

Gongylonema infestation in the oesophagus was discovered at necropsy when a roe deer was found dead in the wild after attack by a predator and examined as part of a national passive health surveillance programme of wildlife in Slovenia. The roe deer population in Slovenia is estimated at around 110,000 animals, with a hunting quota of around 30,000–35,000 animals per year (20, 34). Roe deer are considered the most widespread species of free-living wild ruminants and an important source of game meat in Slovenia (20). In twenty years of passive health surveillance of roe deer, a mortality rate of 26% was recorded for parasitic diseases. In addition to ectoparasites, endoparasites such as *Haemonchus contortus*, *Chabertia ovina* and lung parasites (*Protostrongylidae*, *Dictyocaulus viviparus*) were also

FIGURE 3
Maximum likelihood phylogenetic tree of >6,000-bp long *Gongylonema* rDNA sequences. GenBank accession numbers are listed in addition to *Gongylonema* species and country of origin. In total, 32 nucleotide sequences of *Gongylonema pulchrum* ($n = 22$ and one from the present study), *Gongylonema nepalensis* ($n = 4$), *Gongylonema neoplasticum* ($n = 2$) and *Gongylonema aegypti* ($n = 3$) were included. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Stegophorus macronectes* rDNA (GenBank accession no. HE793715.1) was used as an outgroup to root the tree.

detected during the post-mortem examination (35). Until now, not a single *Gongylonema* sp. has been found. The potential infestations in domestic animals and wildlife in Slovenia should be documented as there are no current prevalence reports.

Based on the morphology and the origin of the isolate, *G. pulchrum* was suspected (16). The species was confirmed by sequencing, as the reconstructed 18S rDNA - ITS1-5.8S rDNA - ITS2 - 28S rDNA region was most similar to the rDNA region of *G. pulchrum*. Apart from the isolate obtained in the present study, there are no other *Gongylonema* isolates and corresponding sequences available from ungulates inhabiting Slovenia. Only one *G. pulchrum* rDNA was deposited from our country in 2019 (GenBank accession no. LR215834.1), but it was obtained as part of a report on *G. pulchrum* infestation in a human case (22). In addition, not many studies have generated *Gongylonema* rDNA sequences longer than 6,000 bp, and only two of these sequences (but not belonging to *G. pulchrum*) are from a neighboring country, namely the rDNA of *G. nepalensis* from Italy (GenBank accession nos. LC388743.1 and LC278392.1). The phylogenetic comparison of the rDNA sequences of *G. pulchrum* isolates obtained in Slovenia showed a high genetic similarity between the two sequences, but the same was true for all compared *G. pulchrum* sequences from four geographically distant countries. More sequences (partial rDNA) of *Gongylonema* spp. are available in GenBank, but most of them are shorter and therefore contain much less phylogenetic information (no additional discriminatory power). The high within-species similarity of *Gongylonema* rDNA was also previously described when it was reported that the nucleotide sequences of *G. pulchrum* rDNA were generally well conserved regardless of their host origin (9). We could achieve somewhat greater discriminatory power, if we sequenced the cytochrome c oxidase subunit I (COI) region of mitochondrial DNA (4, 9); the COI sequences of *G. pulchrum* can be further subdivided into several haplotypes (9).

The first and only human case of *G. pulchrum* in Slovenia was self-diagnosed in 2015 and it was later molecularly identified and reported (22). The infestation was described as autochthonous and was most likely due to ingestion of food or water from natural sources thought to be contaminated with the nematode intermediate hosts, dung beetles and cockroaches (26); it was reported that the patient was drinking water from several local springs in the south-eastern part of Slovenia, where there are many grazing areas for livestock (22). Xiaodan et al. (25) reported that the parasite can be overlooked in a patient for more than ten years after infestation. It can also be misdiagnosed as candidiasis, burning mouth syndrome or even a delusional parasitic infestation, as patients report strange crawling sensations in the upper digestive tract (2, 22, 26, 27, 36, 37). The parasite can also contribute to the development of squamous cell carcinoma (28, 29), which can have serious health implications. Therefore, more attention should be paid to *Gongylonema* species, especially *G. pulchrum* with a proven zoonotic potential.

Until the 1980s, *Gongylonema* sp. was frequently reported, with prevalence in domestic ruminants reaching, e.g., 49.7% (276/555) in Iran or up to 96.0% in some regions in Turkey (8, 21). In free-living wild ruminants, a prevalence of 42.8% was reported in 1959 in roe deer from Romania (11, 12) and recently a prevalence of 18.8% (25/133) in European fallow deer (*D. dama*) from Romania (12). In 2013, researchers from Japan reported varying prevalences (from no infestation to a 100% prevalence, depending on sampling location) in sika deer (*C. nippon*) (9). However, over the years, the prevalence in domestic ruminants in the same countries has decreased to, e.g., 4.6% (16/350; Iran) or 0% (0/848, Turkey) in sheep (8, 10) and 16.2% (96/680, Iran), 5.3% (34/638, Japan) or 0.5% (2/380, Turkey) in cattle (7–9). This decrease in prevalence has been attributed to the decline in grazing, the increased use of commercial feeds and the regular use of anthelmintics (8). In Slovenia, *Gongylonema* sp. is mentioned in veterinary parasitology textbooks as a common parasite in the oesophagus of ruminants (30). To our knowledge, there are no published data or reports indicating the prevalence of the parasite. The parasite may be under-reported or under-diagnosed as the clinical signs in animals are usually non-specific, mild and without obvious pathological changes at the site of infestation (7, 10, 25). In this study, the parasite would probably not have been discovered, if the animal had not been attacked by a predator and collected dead by the hunters.

The occurrence of *Gongylonema* sp. in roe deer prompts us to investigate the potential number of cases that may have been overlooked in domestic/captive and wild/free-living animals. This reminds us of the importance of passive health surveillance in wildlife. As regular monitoring activities are associated with high numbers of animals, passive health surveillance of wildlife is particularly important to detect diseases that might otherwise go unnoticed. Slaughterhouse staff, hunters and veterinarians should be educated

about this elusive parasite and be vigilant during evisceration or post-mortem examinations. Further studies are essential to reassess the prevalence of *Gongylonema* species in domestic and wild ruminants in Europe and their zoonotic impact.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at the National Center for Biotechnology Information (NCBI) using accession number PP594418.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements as samples were collected post-mortem.

Author contributions

PB: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. DŽV: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. GV: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. DK: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, 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 relationship 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.2024.1444614/full#supplementary-material

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First microscopic, pathological, epidemiological, and molecular investigation of *Leucocytozoon* (Apicomplexa: *Haemosporida*) parasites in Egyptian pigeons

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Introduction: *Leucocytozoon* is an intracellular blood parasite that affects various bird species globally and is transmitted by blackfly vectors. This parasite is responsible for leucocytozoonosis, a disease that results in significant economic losses due to reduced meat and egg production. There is limited knowledge about the epidemiological pattern of leucocytozoonosis and its causative species in Egypt, particularly in pigeons.

Methods: The current study involved the collection of 203 blood samples from domestic pigeons from various household breeders and local markets across Qena Province, Upper Egypt. Samples were initially examined for potential *Leucocytozoon* infection using blood smears, followed by an evaluation of associated risk factors. Molecular identification of the parasite in selected samples (n = 11), which had initially tested positive via blood smears, was further refined through nested PCR and sequence analysis of the mitochondrial cytochrome b gene to ascertain the *Leucocytozoon* species present. Additionally, histopathological examination of the liver, spleen, and pancreas was conducted on animals that tested positive by blood smears.

Results: Interestingly, 26 out of 203 samples (12.08%) had confirmed *Leucocytozoon* infections based on microscopic analysis. Additionally, all 11 samples that initially tested positive via blood smears were confirmed positive through nested PCR analysis, and their sequencing revealed the presence of *Leucocytozoon sabrazesi*, marking the first report of this parasite in Egypt. The study into potential risk factors unveiled the prevalence of *Leucocytozoon* spp. seems host gender-dependent, with males exhibiting a significantly higher infection rate (33.33%). Additionally, adult birds demonstrated a significantly higher infection prevalence than squabs, suggesting an age-dependent trend in prevalence. Seasonality played a significant role, with the highest occurrence observed during summer (37.25%). Histopathological examination revealed

the presence of numerous megaloschizonts accompanied by lymphocytic infiltration and multiple focal areas of ischemic necrosis.

Conclusion: To our knowledge, this is the first study to shed light on the epidemiological characteristics and molecular characterization of leucocytozoonosis in pigeons in Egypt. Further research endeavors are warranted to curb the resurgence of *Leucocytozoon* parasites in other avian species across Egypt, thereby refining the epidemiological understanding of the disease for more effective control and prevention measures.

KEYWORDS

***Leucocytozoon*, pigeon, Egypt, epidemiology, molecular, phylogenetic, histopathology**

1 Introduction

Pigeons are abundant and ubiquitous avian species, often found in urban environments. Since ancient times, pigeons have been regarded as symbols of various concepts, including deities, peace, messengers, pets, food, and spiritual sacrifice. In Egypt, pigeons are primarily raised to meet the protein needs of families during special occasions, serve as a source of income, for gaming, and ornamental purposes. However, pigeons can host numerous pathogens and serve as reservoirs for parasitic infections (1). Parasitism is a significant concern affecting bird production, leading to issues such as growth retardation, decreased vitality, blood loss, toxicosis, and poor health conditions. Ultimately, this reduces the quality and quantity of meat and egg production. Among various parasitic diseases affecting avian species, haemoprotozoan infections are predominant (2).

Avian haematozoa comprise a class of vector-borne parasites belonging to the apicomplexan group, which includes genera such as *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. These parasites are transmitted by blood-sucking dipteran vectors, including species of ceratopogonids (genus *Culicoides*), blood-sucking culicine mosquitoes (*Culicidae*), blackflies (*Simuliidae*), and hippoboscid flies, with birds acting as intermediate hosts (2). Among others, leucocytozoonosis is considered the most significant blood protozoan disease affecting birds, caused by approximately 60 species of parasitic protozoa of the genus *Leucocytozoon*. It affects wild and domestic avian species and is transmitted by biting blackflies such as *Simulium venustum*, *S. croxtoni*, *S. euradminiculum*, and *S. rugglesi* (3). The life cycle of *Leucocytozoon* species is complex, involving development both in tissues (exo-erythrocytic merogony) and blood cells. Before infecting blood cells and forming gametocytes, *Leucocytozoon* species undergo exo-erythrocytic merogony, which produces meronts in tissue cells. These meronts are the infective stage for vectors. Subsequently, sexual processes and sporogony occur in dipteran insects, producing infective sporozoites, which initiate new infections in vertebrate hosts (2, 4, 5). The pathogenic impact of *Leucocytozoon* infection on the host can potentially jeopardize productivity, reducing egg production and increasing mortality rates. Clinical signs of infection may include anemia, anorexia, green feces, and ataxia, although infections can be asymptomatic. Upon necropsy, common findings include fatty liver, splenomegaly, regressive reproductive organs, and other characteristic lesions. This can result in a significant loss of production value in industrial settings and group deaths, as reported in various avian species (6, 7).

Direct microscopic examination of Giemsa-stained blood films was considered the most conservative diagnostic approach for detecting *Leucocytozoon* sp. infection. Additionally, identifying the parasite's genome using polymerase chain reaction (PCR) with primers derived from mitochondrial genes offers a more sensitive and accurate method widely employed in laboratory settings for precise analysis of infections. This molecular approach can provide high accuracy even in cases where blood smears are negative due to low parasitemia or early stages of infection in avian hosts (8–10). Understanding the epidemiological patterns of parasitic infections is essential for devising and implementing effective prevention and control strategies. Investigating the previous literature, very scant information is available on Egypt's *Leucocytozoon* sp. infection. Only one previous study in northern Egypt (lower Egypt) revealed the natural co-infection of poultry farms with *Leucocytozoon caulleryi* and chicken anemia virus (11). However, to the best of our knowledge, no specific investigations have been conducted to explore the incidence of blood parasitic infections, particularly *Leucocytozoon* species, in the country's southern region (Upper Egypt). Therefore, the current study was conducted to identify and determine the prevalence of *Leucocytozoon* species in pigeons from Upper Egypt and assess the associated risk factors through microscopic examination of stained blood smears. Additionally, this investigation examined the taxonomy of the identified leucocytozoids at the species level by analyzing the phenotypic characteristics of the cytochrome b gene (*cytb*) and reporting the major histopathological findings of the examined animals.

2 Materials and methods

2.1 Ethical statements

The present study received approval from the Ethics Committee of the Faculty of Veterinary Medicine at South Valley University, Egypt, per ethical regulations and animal research guidelines (permit code No. 84). Written and oral consent was obtained from each owner of the surveyed pigeons.

2.2 Study area

The study was conducted in Qena Province, situated in southern Egypt at coordinates 26°10′12″N 32°43′38″E. Renowned for its pottery, imposing mountains, and lush green landscapes, the province experiences a hot desert climate characterized by scorching summers and minimal yearly precipitation.

2.3 Birds and sample processing

Blood samples ($n = 203$) were randomly collected from apparently healthy pigeons in Qena Province between November 2020 and October 2021, sourced from different household breeders and local markets. Information on age, sex, and sampling season was documented to evaluate potential associations with the presence and abundance of blood parasites. Each bird's sample (3 mL) was gathered in an anticoagulated test tube from the brachial wing vein using a sterile syringe and needle. These samples were transported to the Parasitology Laboratory at the Faculty of Veterinary Medicine, South Valley University, for parasitological analysis.

2.4 Laboratory analysis

Following collection, thin blood films were immediately prepared from each sample to identify blood protozoa. The smears were air-dried, fixed in absolute methanol three times for 10 s each, and stained with Giemsa's stain (30%) for 10 min. Subsequently, the slides were gently washed under running tap water, air-dried, and then subjected to microscopic examination, following established laboratory protocols (12). The stained slides were examined using an Olympus CX31 microscope at higher magnification (100X) to detect infections. The identification and intensity of recovered haemoprotozoa were recorded following established keys and descriptions outlined by Soulsby (13) and Levine (14).

2.5 Histopathological analysis

The examined pigeons were anesthetized using an equal mixture of ketamine and xylazine (0.0044 cc/kg), administered via injection into the pectoral muscle (15), then left to ensure complete euthanasia. Tissue samples, mainly liver, spleen, and pancreas, were then excised and prepared for histopathological examination (16). Approximately 1 cm sections of each tissue were collected and fixed in 10% neutral buffered formalin (pH = 7.4). They underwent processing through ascending grades of alcohols, were embedded in paraffin wax, sectioned at a thickness of 5 µm, and then stained with histochemical stains (Harries hematoxylin and eosin, Sigma-Aldrich) (17). The preparations were examined using a microscope (Olympus BX51, Tokyo, Japan) equipped with a camera (Olympus E-182330, Olympus Optical Co., Ltd., Japan), with five slides inspected for each block.

2.6 Molecular identification

2.6.1 DNA extraction

DNA was extracted successfully from 11 positive blood samples via microscopic examination. This extraction was performed using a QIAamp DNA mini kit (1,043,368, Qiagen, United States) following the manufacturer's instructions, and the extracted DNA was stored at −20°C until PCR analysis.

2.6.2 PCR amplification

Two pairs of specific primers from Macrogen (Korea) were utilized to amplify the *cytb* gene via nested PCR. The first step of amplification employed the primers LsF1 (5′-CATATATAAGAGAATTATGGAG-3′) and LsR1 (5′-ATAAAATGYTAAGAAATACCATTC-3′). In the second step, the primers LsF2 (5′-TAATCACATGGGTTTGTGGA-3′) and LsR2 (5′-GCTTTGGGCTAAGAATAATACC-3′) were utilized. The expected size of amplification products was 248 bp. The reaction was conducted in a 25 µL volume containing 12.5 µL of DreamTaq Green PCR Master Mix (2X) (K1081, ThermoFisher, United States), 1 µL of each primer (20 pmol), 5.5 µL of water, and 5 µL of DNA. PCR was performed using an Applied Biosystems 2,720 thermal cycler with the following program: 40 cycles of denaturation at 94°C for 1 min (1st step) and 20 s (2nd step), annealing at 50°C for 1 min (1st step) and 53°C for 20 s (2nd step), extension at 68°C for 1 min (1st step) and 30 s (2nd step), followed by a final extension at 68°C for 5 min (1st and 2nd steps).

2.6.3 Visualization of the PCR outcomes

The PCR products were subjected to electrophoresis on a 1.5% agarose gel (Agarose, Universal, PeqGold, Peqlab. Germany) in 1x TBE buffer. About 20 µL of the PCR products was loaded onto the gel. A 50 bp DNA ladder gene marker (PeqGold 2 kb DNA-Ladder, Peqlab., VWR) was utilized to determine the size of the amplicons. The gel was stained with ethidium bromide (0.5 µg/mL) and visualized under UV light. The results were documented using a gel documentation system (Geldoc-it, UVP, England).

2.6.4 Sequencing and phylogenetic analysis

The PCR products were purified using a QIAquick PCR product extraction kit (Qiagen, Valencia) and Centrisep spin columns. The gel documentation system (Geldoc-it, UVP, England) was utilized to capture the sequence reaction, and analysis was conducted using Totallab analysis software.[1] The identity of the obtained DNA sequences from the ABI PRISM® 3,100 Genetic Analyzer (Micron-Corp. Korea) was confirmed through BLAST analysis (18). The phylogenetic tree was constructed using the MegAlign module of Lasergene DNA Star version 1.83 software, based on the *cytb* gene sequences (19). The analysis was conducted in MEGA11 using the accession number (ON399180) with maximum likelihood method (20). The sequences, along with their corresponding host and locality or country, were downloaded from GenBank and used in the tree construction, as depicted in Table 1.

2.7 Statistical analysis

Statistical analysis was conducted to assess the variation in *Leucocytozoon* spp. incidence among pigeons, considering the epidemiological data. The chi-square (χ^2) test was employed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk,

1 www.totallab.com, Ver. 1.0.1

TABLE 1 The accession numbers used for construction of the phylogenetic tree, along with their species, hosts, and locations.

Accession number	Species	Host origin	Location/country
MW316431	*Leucocytozoon sabrazesi*	Chicken	Thailand
MW316432	*Leucocytozoon sabrazesi*	Chicken	Thailand
MW316434	*Leucocytozoon sabrazesi*	Chicken	Thailand
KT290930	*Leucocytozoon sabrazesi*	*Gallus gallus spadiceus*	Malaysia
MZ634390	*Leucocytozoon sabrazesi*	*Gallus gallus*	Thailand
KT290929	*Leucocytozoon sabrazesi*	*Gallus gallus domesticus*	Malaysia
AB299369	*Leucocytozoon sabrazesi*	Chicken	Malaysia
LC550031	*Leucocytozoon sabrazesi*	Chicken	Myanma
MW600919	*Leucocytozoon caulleryi*	*Gallus gallus*	Thailand
MN540144	*Plasmodium kentropyxi*	Lizards	Brazil
MW296834	*Haemoproteus* sp.	Avian	Korea
JQ988310	*Parahaemoproteus* sp.	*Coeligena torquata*	Peru
MK721052	*Leucocytozoon* sp.	*Emberiza godlewskii*	China
MK061720	*Haemoproteus* sp.	*Pachycephala hyperythra*	Papua New Guinea
GU59370	*Eimeria acervulina*	*Gallus gallus*	United States

TABLE 2 Prevalence of *Leucocytozoon* spp. in relation to the age, sex, and season of the pigeons inspected in the present study.

Variables	No. of examined cases	No. of positive cases (%)	Pearson Chi-Square χ^2 (*p* value)
Age			19.063 (< 0.0001)*
< 2 months	86	0 (0)	
> 2 months	117	26 (22.22)	
Sex			8.836 (0.002)*
Male	21	7 (33.33)	
Female	182	19 (10.43)	
Season			36.593 (<0.05)*
Winter	47	0 (0)	
Spring	52	6 (1.92)	
Summer	51	19 (37.25)	
Autumn	53	1 (1.88)	

*Superscript indicates the significant difference at $p < 0.05$.

NY, United States). A significance level of $p \leq 0.05$ was considered indicative of statistical significance (21).

3 Results

3.1 Occurrence of *Leucocytozoon* species and potential risk factors

Out of 203 inspected pigeons, 26 were infected with *Leucocytozoon* species, yielding an overall prevalence of 12.80%. Regarding infection frequency (Table 2), *Leucocytozoon* spp. showed significantly higher rates in the age group of >2 months (22.2%, $\chi^2 = 19.063$, $p < 0.05$), while the other age category remained unaffected, suggesting that the risk of infection rises with age. Likewise, the current investigation revealed that the occurrence percentage of *Leucocytozoon* spp. was 10.43% in females, whereas it was 33.33% in males, indicating a statistically significant gender disparity in infection rates ($\chi^2 = 8.836$, $p = 0.002$) as depicted in Table 2. Additionally, the same table presents the prevalence of parasite infections relative to both age and sex groups in examined pigeons. It is intriguing that the proportion the occurrence rate of *Leucocytozoon* infection was notably higher in males (46.66%) compared to females (18.62%) within the same age category (>2 months), which demonstrated a statistically significant difference ($\chi^2 = 13.079$, $p = 0.004$). Conversely, no infections were observed in either male or female pigeons in the younger class (<2 months).

Investigation of seasonal dynamic of infection with *Leucocytozoon* species showed that the summer season exhibiting the highest prevalence of *Leucocytozoon* infections (37.25%), followed by spring (1.92%) and autumn (1.88%). No infection was observed during the winter (0%), indicating a notable difference in infection seasonality, as shown in Table 2. Additionally, the variations in infection rates between seasons were statistically significant ($\chi^2 = 36.593$, $p < 0.05$).

3.2 Morphological description

Gametocytes of *L. sabrazesi* were detected in positive Giemsa-stained blood films measuring 7.75 × 9.20 µm. Mature gametocytes appeared as a distinct parasite stage (Figure 1), occupying the entire cellular space and replacing the cell cytoplasm, occasionally forming elongated "horns." Macrogametocytes appeared darker, with small nuclei, dark blue cytoplasm, light red nuclei, small vacuoles, and magenta volutin cytoplasmic granules. In contrast, microgametocytes showed lighter blue staining, with extremely pale cytoplasm and pale pink nuclei (Figure 2). Additionally, an average of 3 ± 0.4 *Leucocytozoon* sp. infected cells per field were recorded.

3.3 Gross lesions and histopathological findings

During necropsy, the examined pigeons typically exhibited no noticeable gross lesions. However, microscopic examination documented the presence of *Leucocytozoon* spp. in different organs, including the liver, spleen, and pancreas.

Histopathological examination of liver tissue (Figure 3) revealed focal areas of coagulative necrosis with distortion of hepatocytes. The periportal areas exhibited heavy infection with multiple variable-sized megaloschizonts containing numerous basophilic schizonts. These schizonts were observed solitarily or in groups, often surrounded by

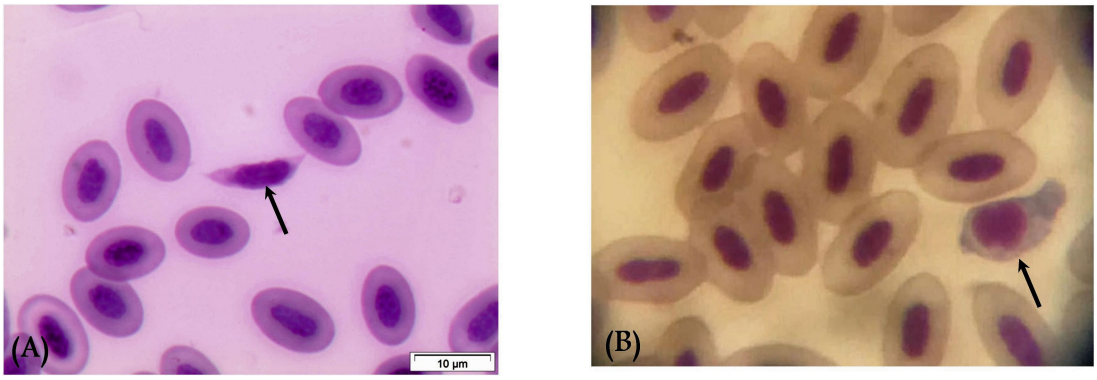

FIGURE 1
Mature macrogametocyte **(A)** and microgametocyte **(B)** of *Leucocytozoon sabrazesi* (arrows).

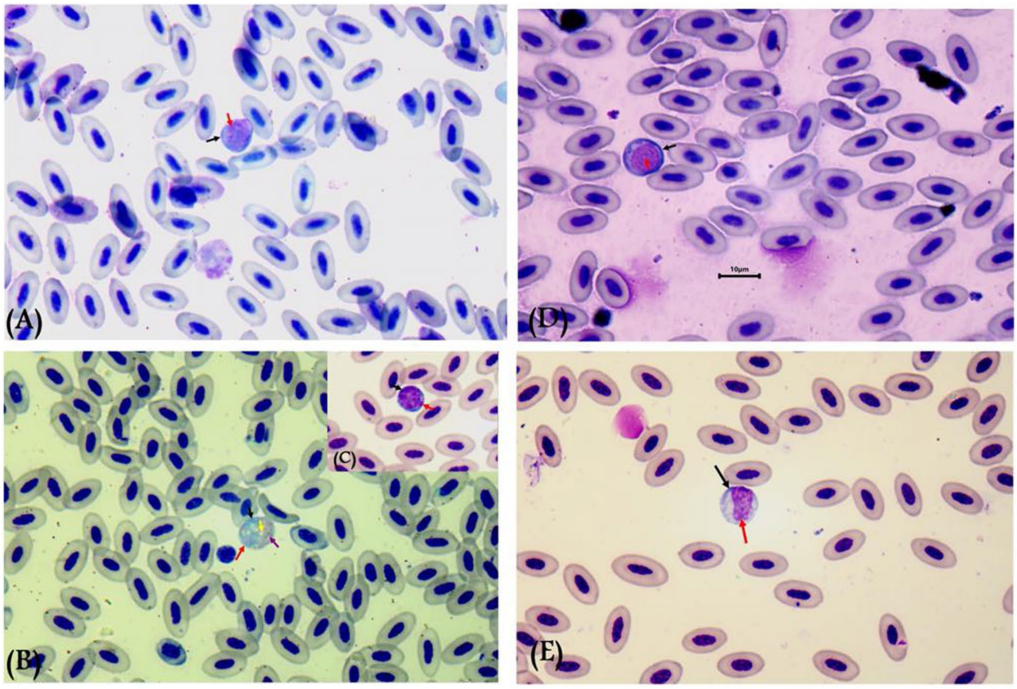

FIGURE 2
Leucocytozoon sabrazesi macrogametocytes **(A–C)** and microgametocytes **(D,E)** in pigeon's cells. **(A,E)** gametocytes in elongated form and **(C,D)** gametocytes in round form in pigeon's leukocyte cells **(C,D)**. The black arrow indicates host cell nuclei, the red arrow indicates nuclei of parasites, the yellow arrow indicates vacuoles, and the violet arrow indicates volutin granules. Scale bar = 10 µm.

well-defined intact or depleted capsules. Additionally, there was notable infiltration of lymphocytes and macrophages around the schizonts' periphery, contributing to hepatic cell necrosis near the portal areas. Other notable findings included portal vein congestion, blood sinusoid widening, fatty degeneration, and multiple thromboses in small and medium-sized hepatic vessels. Furthermore, congestion of the hepatic artery with thickening of its wall accompanied by amorphous eosinophilic infiltration was observed (Figure 3).

In the case of splenic infection (Figure 4), fewer megaloschizonts were observed in the interstitial tissue, accompanied by reticular hyperplasia. Intracellular hemosiderosis was noted as evidence for the destruction of erythrocytes. Moreover, lymphoid depletion and

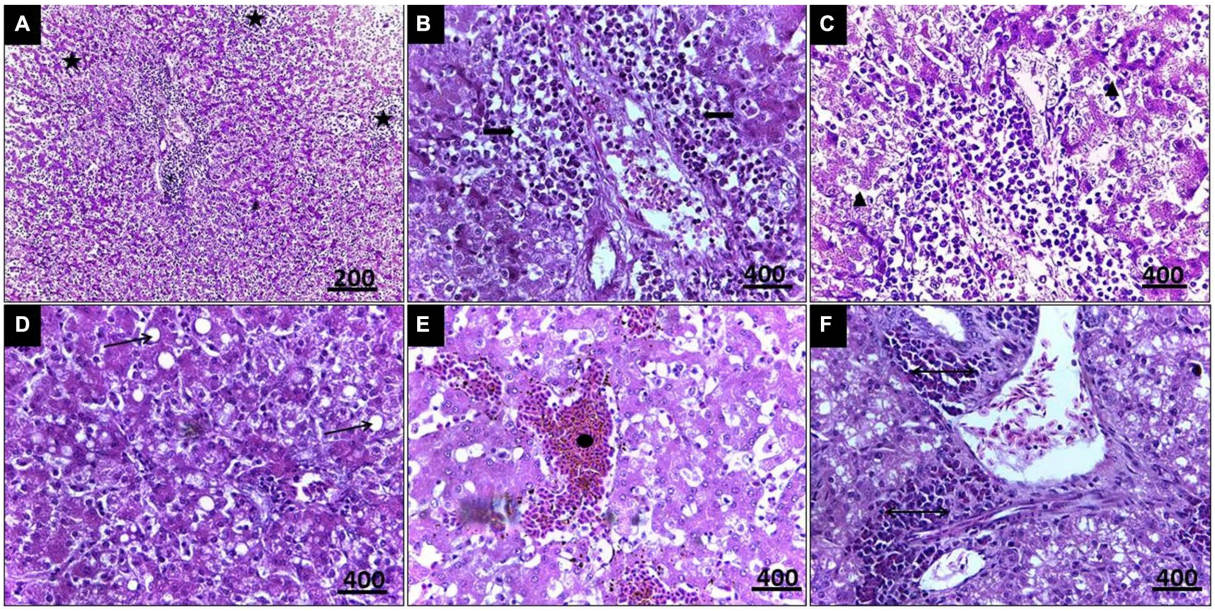

FIGURE 3
(A) Histopathological lesions of *Leucocytozoon* infection in a pigeon's liver illustrate multiple necrosis foci (5-Point Star). **(B)** Numerous developing megaloschizont aggregations in the portal area (Left & Right Arrows). **(C)** Dilated blood sinusoids (Isosceles Triangle). **(D)** Fatty degeneration (Arrow). **(E)** Blood thrombosis (Oval). **(F)** Eosinophilic infiltration (Double arrow), H&E stain.

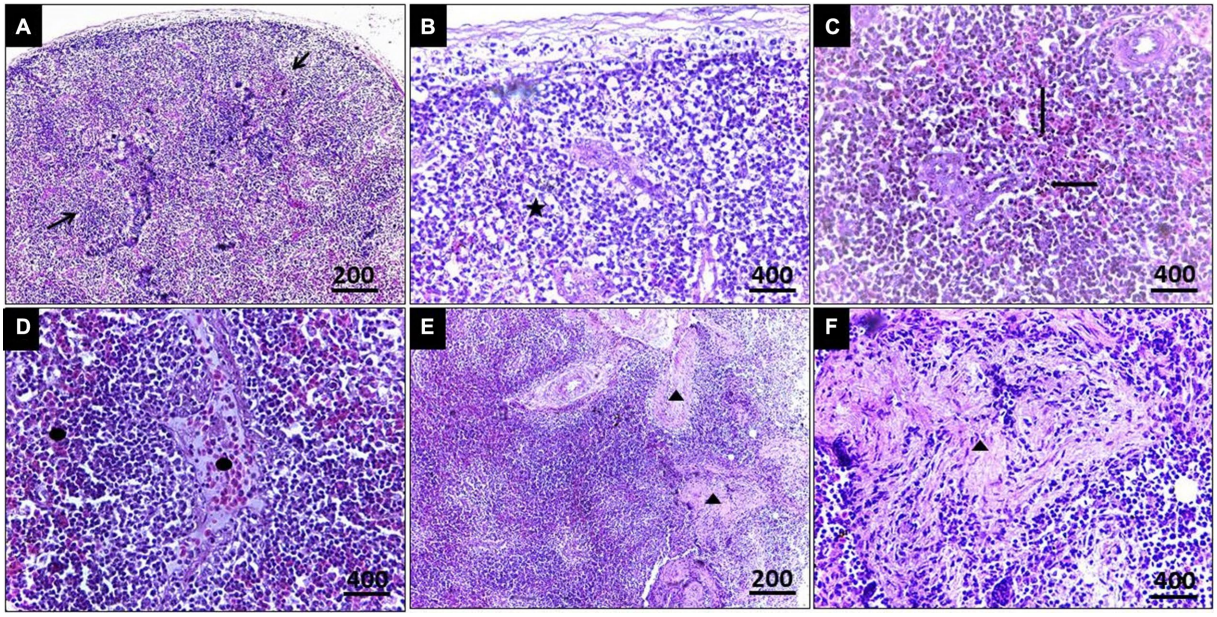

FIGURE 4
(A) Histopathological lesions of *Leucocytozoon* infection in the spleen of a pigeon illustrating necrotic areas with lymphocytic depletion (Arrows). **(B)** Few megaloschizonts distribution (5-Point Star). **(C)** Heamosedrosis (Left & down Arrows). **(D)** Lymphocytic cell infiltration and eosinophilic structures with vascular congestion (Oval). **(E,F)** Bands of fibrous tissue (Isosceles Triangle), H&E stain.

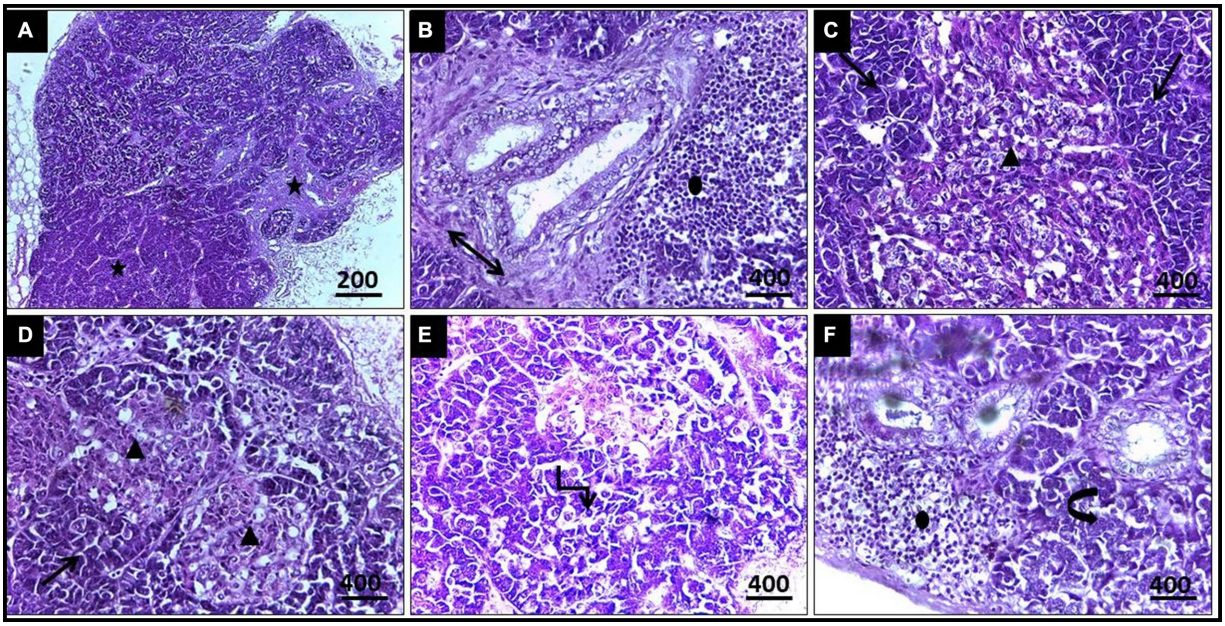

FIGURE 5
(A,B) Histopathological lesions of *Leucocytozoon* infection in a pigeon's pancreas illustrate obvious necrosis areas with mild fibrous tissue proliferation (5-Point Star & Double Arrow). **(B)** Lymphocytic infiltration (Oval). **(C)** Numerous megaloschizonts (Isosceles Triangles). **(D)** Zymogen granules depletion (Arrows). **(E)** Apoptotic acinar cells (Elbow Arrow Connector). **(F)** Disorganized acinar cells (Curved Right Arrow), H&E stain.

spleen atrophy with numerous eosinophilic structures (due to destructed megaloschizonts) were observed adjacent to the congested splenic artery. Bands of fibrous tissue were detected among splenic tissue.

Regarding histopathological lesions in the pancreas (Figure 5), acute pancreatic necrosis was observed, represented by zymogen granules depletion and shrinkage in the exocrine cells with degeneration in islets of Langerhans, resulting from megaloschizonts distribution. Bands of fibrous tissue were observed to separate the pancreatic ductular system, accompanied by multifocal mononuclear cell infiltrations consisting of lymphocytes and eosinophils. These features resulted in an apparent loss of acinar arrangement. Additionally, some acinar cells exhibited apoptotic vacuoles as a result of parasitism.

3.4 Molecular confirmation of *Leucocytozoon* by molecular methods

In the present investigation, a single, homogenous electrophoretic band of 248 bp was yielded by polymerase chain reaction, resulting from the amplification of the mitochondrial DNA genome (*cytb* gene) within the nuclear ribosomal gene complex. Sequence analysis, depicted in Figure 6, revealed that all DNA sequences were identical to the *cytb* gene of *L. sabrazesi*. The obtained sequence was then deposited in GenBank with the accession number ON399180. BLASTN sequence analysis revealed 100% nucleotide sequence homology with the reference isolates. Additionally, nucleotide sequence homologies of 98.16, 95.85, and 93.55% were reported with sequences of *L. sabrazesi* isolates from chicken in Thailand (MW316432; MW316434) and Malaysia (AB299369), respectively (Figure 6).

4 Discussion

Leucocytozoonosis is a significant parasitic disease affecting avian hosts globally. This disease can lead to severe pathology and economic losses, including decreased meat yield and egg production. Previous literature has documented a limited number of reports on *Leucocytozoon* infection in various avian species in Africa (22, 23), New Zealand (24), and Turkey (25, 26). However, to our knowledge, no previous investigations have explored this parasite among pigeons in Egypt. Therefore, this study is the first to examine *Leucocytozoon* species' prevalence and phenotypic characteristics among Egyptian pigeons.

The findings showed that 12.80% of examined birds were infected with *Leucocytozoon* species, highlighting the significant challenge of haemoparasitic infections in the studied area. This finding is consistent with the observation of Gocok et al. (26), who noted an infection rate of 13% for *Leucocytozoon* spp. among Turkish pigeons in Ankara province. However, a substantial disparity in the incidence of *Leucocytozoon* infection was observed compared to those recorded worldwide. Notably, very low prevalence rates of 2, 2.16, and 6.4% were reported among domestic pigeons from Bangladesh, India, and Nigeria, respectively (27–29). On the contrary, other surveys have reported higher infection rates, such as 30% in Pink pigeons (*Columba mayeri*) from the island of Mauritius in the Indian Ocean (30), 20% in pigeons from the Mymensingh district in Bangladesh (6), and 25% in various bird species from Europe, Africa, and North America (12). The significant fluctuations observed among the prevalence rates could be attributed to various factors, including differences in geographical locations, climatic conditions, bird breeds, management practices, sample sizes, detection methods employed, the presence of vectors, and study design (31–34).

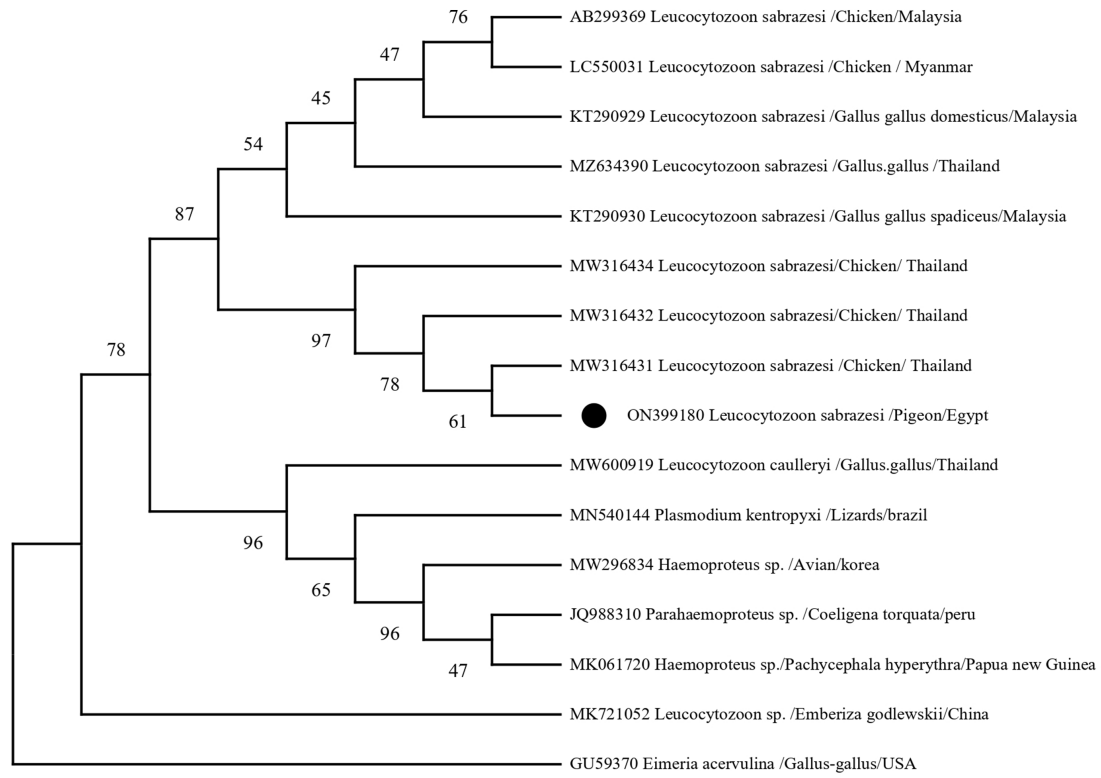

FIGURE 6
A phylogram of the maximum likelihood analysis of the *cytb* gene sequences of the *Leucocytozoon* spp. infecting poultry, depicting the robust association of the currently identified isolates of *Leucocytozoon sabrazesi* from pigeons marked by black circles (ON399180). The Maximum Likelihood Tree was implemented in MEGA software version 11 using *Eimeria acervulina* as outgroup. Bootstrap confident values were calculated with 500 repetitions.

The current investigation showed a significantly higher incidence of infection in adult birds than in young squabs, corroborating the findings of Garvin and Greiner (35). The same observation was documented in previous reports, revealing a significantly higher occurrence of avian haemoparasites in adults than grower birds in Bangladesh and Ethiopia (36, 37). However, our findings contradict previous reports from Kenya and Pakistan, which indicated that grower birds had a higher prevalence of avian haemoparasites than older birds (38, 39). Likewise, Van Oers et al. (40) and Castro et al. (41) observed a noticeably higher prevalence in young birds than in other age categories. On the other hand, other surveys concerned with blood parasites in various avian species have not found any significant association between the infection rate and host age (41–44). The positive correlation between the prevalence and age of screened birds could be accounted for higher mortality rates among young birds (2), declined immune response of adults (45), or increased exposure to the vectors (9).

The present results showed that the prevalence of *Leucocytozoon* spp. is influenced by sex, with a significantly higher infection rate in males (33.33%) than females (10.43%). This observation was supported by various previous reports (6, 46, 47). This observation is consistent with previous discussions by several authors who have suggested that higher testosterone levels and factors like stress during the courtship period play a significant role in immunosuppression in males, rendering them more susceptible to infection (48, 49). In contrast, Krone et al. (50) and Nath et al. (27) suggested that the highest peak of *Leucocytozoon* spp. prevalence rate is in female birds as compared to male pigeons.

According to the present findings, the incidence of *Leucocytozoon* parasite infection in pigeons in winter was the lowest (0%), and the difference between the winter and other seasons was statistically significant. Similarly, Nath and Bhuiyan (51) in Bangladesh demonstrated that the incidence of *Leucocytozoon* infections in pigeons during the summer was 60.6%, significantly lower in other seasons. Additionally, other studies on weaver birds of South Africa (52) and rock pigeons in India by Gupta et al. (53) were in the same line and documented that the summer season had the highest peak as compared to other seasons. However, the present finding disagrees with Senlik et al. (54), who demonstrated that the highest infection rate was recorded in the autumn season (44%), while the lowest rate was observed in the spring season in Iran. Moreover, Lawal et al. (55) revealed that the highest infection rates of haemoparasites, including *Leucocytozoon* spp., occurred during the rainy season (39.3%), followed by the cold dry (12.5%) season and the hot, dry season (7.7%). The potential explanation for the higher prevalence of *Leucocytozoon* in the dry season could be attributed to the warm climatic conditions that support the abundance of vector, *Simuliid* blackflies, which are widely distributed throughout the surrounding environment during this time of year (56–58).

In the current study, histopathological exploration revealed various lesions caused by *Leucocytozoon* spp. in the liver, spleen, and pancreas, including necrotic foci and loss of normal

organization. Numerous megaloschizonts infiltrated the interstitial spaces, some displaying intact capsules with nuclei while others showed signs of degeneration. Surrounding the distribution of schizonts, a lymphocytic reaction with eosinophilic structures was observed, indicating a host defense response. Fatty degeneration was evident in the liver, indicating *Leucocytozoon* infection, pronounced hemosiderosis, lymphoid depletion in the spleen, and depletion of zymogen granules in the pancreas. Additionally, vascular congestion and thrombosis were diagnosed in some cases. Histopathology proved to be a definitive diagnostic tool for *Leucocytozoon* tissue reaction, with numerous megaloschizonts detected in highly vascularized organs such as the pancreas, lungs, pectoral muscles, liver, spleen, and heart. Our findings align with previously reported worldwide studies (59–61).

It should be noted that one of the most characteristic pathological lesions associated with *Leucocytozoon* infections includes fatty degeneration, which may sometimes be mistaken for fatty liver haemorrhagic syndrome. However, in cases of *Leucocytozoon* infection, distinct megaloschizonts are observed in various organs (62). Additionally, microscopic examination of spleens invaded by *Leucocytozoon* documented chronic inflammation characterized by aggregations of mononuclear cells and disorganized tissue with unclear boundaries between splenic pulps, resulting from merozoite invasion of erythrocytes (63). Furthermore, *Leucocytozoon* spp. colonization, blockage, and thrombosis lead to multiple focal areas of necrosis and ischemia, followed by cardio-respiratory failure and death (64).

Interestingly, our study marks the first molecular characterization of *Leucocytozoon* parasites in domestic pigeons from Egypt, shedding light on the presence of this parasite in avian hosts and expanding our understanding of blood parasites infecting Egyptian pigeons, addressing gaps in their phylogeny. Through sequencing analysis, we identified *Leucocytozoon sabrazesi* in pigeons. Furthermore, the isolate recovered in our study exhibited a 98.62% similarity to reference sequences (MW316431) previously identified in chickens. Similarly, research by Chawengkirttikul et al. (3) indicated a low diversity of *L. sabrazesi* populations in Thailand, with similarity values ranging from 89.5 to 100% with sequences from Malaysia and Myanmar. Additionally, genetic diversity studies of *Leucocytozoon* sp. based on *cytb* gene sequences have been conducted in various countries (7, 10, 23, 65–68), highlighting the *cytb* gene's utility as an effective marker for phylogenetic taxonomy on a large scale and as a valuable tool for epidemiological analysis of leucocytozoonosis. Another survey in Egypt (11) reported the presence of *L. caulleryi* in broiler chicken flocks for the first time, showing a 99.14% similarity to strains recovered from Asian isolates in India, Japan, Malaysia, South Korea, Taiwan, and Thailand.

5 Conclusion

The current investigation marks the inaugural molecular study of haemosporidian parasites in pigeons in Egypt. Notably, our study stands as the pioneering genetic characterization of *L. sabrazesi* infection among pigeons, marking a national and global milestone. Additionally, our study has uncovered a significant statistical association between the infection prevalence of the parasite and various potential epidemiological factors, such as the age and sex of screened birds, with notable seasonal fluctuations observed throughout the year. Further studies are suggested to explore potential vectors at the national level, aiming to identify optimal preventive and therapeutic strategies against leucocytozoonosis, thereby mitigating or eradicating its detrimental impact on the bird industry. Additional large-scale surveys about the occurrence of the parasite within the avifauna of other regions in Egypt could provide valuable insights into blood parasite–host relationships and distribution patterns.

Data availability statement

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

Ethics statement

The animal studies were approved by the Ethics Committee, Faculty of Veterinary Medicine, South Valley University, Egypt according to the ethical regulations and guidelines for using animals in research (under permit code No. 84). Written and oral informed consent was obtained from the owners for the participation of their animals in this study. 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

IE: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. EM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing – review & editing. AM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. DS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. EA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. AA: Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Software, Validation, Visualization, Writing – review & editing. HA: Data curation, Formal analysis, Funding acquisition, Investigation, Resources, Software, Validation, Writing – review & editing. EE: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Optimizing sheep B-cell epitopes in *Echinococcus granulosus* recombinant antigen P29 for vaccine development

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Background: *Echinococcus granulosus* is a widespread zoonotic parasitic disease, significantly impacting human health and livestock development; however, no vaccine is currently available for humans. Our preliminary studies indicate that recombinant antigen P29 (rEg.P29) is a promising candidate for vaccine.

Methods: Sheep were immunized with rEg.P29, and venous blood was collected at various time points. Serum was isolated, and the presence of specific antibodies was detected using ELISA. We designed and synthesized a total of 45 B cell monopeptides covering rEg.P29 using the overlap method. ELISA was employed to assess the serum antibodies of the immunized sheep for recognition of these overlapping peptides, leading to the preliminary identification of B cell epitopes. Utilizing these identified epitopes, new single peptides were designed, synthesized, and used to optimize and confirm B-cell epitopes.

Results: rEg.P29 effectively induces a sustained antibody response in sheep, particularly characterized by high and stable levels of IgG. Eight B-cell epitopes of were identified, which were mainly distributed in three regions of rEg.P29. Finally, three B cell epitopes were identified and optimized: rEg.P29$_{71\text{-}90}$, rEg.P29$_{151\text{-}175}$, and rEg.P29$_{211\text{-}235}$. These optimized epitopes were well recognized by antibodies in sheep and mice, and the efficacy of these three epitopes significantly increased when they were linked in tandem.

Conclusion: Three B-cell epitopes were identified and optimized, and the efficacy of these epitopes was significantly enhanced by tandem connection, which indicated the feasibility of tandem peptide vaccine research. This laid a solid foundation for the development of epitope peptide vaccine for *Echinococcus granulosus*.

KEYWORDS

***Echinococcus granulosus*, sheep, recombinant antigen P29, B cell epitopes, vaccine**

1 Introduction

Echinococcus granulosus is a zoonotic parasitic disease caused by the larvae of the *Echinococcus* tapeworm, which parasitizes animals, including humans. It is globally distributed and prevalent in regions like Eastern Europe, East Africa, the Middle East, and Central Asia, particularly in areas with advanced animal husbandry (1, 2). This disease not only poses a severe threat to human health but also adversely affects the development of animal husbandry, leading to substantial medical and economic losses (3–5). Vaccines are a crucial and effective method for the prevention and control of epidemics, offering benefits such as high safety, no residue, and no withdrawal period for animals (6). The main vaccine types researched for *Echinococcus granulosus* include traditional, genetically engineered, nucleic acid, and peptide vaccines. Peptide vaccines are immunogenic vaccines designed and synthesized based on the amino acid sequence of an epitope from a known or predicted effective protective antigen (7, 8). Their simplicity in preparation, relatively stable structure, and absence of infection risk makes them a focal point in new vaccine research.

Screening and identifying dominant epitopes are essential for developing epitope-based vaccines. Optimizing antigen screening at the epitope level can induce a more effective immune response, ensuring immune specificity and safety (9, 10). Our group successfully cloned and constructed the recombinant antigen P29 (rEg.P29) earlier, which induced superior cellular and humoral immune responses in mice and sheep, providing 96.6% and 94.8% immune protection, respectively. These findings suggest that rEg.P29 is a promising candidate vaccine against *Echinococcus granulosus* (11, 12). We conducted rEg.P29 epitope peptide vaccine studies in mice, identifying T-cell and B-cell epitopes (13, 14), that elicited strong cellular and humoral immune responses in mice (15). However, data on peptide vaccines for sheep, the most suitable hosts for *Echinococcus granulosus*, are lacking. Developing and promoting the rEg.P29 peptide vaccine for sheep holds significant practical value for disease prevention and control.

In this study, we designed and synthesized single amino acid peptides covering rEg.P29 using the overlap method. We used enzyme linked immunosorbent assay (ELISA) to detect antibody recognition of overlapping peptides and initially screened B-cell epitopes. Following this, new peptides were designed and synthesized, with the B cell epitopes being finalized through further optimization and characterization.

2 Materials and methods

2.1 Preparation of antigen

rEg.P29 was prepared using a recombinant expression plasmid stored in our laboratory, following the specific protocol previously described (16). Briefly, sterile LB liquid medium was prepared, containing 0.1 mM isopropyl β-D-thiogalactoside (IPTG, Invitrogen, Waltham, USA). The preserved strain was inoculated into the LB liquid medium and incubated at 37°C for 10 h. rEg.P29 was then purified using a Ni-NTA His-Tag purification kit (Merck, Kenilworth, USA), and finally the protein was eluted and dissolved by Elution Buffer containing urea, and endotoxins were eliminated with an endotoxin removal kit (Genscript, Nanjing, China). The endotoxin-free purified rEg.P29 underwent protein concentration assessment using a BCA kit (KeyGen Biotech, Nanjing, China), and protein purity was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.2 Animal immunization and sample collection

Thirty-six female Chinese Yan chi Tan sheep, aged 4-6 months, were randomly divided into four groups. Each group received subcutaneous immunizations with primary and booster doses at 4-week intervals: PBS group (1 mL PBS), Quil A adjuvant group (1 mg Quil A, InvivoGen, San Diego, USA), rEg.P29 group (50 μg rEg.P29), and rEg.P29+Quil A group (50 μg rEg.P29 with 1 mg Quil A). Sheep peripheral blood was collected via the jugular vein at different time points, and serum was isolated. Fifteen 6–8-week-old female C57BL/6 mice were randomly divided into three groups: PBS group (1 mL PBS), Freund's adjuvant group (Freund's adjuvant), and rEg.P29+Freund's adjuvant group (20 μg rEg.P29 with Freund's adjuvant). Mice received booster immunizations two weeks after the initial dose, using Freund's complete and incomplete adjuvants (Sigma-Aldrich, St. Louis, USA). Mice were anesthetized with sodium pentobarbital via tail vein injection for serum collection. Details of animal immunization and sample collection are illustrated in Figures 1A, B.

2.3 Epitope peptide design and synthesis

Following the overlapping peptides design principle, each designed single peptide spanned 15 amino acids, with a step size of 5 amino acids and an overlap of 10 amino acids. We designed a total of 45 overlapping single peptides to encode the 238 amino acids of rEg.P29 (Figure 1C; Table 1). To aid in the screening of single peptides, three adjacent single peptides were mixed in equal proportions, resulting in 15 mixed peptide groups and one comprehensive mix of all single peptides. Sangon Biotech (Shanghai, China) synthesized the designed peptides with a purity of ≥ 98%, ensuring they were sterile and endotoxin-free. According to the buffer recommended in the peptide synthesis report, the peptides were dissolved in a 1 mg/mL solution and stored at -80°C for use.

2.4 Enzyme-linked immunosorbent assay

rEg.P29 (recognition antibody, positive control), along with single or mixed peptides, were diluted to 5 μg/mL by carbonate buffer (pH 9.6) and incubated overnight at 4°C in enzyme-labeled plates for encapsulation. The plates were then washed five times with PBST (PBS with 0.05% Tween-20) and subsequently blocked with 5% skim milk powder for 2 h at 37°C. After washing, diluted sheep or mouse serum, serving as the primary antibody, was added

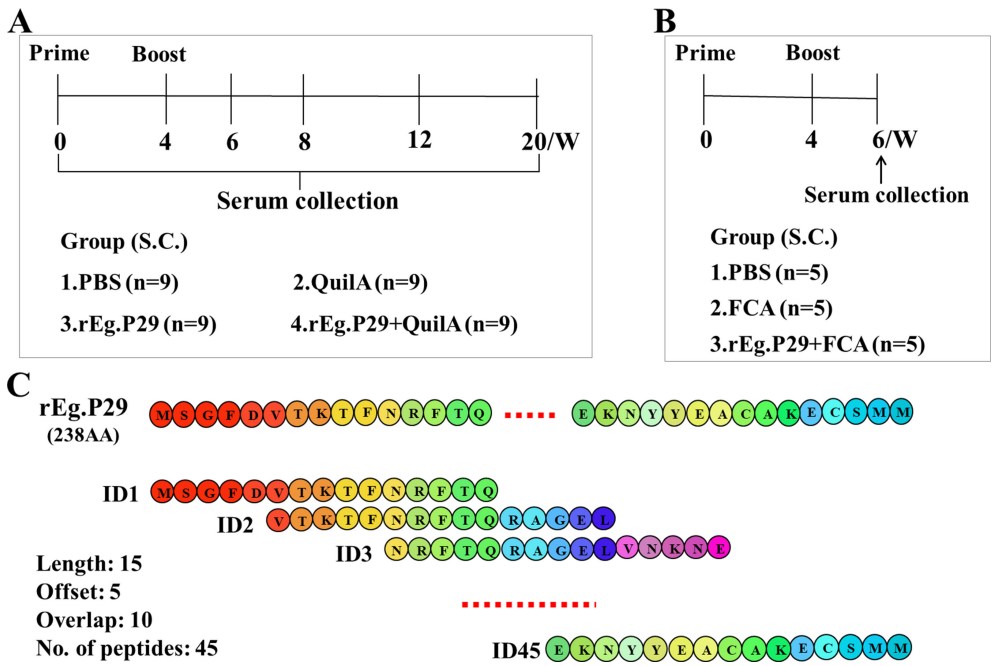

FIGURE 1
Animal immune grouping and epitope peptide design strategies. **(A)** Sheep (n=9/group) were immunized by subcutaneous injection in the abdomen, 4 weeks between initial and booster vaccination. Peripheral blood was collected at the designated times. **(B)** Mice were immunized subcutaneously via the abdomen, with an interval of 4 weeks between the initial and booster immunizations, and serum was collected two weeks after the booster immunization. **(C)** The 45 overlapping single peptides were designed with a length of 15 amino acids, a step of 5 amino acids and an overlap of 10 amino acids, covering 238 amino acids of rEg.P29.

to the plate, and incubated for 1 hour at 37°C. Horseradish peroxidase (HRP)-conjugated anti-sheep immunoglobulin G (IgG), IgM, IgA, IgE, IgG1, IgG2 (ABD Serotec, Kidlington, UK), or anti-mouse IgG, IgM, IgA, IgE, IgG1, IgG2a, IgG2b, IgG2c, and IgG3 (Abcam, Cambridge, USA) were added and incubated for another hour at 37°C. Following this, the plate was washed, and 3,3',5,5'-Tetramethylbenzidine (TMB) was introduced. The reaction was terminated with 2 M H2SO4. Absorbance was measured at 450 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, MA, USA).

TABLE 1 Designed overlapping peptides information in the study.

Peptide No.	Amino acid positions	Sequences	Length	Hydrophilic residue ratio	Basic/acidic
ID1	rEg.P29$_{1-15}$	MSGFDVTKTFNRFTQ	15	40%	neutral
ID2	rEg.P29$_{6-20}$	VTKTFNRFTQRAGEL	15	40%	basic
ID3	rEg.P29$_{11-25}$	NRFTQRAGELVNKNE	15	60%	neutral
ID4	rEg.P29$_{16-30}$	RAGELVNKNEKTSYP	15	53%	neutral
ID5	rEg.P29$_{21-35}$	VNKNEKTSYPTRTSD	15	60%	neutral
ID6	rEg.P29$_{26-40}$	KTSYPTRTSDLIHEI	15	40%	neutral
ID7	rEg.P29$_{31-45}$	TRTSDLIHEIDQMKA	15	47%	neutral
ID8	rEg.P29$_{36-50}$	LIHEIDQMKAWISKI	15	40%	neutral
ID9	rEg.P29$_{41-55}$	DQMKAWISKIITATE	15	40%	neutral
ID10	rEg.P29$_{46-60}$	WISKIITATEEFVDI	15	33%	acidic
ID11	rEg.P29$_{51-65}$	ITATEEFVDINIASK	15	40%	acidic
ID12	rEg.P29$_{56-70}$	EFVDINIASKVADAF	15	40%	acidic
ID13	rEg.P29$_{61-75}$	NIASKVADAFQKNKE	15	60%	neutral
ID14	rEg.P29$_{66-80}$	VADAFQKNKEKITTT	15	60%	neutral

(Continued)

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TABLE 1 Continued

Peptide No.	Amino acid positions	Sequences	Length	Hydrophilic residue ratio	Basic/acidic
ID15	rEg.P29$_{71-85}$	QKNKEKITTTDKLGT	15	53%	basic
ID16	rEg.P29$_{76-90}$	KITTTDKLGTALEQV	15	33%	neutral
ID17	rEg.P29$_{81-95}$	DKLGTALEQVASQSE	15	53%	acidic
ID18	rEg.P29$_{86-100}$	ALEQVASQSEKAAPQ	15	53%	acidic
ID19	rEg.P29$_{91-105}$	ASQSEKAAPQLSKML	15	53%	neutral
ID20	rEg.P29$_{96-110}$	KAAPQLSKMLTEASD	15	53%	neutral
ID21	rEg.P29$_{101-115}$	LSKMLTEASDVHQRM	15	47%	neutral
ID22	rEg.P29$_{106-120}$	TEASDVHQRMATARK	15	47%	basic
ID23	rEg.P29$_{111-125}$	VHQRMATARKNFNSE	15	53%	basic
ID24	rEg.P29$_{116-130}$	ATARKNFNSEVNTTF	15	47%	neutral
ID25	rEg.P29$_{121-135}$	NFNSEVNTTFIEDLK	15	53%	acidic
ID26	rEg.P29$_{126-140}$	VNTTFIEDLKNFLNT	15	40%	acidic
ID27	rEg.P29$_{131-145}$	IEDLKNFLNTTLSEA	15	47%	acidic
ID28	rEg.P29$_{136-150}$	NFLNTTLSEAQKAKT	15	47%	neutral
ID29	rEg.P29$_{141-155}$	TLSEAQKAKTKLEEV	15	53%	neutral
ID30	rEg.P29$_{146-160}$	QKAKTKLEEVRLDLD	15	60%	neutral
ID31	rEg.P29$_{151-165}$	KLEEVRLDLDSDKTK	15	67%	acidic
ID32	rEg.P29$_{156-170}$	RLDLDSDKTKLKNAK	15	67%	basic
ID33	rEg.P29$_{161-175}$	SDKTKLKNAKTAEQK	15	67%	basic
ID34	rEg.P29$_{166-180}$	LKNAKTAEQKAKWEA	15	53%	basic
ID35	rEg.P29$_{171-185}$	TAEQKAKWEAEVRKD	15	60%	neutral
ID36	rEg.P29$_{176-190}$	AKWEAEVRKDESDFD	15	67%	acidic
ID37	rEg.P29$_{181-195}$	EVRKDESDFDRVHQE	15	73%	acidic
ID38	rEg.P29$_{186-200}$	ESDFDRVHQESLTIF	15	53%	acidic
ID39	rEg.P29$_{191-205}$	RVHQESLTIFEKTCK	15	47%	basic
ID40	rEg.P29$_{196-210}$	SLTIFEKTCKEFDGL	15	40%	acidic
ID41	rEg.P29$_{201-215}$	EKTCKEFDGLSVQLL	15	47%	acidic
ID42	rEg.P29$_{206-220}$	EFDGLSVQLLDLIRA	15	40%	acidic
ID43	rEg.P29$_{211-225}$	SVQLLDLIRAEKNYY	15	47%	neutral
ID44	rEg.P29$_{216-230}$	DLIRAEKNYYEACAK	15	47%	neutral
ID45	rEg.P29$_{221-235}$	EKNYYEACAKECSMM	15	47%	acidic

Peptide information includes number, amino acid positions, sequences, length, hydrophilic residue ratio, and acid-base property.

2.5 B-cell epitopes screening

Single- and mixed-peptide screening was conducted using sheep serum samples exhibiting the highest IgG antibody levels. Plates were coated with either peptides or rEg.P29, and the serum acted as the primary antibody. B-cell mixed peptides were identified by assessing IgG antibodies' recognition using the ELISA method, as previously described. Subsequently, the corresponding single peptides of these mixed peptides were screened to pinpoint the B-cell epitopes.

2.6 B-cell epitopes identification and optimization

Based on the locations and amino acid sequences of the initially identified epitopes, new peptides were methodically designed, optimized, and subsequently synthesized. ELISA plates were coated with these newly synthesized peptides alongside rEg.P29, employing the same ELISA procedure as described previously for the screening of B-cell epitopes.

Ultimately, this process led to the identification and optimization of sheep B-cell epitopes.

2.7 Statistical analysis

Data analysis and graphing were conducted using the Statistical Package for the GraphPad Prism 8.0 graphing software (SPSS) version 22.0. Comparisons between two groups were executed using an unpaired t-test, while comparisons involving two or more groups employed one-way ANOVA. Data are presented as either mean or mean ± standard deviation (SD). $P < 0.05$ is considered statistically significant.

3 Results

3.1 rEg.P29 induces a sustained and strong antibody response in sheep

Analysis of serum antigen-specific antibodies in sheep at various time points post-immunization revealed that immunization with rEg.P29, particularly when supplemented with the adjuvant QuilA, elicited high levels of specific IgG, IgM, IgE, IgG1, and IgG2 (Figures 2A, B, D–F). A modest amount of IgA was also detected (Figure 2C), with IgG showing the highest and most rapid increase. Notably, immunization with rEg.P29 alone also induced some level of IgG production (Figure 2A). All antibody types demonstrated a rapid increase following immunization, reaching a peak two weeks post-booster immunization. Over time, antibody levels gradually declined, with IgA and IgM decreasing more rapidly compared to a slower decline in IgG.

Sera collected at weeks 4, 6, and 8 were diluted from 1:1,000 to 1:256,000. Remarkably, even at a 256,000-fold dilution, high IgG titers were maintained (Figures 3A–C), particularly evident at week 6 (Figure 3B), which corresponds to two weeks post-booster immunization. At a 64,000-fold dilution of the week 6 serum, the levels of IgG subtypes IgG1 and IgG2 remained relatively high, with IgG1 levels surpassing those of IgG2 (Figures 3D–F). These findings strongly suggest that rEg.P29 effectively induces a sustained antibody response in sheep, particularly characterized by high and stable levels of IgG.

3.2 Preliminary screening of eight B-cell dominant epitopes

In this phase, fifteen pools of 3 epitope peptides (Table 1), each comprising three adjacent single peptides, were screened. Five pools of 3 epitope peptides were identified: ID13-15, ID16-18, ID31-33, ID34-36, and ID43-45 (Figure 4A). Notably, ID13-15 and ID16-18 elicited higher responses, significantly differing from the other pools of 3 epitope peptides. These five pools of 3 epitope peptides collectively encompass fifteen single peptides: ID13, ID14, ID15,

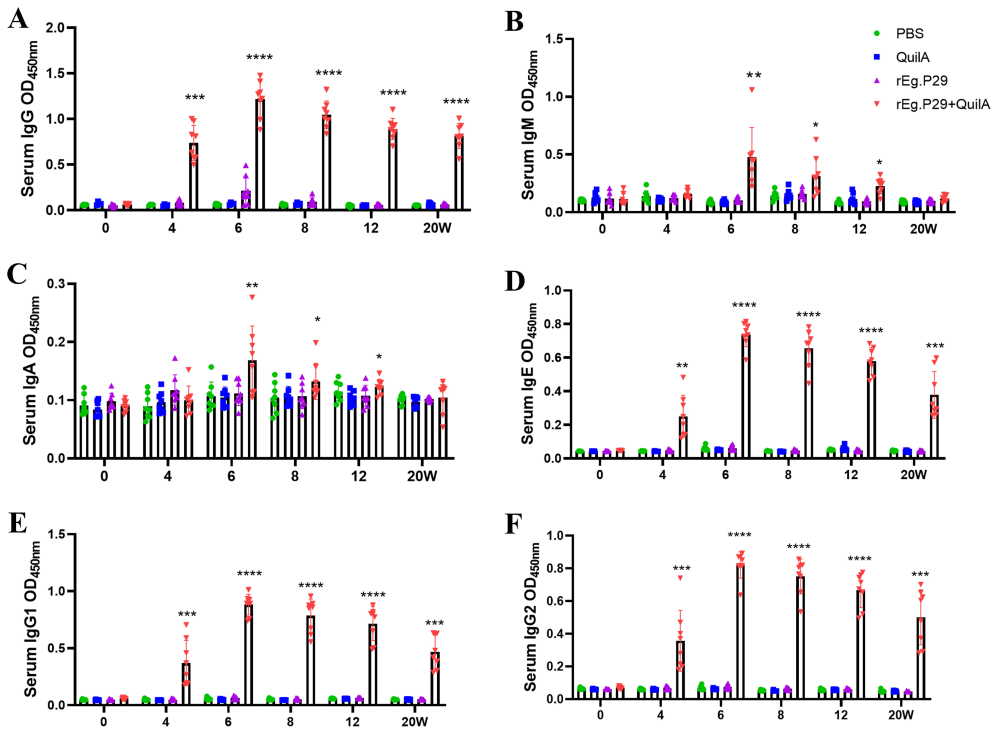

FIGURE 2
Serum antibody expression in rEg.P29-immunized sheep. **(A)** Expression of serum specific antibody IgG. **(B)** Expression of serum specific antibody IgM. **(C)** Expression of serum specific antibody IgA. **(D)** Expression of serum specific antibody IgE. **(E)** Expression of serum specific antibody IgG1. **(F)** Expression of serum specific antibody IgG2. 0 weeks represents primary immunization. Results presented as mean ± SD (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

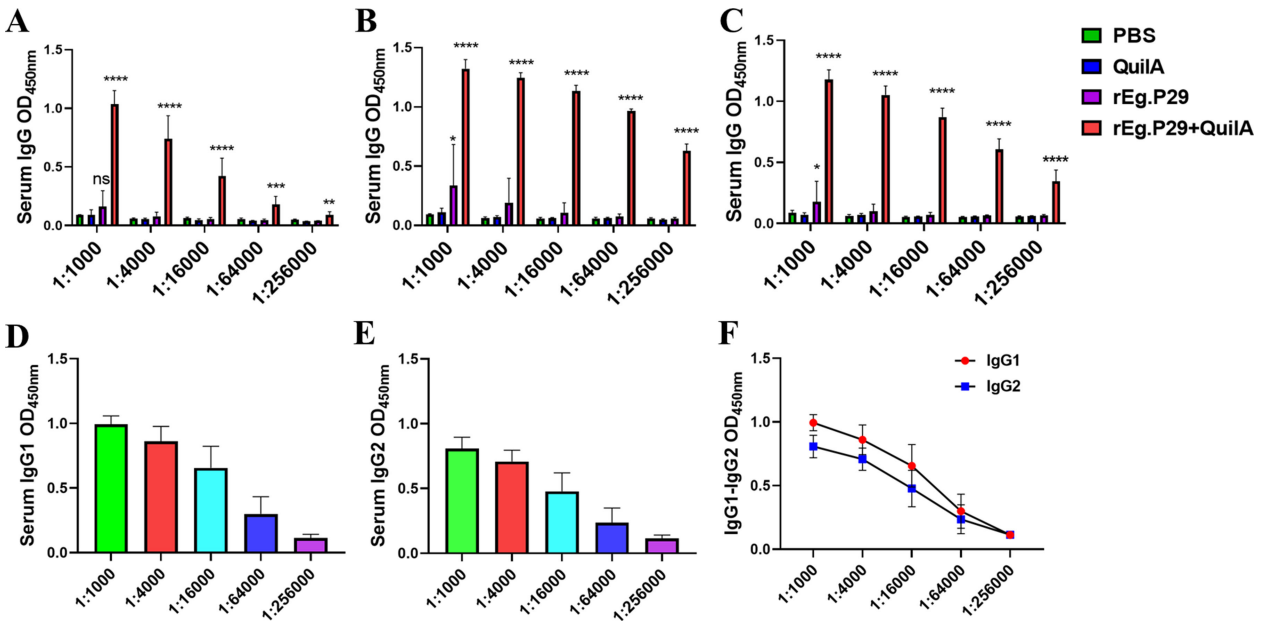

FIGURE 3
Serum IgG antibody titers determination in immunized sheep. **(A)** IgG antibody expression after doubling dilution of serum samples at week 4. **(B)** IgG antibody expression after doubling dilution of serum samples at week 6. **(C)** IgG antibody expression after doubling dilution of serum samples at week 8. **(D)** IgG1 antibody expression after doubling dilution of serum samples at week 6. **(E)** IgG2 antibody expression after doubling dilution of serum samples at week 6. **(F)** Comparison of IgG1 and IgG2 antibody expression after doubling dilution of serum samples at week 6. Results presented as mean ± SD (ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

ID16, ID17, ID18, ID31, ID32, ID33, ID34, ID35, ID36, ID43, ID44, and ID45. A subsequent screening was conducted on these 15 peptides.

Further analysis revealed that eight peptides were recognized in the immunoserum: ID15, ID16, ID31, ID32, ID33, ID43, ID44, ID45 (Figure 4B). These peptides correspond to the 71-85AA, 76-90AA, 151-165AA, 156-170AA, 161-175AA, 211-225AA, 216-230AA, and 221-235AA regions of rEg.P29, initially considered as potential linear B-cell epitopes. The identified epitopes were in three distinct regions of rEg.P29: ID15 and ID16 in the 71-90AA region, ID31, ID32, and ID33 in the 151-175AA region, and ID43, ID44, and ID45 in the 211-235AA region. The question arose whether these three regional peptides are more effective than the corresponding single peptides. To address this, further optimization, verification, and identification were undertaken.

3.3 Identification and optimization of three B-cell dominant epitopes

New single peptides corresponding to the regions 71-90AA, 151-175AA, and 211-235AA were synthesized. Additionally, three peptides were created by tandemly connecting two regions each, with GSGSGS tandem sequences inserted between them. This process resulted in six new single peptides (P1-P6), as depicted in Figure 5A. Antibody recognition tests revealed that P1 (71-90AA), P2 (151-175AA), and P3 (211-235AA) demonstrated markedly enhanced recognition compared to their respective individual peptides within each region. Notably, P3 exhibited superior efficacy (Figures 5B, C). The number of amino acids increased when P1, P2, and P3 were linked in tandem, enhancing their recognition beyond the pre-tandem levels. However, no significant difference in recognition was observed among the three tandem peptides (P4, P5, and P6), as shown in Figure 5B. Consequently, epitope peptides P1, P2, and P3 were identified as the three principal B-cell epitopes. It was also observed that the efficacy of these three epitopes significantly increased when they were linked in tandem.

3.4 Identified B-cell epitopes efficiently recognize antibodies in sheep and mice

The recognition of six single peptides, P1-P6, by IgM, IgA, IgE, IgG1, and IgG2 antibodies in sheep serum was observed. The results indicated that these peptides could not recognize IgM and IgA antibodies (Figures 6A, B), but they effectively recognized IgE, IgG1, and IgG2 antibodies. Notably, peptides P4, P5, and P6 showed superior recognition effects compared to P1, P2, and P3, with P5 demonstrating the most significant recognition impact on the three antibodies (Figures 6C–E).

Similarly, the interaction of these six single peptides, P1-P6, with various antibodies in mouse serum was examined. The findings revealed that they could recognize IgG, IgM, IgG1, and IgG2b antibodies (Figures 7A, B, E, G), but failed to recognize IgA, IgE, and IgG3 antibodies (Figures 7C, D, I). Peptides P4, P5, and P6 exhibited enhanced recognition effects compared to P1, P2, and P3. Additionally, P4, P5, and P6 were able to recognize IgG2a and IgG2c antibodies, unlike P1, P2, and P3 (Figures 7F, H). These

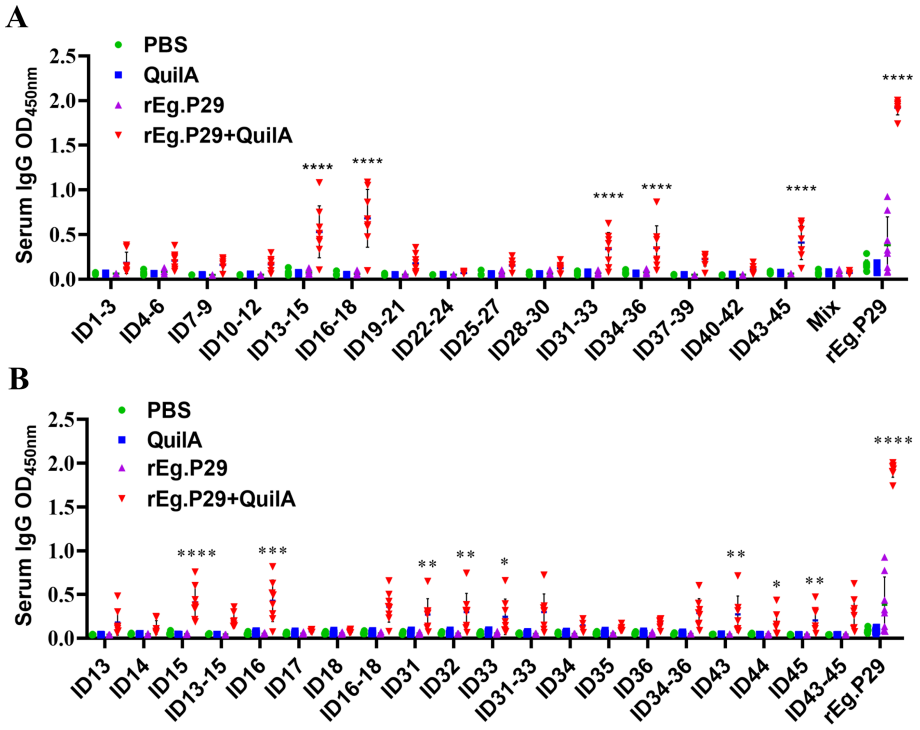

FIGURE 4
Preliminary screening of B-cell mixed peptides and single peptides. **(A)** Screening of B-cell mixed peptides by recognition of IgG antibodies. "Mix" means pools of 15 epitope peptides. **(B)** Screening of B-cell monopeptides by recognition with IgG antibodies. Results presented as mean ± SD (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

results demonstrate that the three B-cell epitopes, P4, P5, and P6, optimized through this study, can effectively recognize antibodies in both sheep and mice, qualifying them as dominant B-cell epitopes.

4 Discussion

Antigen-specific antibodies are crucial in antiparasitic infections and serve as a key index for evaluating humoral immunity (17, 18). Post-immunization of sheep with rEg.P29, the serum exhibited high titers of IgG, IgG1, and IgG2 antibodies, with levels rapidly increasing after booster immunization and remaining elevated until the 20th week. In earlier stages, our group employed rEg.P29 in conjunction with Freund's complete/incomplete adjuvant for sheep immunization and infection (12), which is consistent with the current research results. Lalramhluna et al. infected two types of sheep with Haemonchus contortus and noted a significant increase in IgG1 and IgG2 levels in the serum of resistant sheep, indicating a more robust humoral immune response (19). Valizadeh et al. used the envelope antigen extracted from live Protocercaria for sheep immunization and observed a notable elevation in IgG antibody titer in the immunized group (20). Heath et al. established the correlation between IgG antibody levels and immune protection in organisms (21). The high and sustained levels of IgG, IgG1, and IgG2 antibodies induced by the vaccine are vital for resistance to parasitic infections (22, 23), which is corroborated by the antibody responses observed in this study. These findings suggest that rEg.P29 is effective in inducing humoral immune responses in sheep.

On the surface of an antigenic molecule, certain specific chemical groups determine the antigen's specificity. These groups are known as antigenic determining groups or antigenic epitopes (24, 25), which act as functional units for antigen-receptor binding and play various roles in eliciting humoral and cellular immune responses (26, 27). In vaccine-induced protective immune responses, antigen-specific epitopes are predominantly involved, with the body's immune response primarily targeting antigen-dominant epitopes (28, 29). Peptide vaccines, based on antigenic epitopes, are crucial in disease prevention (30, 31). Identifying antigenically dominant epitopes with protective effects is fundamental for developing epitope-based peptide vaccines, making the study of antigenic epitopes a critical methodology. The overlapping synthetic peptide method is commonly used for identifying cellular epitopes (32, 33), which is adopted in this study.

Peptide vaccines designed to effectively elicit humoral and/or cellular immune responses must incorporate epitopes capable of triggering the desired immune reaction. B-cell epitopes are typically categorized as either linear or conformational epitopes (34, 35). Due to the challenges in identifying conformational epitopes and the widespread use of linear epitopes, the latter have garnered more attention. In dogs, fine-grained *Echinococcus granulosus* tapeworm infections are chiefly mediated by antibody-specific B-cell antibodies (36), making protective B-cell epitope peptides critical for peptide vaccine development (37). Researchers have conducted extensive studies on echinococcosis peptide vaccines, focusing primarily on informatics analysis and the epitope peptides of the Eg95 gene. Woollard et al. synthesized four epitope peptides of

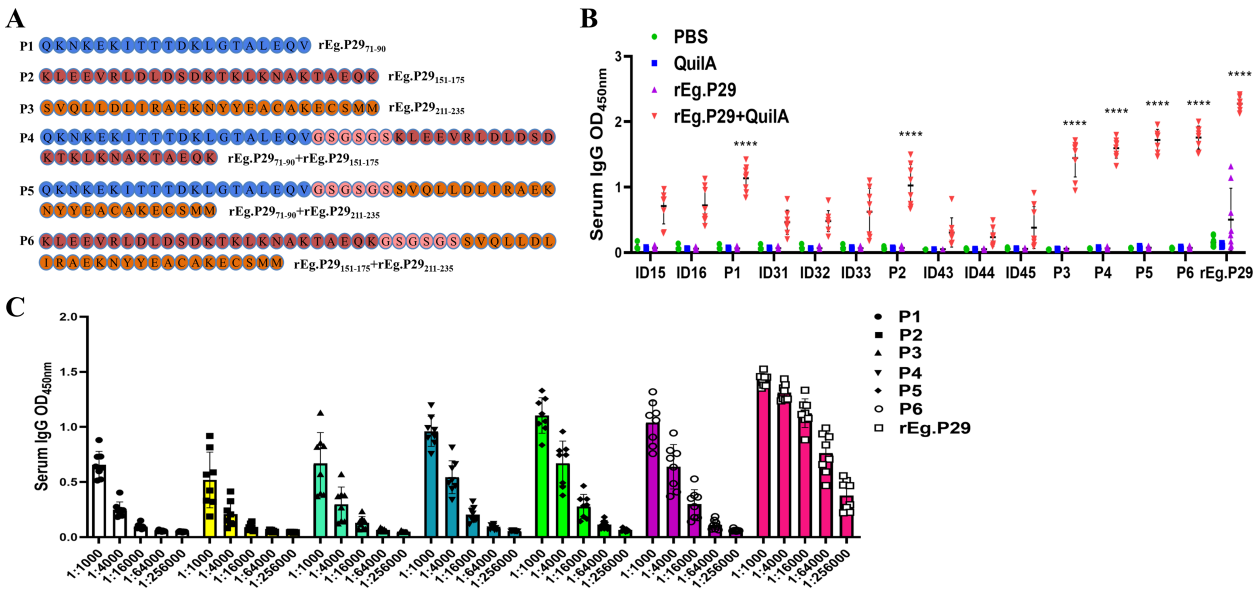

FIGURE 5
Optimized design strategies and identification of B-cell epitopes. **(A)** Three new single peptides were designed and connected in series two by two with GSGSGS in the middle. **(B)** Optimization and identification of B-cell epitopes by IgG antibody recognition (Statistical analysis was made within and between groups). **(C)** Doubling dilution determination of IgG antibodies to six B epitopes. Data from 9 sheep, results presented as mean ± SD (*****P* < 0.0001).

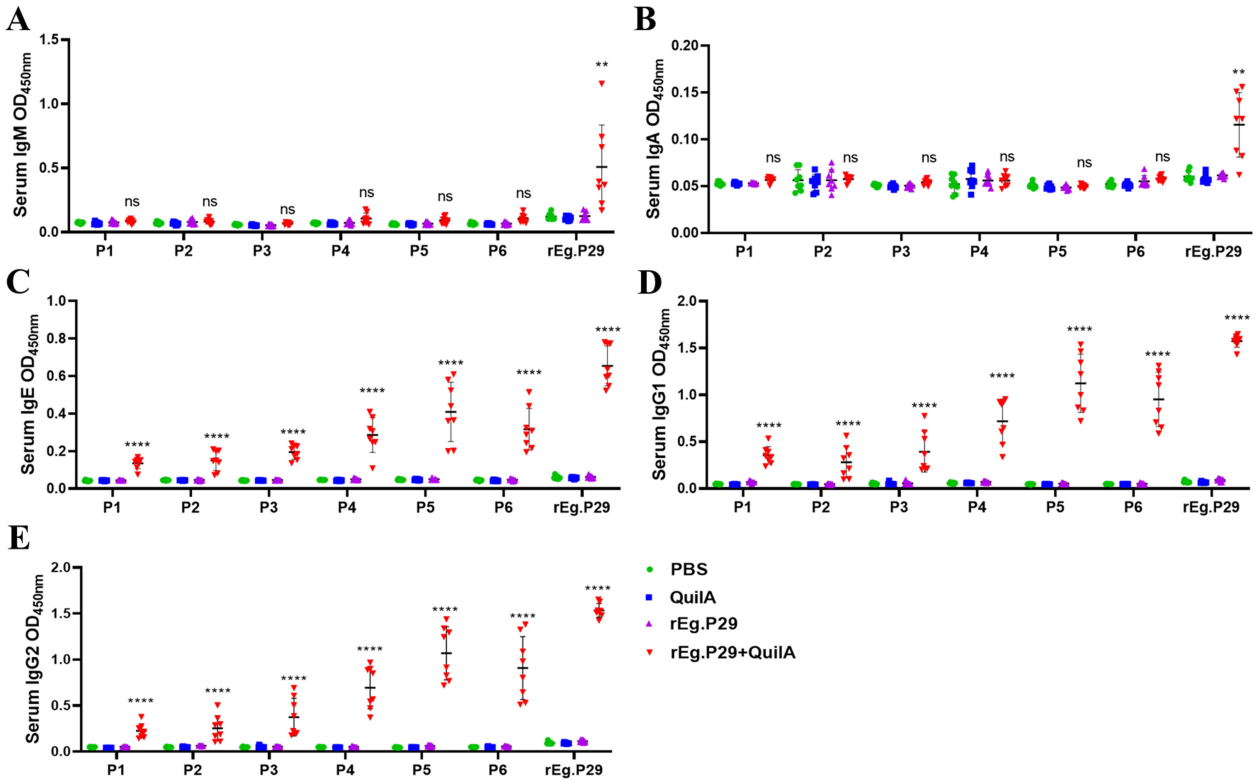

FIGURE 6
Recognition of sheep antibodies by optimized B cell epitopes. **(A)** Recognition of IgM by B cell epitopes. **(B)** Recognition of IgA by B cell epitopes. **(C)** Recognition of IgE by B cell epitopes. **(D)** Recognition of IgG1 by B cell epitopes. **(E)** Recognition of IgG2 by B cell epitopes. Results presented as mean ± SD (ns, *P* > 0.05; ***P* < 0.01; *****P* < 0.0001).

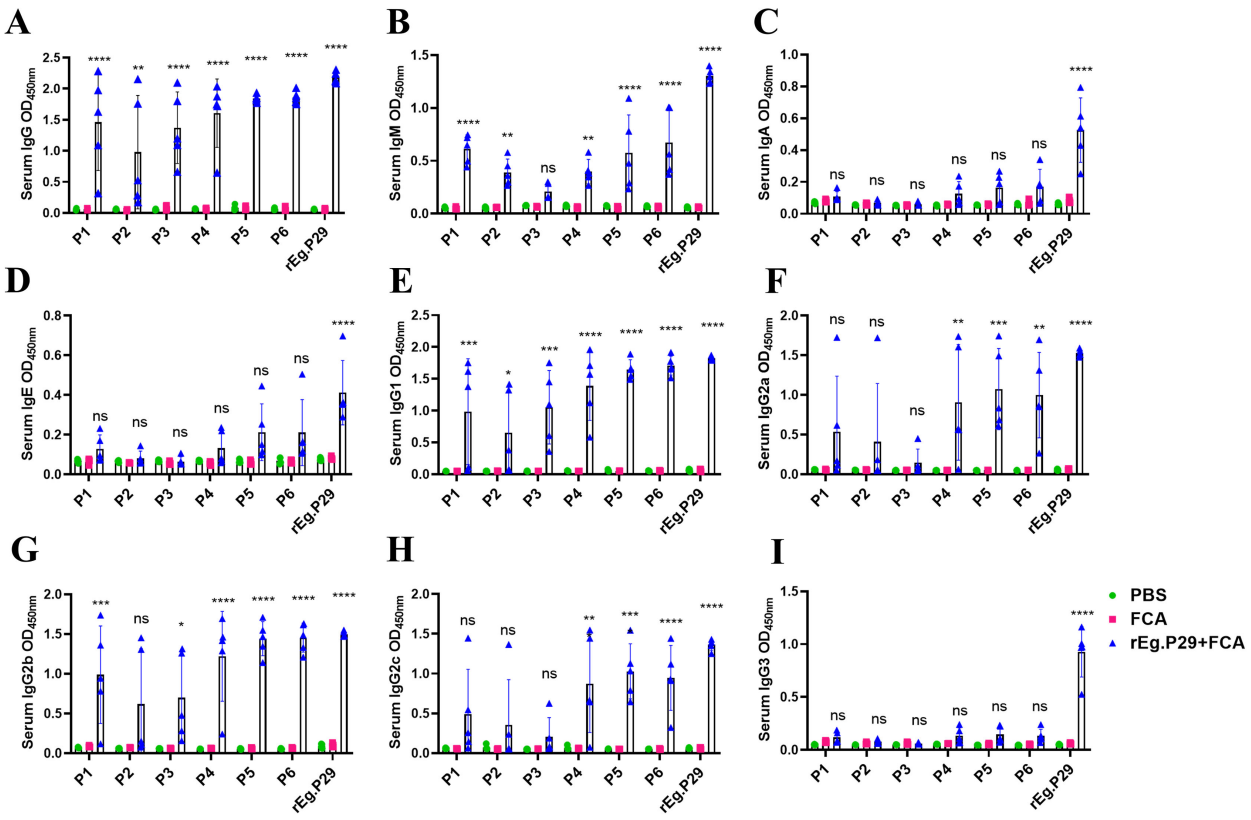

FIGURE 7
Recognition of mouse antibodies by optimized B cell epitopes. **(A)** Recognition of IgG by B cell epitopes. **(B)** Recognition of IgM by B cell epitopes. **(C)** Recognition of IgA by B cell epitopes. **(D)** Recognition of IgE by B cell epitopes. **(E)** Recognition of IgG1 by B cell epitopes. **(F)** Recognition of IgG2a by B cell epitopes. **(G)** Recognition of IgG2b by B cell epitopes. **(H)** Recognition of IgG2c by B cell epitopes. **(I)** Recognition of IgG3 by B cell epitopes. Results presented as mean ± SD (ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

Eg95, demonstrating their strong immunogenicity in inducing IgG1 and IgG2 antibodies in sheep. However, these peptides did not confer immune protection in sheep, indicating a need for further investigation into the mechanism of immune protection by epitope peptides (38). Esmaelizad et al. integrated five T-cell epitopes into a multicell epitope antigen, which induced mice achieved a protection rate of 99.6% (39). Currently, peptide vaccines for echinococcosis remain in the stages of informatics prediction and laboratory validation (40–42), with no mature peptide vaccines available yet for the prevention and treatment of echinococcosis. Our efforts are directed towards developing a multi-epitope peptide vaccine with effective immune-protective properties.

In this study, we screened and optimized B-cell epitopes by examining the interaction of overlapping peptides with specific IgG antibodies using ELISA. The dominant B-cell epitopes were identified as rEg.P29$_{71\text{-}90}$ (P1), rEg.P29$_{151\text{-}175}$ (P2), and rEg.P29$_{211\text{-}235}$ (P3). It was confirmed that these dominant B-cell epitopes could recognize sheep-specific IgE, IgG1, and IgG2 antibodies, but they did not bind to specific IgM and IgA antibodies. This may be attributed to the relatively low levels of these two antibodies in the serum. When the three dominant epitopes were linked in tandem, their peptide recognition efficacy significantly exceeded that of the individual dominant epitopes. This improved recognition is likely due to the broader range of epitopes presented by a larger number of amino acids, suggesting the potential for developing multi-epitope peptide vaccines. Moreover, various peptides and peptides in tandem also identified mouse-specific IgG, IgM, IgG1, and IgG2b antibodies. Additionally, the tandem peptides were able to recognize mouse-specific IgG2a and IgG2c. This indicates that the B-cell epitopes screened and identified using sheep might also be effectively applicable in mice.

Screening of the few peptides identified did produce high OD values, but we must be concerned that the coating efficiency of peptides affects the results of peptide identification. This requires us to use certain methods to make the peptide encapsulation as homogeneous as possible, such as the use of labelling and antibody capture methods. Inhibition experiments can be performed to control the binding of rEg.P29 antibody bound to the enzyme labelled plate, allowing better control of the encapsulation efficiency. Antibodies that are highly reactive to peptides in ELISA are not necessarily neutralizing antibodies and may not be immunoprotective. At the same time, some of the peptides identified by the screen may be aggregated, resulting in obtaining peptides that may not be the results we desire. Through the mouse animal model, our research group screened and confirmed the dominant epitopes of T cells and B cells of rEg.P29. Immunizing mice with combined epitopes produced strong humoral and cellular immune effects, especially B cell

epitopes (15). The protective effect of combined epitope vaccine on mouse infection model is being studied. Screening and identifying peptides with good immunoprotective effects is our goal. The binding of peptide-inducing antibodies to natural rEg.P29 would be a good indicator of potential efficacy. rEg.P29 is known to bind tightly to lipids, which may affect its ability to bind to peptide-inducing antibodies. This provides a better reference for our subsequent studies. Therefore, combined with the results of the mouse animal model, in the follow-up study, we will carry out the peptide vaccine protection effect study targeting B-cell epitopes.

5 Conclusion

Three dominant B-cell epitopes of rEg.P29 were successfully identified: rEg.P29$_{71\text{-}90}$, rEg.P29$_{151\text{-}175}$, and rEg.P29$_{211\text{-}235}$. The efficacy of these epitopes was notably enhanced through tandem linkage, indicating the feasibility of conducting research on tandem peptide vaccines. This advancement lays a solid groundwork for the development of epitope-based peptide vaccine.

Data availability statement

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

Ethics statement

The animal study was approved by Ethics Committee of Ningxia Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JY: Writing – original draft, Writing – review & editing. YL: Writing – original draft. YZZ: Writing – original draft. JS: Writing – original draft. MZ: Writing – review & editing. CW: Writing – review & editing. YF: Writing – original draft. WZ: Writing – review & editing. YQZ: 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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A comparative field efficacy trial of three treatment programs against endo- and ectoparasites in naturally infected dogs

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Introduction: Tropical climates in remote Aboriginal and Torres Strait Islander communities in northern Australia are conducive to the transmission of canine helminths such as hookworms, as well as ectoparasites such as fleas and ticks. In addition to their veterinary importance, these parasites may present a zoonotic risk either directly, or as potential vectors for bacterial pathogens. These factors necessitate efficacious and effective antiparasitic treatment programs for community dogs.

Methods: A cluster-randomised trial was performed on three islands in the Torres Strait to examine the short-term efficacy and medium-term effectiveness of three treatment programs. Treatments administered included oral oxibendazole/praziquantel (Paragard®) and oral afoxolaner (Nexgard®); topical moxidectin/imidacloprid (Advocate®) and imidacloprid/flumethrin collars (Seresto®); and off-label oral ivermectin (Bomectin®). Canine faecal samples were collected and examined for endoparasites by faecal flotation and real-time PCR at baseline, 7–11 days after treatment and 6 months later.

Results: The proportion of dogs positive for *Ancylostoma caninum* at baseline and negative at day 7–11 was 9% (95% CI 4.4–17.4) for dogs treated with oxibendazole, 56.4% (95% CI 41–70.7) for moxidectin, and 89.7% (95% CI 73.6–96.4) for ivermectin. Faecal flotation results showed a greater than 90% egg reduction in 29.2% (95% CI 19.9–40.5) of dogs treated with oxibendazole, 79.4% (95% CI 63.2–89.7) for moxidectin, and 95% (95% CI 76.4–99.1) for off-label ivermectin. Elimination of ectoparasite infestation was observed at day 7–11 in 69.9% (95% CI 56.7–80.1) of dogs treated with afoxolaner, 80% (95% CI 60.9–91.1) with imidacloprid/flumethrin collars, and 0% (95% CI 0–11.7) for off-label ivermectin. Mixed effects modelling revealed only treatment group to be significantly associated with outcome measures.

Discussion: Based on these study results, the poor efficacy of oxibendazole against *A. caninum* renders it inept for treatment, while ivermectin and moxidectin were suitable. Ivermectin was unsuitable for ectoparasite treatment due to its poor efficacy, while afoxolaner and imidacloprid/flumethrin collars appear suitable.

KEYWORDS

canine, ivermectin, moxidectin, oxibendazole, afoxolaner, flumethrin, hookworm

1 Introduction

In tropical climates, and particularly in remote community settings, canine endoparasites and ectoparasites and the diseases they vector cause significant morbidity and mortality in dogs and are also responsible for some of the most important and well recognised zoonoses affecting humans (1–4). Endoparasites such as hookworms of the

genus *Ancylostoma* spp., threadworms (*Strongyloides* spp.) and roundworms (*Toxocara canis*) constitute some of the most prevalent canine zoonotic helminths of stray, semi-domesticated and pet dogs throughout tropical regions of the world (5, 6). Infections with these parasites can result in asymptomatic to serious clinical manifestations in dogs and people. For example, *Ancylostoma* spp. infections can cause profound haemorrhagic enteritis and anaemia in dogs, depending on parasite species and worm burden. *Ancylostoma* spp. infection in humans may cause cutaneous larva migrans, or in the case of *Ancylostoma caninum*, eosinophilic enterocolitis (5, 7). While most human intestinal infections with *A. caninum* were found to be caused by a single adult worm, more recent evidence suggests that patent infections are potentially possible (8). Infection with *Toxocara canis* may manifest as ocular toxocariasis with vision loss or retinal damage or as visceral toxocariasis with wheezing, asthma, fever, or abdominal pain (9).

High burdens of fleas (*Ctenocephalides felis*) and brown dog ticks (*Rhipicephalus linnaei*) in community dogs contribute to the spread of tick-borne diseases ehrlichiosis, hepatozoonosis, babesiosis and anaplasmosis, while fleas may pose a zoonotic risk for the transmission of bartonellosis and flea-borne spotted fever (10–13). In addition to the risk of vector-borne diseases, pruritis caused by even transient flea or tick infestations or bites may predispose humans to chronic secondary skin infections with potential sequelae of impetigo, rheumatic fever, or rheumatic heart disease (14, 15).

As in Aboriginal communities across other parts of Australia, dogs in Torres Strait Islander communities may have many different roles including companion, hunting partner, source of protection, or cultural or spiritual roles (16–18). These important roles, as well as the often free-roaming nature and large populations of dogs in these communities, may place community members at risk of acquiring parasite and flea-borne zoonotic pathogens either directly through close contact, or indirectly through contact with, or ingestion of parasitic stages in contaminated soil and bedding (19).

Efficacious endo- and ectoparasitic treatments are essential to mitigate the morbidity related to canine parasites. The remoteness of many Australian Aboriginal and Torres Strait Islander communities means that veterinary visits may be limited, sporadic or ultimately unattainable due to logistical or financial barriers. As such, identifying effective antiparasitic treatment programs which can be administered regularly without the need for veterinary oversight is of value to these communities. Off-label treatments require veterinary oversight to be administered as they are being used outside of the registered and labelled use (20). Such treatments have formed the mainstay of remote community veterinary antiparasitic treatment despite scarcity of evidence of their effectiveness in these settings. Evaluating the efficacy of off-label treatment is therefore of value, particularly to the veterinarians, local government departments or non-government organisations (NGOs) owing to their potential cost effectiveness (21, 22). With these factors in mind, the aim of this study is to examine the short-term efficacy and medium-term effectiveness of two labelled antiparasitic treatment programs in comparison to the off-label usage of ivermectin in a remote Torres Strait Islander community setting. The resulting evidence will inform antiparasitic programs which can be administered by community members either with or without veterinary oversight.

2 Materials and methods

2.1 Study setting and population

The Torres Strait Islands comprise over 270 small islands in the Torres Strait between the northernmost tip of mainland Australia in the state of Queensland and Papua New Guinea spanning an area of over 48,000 km^2. Sitting at the border of equatorial savanna and monsoonal climate regions based on a modified Köppen climate classification system (23), the primary weather station for the islands recorded a mean annual rainfall of 1736 mm and mean temperature range of 24.7–30.5°C between 1995 and 2023 (24).

Dogs on three remote islands were enrolled in this cluster-randomised trial. Islands were selected based on recommendations from the Torres Strait Islands Regional Council regarding adequate dog numbers present as well as community consultation and acceptance of the proposed study. Locations of the selected islands are shown in Figure 1. Torres Strait Islander community engagement and leadership was crucial to this study. In-person consultation was conducted with local Environmental Health Worker staff to ascertain what was important to the community and to develop a feasible study methodology. This was followed by consultation with elders and elected council representatives of all island groups regardless of their inclusion in the study, and approval of a formal research proposal. This study was also approved by the University of Melbourne Animal Ethics Committee (ID: 10298).

All dog owners on each selected island were approached to provide verbal and written consent to have their dogs recruited into the study. Dogs were not recruited if owners did not consent or were not present to provide consent. Dogs on each island were assigned to the same treatment arm to ensure consistent administration of ongoing treatments and to reduce the risk of environmental contamination influencing other treatment group outcome measures. Treatment arms consisted of; Group A—oral tablets administered at 22.5 mg oxibendazole/5 mg praziquantel per kilogram bodyweight (Paragard®, Boehringer Ingelheim) and oral chews administered at 2.5 mg afoxolaner per kilogram bodyweight (Nexgard®, Boehringer Ingelheim); Group B—topical 1% moxidectin/10% imidacloprid applied at 0.1 mL per kilogram bodyweight (Advocate®, Elanco) and a 10% imidacloprid/4.5% flumethrin polymer matrix collar (Seresto®, Elanco) administered according to the labelled instructions and; Group C—off-label oral ivermectin (Bomectin®, Elanco) administered at 200 µg/kg in bread with flavoured paste. As ivermectin administration in this context is off-label usage, it required oversight from a registered veterinary practitioner.

2.2 Data collection

At baseline, dog and owner names and address details were collected for the purpose of follow-up reidentification. Other dog details recorded at the time of enrolment included sex, sterilisation status, estimated weight, and age group. Age group information was provided by dog owners at the time of enrolment or was estimated by a veterinarian on examination of the dog. Age group classifications consisted of puppies which were less than 6 months old, young dogs which were 6 months to 2 years old, adults which

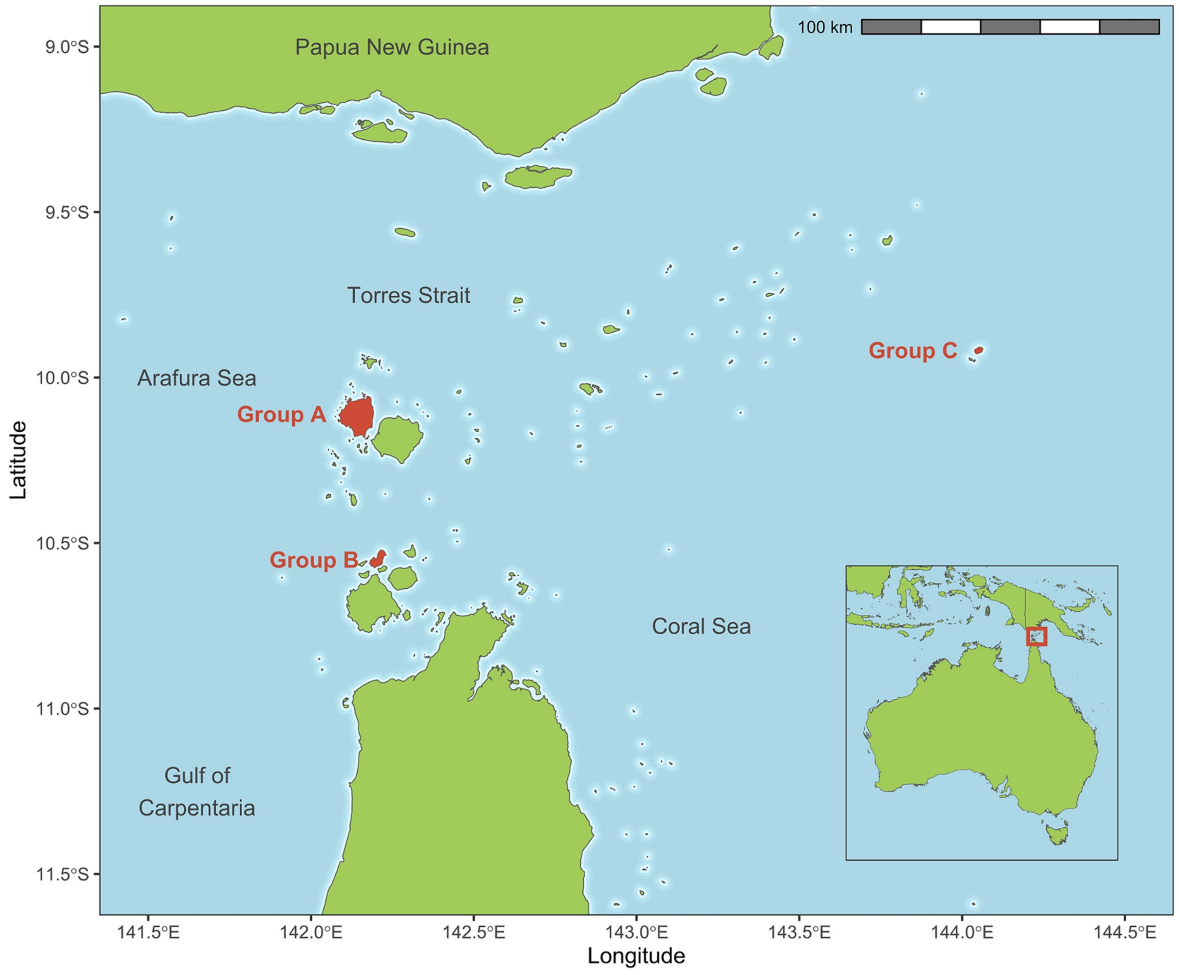

FIGURE 1
Torres Strait region map with shaded and labelled study islands.

were 2–8 years old, and old dogs which were greater than 8 years old. Any overt skin lesions were noted, and a targeted patch examination technique of predilection sites was used to establish a semi-quantitative measure of tick burden on each dog as described by Brianti et al. (25). Briefly, a tick score of zero indicates no ticks detected, a score of 1 indicates between 1 and 5 ticks detected, a score of 2 indicates 6–20 ticks detected, a score of 3 indicates 21–50 ticks detected, a score of 4 indicates 51–100 ticks detected and a score of 5 indicates over 100 ticks detected. The same system was employed to determine flea burden. Single faecal samples were collected from each dog rectally, or from the ground if rectal collection was not possible and a fresh ground sample identifiable to the dog was available. All faecal samples were immediately stored in DNA/RNA Shield (Zymo Research, Irvine, USA) at a 1:2 ratio for transport at room temperature to the University of Melbourne for laboratory analysis. At this point, treatments were administered per specified treatment arm and dogs remained under their owners' care thereafter.

Follow-up sampling was conducted by the same method 7–11 days post-treatment. This timeframe allows detection of reduction or cure of initial infection whilst avoiding new or re-infections as it is shorter than the prepatent period of *Ancylostoma* spp. Dogs were reidentified from recorded data to allow comparison of baseline and post-treatment data. Repeat measures of flea and tick count were also recorded.

Dogs again remained in their owners' care and were treated according to their treatment arm 3 months post-baseline. Treatments were administered by trained local Environmental Health Workers. Six months post-baseline, dogs were reidentified and underwent repeat faecal sampling and flea and tick counting.

2.3 Coproscopic and molecular methods

One gram of faeces was subjected to a quantitative faecal float using a centrifugal faecal flotation (CFF) method with saturated sodium chloride and sucrose (specific gravity 1.27). Parasite eggs were manually counted and converted to eggs per gram (EPG) by multiplying counts by the inverse of the faecal sediment measured in the centrifuge tube to allow sample comparison.

DNA was extracted from 200 mg of faeces of each sample using the Maxwell® RSC PureFood GMO and Authentication Kit (Catalog no. AS1600, Promega Corporation, Madison, USA) with the Maxwell® RSC 48 Instrument (Catalog no. AS8500, Promega Corporation,

Madison, USA) using a modified method as described by Massetti et al. (26).

Extracted DNA was subjected to multiplex qPCR assays for the detection of four species of canine hookworm including *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Uncinaria stenocephala*, and *Ancylostoma braziliense* as well as *Strongyloides* spp. according to published protocols (26, 27). Internal amplification controls were performed using equine herpes virus (EHV4) primers (EHV-F, EHV-R), probe (EHV probe) and EHV4 synthetic DNA fragments containing the target sequence (gBlock® Gene Fragments, IDT® Technologies, Skokie, USA). DNA extraction controls were performed with mammalian primers (MAM-F, MAM-R) and probe (MAM probe) (27–29). Synthetic DNA fragments containing the target sequence of each parasite species (gBlock® Gene Fragments, IDT® Technologies, Skokie, USA) were used as positive controls and no-template negative controls were included in all runs. A five channel AriaMx Real-time PCR System (Agilent, Santa Clara, USA) was used for the amplification, detection, and data analysis of all samples (Agilent Aria software).

2.4 Statistical analysis

Demographic and physical examination and laboratory data were recorded on paper then transferred, cleaned, and validated in an electronic spreadsheet (Microsoft Excel v. 1908, Microsoft Corporation, Redlands, USA). Recoding of variables was conducted where necessary and data was analysed and plotted in R (v. 4.2.2) (30) using RStudio and contributed packages lme4 (v. 1.1–34) (31), emmeans (v. 1.8.7) (32), ggplot2 (v. 3.4.2) (33), epiR (v. 2.0.60) (34), and terra (v.1.7–55) (35). Flea and tick scores were combined to an ectoparasite score and subsequently used as a binary variable (present/absent) to account for low frequencies. Similarly, age group categories were collapsed to dogs under 1 year of age and dogs over 1 year of age to account for low frequencies.

Dog demographic data including age group, sex and desexed status as well as qPCR-based endoparasite prevalence, hookworm EPG distributions and ectoparasite prevalence were described for each treatment arm. Short-term data between baseline and day 7–11 post-treatment permitted the calculation of efficacy measures for each treatment; those being the performance of each treatment under close to ideal conditions which do not include new re-infections. Cure rates (CR) were calculated as a percentage in which the number of dogs qPCR-positive for a parasite species pre-treatment and negative 7–11 days post-treatment was divided by the total number of dogs positive for the parasite species pre-treatment. 95% confidence intervals were calculated for prevalence and CR estimates using the epi.conf function in the epiR package. Cure rates for ectoparasites were also conducted in the same manner for each treatment arm and demographic group.

For dogs testing positive for hookworm eggs at baseline, egg reduction rates (ERR) were calculated as a percentage, where the 7–11 days post-treatment count was subtracted from the baseline count and divided by the baseline count. Per World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines and International Co-operation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guidelines as adopted by the Australian Pesticides and Veterinary Medicines Authority (APVMA), a 90% ERR threshold was used to indicate an anthelmintic as efficacious for label claim requirements (36–38). In the present study, the proportion of dogs achieving a 90% or greater ERR in each treatment and demographic group was calculated along with 95% confidence intervals.

Considering all timepoint data up to 6 months permits the calculation of effectiveness measures which, in contrast to efficacy measures, are inclusive of real-world influences such as re-infection. Generalised linear mixed models were used to assess associations between treatment group and EPG and treatment group and ectoparasite infestation based on Poisson and binomial family models, respectively. Individual dog and island were included as random effects and age group, sex and desexed status were included as fixed effects. Backward stepwise variable selection was used to arrive at the final model considering a p-value of <0.05 significant. A data dispersion ratio was calculated from the sum of residual squares divided by the number of observations. R code for this analysis is included in Supplementary File S1.

3 Results

Treatment arms consisted of 80 dogs in Group A, 51 in Group B and 44 in Group C at baseline. Populations varied in each treatment group with respect to the representation of age group, sex and desexed status. Demographic data for dogs in each treatment group at baseline are shown in Table 1. Adult dogs were the largest age group in each treatment group followed by young dogs. No puppies were included in Group C. Proportions of male dogs were higher in Groups A and C, while more females were found in Group B. More desexed dogs were present in Group C while more entire dogs were present in Groups A and B. One dog in Group B was not present for resampling at post-treatment follow up and was therefore excluded from efficacy analysis. 18 dogs from Group A, 15 dogs from Group B and 20 dogs from Group C had either died or were not present for sampling at the six-month timepoint and were therefore excluded from medium-term

TABLE 1 Dog demographic data from each treatment group.

Variable and category	Total *n* (%)	Group A *n* (%)	Group B *n* (%)	Group C *n* (%)
Age group				
Puppy	11 (6.3)	4 (5)	7 (13.7)	0 (0)
Young	45 (25.7)	18 (22.5)	10 (19.6)	17 (38.6)
Adult	103 (58.9)	53 (66.2)	32 (62.7)	18 (40.9)
Old	16 (9.1)	5 (6.2)	2 (3.9)	9 (20.5)
Sex				
Female	74 (42.3)	33 (41.2)	26 (51)	15 (34.1)
Male	101 (57.7)	47 (58.8)	25 (49)	29 (65.9)
Desexed				
Yes	69 (39.4)	27 (33.8)	19 (37.3)	23 (52.3)
No	106 (60.6)	53 (66.2)	32 (62.7)	21 (47.7)

effectiveness analysis. All other dogs were present for sampling at all time points.

Mammalian DNA extraction controls were positive for all samples. Only *A. caninum* and *Strongyloides* spp. were detected by the multiplex qPCR and only *A. caninum* was detected at levels allowing for before-and-after comparison in individual dogs. Overall baseline qPCR-based prevalence of *A. caninum* was 83.9% (95% CI 77.7–88.6) with 97.5% (95% CI 91.3–99.3) in Group A, 78.4% (95% CI 65.4–87.5) in Group B and 65.9% (95% CI 51.1–78.1) in Group C. Baseline microscopy-based EPG varied widely, with a geometric mean of 219 (range 0–14,430) and high degrees of skewness (4.85) and kurtosis (26.04). Baseline EPG was highest in puppies, with three puppies (and a single adult) shedding more than 10,000 EPG. Individual dog *A. caninum* EPG counts, flea score and tick score at each time point are presented in Figure 2.

Cure rates and ERR results for dogs which tested positive to *A. caninum* via qPCR CFF and positive for ectoparasites via patch examination are shown in Table 2.

Baseline prevalence for fleas was 37.5% (95% CI 27.7–48.5) for dogs in Group A, 23.5% (95% CI 14–36.8) for Group B and 36.4% (95% CI 23.8–51.1) for Group C. Baseline prevalence for ticks was 65% (95% CI 54.1–74.5) for dogs in Group A, 39.2% (95% CI 27–52.9) for Group B and 52.3% (95% CI 37.9–66.2) for Group C. Positive or negative ectoparasite status derived from this led to the calculation of cure rates presented in Table 2.

Coefficient estimates for the final EPG Poisson and ectoparasite infestation binomial models are presented in Table 3. Neither age group, sex nor desexed status were significantly associated with EPG or ectoparasite infestation between baseline and day 7–11 or between this time point and 6-months and were thus removed from the final model. Intraclass correlations were calculated, with greater than 99.9% of the variation in EPG and ectoparasite infestation attributable to differences between dogs, rather than island clusters. Overdispersion was present in the EPG model with a data dispersion ratio of 406.

4 Discussion

This study found that the treatment administered to each animal group was the most significant factor associated with reductions in *A. caninum* egg shedding (EPG) as well as presence or absence of ectoparasite infestation. Results indicate that demographic variables of age group, sex and desexing status are not associated with anti-parasiticide efficacy and effectiveness in this setting.

For treatment of *A. caninum*, off-label ivermectin performed best in terms of both qPCR CR and 90% ERR. This supports the findings of a treatment trial by Bhanjadeo et al. (39), for which ivermectin administered at 200 µg/kg body weight to 12 dogs infected with *A. caninum* with a mean EPG of 1,725 at baseline, produced a CR and EPG reduction of 100% at day 15 post-treatment. Studies have also demonstrated high efficacy of ivermectin against *A. caninum* at doses as low as 10 µg/kg (40, 41). In Australia, administration of ivermectin in dogs at doses above 6 µg/kg body weight represents off-label use. The lack of registered treatments may be partly due to the presence of the ABCB1 gene mutation often present in collie breeds and their crosses, which makes them more sensitive to toxic effects of ivermectin at doses used to target gastrointestinal helminths (42). In the author's experience, the dogs living in remote Aboriginal and Torres Strait Islander communities tend to be medium-sized crossbreeds often known as 'Australian camp dogs,' and very rarely include collie dog genetics. The risk for these dogs is low and testing for gene mutations is not necessary (43). Nonetheless, care must be taken in populations which may have the ABCB1 gene mutation. Off-label drugs cannot be purchased by dog owners and require veterinary oversight, which comes at greater cost either at an individual dog level or in community-level animal health programs. Off-label usage also means that there may be less standardisation in the method and dose administered compared to commercially produced animal treatments, especially oral treatments. In this study, ivermectin was soaked into bread and covered with peanut butter for palatability, which was well accepted by the dogs, though palatability is often a challenge in these settings. However, acceptance cannot always be relied upon for any oral treatment in any dog whether it be commercially available or off-label.

While a study by Hellmann et al. (44) of the efficacy of topical moxidectin/imidacloprid in 131 naturally hookworm infected dogs found a geometric mean ERR of 99.92% 8 to 13 days following treatment, the proportion (29.2%) of dogs achieving a 90% ERR in the present study and low CR of 56.4% did not support this treatment's efficacy to the same degree. One possible explanation for the reduced efficacy may be the inability to control for the application of the product to a dry coat and the avoidance of wetting the coat within 24 hours of application (45). Since dogs could not be supervised after treatment, it is possible that the rapid skin absorption of the moxidectin component of the product may have been disrupted. Moreover, individual clearance of moxidectin from the system may vary between dogs of different body condition score owing to their differing levels of adipose tissue, though this would be more relevant to moxidectin's sustained larvicidal effect than its immediate adulticidal efficacy (46). Furthermore, differences in the distribution of body condition score did not differ significantly between treatment groups and would not sufficiently explain any differences in observed treatment effects.

Efficacy of oxibendazole based on this study was demonstrated to be poor against *A. caninum*. While tableting of dogs is the most difficult of the three endoparasitic treatments to administer in this study and is generally prone to failure due to dogs not accepting tablets, these treatments were all administered by a trained, registered veterinarian and all treatments were confirmed to have been swallowed. Individual dog data in Figure 2 shows multiple cases in which dogs were not only without cure or egg reduction but appear to have increases in egg counts following treatment. Several confounding factors are known to influence successive faecal egg counts in the same individual such as time of sampling, faecal consistency, and host diet (47). These effects may have been masked in the other treatment groups by treatment effects but were more evident in Group A due to a lack of efficacy.

Only a single study is known to have examined the efficacy of oxibendazole against hookworms in dogs. In this study, oral oxibendazole at a dose rate of 15 mg/kg administered to naturally infected dogs found a 94.6% reduction of *A. caninum* based on the reduction in the arithmetic mean EPG from baseline to 8–10 days post-treatment (48). The finding of such a high arithmetic mean ERR is surprising compared to the findings of the present study which used a higher dose rate of 22.5 mg/kg. The fact that only 11 dogs were initially infected with *A. caninum* in the Overgaauw and Boersema study, along with a lack of reported confidence intervals and accurate

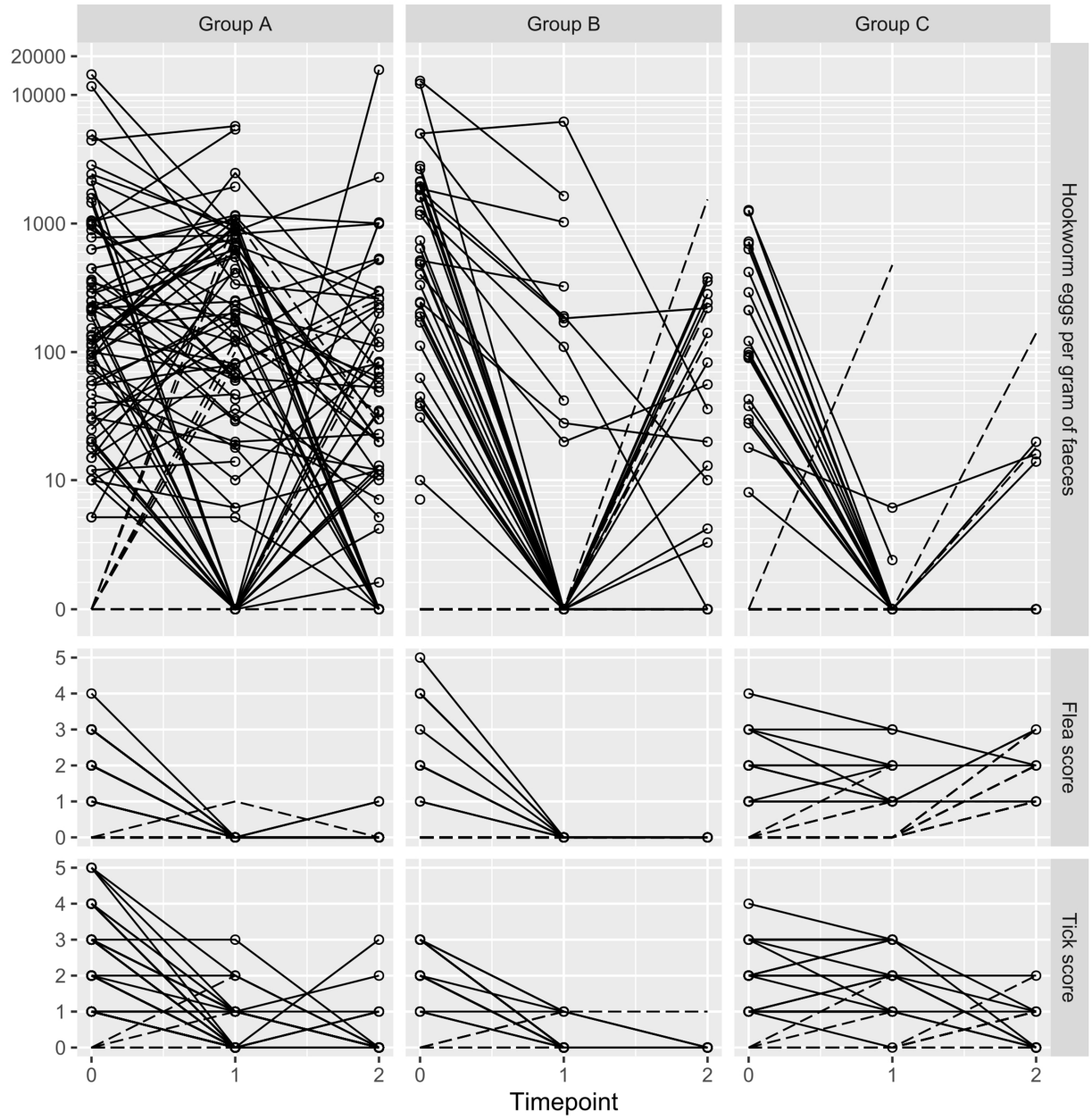

FIGURE 2
Trellis plot of hookworm eggs per gram, flea score and tick score at baseline (timepoint 0), 7–11 days following treatment (timepoint 1) and 6 months later (timepoint 2). Each line shows results of an individual dog. Dotted lines represent dogs with a baseline count or score of zero.

demographic data calls the validity of the presented results into question. Poor efficacy of oxibendazole, as with other benzimidazoles, may be related to its low aqueous solubility further compounded by the relatively rapid gut transit times of dogs (49). For that reason, efficacy of benzimidazoles is predominately time- rather than dose-dependent, with optimal efficacy typically only seen after repeated doses over 3–5 days (50). By contrast, in a recent study involving the development of an *in vitro* egg hatching assay to determine the ovicidal effects of anthelmintics it was revealed that oxibendazole, despite its poor adulticidal and larvicidal properties, demonstrated high potency against hookworm eggs, while eggs exposed to moxidectin or ivermectin showed relatively unchanged levels of maturation and hatching (51). This may point to a potential use for benzimidazoles in combination with an efficacious adulticidal and larvicidal treatment to immediately reduce environmental shedding of viable eggs, though further *in vivo* studies are necessary. Further studies are also required to investigate the potential for resistance to benzimidazole anthelminthics, and indeed all anthelmintics used for mass drug administration to treat *A. caninum*, especially with mounting evidence of β-tubulin gene fenbendazole resistance in this species (42).

Analysis of ectoparasite cure rates found oral afoxolaner given to Group A and imidacloprid/flumethrin collars given to Group B to be highly efficacious, which supports the findings of previous

TABLE 2 Endoparasite and ectoparasite outcome measures at 7–11 days post-treatment by treatment and demographic group for dogs which were positive at baseline.

Variable and category	*A. caninum* qPCR cure rate		Dogs achieving *A. caninum* 90% egg reduction rate		Ectoparasite cure rate	
	n	% (95% CI)	*n*	% (95% CI)	*n*	% (95% CI)
Total	146	37.7 (30.2–45.8)	126	53.2 (44.5–61.7)	110	53.6 (44.4–62.7)
Treatment						
Group A	78	9 (4.4–17.4)	72	29.2 (19.9–40.5)	56	69.6 (56.7–80.1)
Group B	39	56.4 (41–70.7)	34	79.4 (63.2–89.7)	25	80 (60.9–91.1)
Group C	29	89.7 (73.6–96.4)	20	95 (76.4–99.1)	29	0 (0–11.7)
Age group						
Puppy	9	22.2 (6.3–54.7)	9	55.6 (26.7–81.1)	8	75 (40.9–92.9)
Young	38	47.4 (32.5–62.7)	32	46.9 (30.9–63.6)	27	40.7 (24.5–59.3)
Adult	89	32.6 (23.7–42.9)	78	55.1 (44.1–65.7)	64	60.9 (48.7–71.9)
Old	10	60 (31.3–83.2)	7	57.1 (25–84.2)	11	27.3 (9.7–56.6)
Sex						
Female	63	39.7 (28.5–52)	54	53.7 (40.6–66.3)	48	56.2 (42.3–69.3)
Male	83	36.1 (26.6–46.9)	72	52.8 (41.4–63.9)	62	51.6 (39.4–63.6)
Desexed						
Yes	54	44.4 (32–57.6)	44	48.8 (38.3–59.4)	38	44.7 (30.1–60.3)
No	92	33.7 (24.9–43.8)	82	61.4 (46.6–74.3)	72	58.3 (46.8–69)

studies by Brianti et al. (25) and Fankhauser et al. (52). While the product label of Advocate® and Seresto® state that the products are still efficacious against ectoparasites after swimming, free-roaming dogs in these island settings frequently swim in salt water. Nevertheless, regular wetting of the coat did not appear to reduce efficacy of the imidacloprid and flumethrin concentrations within the coat in the hours to days after application in this study. Group C demonstrated very poor ectoparasitic efficacy, and although macrocyclic lactones are known to have lethal paralytic effects on arthropods at the time of exposure, this could not be observed at the time of follow-up and either the same or new flea and tick burdens were observed (50).

Random effects variance for island clusters in mixed effects modelling in this study was very low. While a lack of treatment randomisation would ordinarily be a limitation in many treatment trials, here it was a necessary study design feature. The impact of mass treatments was being assessed on a community, rather than individual animal level, including the ability of mass treatment to reduce environmental shedding and in turn re-infection rates. Had dogs been randomly allocated on each island, treatments with poor efficacy could have led to greater environmental contamination with parasites and greater chances of reinfection for all dogs over time, which may have reduced apparent medium-term effectiveness for what were otherwise more effective treatments. Realistically, differences in location in terms of veterinary and owner care would have been negligible and given that the time between pre-treatment and post-treatment sampling was insufficient to allow new patent reinfections, any differences based on location would have been minimal.

Random effects variance for individual dogs was, in comparison to island clusters, much higher. To allow maximal inclusion of dogs from areas with limited populations for the sake of statistical power, all dogs from all demographics were enrolled. Ideally at least 80 dogs would have been included in each treatment arm with a more equal distribution of age groups. While attempts were made to choose islands with the largest dog populations, a wave of parvovirus in the study islands leading to the deaths of several dogs immediately prior to initial sampling precluded reaching the planned sample size. Low numbers of dogs in the puppy and old age categories meant that collapsing these categories was necessary and that more detailed examination of age group associations with changes in outcome were not possible in mixed effects modelling. While puppies had the highest baseline EPG, it is biologically doubtful that age group alone would affect the clearance of infection holding all other variables constant. Other factors and comorbidities affecting young or old animals may affect their susceptibility to infection, however.

The Poisson model showed a high degree of overdispersion, which may be expected from field based faecal egg count data in which a large proportion of counts were zero along with some counts above 14,000 EPG. This overdispersion made for challenging model selection and meant that model fit parameters remained imperfect, even when other distributional assumptions were used. The presented final model selection and structure, however, is sufficient to demonstrate that associations between treatment and EPG or ectoparasite infestation were significant and that associations with demographic factors and cluster groups were not.

Access to efficacious and effective antiparasitic treatments is important in any setting, but particularly in remote Aboriginal and Torres Strait Islander communities where access to veterinary care and animal health products can have additional barriers and where

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TABLE 3 Mixed effects model outputs for associations with changes in eggs per gram of faeces and ectoparasite infestation.

		Association with *A. caninum* EPG of faeces			Association with ectoparasite infestation		
Variable	Category	Coefficient estimate	Standard error	*p*-value	Coefficient estimate	Standard error	*p*-value
Fixed effects							
Treatment group				<0.001*			<0.001*
	Group A	*Reference*			*Reference*		
	Group B	−0.90	0.54	0.097	−1.62	0.71	0.023
	Group C	−3.81	0.58	<0.001	−0.15	0.73	0.84
Timepoint				<0.001*			<0.001*
	Baseline	*Reference*			*Reference*		
	Day 7–11	−0.53	0.01	<0.001	−3.62	0.70	<0.001
	6 months	−0.54	0.01	<0.001	−5.23	0.94	<0.001
Treatment × timepoint				<0.001*			0.002*
	Group A × baseline	*Reference*			*Reference*		
	Group B × Day 7–11	−1.29	0.01	<0.001	0.21	0.86	0.803
	Group C × Day 7–11	−2.05	0.05	<0.001	3.81	0.96	<0.001
	Group B × 6 months	−1.22	0.02	<0.001	−0.08	1.54	0.957
	Group C × 6 months	−2.20	0.07	<0.001	24.2	209.02	0.908
Random effects							
	Individual dog variance	8.94			5.28		
	Island cluster variance	<0.001			<0.001		

*Variable level *p*-values were calculated using the joint_tests function from the emmeans package.

the potential risks of zoonotic disease are especially relevant. The results of this study demonstrate that single-dose oxibendazole/praziquantel (Paragard®) has poor efficacy against the zoonotic dog hookworm *A. caninum*, while moxidectin/imidacloprid (Advocate®) and off-label ivermectin at 200 µg/kg appear efficacious. Furthermore, afoxolaner chews (Nexgard®) and imidacloprid/flumethrin collars (Seresto®) are efficacious against flea and tick infestation and may aid in preventing the spread of vector-borne diseases.

With the benefit of up-to-date efficacy data relevant to remote community field sites, local organisations can make informed decisions to help develop effective One Health programs and manage the risks of parasitic disease for all human and animal community members.

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 University of Melbourne Animal Ethics Committee. 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

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

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Conflict of interest

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

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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.2024.1460452/full#supplementary-material

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Molecular identification of *Baylisascaris melis* (Gedoelst, 1920) from the Eurasian badger (*Meles meles*) and ascarids from other wild carnivores in Kazakhstan

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Introduction: The presence of gastrointestinal nematodes, including zoonotic ascarids, in wild canids, felids and mustelids as definitive hosts in Central Asian countries has been documented in many studies based on traditional morphological methods. In contrast, relevant data for the badger are scarce. The aim of this study was the molecular identification of ascarid nematodes from five wild carnivore species in different regions of Kazakhstan.

Methods: A total of 211 adult ascarids were collected from gray wolves (*Canis lupus*, 8 of 83 infected with 2–6 *Toxascaris leonina*), red foxes (*Vulpes vulpes*, 26 of 53, with 2–8 *Toxascaris leonina*), corsac foxes (*Vulpes corsac*, 6 of 11, 3–6 *Toxascaris leonina*), lynx (*Lynx lynx*, 2 of 3, with 2–5 *Toxocara cati*) and badgers (*Meles meles*, 2 of 4, with 2–7 *Baylisascaris melis*). Genomic DNA was extracted from the worms and ribosomal DNA, including the first and second internal transcribed spacer genes, was amplified by polymerase chain reaction using specific oligonucleotide primers and then sequenced.

Results: *Toxascaris leonina*, but not *Toxocara canis*, was molecularly identified in the wild canids, *Toxocara cati* in the lynx and *Baylisascaris melis* in the badger. The maximum likelihood phylogenetic tree showed three distinct clades: the canid *Toxascaris leonina* was placed in one clade, *Toxocara cati* in another and *Baylisascaris melis* in a third.

Discussion: The study provides the world's first molecular data and phylogenetic analysis of *Baylisascaris melis*, identified for the second time since its description over 100 years ago. This species was shown to be genetically distinct from other *Baylisascaris* spp. (*B. columnaris*, *B. procyonis*, *B. transfuga*, *B. devosi*). The possible zoonotic significance of ascarids from wild carnivores is discussed in the light of conditions in Central Asia.

KEYWORDS

***Baylisascaris melis*, *Toxascaris leonina*, *Toxocara cati*, wild carnivores, mustelids, molecular identification, phylogeny, Kazakhstan**

1 Introduction

Members of the genera *Toxocara*, *Toxascaris*, and *Baylisascaris* comprise the spectrum of ascarid nematodes (order Ascaridida: family Ascarididae) of terrestrial mammals, including the carnivores Canidae, Felidae, and Mustelidae (1, 2). Their adult stages parasitize the small intestines of the definitive host, which contaminates the environment by excreting worm eggs in feces. The eggs embryonate, can survive for months or years, and are ingested by another animal. Paratenic hosts (e.g., in *Toxocara* spp.) or intermediate hosts (in *Baylisascaris* spp.) may be facultatively involved, e.g., prey rodents. After oral ingestion of infective eggs, larvae penetrate the intestinal mucosa and migrate to the liver and other tissues, including the brain (3, 4). The infection can also be transmitted to humans (known as 'toxocariasis') (5). For example, the seroprevalence of toxocariasis in humans has been reported to be 11% in eastern Kazakhstan (6) and up to 54% in western Siberian regions of Russia (7). Depending on the ascarid species and the number of eggs ingested, the infection may be latent, but may also cause clinical symptoms (larva migrans syndrome) (4, 8). Contamination of the environment with ascarid eggs by domestic and wild carnivores is known in principle (4, 9, 10), but its impact in Central Asia is still unknown.

A number of studies have documented the occurrence and prevalence of helminth infections, including ascarids, in wild canids and felids in Kazakhstan [e.g., (11–14)] and neighboring countries [e.g., (15–19)]. In these studies, for example, wolves and red foxes were infected with *Toxocara canis* in 39% and 8–30% respectively, and with *Toxascaris* (*T.*) *leonina* in 38% and 6–78% respectively; *Toxocara cati* was present in 86% of lynx. In contrast, there are only two reports on the helminth fauna of badgers from Uzbekistan (17, 18), but no data from Kazakhstan. All these studies were carried out using traditional morphological methods. However, in field studies where the species identification of roundworms is based solely on their morphological features, the diagnosis is sometimes at least questionable, e.g., in badger (18–20). These diagnostic problems can be solved using molecular methods that have been available for many years. Such methods confirm or modify the taxonomic classification and can also be used to study the phylogenetic relationships of parasites such as ascarids, detect their genetic diversity and explain epidemiological results [e.g., (2, 21–24)]. Therefore, the aim of the present study was to molecularly confirm the morphological species diagnosis of roundworms from five wild carnivore species in different regions of Kazakhstan, including wolf, red fox, corsac fox, lynx and badger, and to provide baseline data for future investigations.

2 Materials and methods

2.1 Ethical approval

The study had been approved by the local Animal Ethics Committee (extract from Protocol No. 1 dated 24 July 2019) prior to commencement and was conducted in accordance with the World Medical Association Code of Ethics (Declaration of Helsinki) for animal research.[1]

1 http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm

2.2 Sample collection

Adult wild carnivores, including 83 gray wolves (*Canis lupus*), 53 red foxes (*Vulpes vulpes*), 11 corsac foxes (*Vulpes corsac*), 3 European lynx (*Lynx lynx*) and 4 badgers (*Meles meles*) were available for this study. They had been shot by hunters in different regions of Kazakhstan (Figure 1) between December 2019 and October 2023. The gastrointestinal tract of each animal, frozen until examination, was examined for helminths as described by Skrjabin (25). Adult roundworms were collected, washed in physiological saline, morphologically identified to species (26, 27) and preserved in 70% ethanol.

2.3 DNA extraction

Following morphological specification, one worm from each ascarid-positive animal was randomly selected for molecular analysis. A small piece of this specimen was cut off and homogenized, and the homogenate was subjected to the standard phenol-chloroform method supplemented with proteinase K, to extract genomic DNA (gDNA). The DNA was then precipitated with ethanol (28), purified, dissolved in ddH_2O and stored at −70°C for subsequent analysis.

2.4 PCR analysis

First, a polymerase chain reaction (PCR) was performed using the universal NC13/NC2 primer pair to amplify worm gDNA (21). PCR was performed in a 25 µL reaction mixture containing 10× Taq buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase and 200 µM dNTPs (Thermo Scientific, Carlsbad, CA, USA), 10 pmol of each primer and 20 ng of extracted gDNA as a template. DNA segments were amplified using thermal cycling reactions for 30 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 30 s). The resulting amplification products were separated by electrophoresis on a 1.5% agarose gel prepared with 1× TAE buffer solution containing 8 ng/µL ethidium bromide. This was followed by species-specific PCR targeting the partial internal transcribed spacer 2 (ITS2) ribosomal DNA (rDNA) gene of *Toxocara canis*, *Toxocara cati* and *T. leonina* using the primer pairs Tcan1/NC2, Tcat1/NC2 and Tleo1/NC2, respectively, (21). All PCRs were performed as described by Jacobs et al. (21). For the identification of *Baylisascaris* sp. a primer pair targeting the ITS1-5.8S-ITS2 rDNA genes was used under the conditions described by Franssen et al. (29). The sequences of all primers used are shown in Table 1.

2.5 Sequencing analysis and phylogeny

Two positive amplification products were randomly selected from each host species for sequencing and genotyping. The respective amplicons were purified using a Quick PCR Purification Kit (Invitrogen, Lithuania) according to the manufacturer's protocols. Sequencing was performed according to the Seq Studio Genetic Analyzer manual (Thermo Fisher Scientific Applied Biosystems, USA). The nucleotide sequences were visually checked using the Bio Capt program (version 11.0) and then analyzed by

FIGURE 1
Map of Kazakhstan showing the provinces and the geographical origin of the host species collected.

TABLE 1 List of primers used in this study.

Parasite	Target gene	Primer name	Primer sequence (5′–3′)	Reference
Universal nematode	5.8S	NC13	F: ATCGATGAAGAACGCAGC	(21)
		NC2	R: TTAGTTTCTTTTCCTCCGCT	
Toxocara canis	ITS2	Tcan1	F: AGTATGATGGGCGCGCCAAT	(21)
		NC2	R: TTAGTTTCTTTTCCTCCGCT	
Toxocara cati	ITS2	Tcat1	F: GGAGAAGTAAGATCGTGGCACGCGT	(21)
		NC2	R: TTAGTTTCTTTTCCTCCGCT	
Toxascaris leonina	ITS2	Tleo1	F: CGAACGCTCATATAACGGCATACTC	(21)
		NC2	R: TTAGTTTCTTTTCCTCCGCT	
Baylisascaris spp.	ITS1-5.8S-ITS2	ITS1-5.8S-IT2-F	F: ATAGTGAGTTGCACACTAATGT	(29)
		ITS1-5.8S-ITS2-R	R: TTATATGCTTAAATTCAGCGGG	

F, forward primer; R, reverse primer.

TABLE 2 Prevalence, intensity and abundance of adult ascarid species on the basis of morphology in wild carnivores in Kazakhstan.

Host	N infected/N examined	% prevalence (95% CI)	N worms found	Range of intensity	Mean (SD) intensity	Mean (SD) abundance	Ascarid species identified
Wolf	8/83	9.6 (4.3–18.1)	34	2–6	4.3 (1.3)	0.4 (1.3)	*Toxascaris leonina*
Red fox	26/53	49.1 (35.1–63.2)	134	2–8	5.1 (1.7)	2.5 (2.9)	*Toxascaris leonina*
Corsac fox	6/11	55 (23–83)	27	3–6	4.5 (1.0)	2.6 (2.5)	*Toxascaris leonina*
Lynx	2/3	66 (9–99)	7	2–5	3.5 (2.3)	2.1 (2.5)	*Toxocara cati*
Badger	2/4	50 (0.7–93)	9	2–7	4.5 (3.5)	2.3 (3.3)	*Baylisascaris melis*

95% CI, 95% confidence interval; SD, standard deviation.

BLAST search against the GenBank database.[2] Finally, the nucleotide sequences were aligned using the Clustal W program, and the relationships of the taxa were analyzed with 1,000 bootstrap replicates by the maximum likelihood method with MEGA11 (30). For the inference method, the nearest neighbor Interaction (NNI) was used. The tree for *Baylisascaris* species was rooted by the outgroup *Anisakis nascettii* (JX486104).

2 https://www.ncbi.nlm.nih.gov/

2.6 Statistical analysis

Explorative data analysis was performed using the BIAS statistical software (31). The observed prevalence, mean intensity and abundance of each ascarid species were calculated as described by Bush et al. (32).

3 Results

A total of 211 adult ascarids were collected from 154 host animals. Based on morphology, three species were identified: wolves (9.6% infected), red foxes (49.1%) and corsac foxes (55%) were infected only with *T. leonina*, lynx (66%) and badgers (50%) were infected only with *Toxocara cati* and *Baylisascaris* (*B.*) *melis*, respectively. Their mean intensity and abundance were low (Table 2). Adult *Toxocara canis* were not found in any of the hosts.

The first PCR performed with the universal primer pair NC13/NC2 showed that the length of the PCR products from the ascarids of canids (wolf, red fox, and corsac fox) was different from that of the PCR products from the worms of lynx and badger (Figure 2).

The second PCR, performed with the respective species-specific primer pairs targeting the ITS2 rDNA region, identified *T. leonina* in canids and *Toxocara cati* in lynx (Figure 3). The primer pair specific for *Toxocara canis* gave no results in any sample (data not shown). Ribosomal ITS2 amplicons were obtained from six *T. leonina* isolates (232–261 bp), two each from wolf, red fox and corsac fox, and from two *Toxocara cati* isolates (375 and 434 bp) from lynx. The badger ascarids were identified as *Baylisascaris* sp. using a primer on the ribosomal ITS1-5.8S-ITS2 region and by comparison of the nucleotide sequences obtained with references from the GenBank database. Ribosomal ITS1-5.8S-ITS2 amplicons of 511 bp and 842 bp in length were obtained from two *B. melis* isolates. Nucleotide sequence data for all isolates have been deposited in the NCBI GenBank database under the accession numbers shown in Table 3.

Nucleotide sequences from representative ascarid samples of the five host species were used to construct the maximum likelihood phylogenetic tree. Three distinct clades were identified: *T. leonina* from canids was placed in one clade with bootstrap values ranging from 46 to 96, *Toxocara cati* from lynx in another and *B. melis* from badgers in a third (Figure 4). Maximum tree analyses of the ribosomal ITS1-5.8S-ITS2 gene sequence showed that the two *B. melis* isolates formed a clade with the four reference species *Baylisascaris columnaris, Baylisascaris procyonis, Baylisascaris transfuga*, and *Baylisascaris devosi*. Both *B. melis* isolates showed slight genetic differences (Figure 5).

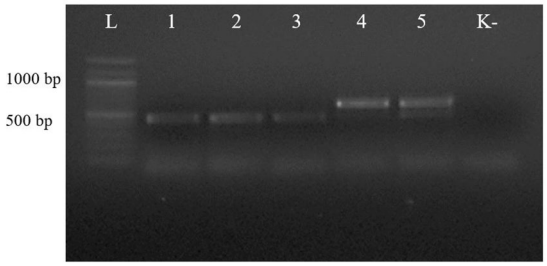

FIGURE 2
Electrophoresis of PCR products of gDNA from representative ascarid samples using the universal primer pair NC13/NC2: lane L: DNA marker; lanes 1–5: gDNA from ascarids collected from red fox (1), wolf (2), corsac fox (3), lynx (4) and badger (5); lane K: negative control (ddH_2O).

4 Discussion

In this study, five wild carnivore species in Kazakhstan were examined for their respective ascarid species. The species found, their prevalence, intensity and abundance partly differ from those of other Kazakh studies. This is not surprising as the regions of origin of the sampled hosts were different. It should also be noted that the data presented (as from previous studies) are not representative. They are based on a relatively small number of non-randomly selected hosts in a few regions of Kazakhstan, a large country of 2,725,000 km^2, where, for example, the wolf and red fox populations are estimated to be 30,000 and 75,000, respectively, (33, 34). It is also well documented that the ascarid fauna of wild carnivores varies between landscapes (e.g., steppe, foothills, mountains) (19, 35, 36), which may be explained by local differences in prey availability (10). Furthermore, lynx are protected species and their killing requires justified exemptions. It is therefore quite difficult to study representative samples of these wild carnivores in such large countries.

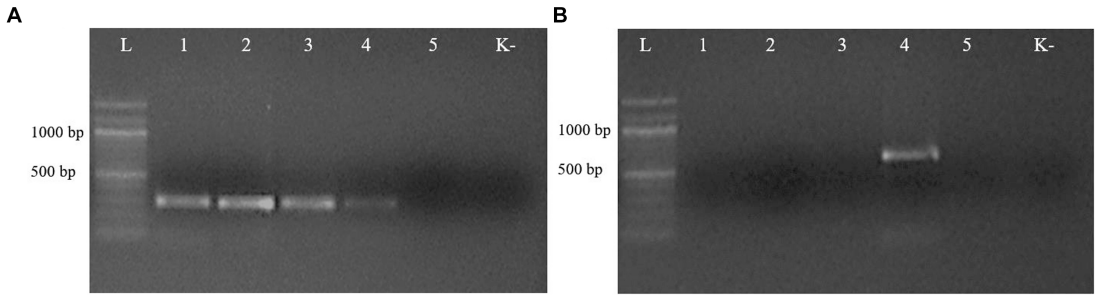

FIGURE 3
Electrophoresis of PCR products of gDNA from representative ascarid samples using the primer pairs Tleo1/NC2 **(A)** and Tcat1/NC2 **(B)**, species-specific for *Toxascaris leonina* and *Toxocara cati*, respectively. Lane L: DNA marker; lanes 1–5: DNA from ascarids collected from red fox (1), wolf (2), corsac fox (3), lynx (4) and badger (5); lane K: negative control (ddH_2O).

Nevertheless, it is the first study to use molecular methods to identify ascarid nematodes from Central Asian countries. Phylogenetic analysis revealed three distinct species: *Toxocara cati*, *T. leonina*, and *B. melis* (Figure 4), confirming the morphological diagnosis.

TABLE 3 GenBank accession no. and number of nucleotide base pairs of representative samples of adult ascarids from this study.

Species	Host	Accession no.	N bp
Toxascaris leonina	Wolf	OR647588	261
	Wolf	OR647594	241
	Red fox	OR647692	242
	Red fox	OR647694	232
	Corsac fox	OR647689	234
	Corsac fox	OR647691	235
Toxocara cati	Lynx	OQ975261	434
	Lynx	OQ975262	375
Baylisascaris melis	Badger	PP333110	842
	Badger	PP333114	511

In the three canid hosts, only *T. leonina* was identified, but not *Toxocara canis*. This is consistent with previous findings, based on traditional morphological methods, that *T. leonina* was the dominant ascarid species in corsac foxes in Kazakhstan (11), wild canids in southern Siberia (15), and stray dogs in Eurasian regions (37). It may be due to the higher cold tolerance of *T. leonina* eggs compared to *Toxocara canis* eggs, which favors this roundworm species in colder regions (37). However, it should be noted that the worms in the present study were obtained from adult hosts. This may have biased the results, as *Toxocara canis* is known to be mainly found in young canids (1, 26). In fact, other studies in Kazakhstan and neighboring countries have reported that wolves, red foxes or corsac foxes are infected with both ascarid species (12–14, 16–18).

Toxocara cati was the only ascarid species found in lynx. This was consistent with most reports from different countries (10, 15, 18), although occasionally *T. leonina* was also reported from this felid species (2, 38).

Wild canids and felids (as well as their domestic relatives) infected with ascarids contaminate the environment by excreting worm eggs in feces. The embryonated eggs are a potential source of infection for domestic dogs and cats and for paratenic hosts, including humans,

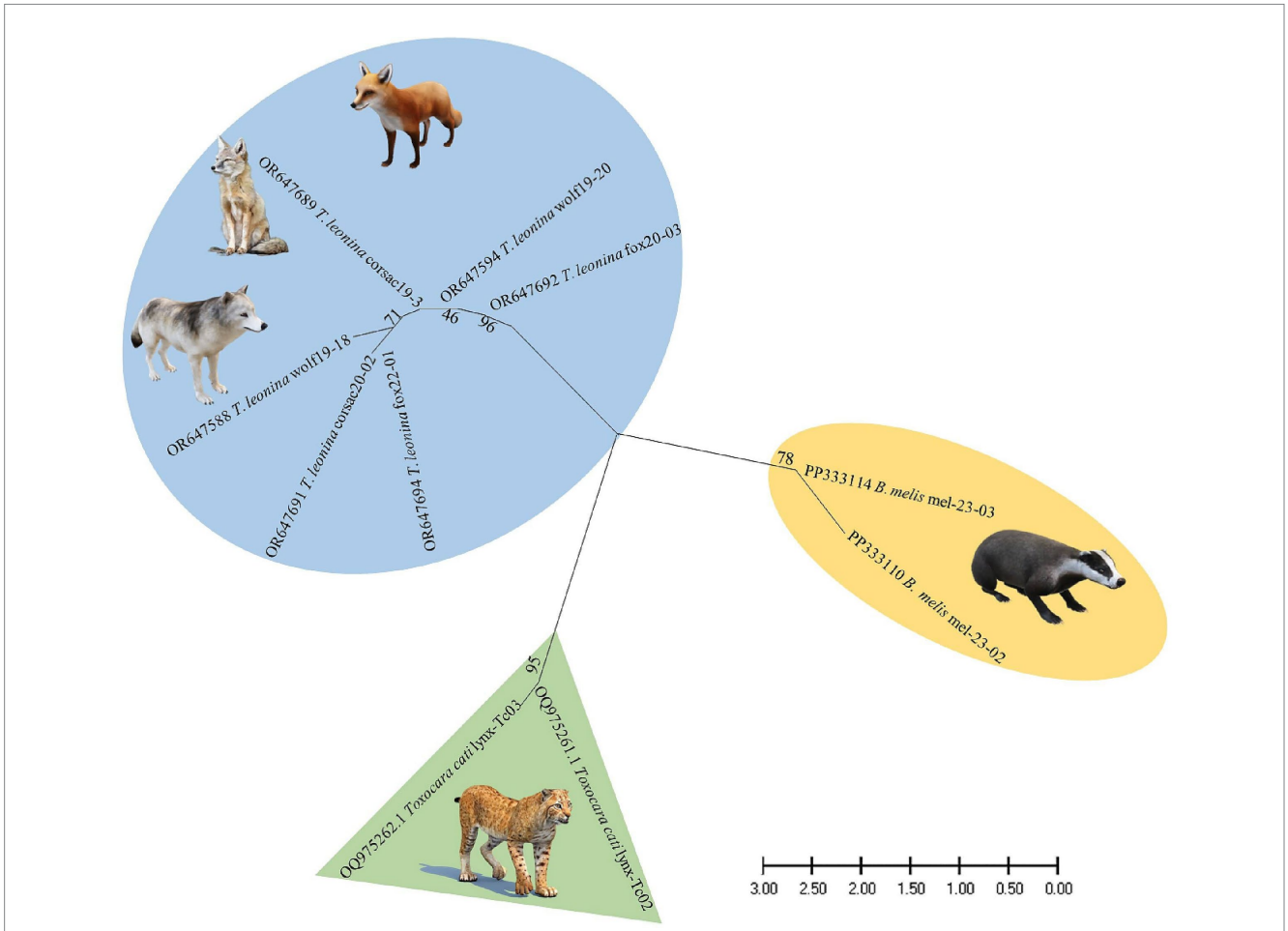

FIGURE 4
Maximum likelihood phylogeny constructed from nucleotide data of representative ascarid samples from wild carnivores in Kazakhstan. Numbers along the branches show bootstrap values resulting from different analyses in percent; scale: estimated number of nucleotide substitutions per site; species name and host species after the GenBank accession no.

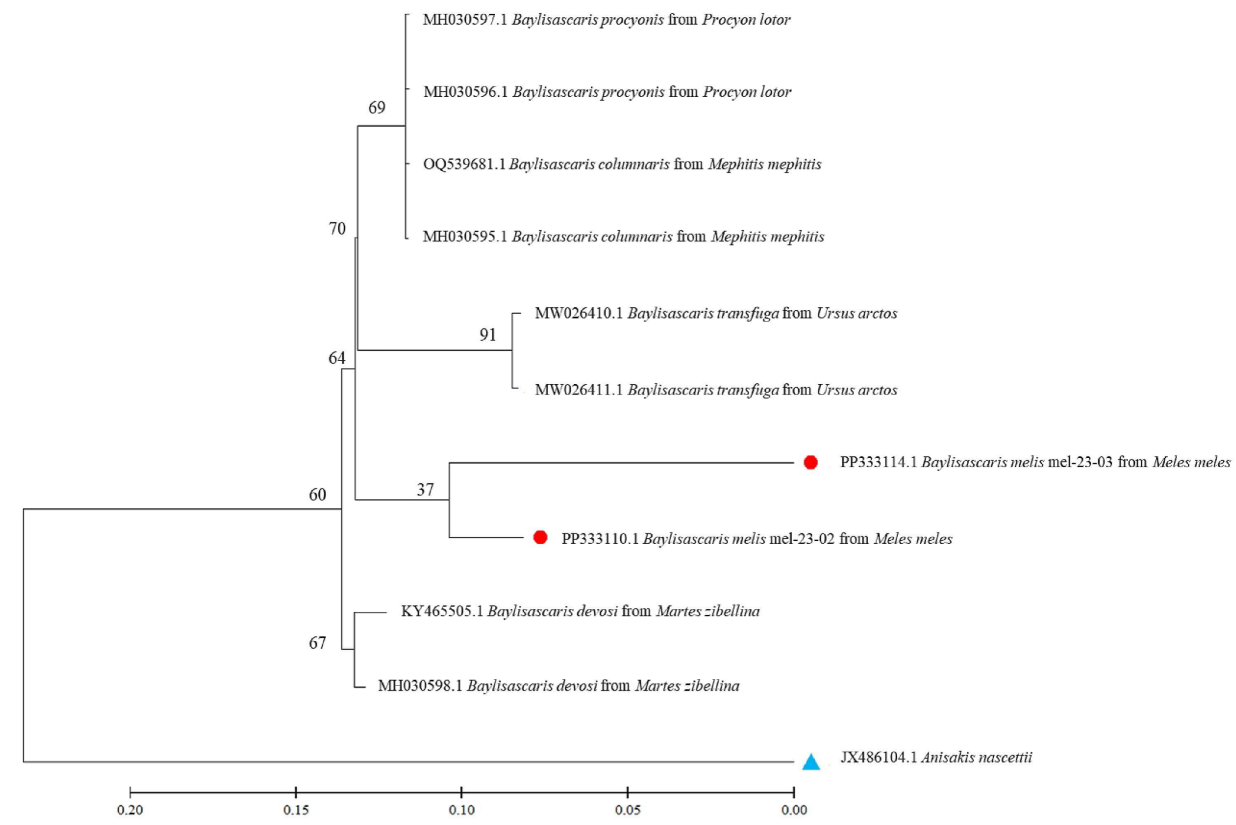

FIGURE 5
Maximum likelihood tree based on ribosomal ITS1-5.8S-ITS2 gene regions from several *Baylisascaris* spp. available in the GenBank and two isolates from this study. Numbers along the branches show bootstrap values resulting from different analyses in percent; scale: estimated number of nucleotide substitutions per site; species name and host species after the GenBank accession no.; red dots: isolates from this study; blue triangle: outgroup.

who may ingest these eggs (1, 9, 10). In the light of the results presented, this infection risk can be assessed as follows: (a) *T. leonina* is considered a negligible parasite from a veterinary and zoonotic point of view (37). (b) In contrast, *Toxocara cati* is pathogenic in its definitive feline host (39) and also in paratenic hosts: Experimental studies have shown that after ingestion of *Toxocara cati* eggs, larvae migrate into tissues, including the brain, causing pathomorphological alterations in mice and pigs and abnormal neurobehaviour in mice (40, 41). It should therefore be considered as a potential cause of neural larval migrans symptoms in humans (42). However, lynx are likely to be a negligible source of *Toxocara cati* infection to humans, at least in Central Asia. This is because the lynx prefers to live in forested areas, which provide sufficient cover for hunting and abundant prey without much contact with human settlements (43). (c) *Toxocara canis* may be present in wolves and red foxes (see above), although not in this study. These wild canids are more synanthropic than the lynx, and their range extends close to human settlements (10, 44). This increases the risk of successful transmission of their parasites, including the zoonotic *Toxocara canis*, to domestic animals and humans (1, 9, 10).

This study also presents the first molecular data and provides the first phylogenetic analysis of *B. melis* worldwide. The badger ascarid was shown to be genetically distinct from *Baylisascaris* spp. of other carnivores: *B. columnaris* (definitive host: skunk [*Mephitis* spp.]), *B. procyonis* (raccoon [*Procyon lotor*]), *B. transfuga* (bears [*Ursus* spp.]) and *B. devosi* (marten [*Martes* spp.], fisher [*Pekania pennanti*], wolverine [*Gulo gulo*]) (Figure 5). This also confirms the morphological differentiation by Sprent (45) and supports the hypothesis (46) that the ascarids found in North American badgers (*Taxidea taxus*), which have been described as *B. columnaris*, are in fact *B. melis*. The significance of the slight genetic differences between the two *B. melis* isolates analyzed remains to be investigated. The phylogenetic analysis also showed that *B. procyonis* and *B. columnaris* form a clade. This confirms previous results suggesting that they are closely related species or that the former is even a synonym of the latter (47, 48).

Interestingly, there is little information on the geographical distribution and prevalence of *B. melis* in badger populations in Eurasia. First described over 100 years ago in Belgium (49), this is the second unequivocal identification of this species. This nematode had not been mentioned in any relevant study in central, western or southern European countries. There are two studies from Italy and Switzerland reporting only unspecified "ascarid" eggs or worms in a few badgers (Table 4). In contrast, ascarids have been collected from badgers in Uzbekistan, Azerbaijan and Caucasian Russia and morphologically identified as *Toxocara canis*, *B. columnaris* or *B. devosi* (Table 4). However, it is most likely that these worms were misidentified and were

TABLE 4 Results of previous studies on intestinal helminths, including ascarids, in badgers in Eurasia.

Country	N ascarid positive/N examined	Method used	Reference
Uzbekistan	0/19	Nec	(17)
	4/25 "*Toxocara canis*"	Nec	(18)
Azerbaijan	10/43 "*B. columnaris*"	Nec	(20)
	4/43 "*B. devosi*"		
Russia (Caucasus)	3/60 "*B. columnaris*"	Nec	(19)
Poland	0/17	Cop	(51)
Slovenia	0/18	Nec	(52)
Croatia	0/13	Nec	(53)
Austria	0/20	Nec	(54)
Germany	0/16	Nec	(55)
	0/84	Nec	(56)
Switzerland	2/249 "ascarids"	Nec	(57)
Italy	0/19	Nec	(58)
	1/43 "ascarid egg"	Cop	(59)
	0/18	Nec	(60)
Spain	0/85	Nec	(61)
	0/26	Nec	(62)
Portugal	0/163	Cop	(63)
Great Britain	0/118	Nec	(64)
Ireland	0/50	Cop	(65)
	0/289	Nec	(66)

Nec, necropsy; Cop, coproscopy.

actually *B. melis*; the molecular results support this assumption. Thus, data from the literature and the results presented here suggest that *B. melis* may occur primarily, if not exclusively, in badger populations of western and central Asia. The reasons for this are still unknown.

It should be noted that *B. melis* is able to infect rodents (facultative intermediate hosts) under experimental conditions: It was highly pathogenic and caused fatal neural larva migrans symptoms in the American ground squirrel (*Urocitellus armatus*); mice (*Mus musculus*) did not develop clinical symptoms, but their brains and other tissues contained *B. melis* larvae (50). Whether this can also occur in Central Asian ground squirrel species (*Spermophilus* spp.) or other rodents under natural conditions does not seem impossible and requires further study. In any case, based on the clinical and pathological findings in rodents, a zoonotic significance of *B. melis* cannot be excluded and should be further investigated.

This study concludes by identifying ascarid nematodes from five distinct wild carnivore species in Central Asia within the phylogenetic framework. The study also presents the world's first molecular data on *B. melis* from badger. It provides further insights into the classification and genetic diversity of ascarids. It reiterates the need for molecular methods to complement traditional morphological methods as a basic diagnostic tool in the future, for example in studies of the fauna, diversity, ecology and epidemiology of wildlife parasites, especially potential zoonotic agents. For future research, we are also considering collecting feces from wild carnivores to detect roundworm infection, which would increase the sample size.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/nuccore/OR647588, OR647588; https://www.ncbi.nlm.nih.gov/nuccore/OR647594, OR647594; https://www.ncbi.nlm.nih.gov/nuccore/OR647692, OR647692; https://www.ncbi.nlm.nih.gov/nuccore/OR647694, OR647694; https://www.ncbi.nlm.nih.gov/nuccore/OR647689, OR647689; https://www.ncbi.nlm.nih.gov/nuccore/OR647691, OR647691; https://www.ncbi.nlm.nih.gov/nuccore/OQ975261, OQ975261; https://www.ncbi.nlm.nih.gov/nuccore/OQ975262, OQ975262; https://www.ncbi.nlm.nih.gov/nuccore/PP333110, PP333110; https://www.ncbi.nlm.nih.gov/nuccore/PP333114, PP333114.

Ethics statement

The animal study was approved by the local Animal Ethics Committee (extract from Protocol No. 1 dated 24 July 2019) prior to commencement and was conducted in accordance with the World Medical Association Code of Ethics (Declaration of Helsinki) for animal research (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RU: Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. ASm: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. LL: Data curation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. ASh: Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. AAB: Data curation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. APB: Data curation, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. CB: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. VK: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, 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.

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Seasonality of anti-*Leishmania infantum* titers in dogs: a crucial factor for designing effective clinical trials

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1 Introduction

Canine leishmaniosis (CanL), caused by *Leishmania infantum*, remains a significant focus in veterinary parasitology, with a worldwide distribution and an estimated 2.5 million infected dogs in southwestern Europe (1–4). From a public health perspective, this sandfly-borne disease also represents a significant global health issue due to its zoonotic nature (5).

Therefore, it is not surprising that research on CanL has grown exponentially over the past two decades, with over 3,900 scientific papers published on diverse aspects of the disease (PubMed database, accessed on 7 August 2024). This surge in scholarly activity underscores the complexity and importance of understanding CanL, from its epidemiology and pathophysiology to its treatment and prevention. Research efforts have particularly focused on the latter two areas, with several clinical trials being conducted to evaluate the efficacy of therapies and preventive measures for CanL. These efforts have been crucial for significantly reducing the disease burden and preventing the spread of the protozoan in endemic and non-endemic regions, respectively. To date, due to the various research endeavors, we know that the all-around control of *L. infantum* infection can be achieved through an integrating approach. This includes the use of sandfly repellents as well as three main areas of intervention: chemotherapy, immunotherapy, and immunoprophylaxis (6).

2 Subsection relevant to the subject

The World Association for the Advancement of Veterinary Parasitology (WAAVP) has always recognized the significance of leishmaniosis among canine vector-borne diseases (VBDs). In 2021, the WAAVP developed guidelines that provide comprehensive recommendations for conducting studies aimed at evaluating the efficacy of parasiticides in reducing vector-borne pathogen (VBP) transmission risks in dogs and cats (7). These guidelines serve as a valuable resource for researchers, pharmaceutical companies, and regulatory authorities involved in VBD research, including CanL (7). In this regard, according to the WAAVP guidelines, field studies aiming to assess the efficacy of products for preventing *L. infantum* transmission in companion animals should adhere to strict inclusion criteria (e.g., equal distribution between control and treated dogs, randomization, and allocation by household) (7). Moreover, animals should be followed up for at least 1 year, with assessments conducted before inclusion, at the end of the efficacy period of the investigational product, and at the end of the observational period (7). If feasible, intermediate assessments should be conducted every 3–4 months (7).

3 Discussion

Despite the thoroughness of the WAAVP guidelines, the present opinion article aims to focus on a crucial aspect of the host–parasite relationship that can have a significant impact on the design and results of clinical trials, particularly in regions with distinct climatic patterns: the seasonality of anti-*L. infantum* antibody titers in dogs. Indeed, shortly after the WAAVP guidelines were published, an article by Cavalera et al. showed that *L. infantum* antibody titers can vary significantly between the transmission and non-transmission seasons in dogs from a hyperendemic area for CanL (i.e., Apulia region, Southern Italy) (8). For the sake of clarity, it should be noted that in temperate regions, the transmission of *Leishmania* is highly seasonal, with higher infection rates during warmer months when sandflies are most active, the so-called "transmission period" or "sandfly season." In the article cited above, most of the enrolled dogs ($n = 36/65$; 55.4%) experienced a reduction in anti-*L. infantum* antibody titers, as measured by the indirect fluorescent antibody test (IFAT), during the non-transmission season. Nearly half of these dogs ($n = 16/36$; 44%) became seronegative. Similarly, seasonal variations in *Leishmania* antibody titers during sand fly transmission and non-transmission periods were observed in domestic ferrets in Spain (9). It has been hypothesized that the reduction of anti-*L. infantum* antibody titers during the non-sand fly period may be related to the progressive reduction of exposure to vectors. More specifically, the immune response of the host could be upregulated during the transmission period because of uninfected and *L. infantum*-infected sand fly bites and the immunogenic effect of the parasite. It should be considered that the measurement of antibody titers in dogs is a crucial and ever-present practice in clinical/parasitological trials for the diagnosis and therapeutic monitoring of this parasitosis, as outlined in the currently available guidelines (10, 11). Moreover, among the serology techniques for *L. infantum*, IFAT remains the most suitable assay used for detecting anti-*L. infantum* antibodies, as recommended by the World Organization for Animal Health (12). Ignoring the seasonality of antibody titers can lead to significant biases in clinical trial results evaluating the efficacy of new therapeutic strategies or preventive measures as well as the prevalence/incidence of CanL in the canine population. For example, trials starting during the transmission season and ending during the non-transmission season could lead to an "inflated" efficacy of the molecule(s) under investigation, if any reduction in antibody titers is entirely (and wrongly) attributed to the treatment effect. Similarly, the assessment of the prevalence or incidence of CanL in a dog population may yield diametrically opposed results depending on the season chosen for the study.

With regard to the design of trials to evaluate products capable of preventing *L. infantum* infection, the authors believe that it would be advisable to perform the enrolment at the end of the non-transmission season and to conclude the study at the end of the next transmission season (considering a study period of 18 months). This approach would enable the inclusion of dogs that can be considered "truly *L. infantum* seronegative" at the outset of the study and allow for an assessment of how many of these dogs have actually been protected from exposure. An alternative approach would be to enroll clinically healthy *L. infantum* seropositive dogs (i.e., those previously exposed to the protozoan) at the end of the non-transmission season and evaluate them at the end of the following transmission season, taking advantage of the "seasonality effect." If, at the end of the transmission season, anti-*L. infantum* antibody titers are elevated—even in the absence of clinical signs and laboratory abnormalities (such as increased C-reactive protein and/or ferritin, elevated total protein with hypergammaglobulinemia, and a decreased albumin/globulin ratio) consistent with CanL (11, 13–15)—it can be posited that the animal has been exposed to the sand fly bites despite the use of the repellent product.

In addition, it is important to consider that in both countries where the seasonal variation of *L. infantum* antibodies was detected (i.e., Italy and Spain), a confluent bi-modal trend in the seasonal dynamics of *Phlebotomus perniciosus* was observed (16). Therefore, before applying the suggested indications to set up clinical studies, it would be appropriate to consider the seasonal dynamics of the Mediterranean *L. infantum* vectors described (16).

In conclusion, seasonality of anti-*L. infantum* titers in dogs can represent a critical factor that should not be overlooked in the design of clinical trials aimed at evaluating treatments and preventive measures for CanL. Incorporating this variable will ensure more accurate and reliable results, which will ultimately contribute to more effective control strategies for this potentially life-threatening disease for dogs.

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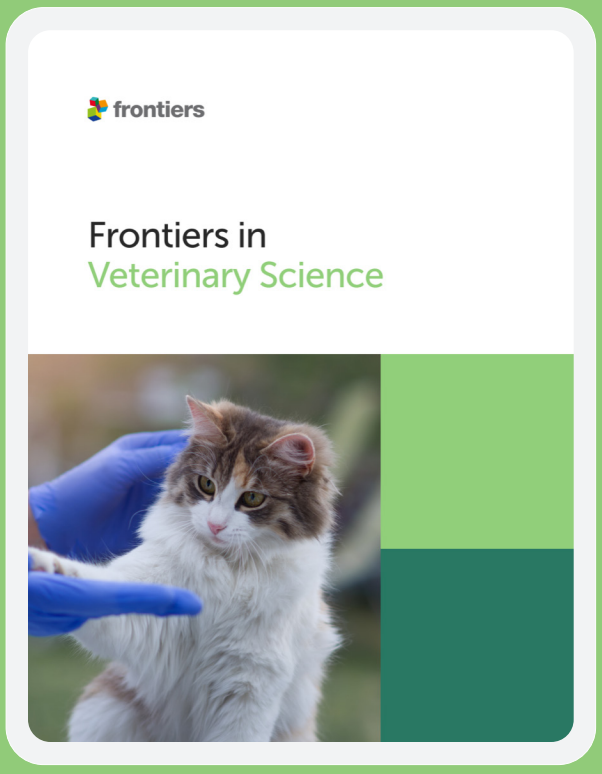

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