ZOONOTIC DISEASES: THEIR HOSTS AND VECTORS

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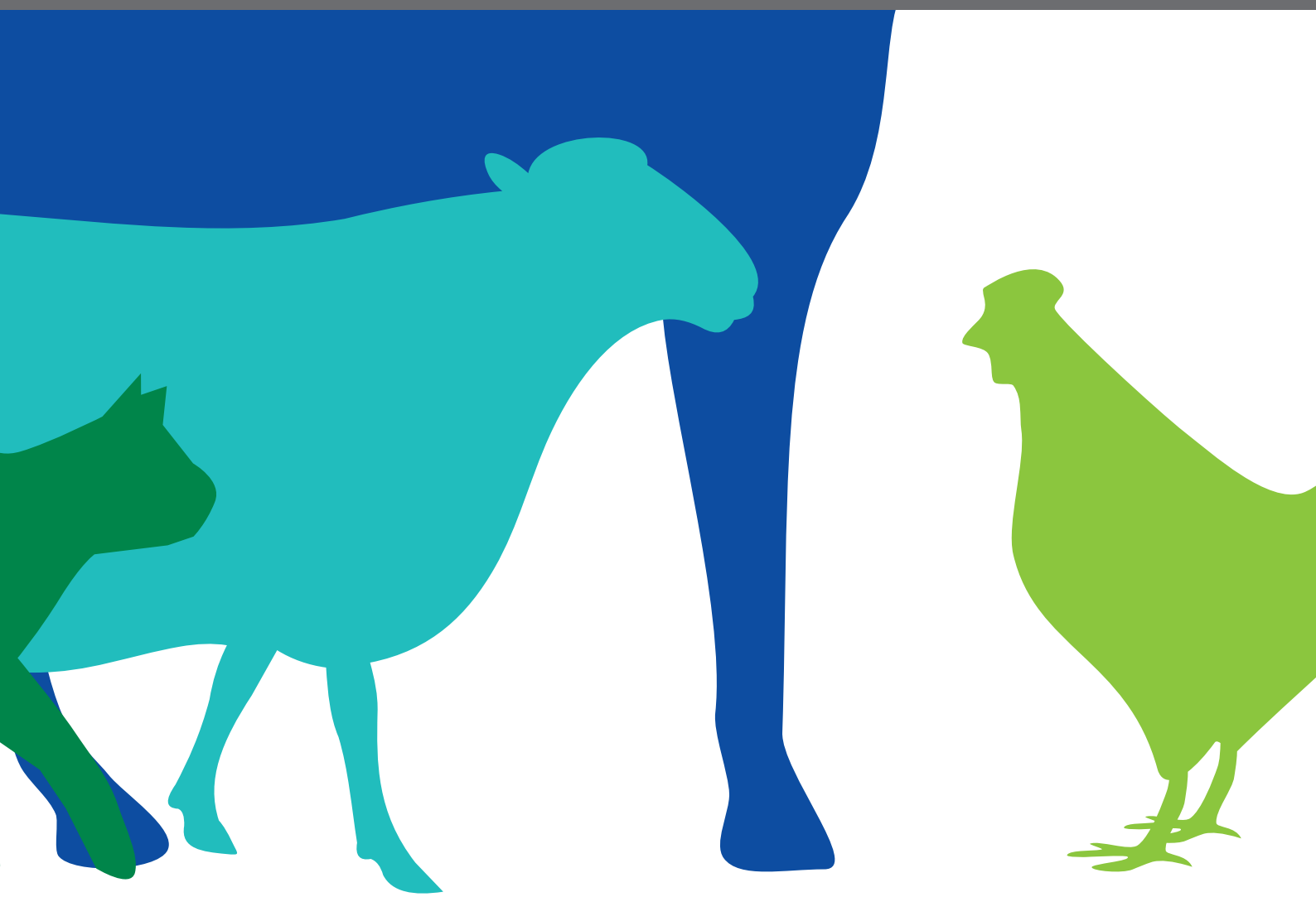

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ZOONOTIC DISEASES: THEIR HOSTS AND VECTORS

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EDITORIAL
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Editorial: Zoonotic Diseases: Their Host and Vectors

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Editorial on the Research Topic

Zoonotic Diseases: Their Host and Vectors

When Frontiers in Veterinary Science asked us to produce a Research Topic, we were aware of the importance and dissemination it could have, so we tried to address an interesting, attractive, and practical topic that would be of help to the scientific community and the general public. Themes dealing with the One *Health* concept and zoonotic diseases are on the rise. The One *Health* concept, involving collaboration between veterinary and medical scientists, policy makers, and public health officials, is necessary to foster joint cooperation and control of emerging zoonotic diseases. Zoonotic diseases, which are caused by a wide range of arthropods, helminths, protozoa, bacteria and viruses, can cause severe and even fatal clinical conditions in animals and seriously affect the infected humans. The main zoonoses are related to interactions between livestock and wildlife, as well as between dogs and cats and human populations. Humans are accidentally infected in endemic areas, where animals act as *reservoirs* and climatic conditions favor the proliferation of vectors. The influence of other variables, such as temperature, humidity, presence of irrigated areas, introduction of new vector species, climate change, increasing human activity, travel with pets to/from endemic countries and the presence of these diseases in areas previously not described as endemic, are important factors to consider in the establishment of new zoonotic diseases in areas where, until then, were considered free of the disease. Approximately 60% of human diseases are zoonotic and at least 75% of the emerging pathogens of human infections are of animal origin. Currently, most of these diseases are neglected despite causing a potentially global problem.

Therefore, this Research Topic entitled *Zoonotic Diseases: Their Host and Vectors* was proposed with the aim of providing state-of-the-art research focused on preventing and controlling zoonotic diseases, both through the control of the vectors and their animal reservoirs. It contains a total of 16 contributions from parasitologists, immunologists, entomologists, veterinarians, virologists, and microbiologists from all continents, who have addressed the study of different zoonotic diseases, dealing with topics such as the relationship between the human population, domestic animals and wildlife, the role of invasive alien species, the epidemiology of zoonotic infections, different strategies in the monitoring and control, programmes for treatment and prevention, vector dynamics, vector life cycles, and immune response in their hosts.

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RM, RB-M, LR, and EC wrote the editorial. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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BRIEF RESEARCH REPORT
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Mosquitoes in an Urban Zoo: Identification of Blood Meals, Flight Distances of Engorged Females, and Avian Malaria Infections

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Zoological gardens are home to a large number of vertebrate species and as such are suitable sites for both mosquito breeding and maintenance. They are excellent places for entomological studies of mosquito phenology, diversity, and blood-feeding patterns, as well as for xenomonitoring. During 2016, we sampled mosquitoes in Barcelona Zoo and used molecular methods to determine their blood-feeding patterns and the prevalence and diversity of avian malaria parasites. We also estimated the flight distance of engorged mosquitoes in the area. Overall, 1,384 adult *Culex pipiens* s.l., *Culiseta longiareolata*, and *Aedes albopictus* were captured. Birds dominated the diet of *Cx. pipiens* s.l. ($n = 87$) and *Cs. longiareolata* ($n = 6$), while humans were the only blood-meal source of *Ae. albopictus* ($n = 3$). Mosquitoes had a mean flight distance of 95.67 m after feeding on blood (range 38.71–168.51 m). Blood parasites were detected in the abdomen of 13 engorged *Cx. pipiens* s.l., eight of which had fed on magpies. Four *Plasmodium* lineages and a single lineage of the malaria-like parasite *Haemoproteus* were identified. These results suggest that *Cx. pipiens* s.l. is involved in the local transmission of avian *Plasmodium*, which potentially affects the circulation of parasites between and within wildlife and enclosed animals. Vigilance regarding possible mosquito breeding sites in this zoo is thus recommended.

Keywords: ***Aedes albopictus***, **avian** ***Plasmodium***, ***Culex pipiens***, **malaria, vectors**

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INTRODUCTION

Mosquitoes transmit a diversity of vector-borne pathogens affecting humans, livestock, and wildlife (1, 2). In addition to native species, invasive mosquitoes such as alien *Aedes* mosquitoes are involved in the circulation of both imported and locally circulating pathogens (3). This is the case of the invasive Asian tiger mosquito *Aedes albopictus*, which is associated with the local transmission of pathogens such as filarioid worms (e.g., *Dirofilaria* spp.), protozoa (e.g., avian malaria parasites), and viruses (e.g., Dengue virus) (4–6). This species has a broad global distribution and is present in countries outside its native range in America, Europe, Oceania, and Africa (7). In Spain, *Ae. albopictus* was first recorded in 2004 in Catalonia and since then has progressively colonized different parts of this region (8).

Zoos and wildlife parks with non-autochthonous and stabled fauna are excellent sites for studying the ecology and epidemiology of vector-borne pathogens, as studies of sand flies (9, 10), biting midges *Culicoides* (11), and mosquitoes (12–15) have previously shown. Given that captive animals are housed in known locations, the flight distances of captured insect vectors containing blood from these animals can be accurately estimated (10). In addition, these areas are frequented by human visitors and there are often both freely moving animals and animals maintained in captivity, all of which are potentially exposed to the transmission of various pathogens (15). This is especially the case of avian malaria parasites of the genus *Plasmodium*, a group of haemosporidians that naturally circulates between birds and mosquitoes (16) that can severely affect the health and survival of birds in zoos and recovery centers (17–22). However, in spite of their veterinary importance, the transmission dynamics of mosquito-borne avian *Plasmodium* in these particular ecosystems are still poorly understood [e.g., (12, 15, 23, 24)].

Here, we sampled mosquitoes in Barcelona Zoo, a site with a great diversity of mosquito and bird species, many of which are infected by avian malaria parasites (25). We employed a comprehensive approach based on the analysis of mosquito blood-feeding patterns, xenomonitoring (defined as the identification of pathogens in mosquito vectors), and flight distances of engorged females. We used molecular methods to identify the blood-meal sources of both native and alien engorged mosquitoes and to screen for the presence of avian *Plasmodium* in the abdomens of engorged mosquitoes to assess contact rates between potential vectors and parasites in the area.

METHODS

Study Area, Mosquito Sampling, and Species Identification

Mosquitoes were collected in June–November 2016 in Barcelona Zoo using both passive and active trapping techniques (**Figure 1**). BG-Sentinel traps (Biogents GmbH, Regensburg, Germany) were installed for 24 h in three different sites in the zoo, namely, the aviary, the farm, and the terrarium. These sites were selected according to criteria related to host proximity, distance between traps, and cover and protection for the traps. Every 2 weeks, mosquitoes were sampled using BG-Sentinel traps baited with CO_2 during 24 h. In addition, we used entomological aspirators (Improved Prokopack Aspirator, Mod. 1419, John W. Hock Company, FL, USA, and CDC Backpack Aspirator Mod. 2846, BioQuip, CA, USA) to collect mosquitoes resting on vegetation, bins, and animal cages. Aspirations were performed in six pre-established sites distributed throughout the whole zoo (13.5 ha) for 5 min in each area to standardize active adult mosquito sampling and increase the overall number of blood-fed mosquitoes collected. Additionally, 10 standard ovitraps (350 ml water capacity) were installed and monitored weekly to obtain information on mosquito phenology. Larval samplings were conducted occasionally to check for the presence/absence of other species that had not been collected by the above-described methodologies. Mosquitoes were identified using morphological keys (26); females containing any remains of blood meals in their abdomens were stored individually at −80°C until subsequent molecular analyses.

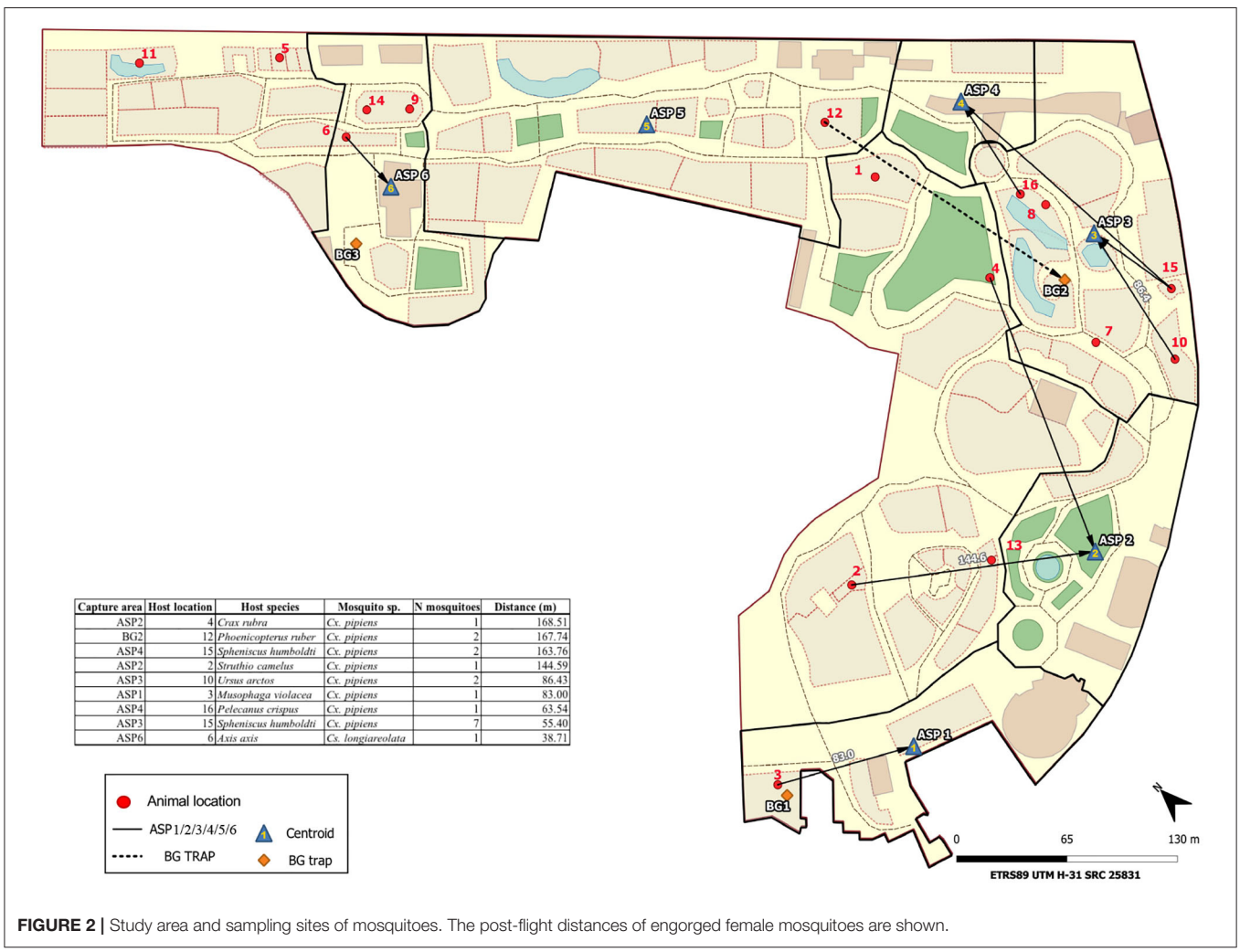
FIGURE 1 | Sampling methods used in this study including direct aspiration in mosquito resting areas **(A)**, BG-Sentinel traps **(B)**, and ovitraps **(C)**.

Molecular Identification of Blood-Meal Sources and Parasites in Engorged Mosquitoes

The abdomen of each engorged mosquito was separated from the head–thorax using sterile tips on Petri dishes. Genomic DNA was isolated from these abdomens using Maxwell® 16 LEV System Research Kit (Promega, Madison, WI). The protocol described by Alcaide et al. (27) involving the amplification of the barcoding region of the cytochrome oxidase subunit 1 (COI) gene was used to identify the blood-meal origin of mosquitoes [see also (28) for the adaptation of the protocol for *Ae. albopictus*]. The presence of avian *Plasmodium*/*Haemoproteus* parasites was screened for following Hellgren et al. (29). Amplified fragments were sequenced using the Macrogen Inc. facilities (Amsterdam, The Netherlands), and the resulting sequences were edited using Sequencher™ v4.9 (Gene Codes Corp.,© 1991–2009, Ann Arbor, MI 48108, USA). Blood-meal sources were identified by comparing sequences obtained with those deposited in public-access databases (GenBank DNA, National Center for Biotechnology Information Blast and the Barcode of Life Data Systems), bearing in mind the species potentially present in the study area. Parasite lineages and, whenever possible, their respective morpho-species were identified by Blast comparison with sequences in GenBank and/or MalAvi (30).

Analyses of Flight Distances of Blood-Fed Female Mosquitoes

We estimated the minimum flight distances of mosquitoes using data concerning where mosquitoes were captured and the locations of caged host species (**Figure 2**). Mosquito trapping areas and enclosures within the zoo of identified vertebrate host species were georeferenced. Only vertebrate species in enclosures in the zoo were considered in the analyses. Freely moving hosts (e.g., humans) were excluded from the analyses. Based on host identification, distances between the centroids of the areas enclosing animals and mosquito sampling points (BG-Sentinel stations or entomological aspiration points) were measured to calculate flight distances. Analyses were carried out with QGIS 3.12 (QGIS Development Team. http://qgis.osgeo.org).



Capture area	Host location	Host species	Mosquito sp.	N mosquitoes	Distance (m)
ASP2	4	*Crax rubra*	*Cx. pipiens*	1	168.51
BG2	12	*Phoenicopterus ruber*	*Cx. pipiens*	2	167.74
ASP4	15	*Spheniscus humboldti*	*Cx. pipiens*	2	163.76
ASP2	2	*Struthio camelus*	*Cx. pipiens*	1	144.59
ASP3	10	*Ursus arctos*	*Cx. pipiens*	2	86.43
ASP1	3	*Musophaga violacea*	*Cx. pipiens*	1	83.00
ASP4	16	*Pelecanus crispus*	*Cx. pipiens*	1	63.54
ASP3	15	*Spheniscus humboldti*	*Cx. pipiens*	7	55.40
ASP6	6	*Axis axis*	*Cs. longiareolata*	1	38.71

FIGURE 2 | Study area and sampling sites of mosquitoes. The post-flight distances of engorged female mosquitoes are shown.

RESULTS

We trapped 1,384 adult *Culex pipiens* s.l. ($n = 733$; 52.96%), *Ae. albopictus* ($n = 490$; 35.40%), and *Culiseta longiareolata* ($n = 161$; 11.63%). In total, 63.29% ($n = 876$) of these mosquitoes were collected using entomological aspirators and the remaining 36.71% ($n = 508$) using BG-Sentinel traps. Catches of *Cx. pipiens* s.l. peaked in June–July, while those of *Ae. albopictus* adults peaked in September–October; these results were supported by both direct collection by entomological aspirators and the BG-Sentinel traps. In addition, we collected 9,658 *Ae. albopictus* eggs, with a maximum in July–October and a peak in August (3,965 eggs).

In all, 137 (9.90%) mosquitoes had some blood remains in their abdomens, of which the vertebrate hosts of 86 (62.32%) were successfully identified (birds and mammals) (**Table 1**). Birds dominated the diet of *Cx. pipiens* s.l. (92.21%; $n = 77$). The three identified *Ae. albopictus* blood meals came from humans, while the six blood meals from *Cs. longiareolata* corresponded to birds. Post-blood-feeding flight distances were calculated for the 18 mosquitoes that had fed on enclosed animals (17 *Cx. pipiens* s.l. and a single *Cs. longiareolata*) (**Figure 2**). For *Cx. pipiens* s.l., the mean post-blood feeding flight distance was 99.02 m, with a maximum of 168.51 m. The only distance measured for *Cs. longiareolata* was 38.71 meters. Overall, 13 (9.49%) out of the 137 engorged mosquitoes analyzed were positive for the presence of parasites (**Table 1**). All the parasite lineages found in this study coincided with *Plasmodium* lineages: Delurb4 ($n = 1$), Delurb5 ($n = 3$), Syat05 ($n = 1$, corresponding to *P. vaughani*), SGS1 ($n = 1$, corresponding to *P. relictum*), and the *Haemoproteus* lineage hCIRCUM04 ($n = 5$, also called BLUTI9). Sequences corresponding to each of the five lineages identified in this study were deposited in GenBank (MT568928–MT568932). In addition, one *Plasmodium* parasite and one *Haemoproteus* parasite were detected in mosquitoes, although the low quality of these sequences did not allow us to accurately identify their parasite lineages. Twelve out of the 13 positive mosquitoes corresponded to *Cx. pipiens* s.l. A single *Cs. longiareolata* was positive for *Haemoproteus* lineage hCIRCUM04.

TABLE 1 | Vertebrate hosts of mosquitoes trapped in the zoological garden of Barcelona during 2016.

Host	*Ae. albopictus*	*Cs. longiareolata*	*Cx. pipiens*	Parasites
Birds				
Ardea cinerea			12	
Ardea sp.		1		
Bubulcus ibis		1	18	
Columba livia			1	*Haemoproteus* sp. (1)
Corvus monedula			1	
Egretta garzetta/sp.			4	
Crax rubra			1	
Musophaga violacea			1	
Myiopsitta monachus			3	
Passer domesticus		1		
Pavo sp.			2	
Pelecanus sp.			1	
Phoenicopterus ruber			2	*P. vaughani* SYAT05 (1)
Pica pica		2	8	*Haemoproteus* hCIRCUM04 (3), *Plasmodium* Delurb5 (3), *Plasmodium* Delurb4 (1), *Plasmodium* sp. (1)
Spheniscus humboldti			9	*P. relictum* SGS1 (1)
Streptopelia decaocto			7	*Haemoproteus* hCIRCUM04 (1)
Struthio camelus			1	
Mammals				
Axis axis		1		
Canis lupus			1	
Homo sapiens	3		3	
Ursus arctos			2	

The parasite lineages identified in mosquitoes fed on each animal are shown. One mosquito with an unidentified blood meal was positive for Haemoproteus (hCIRCUM04).

DISCUSSION

Infections by *Plasmodium* parasites and related haemosporidians are commonly found in birds in zoos (31) and can lead to illness and/or lethal diseases (20–22). These cases usually occur in immunologically naïve species such as penguins originating from areas where the parasites circulating in zoos are not present. These animals are frequently bitten by competent vectors of avian malaria parasites, especially of the genus *Culex* (18), and largely suffer the costs of infections (17, 19). Interestingly, we found that a number of *Cx. pipiens* s.l. had fed on Humboldt penguins (*Spheniscus humboldti*), including mosquitoes with *P. relictum*. Different treatments and prophylactic protocols are applied to penguins to minimize the parasite-induced illness (18), as is also common practice in the study area.

Our results regarding the blood-feeding pattern of mosquitoes support the hypothesis that *Cx. pipiens* s.l. females feed mainly on birds (6, 28, 32). Despite the extremely low sample size, we also found support for the ornithophilic behavior of *Cs. longiareolata*; however, *Ae. albopictus* fed exclusively on humans. This latter species is a nuisance for visitors, especially in summer when it is commonest. These results provide valuable information regarding the blood-feeding sources of these species, which is especially relevant in the case of *Ae. albopictus* as this subject has only to date been investigated at a handful of studies in Europe (6, 25, 28, 33). Identification of the blood-meal sources of mosquitoes in zoos allows researchers to investigate additional aspects of vector ecology such as the flight distances of engorged females. Greenberg et al. (13) recorded an average distance of 106.7 m for mosquitoes of the genus *Aedes* (*Ae. vexans*), *Culex* (*Cx. Quinquefasciatus*, and *Cx. tarsalis*), and *Culiseta* (*Cs. inornata*) in USA. Similar values were reported by Tuten et al. (34) in zoos from USA, where *Anopheles* and *Culex* mosquitoes flew a mean distance of 94.1 m after feeding. Ejiri et al. (12) found that the post-blood-meal flight distance of *Cx. pipiens pallens* lay in the range 10–350 m in Japan. Heym et al. (14) estimated the mean post-blood-meal flight distance of *Cx. pipiens* (biotype *pipiens*) in two zoos in Germany as 132.2

and 362.3 m. In this study in a Mediterranean area, engorged *Cx. pipiens* s.l. and *Cs. longiareolata* were captured in average at 95.67 m from their hosts, with a maximum of 168.51 m for *Cx. pipiens* s.l. It is worth highlighting that only a single female of *Cs. longiareolata* was found to have blood from zoo animals and so further studies targeting this species are still required. We were not able to calculate the flight distances of *Ae. albopictus* due to that fact that in this study it fed exclusively on humans.

Our results suggest that encounters between infected birds and competent mosquito vectors frequently occur in the area, which probably facilitates the transmission of avian *Plasmodium* within this zoo (12, 15, 23, 24). Previous studies in the Iberian Peninsula have identified avian *Plasmodium* in mosquitoes of the genera *Culex* and *Aedes* (25, 32, 35, 36), and of them, *Cx. pipiens* probably plays a key role in the transmission of avian *Plasmodium* due to its wide distribution (1), competence for the transmission of different avian *Plasmodium* lineages/species (16, 37), and its ornithophilic behavior (6, 32). A previous study in the same area also identified the presence of avian *Plasmodium* in *Cx. pipiens* s.l. pools (25). Nevertheless, the molecular identification of parasite DNA in mosquitoes does not necessarily imply vector competence (38) and so we were not able to assess the competence of these mosquitoes for avian malaria transmission. This is the case, above all, of *Haemoproteus* parasites, a parasite genus transmitted by *Culicoides* spp. and louse flies (16), that cannot be effectively transmitted by mosquitoes.

CONCLUSION

Barcelona Zoo is a suitable site for the development and maintenance of both native and alien species of mosquitoes. Based on their blood-feeding patterns, *Cx. pipiens* s.l. probably play a role in the local transmission and spread of mosquito-borne pathogens. This ornithophilic mosquito is a well-known vector of avian *Plasmodium* parasites. Thus, *Cx. pipiens* s.l. and the parasites transmitted may have an impact on the health of caged birds and so very likely represent a veterinary concern. In addition, magpies are common hosts of mosquitoes in the area and it is likely that they act as *Plasmodium* spp. reservoirs since high parasite prevalence has been found in the mosquitoes that feed on this bird species.

DATA AVAILABILITY STATEMENT

All data analysed during this study are included in this published article, any further information is available from the corresponding author on reasonable request.

ETHICS STATEMENT

Ethical approval was not required for this study according to national/local legislation because mosquitoes are not protected by any law. Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JM, JF, RB-M, and TM designed the study. JS, RB-M, and TM performed the fieldwork. JM, RS, JF, RB-M, and TM analyzed the samples and data. RS, JF, RB-M, and TM contributed with reagents. JM drafted the first version of the manuscript. All authors read and approved this manuscript.

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Hematological and Biochemical Changes in Dogs Naturally Infected With *Dirofilaria repens*

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Subcutaneous dirofilariosis is a zoonotic disease emerging throughout Europe caused by the filarial nematode *Dirofilaria repens*. Despite its increasing prevalence, there is a large gap in knowledge of the impact of the parasite on the host. Currently classified as being non-pathogenic, recent evidence suggests that skin dirofilariosis is associated with dermatological conditions including concomitant pruritus, neoplastic processes, inflammation, and even blindness in dogs and humans. The aim of this study was to determine if natural canine *D. repens* infection leads to biological changes in the canine host. In a real-life veterinary clinic setting, animals are often presented to clinicians for unrelated issues, and *D. repens* is incidentally identified during inspection. As such, we compared hematological and biochemical parameters of 218 uninfected and 197 dogs naturally infected with *D. repens*. Interestingly, animals infected with *D. repens* had lower numbers of lymphocytes ($p < 0.001$), red blood cells ($p < 0.001$), and thrombocytes ($p = 0.025$), decreased hematocrit ($p < 0.001$), and increased alkaline phosphatase ($p = 0.016$) and creatinine activity ($p = 0.023$) compared to uninfected dogs. We further selected a subpopulation of 214 dogs having *prima facie* hematological and biochemical results within normal reference ranges to evaluate the effect of *D. repens* infections in seemingly healthy dogs. Among these patients, 93 dogs infected with *D. repens* had lower numbers of lymphocytes ($p = 0.031$), red blood cells ($p = 0.025$), and hematocrit ($p = 0.002$), higher glucose levels ($p = 0.023$), and border line elevated alkaline phosphatase levels ($p = 0.054$) compared to 121 uninfected animals. Despite being categorized as asymptomatic, we have observed hematological and biochemical changes associated with *D. repens* infections in dogs, and our data suggest that dirofilariosis may induce a state of chronic stress. These results link the presence of skin dirofilariosis to biological changes in the canine host, suggesting a mechanism for pathogenicity and shedding new light on the host–parasite relationship.

Keywords: *Dirofilaria repens*, subcutaneous dirofilariosis, dog, hematology, biochemistry

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INTRODUCTION

Dirofilaria immitis and *Dirofilaria repens* are both filarial nematodes that have zoonotic potential and cause canine heartworm disease and skin dirofilariosis in dogs, respectively. Despite its predominance throughout Europe and being the primary causative agent of human dirofilariasis, *D. repens* has received much less attention and study than *D. immitis* (1).

Briefly, dirofilariosis is a vector-borne disease transmitted by mosquitos. Mosquitos uptake microfilariae (Mf) circulating in the bloodstream of a definitive host during blood meals. After 2 weeks, Mf develop into infective larvae and are injected into a new host when the mosquito takes another blood meal. In the new host, the L3 larvae penetrate into subcutaneous tissue where they mature into adult skin filarial worms, copulate, and release Mf into the circulating bloodstream. Adult *D. repens* can survive and reproduce in a host for as long as 5–10 years (2) and may actively migrate within the host tissues during this period what considerably hampers their detection (3).

Dirofilaria spp. infections can be divided into microfilaremic and amicrofilaremic (occult). Microfilaremic infections are diagnosed using a modified Knott's test or by examining blood smears; Mf can be differentiated between species by morphology or using multiplex PCR. Although *D. immitis* amicrofilaremic infection may be diagnosed using a standard commercial test for adult female parasite antigens, there is no such rapid test available for the detection of *D. repens* occult infection in dogs (1).

In humans, the adult worms can migrate to subcutaneous tissue of different parts of the body (commonly in the head, mainly the periorbital region and neck), but they can also localize in the epididymis, the spermatic cords, the lungs, the breasts, the visceral cavity, or under the conjunctive tissue, lymph nodes, and muscles (4). In dogs, they are usually incidentally found in the scrotum during castration, but they can reach other locations including the periorbital or ectopic regions, such as mesenteric tissue and pelvic cavity (1). Infection with *D. repens* is usually considered asymptomatic, but a growing body of evidence suggest that it can cause significant morbidity, and increasing cases have been reported of severe disease leading to liver or kidney failure (5). *Dirofilaria repens* infections were also connected to neoplastic, sometimes malignant, processes in dogs (6, 7) and humans (8). Furthermore, the presence of adult *D. repens* and circulating Mf in blood or parenchymal organs can influence the course of coexisting diseases in infected animals (5, 9). General emaciation has been observed during massive *D. repens* infections associated with peritonitis, jaundice, degeneration of the liver, and renal failure (5, 10). In such conditions, numerous Mf were found in histopathology of internal organs (5–7). Microfilariae remaining in capillaries or disseminated into parenchymal tissues may be involved in the pathogenesis of tissue lesions and progress of the ongoing disease (5).

Infections presenting with conspicuous clinical signs and a severe course of disease progression are clear indicators to employ anthelmintic intervention for the welfare of infected host, but those that are asymptomatic should not be neglected. Untreated infection increases the zoonotic risk (1), can lead to the development of predisposition to other diseases, and may cause silent undiagnosed morbidity. Unfortunately, there are currently no biochemical markers to assess the impact of *D. repens* infections.

The aim of this study was to determine if natural *D. repens* infection leads to biological changes, which may be detected in a real-life clinical situation, where animals are presented to clinicians for unrelated issues, and *D. repens* comorbidity is incidentally identified during inspection.

MATERIALS AND METHODS

Study Design

The flow chart of the study design is shown in **Figure 1**.

Animals

A total of 415 dogs of different breeds, sexes, body weights, health, and breeding status, aged 1–17 years were admitted to the study. Those patients were presented to veterinary clinics for regular health issues and/or because they used to live with dogs previously infected with *D. repens*. All examined dogs lived in Poland and had never left the country before the study.

Sampling

Patients underwent routine physical examination and blood test. *Dirofilaria repens* infection was diagnosed by direct analysis of blood smears, stained blood smears, adult worm examination (if obtained surgically, $n = 24$), and multiplex PCR.

Five dogs with confirmed *Babesia canis* infections were excluded from the data analysis to obtain the pattern characteristic for only one vector-borne disease.

Dogs were assigned to the infected group if at least one of the three parasitological examinations was positive for *D. repens*, and the rest of animals with negative results were qualified to the uninfected group.

We initially compared the parameters within all 415 dogs; 197 were infected, and 218 were uninfected with *D. repens*. Then, within the entire study population, we selected a subpopulation of 214 (98 infected and 112 uninfected) dogs having hematological and biochemical blood parameters within normal reference ranges. We believe that this group represents the asymptomatic patients diagnosed with *D. repens* incidentally.

Laboratory Analysis

Parasitological Examination

Adult worms isolated during surgical procedures were examined under a light microscope and, based on their morphology, were identified as *D. repens*. Microfilariae were detected by microscopic examination of blood smear. Subsequently, genomic DNA was isolated from blood samples using the Blood Mini kit

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate transaminase; EDTA, ethylene diamine tetraacetic acid; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PCR, polymerase chain reaction; RBC, red blood cell; PMN, polymorphonuclear leukocytes; WBC, white blood cells: leukocytes; Mf, microfilariae.

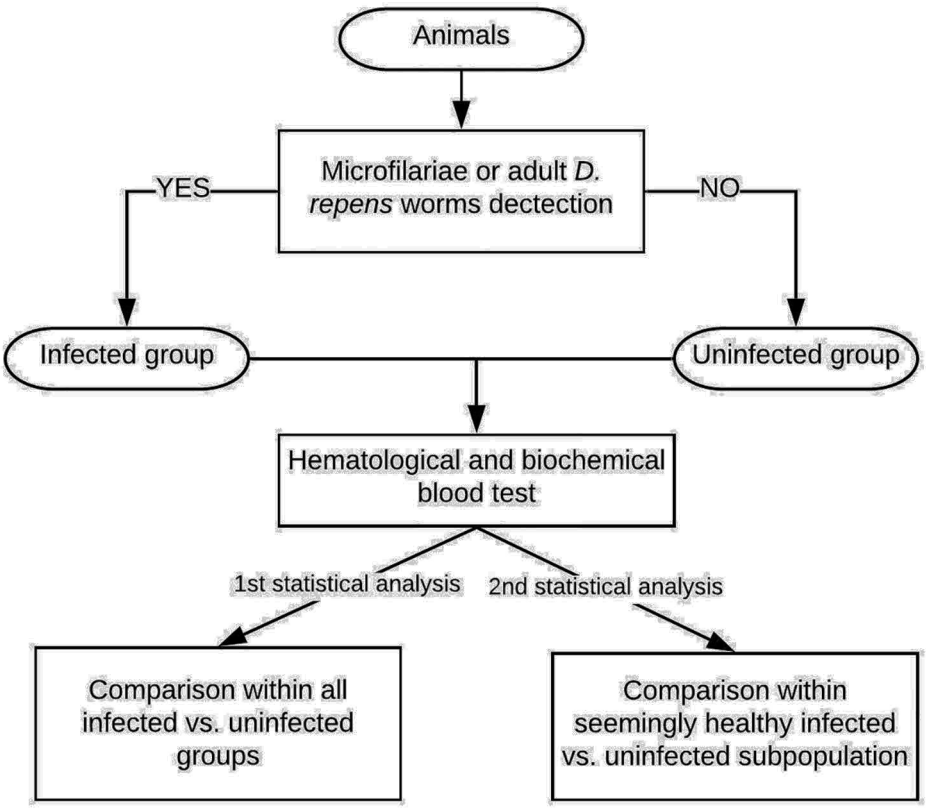

FIGURE 1 | Study design: The examined dogs were classified into the infected group based on the presence of Mf in blood smear/positive result of multiplex PCR/adult *D. repens* parasites found during surgery. Then, blood tests were performed on all individuals. The first statistical analysis included all patients, while the second analysis was performed on a seemingly clinically healthy subpopulation of dogs having blood check-up results within normal reference ranges.

(AA Biotechnology, Poland) and used as a template for multiplex PCR in order to discriminate between *D. immitis* and *D. repens* species according to Gioia et al. (11). PCR results were analyzed by electrophoresis on 2% agarose gels. All infected dogs tested positive for *D. repens* and none for *D. immitis*.

Hematological and Biochemical Analysis

Hematological and biochemical analysis were performed in commercial veterinary diagnostic laboratories. The following standard blood check-up parameters were determined and evaluated according to reference ranges (**Table 1**): white blood cell count (WBC), neutrophil count, lymphocyte count, eosinophil count, red blood cell count (RBC), packed cell volume (hematocrit, HCT), mean corpuscular volume (MCV), platelet count (PLT), enzyme activity [alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP)], and metabolite concentration [total protein (TP), glucose, urea, and creatinine].

TABLE 1 | Normal reference values of investigated hematological and biochemical parameters.

	Unit	Reference intervals
RBC	T/L	5–8
HCT	%	35–60
MCV	fL	55–80
PLT	G/L	100–500
WBC	G/L	5–16
AST	U/L	<100
ALT	U/L	<100
ALP	U/L	<200
TP	g/L	50–80
Glucose	mmol/L	3.8–6.7
Urea	mmol/L	3–12
Creatinine	μmol/L	<150

Statistical Analysis

The number of dogs included in both statistical analyses with respect to their hematological and biochemical blood parameters differed due to missing data of some individuals.

Data were presented as a mean ± standard deviation (SD) or a median and interquartile range (IQR) and compared between groups using the unpaired-sample Student's *t*-test or Mann–Whitney U-test depending on the asymmetry of a variable distribution assessed on the basis of histograms. The range was

always presented. Categorical variables were presented as the number and proportion of dogs in a given group and compared between groups using the Pearson's chi-square test. Ninety-five percent confidence intervals (95% CI) for proportions were calculated with the Wilson score method (12). A two-tailed significance level (α) was set at 0.05. Total study population analysis was performed in Statistica 12 (StatSoft Inc., Tulsa, OK). The analysis of the subpopulation of dog parameters within normal reference range was performed in TIBCO Statistica 13.3.0 (TIBCO StatSoft Inc., Palo Alto, CA).

The Analysis of Check-Up Parameters Within the Total Study Population

This included 415 dogs: 225 males (54.2%) and 190 females (35.8%). Their age ranged from 8 months to 17 years with the median of 7 years (IQR from 4 to 10 years) and did not differ between sexes ($p = 0.655$). There were 56 castrated dogs (13.5%) and 112 (27.0%) pedigree dogs. Their body weight varied from 1 to 75 kg with the median of 16 kg (IQR from 10 to 25 kg).

Two hundred eighteen dogs (52.5%) were allocated to the infected group and 197 (47.5%) to the uninfected group, respectively. Infected dogs were significantly heavier ($p = 0.041$) and more often belonged to a particular breed ($p < 0.001$).

The Analysis of Parameters Within the Subpopulation Having Results Within Normal Range Values

This included 214 out of 415 dogs with blood check-up within or only slightly (by not more than 25% of the upper limit) deviated from the reference intervals. There were 121 males (56.5%) and 93 females (43.5%). Their age ranged from 1 to 14 years with the arithmetic mean (SD) of 6.8 (3.2) years and did not differ significantly between males and females ($p = 0.190$). There were 171 cross-breed dogs (79.9%) and 43 pedigree dogs (20.1%) belonging to the following breeds: German shepherd ($n = 9$), Yorkshire terrier ($n = 5$), Border collie ($n = 4$), Labrador retriever ($n = 3$), French bulldog ($n = 3$), Chesapeake retriever ($n = 2$), Boxer ($n = 2$), Bavarian mountain dog ($n = 2$), Belgian shepherd ($n = 2$), and Alaskan malamute, Amstaff, Polish hound, Black Russia terrier, Akita Inu, Dachshund, Chinese Crested dog, Bernese Mountain dog, Pug, Poodle, and Welsh terrier ($n = 1$ each).

RESULTS

We first compared blood hematology and biochemistry results within the entire study population. Dogs infected with *D. repens* compared to uninfected individuals had significantly lower lymphocyte count ($p < 0.001$), RBC ($p < 0.001$), Ht ($p = 0.001$), and thrombocyte count ($p = 0.025$) and higher ALP ($p = 0.016$) and creatinine ($p = 0.023$) activity (**Table 2**).

In order to determine if observed changes were present only in patients with abnormal blood test results, we decided to select a subpopulation of dogs having parameters within normal values, which we believe mainly represents the undiagnosed dogs infected with skin dirofilariosis in real-clinical practice. Interestingly, in clinically healthy dogs, animals infected with *D. repens* had significantly reduced RBC ($p = 0.025$), Ht ($p = 0.002$), and lymphocyte ($p = 0.031$) count and significantly elevated glucose concentration ($p = 0.023$). ALP activity was boarder line elevated ($p = 0.054$) (**Table 3**).

DISCUSSION

D. repens is by far the most prevalent causative agent of human and canine dirofilariosis in Europe (1). It is generally believed that *D. repens* infections in dogs are asymptomatic, but several case reports (5–7) and the results of blood parameters in our study suggest that the illness is not non-pathogenic but goes rather undetected.

Underdiagnosis of skin dirofilariosis is mainly due to the lack of diagnostic tools to detect occult infections. Amicrofilaremic infections can occur after previous antiparasitic treatment based on macrocyclic lactones, long prepatent time of the infection or monosex infection. Microfilariae periodicity and low intensity of microfilaremia also contribute to obtaining false negative results while investigating a dog for skin dirofilariosis. In contrast, clinicians are left to their own devices for diagnosing and interpreting importance of *D. repens* infections. Thus, having hematological and biochemical biomarker to suggest the presence and severity of infection might be a supportive tool for managing the disease progression, especially in asymptomatic patients.

Our first analysis of all dogs admitted to the study mirrored the real-clinical scenario of dogs brought for different reasons and showed that dogs additionally infected with *D. repens* compared to those uninfected had significantly lower RBC, thrombocyte, and lymphocyte counts, decreased Ht, and increased ALP activity.

The second analysis included a subpopulation of dogs having parameters within normal reference ranges, and in this group, differences were observed in infected animals, such as lower RBC, Ht, lymphocytes, and higher glucose and elevated boarder line ALP activity comparing to the uninfected group. Despite presenting as clinically healthy dogs, these alterations indicate that *D. repens* infection influenced the functioning of their body.

Statistically significant hematological alterations and ALP increase in dogs infected with *D. repens* are similar to those observed in other filarial disease, especially *D. immitis* infections (13), so the pathogenicity, as claimed by other authors (5, 14), is rather correlated with Mf than the presence of adult parasites.

The lower RBC, hematocrit, and hemoglobin characterize anemia in dogs and may be a result of destructive capability of Mf associated with severe intravascular hemolysis as reported in dogs infected with *Diptelonema reconditum* (15). It also might be connected to inflammation-associated production of pro-inflammatory cytokines resulting in suppression of erythrocytes production and inhibition of iron absorption and utilization (16). Lower thrombocyte count may be due to platelet consumption or presence of antiplatelet antibodies observed in dogs infected with other vector-borne diseases (17) or might be assigned to immune-mediated platelet destruction as reported in dogs infected with *D. immitis* (13). Lymphopenia was not only observed in infected patients in this study but also reported

TABLE 2 | Hematological and biochemical parameters in the total study population.

Parameter	Infected		Uninfected		
Hematology	***n***	**Mean ± SD (range)**	***n***	**Mean ± SD (range)**	***t*-test *p*-value**
WBC (G/L)	206	14.9 ± 9.2 (4.7–84.7)	192	14.5 ± 6.2 (3.5–50.4)	0.640
Neutrophils (G/L)	177	10.4 ± 6.5 (1.6–63.8)	189	10.1 ± 5.4 (1.4–39.8)	0.636
Lymphocytes (G/L)	177	2.4 ± 1.5 (0–9.0)	189	3.1 ± 1.6 (0–9.4)	<0.001
Eosinophils (G/L)	177	0.9 ± 0.9 (0–5.1)	189	0.9 ± 1.0 (0–6.2)	0.904
Erythrocytes (T/L)	206	6.7 ± 1.1 (3.6–10.0)	191	7.1 ± 1.0 (3.7–9.9)	<0.001
Haematocrit (%)	206	43.2 ± 6.8 (22–60)	192	45.5 ± 6.3 (22–65)	0.001
MCV (fl)	206	64.8 ± 4.5 (36–77)	192	64.5 ± 3.5 (53–79)	0.404
PLT (G/L)	206	316 ± 160 (13–1000)	192	351 ± 169 (33–1000)	0.025
Biochemistry	***n***	**Median, IQR (range)**	***n***	**Median, IQR (range)**	**Mann-Whitey *U*-test *p*-value**
AST (U/L)	178	32.1, 25.7–43.1 (2.0–689)	167	33.1, 27.5–40.7 (10.5–178)	0.604
ALT (U/L)	182	41.4, 30.0–73.4 (1.3–3026)	167	41.5, 31.2–56.0 (6.9–765)	0.476
ALP (U/L)	168	48.3, 32.3–84.4 (1.0–2015)	167	42.0, 31.3–59.6 (15.0–1302)	0.016
Total protein (g/L)	143	60, 56–64 (40–99)	166	59, 55–63 (38–88)	0.263
Glucose (mmol/L)[a]	152	4.6, 3.8–5.4 (1.1–19.3)	164	4.5, 3.6–5.3 (1.3–17.2)	0.320
Urea (mmol/L)[b]	184	5.4, 4.2–6.9 (1.2–44.6)	165	5.2, 4.3–6.5 (2.0–39.0)	0.663
Creatinine (μmol/L)[c]	184	88.4, 70.7–106.1 (26.5–539.2)	166	79.6, 70.7–97.2 (44.2–592.3)	0.023

[a] *To convert into mg/dL multiply by 18.*
[b] *To convert into mg/dL multiply by 6.03.*
[c] *To convert into mg/dL multiply by 0.011.*

in another dog infected with *D. repens* (5) and during other canine-borne vector diseases. The mechanism of lymphopenia might be associated to endogenous glucocorticoids release that occurs in response to infectious stimuli or stress, which leads to lymphocyte apoptosis or sequestration of lymphocytes in lymphoid organs (18).

The increase in creatinine was observed in the first analysis in the infected group compared to the uninfected group in our study but was not shown to be statistically significant in dogs infected with *D. immitis*. On the contrary, another parameter suggesting renal damage (urea) was increased in *D. immitis*-infected patients but not in our *D. repens*-infected group (13). A case report describing the most severe course of skin dirofilariosis of a dog coinfected with *D. immitis* suggested that only *D. repens* Mf were involved in kidney damage. However, other reports suggest that microfilaremic (not occult) dogs infected with *D. immitis* show marked signs of kidney damage (14, 19, 20).

ALP was significantly higher in infected dogs. Although not being highly liver specific (21), ALP is a marker having a good sensitivity for liver diseases in dogs, suggesting cholestasic disease or chronic hepatitis/cirrhosis. Other parameters suggesting liver injuries, as ALT and AST were reported to be increased in case reports of dogs infected with *D. repens* (5, 22) and in some dogs in our study. This difference, however, was not statistically significant while comparing the infected and uninfected group, suggesting a rather big variability between infected individuals. Interestingly, the ALP increase was also the only liver parameter changed in *D. immitis*-infected dogs (13). The increase in ALP activity, with AST and ALT activity within normal values, makes a hepatocellular damage unlikely and might be rather due to chronic stress associated with increase in endogenous glucocorticoids (23), although, in patients having elevated AST, ALT, and ALP activity, an injury of the liver was very likely.

A higher level of glucose was observed in dogs infected with *D. repens* having all blood parameters within normal ranges. This also may be the result of higher glucocorticoids levels in these animals, which again suggests a correlation between *D. repens* and development of chronic stress response. Interestingly, glucose level was not significantly changed while the whole population was analyzed, which might suggest that, after development of concomitant disease, the host's body reaction to the infection changes.

TABLE 3 | Hematological and biochemical parameters in clinically healthy dogs with blood check-up results within normal reference ranges.

Parameter	Infected		Uninfected		
	n	mean ± SD (range)	*n*	mean ± SD (range)	*t*-test*p*-value
Hematology					
WBC (G/L)	93	11.89 ± 3.39 (5.1–18.4)	121	12.67 ± 3.65 (5.3–19.6)	0.110
Neutrophils (G/L)	93	8.33 ± 2.98 (1.6–16.2)	121	8.74 ± 3.23 (2.7–16.9)	0.343
Lymphocytes (G/L)	93	2.65 ± 1.46 (0.1–6.4)	121	3.07 ± 1.33 (0.6–7.0)	0.031
		2.3 (1.6–3.4)[a]		2.8 (2.1–4.0)[a]	0.007[b]
Eosinophils (G/L)	93	0.93 ± 0.87 (0–3.5)	121	0.86 ± 0.85 (0–5.6)	0.580
		0.7 (0.3–1.2)[a]		0.7 (0.3–1.1)[a]	0.645[b]
RBC (T/L)	93	7.03 ± 0.78 (5.2–8.9)	121	7.25 ± 0.69 (5.6–9.5)	0.025
Ht (%)	93	45.1 ± 4.5 (35–55)	121	47.0 ± 4.3 (36–56)	0.002
MCV (fl)	93	64.4 ± 3.8 (53–74)	121	65.1 ± 3.3 (58–79)	0.173
PLT (G/L)	93	320 ± 103 (110–598)	121	300 ± 98 (99–565)	0.136
Biochemistry					
AST (U/L)	84	31.9 ± 10.3 (13–66)	101	34.4± 10.1 (11-66)	0.095
ALT (U/L)	84	42.1 ± 19.2 (1–99)	101	42.4 ± 17.2 (14–94)	0.900
ALP (U/L)	82	54.3 ± 35.7 (1–163)	101	45.3 ± 23.5 (15–167)	0.054
		43 (30–63)[a]		40 (31–53)[a]	0.258[b]
Total Protein (g/L)	75	60.3 ± 5.8 (50–76)	100	60.2 ± 6.2 (50–77)	0.902
Glucose (mmol/L)[c]	80	4.76 ± 1.08 (1.6–7.6)	100	4.39 ± 1.07 (2.1–7.6)	0.023
Urea (mmol/L)[d]	85	5.29 ± 1.49 (3.0–12.0)	99	5.42 ± 1.46 (2.1–9.0)	0.537
Creatinine (μmol/L)[e]	85	83.83 ± 19.81 (23.9–130.0)	100	84.33 ± 20.84 (43.3–138.8)	0.868

[a] *Median (IQR).*
[b] *Mann-Whitney U-test.*
[c] *To convert into mg/dL multiply by 18.*
[d] *To convert into mg/dL multiply by 6.03.*
[e] *To convert into mg/dL multiply by 0.011.*

Sometimes parasitological coinfection might positively influence the course of the main disease. For example, it has been reported that coinfection of malaria and hookworms seems to increase malaria incidence but at the same time might protect from malaria severe manifestations in humans (24). Moreover, dogs infected solely with *Babesia canis* compared to those coinfected with *D. repens* displayed more pronounced biochemical changes, implying coinfection with *D. repens* was somehow beneficially counteracting against renal and liver damage characteristic for the course of babesiosis in dogs (9), although in the same study, the authors reported that thrombocytopenia and anemia concomitant with babesiosis in dogs were aggravated in individuals coinfected with *D. repens*. Our results show differences in blood parameters between seemingly asymptomatic *D. repens* patients and uninfected dogs. *D. repens* infection seems to promote glucocorticoids release as chronic stress response associated with lymphopenia, glucose level, and ALP activity increase that may predispose infected individuals to develop other infectious, metabolic, or hormonal diseases in the future. This in turn might explain why dogs presented with different diseases and at the same time coinfected with *D. repens* had more serious alternations in blood parameters than uninfected dogs. We suspect that the chronic stress response observed in seemingly healthy dogs may lead to more deepened alternations of concomitant diseases in the future.

There are several limitations in the presented study, such as no checkups or no final diagnosis of concomitant disease available for all admitted dogs. However, we aimed to show a trend in blood parameter changes related to *D. repens* infection. In dogs with different ailments as well as in asymptomatic dogs, significant differences in blood parameters were noted. Our data suggest that *D. repens* infection indeed induces some biological changes in the host. The length of the infection is not known either, but in naturally infected dogs, it cannot be evaluated. However, this mirrors more adequately the real clinical approach to the problem of increasing spread of *D. repens* parasite.

Our results indicate that *D. repens* infection complicates concomitant diseases, and animals develop more serious and detectable changes such as lower numbers of erythrocytes, lymphocytes, and thrombocytes and higher activity of ALP and creatinine. Even among dogs with blood results within normal reference ranges, individuals infected with *D. repens* showed detectable differences compared to uninfected ones, suggesting that seemingly healthy animals may still have altered biological processes or early indications of disease. The analysis of parameters within dogs having results within normal reference ranges, where the differences between groups are indeed within normal values, suggests a trend and perhaps a predisposition of development of other diseases in the future. Further studies with cortisol level evaluation, the measurements of total but also differentiation between liver ALP (L-ALP) and corticosteroid

ALP (C-ALP) isoforms in infected dogs with full follow-ups could be valuable.

CONCLUSIONS

Our paper describes hematological and biochemical changes in dogs naturally infected with *D. repens*. Alterations such as a decrease in RBC, lymphocytes, thrombocytes, hematocrit, as well as increases in ALP and creatinine could be indicators of coinfection with *D. repens,* especially in endemic regions. Furthermore, the results in clinically healthy dogs suggest that the presence of *D. repens* might lead to development of anemia and a state of chronic stress response, characterized by the combination of lymphopenia and increase in glucose level and ALP activity and, as such, predispose infected dogs to other ailments. This could explain the existence of more serious statistically significant blood parameters alternations in dogs with different ailments simultaneously coinfected with *D. repens*.

Our results strongly indicate that *D. repens* infection has a pejorative influence on the health of the canine host independent of their clinical condition. Finally, the pathogenicity of *D. repens* remains a mystery but needs further investigation in order to comprehend its association with chronic stress response in infected individuals.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the blood samples were collected for direct benefit of the patients and the analysis was performed on leftover samples (Act of 15th January 2015 on the protection of animals used for scientific purposes). The director of veterinary services of local dog shelters and the owners of privately owned dogs were informed about the results of Dirofilaria testing of the dogs under their care. Written informed consent for participation was not obtained from the owners because no interventions outside routine care were performed. All blood samples were taken during routine checkups or process of diagnosis in veterinary clinics and only then the results were used to compare the results between *D. repens* infected and uninfected groups of dogs. Multiplex PCR was run on the leftover blood samples. A verbal consent of the owners' to voluntirely test their animals for skin dirofilariosis was present for all dogs admitted in the study. All data were de-indentified before running statistical analysis and the entire anonymity of data has been assured. The director of local dog shelters and the owners of client-owned dogs were informed about the results of Dirofilaria testing of the dogs under their care. Doctors of veterinary medicine were provided with all information that could help them to introduce the best treatment to infected dogs.

AUTHOR CONTRIBUTIONS

AD and MK collected clinical data, parasitic, biological specimens, and performed blood smears. ED and MEW performed PCR. MC performed the statistical analysis of the results. MEW, AD, MK, ED, PJ, and MW planned and discussed the study and the results. MEW wrote the manuscript. All authors read and approved the final version of the manuscript.

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BRIEF RESEARCH REPORT
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Chagas Disease in Pregnant Women in the Peruvian Amazon Basin. Cross-Sectional Study

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Aims: To assess the prevalence of Chagas disease in pregnant women in Iquitos City, Peru.

Material and Methods: Cross-sectional survey in 300 pregnant women in Iquitos (Peru) from 1 May 2019 to 15 June 2019. Women were tested using an ELISA serology test.

Results: Serology was positive in one case (prevalence: 0.33%; 95% confidence interval: 7.1–13.9%), of a 25-year-old woman who lived in a wooden house with a leaf roof in a periurban area of Iquitos. She was familiar with kissing bugs and had chronic, asymptomatic Chagas disease.

Conclusion: The prevalence of Chagas disease is low in the urban and peri-urban area of the city of Iquitos.

Keywords: Chagas disease, *Trypanosoma cruzi*, pregnant, Peru, Amazon

INTRODUCTION

Chagas disease is a systemic chronic parasitic infection caused by *Trypanosoma cruzi* that affects 6–7 million people in Central and South America. It is considered a neglected tropical disease and has a high public health impact in the area.

While mainly vector-borne, Chagas disease can also be spread via blood transfusion, transplantation, or from mother to child (1). The latter, congenital route is frequent and especially relevant in areas where there is no vector transmission by insects or blood transfusion. To accelerate the elimination of this transmission route, strategies and methods should be applied to screen, diagnose, and treat all infected pregnant women as well as their newborns and, where appropriate, other children as soon as possible (2).

Chagas disease is endemic throughout the Pacific southwest of Peru, known as the Greater Southern Region, in the departments of Arequipa, Moquegua, Tacna, Ayacucho and Apurimac.

In the past decade, widespread infestation with the vector *Triatoma infestans* and active transmission of Chagas disease to humans have been documented in this area (3, 4). In addition, the northeastern departments of Cajamarca, Amazonas, San Martín, and Ucayali (400–1,000 m above sea level) have also detected the thriving presence of the *Panstrongylus herreri* vector (3). However, there is little knowledge about the prevalence and epidemiology of *Trypanosoma cruzi* in northern Peru (5); the most significant resources have been allocated toward research and control efforts in the south (5).

Iquitos is a city in the Peruvian Amazon Basin, in the Department of Loreto and near the confluence of the Marañon and Ucayali rivers. A few isolated cases of Chagas disease have been documented in the town and surroundings areas (6), but no survey has ever focused on the pregnant population. As these women can unknowingly transmit the disease to their newborns, its detection is highly recommended (2, 7). The aim of this study was to estimate the prevalence of Chagas disease in pregnant women in Iquitos, Peru, in the Peruvian Amazon basin.

METHODS

Setting and Study Period

We performed a cross-sectional survey of Chagas disease, strongyloidiasis, and human T-cell leukemia-lymphoma virus (HTLV) infection in an urban and periurban area of Iquitos (Peruvian Amazon) (**Figure 1**), from 1 May 2019 to 15 June 2019.

Study Population

We included pregnant women attending the health centers in four districts of the greater Iquitos area, located in the Peruvian Amazon. Participants were adults over the age of 18 years and were selected through convenience sampling (i.e., on days when the researcher was at the health center) when they visited the midwife during prenatal check-ups. All participants were informed about the screening and signed informed consent.

Data Collection

On enrollment, participants were asked about sociodemographic variables and their familiarity with Chagas disease. We obtained a blood sample for serological detection of *T. cruzi* antibodies, *S. stercoralis* IgG antibodies, and HTLV antibodies, and a stool sample to test for parasitic infections. Our group published results on the prevalence of strongyloidiasis and other intestinal parasites in a parallel report (8).

Detection of *T. cruzi* IgG antibodies was performed by two different assays: the Chagatest ELISA lysate (Wiener, Rosario, Argentina) and the Chagatest ELISA recombinant v.4.0 (Wiener, Rosario, Argentina). *T. cruzi* infection was considered confirmed if both tests yielded a positive result, while participants were considered seronegative when both tests yielded negative results. Specimens with only one positive test were considered inconclusive. The Chagatests were completed according to manufacturer's instructions and the threshold for positive results was 0.10 optical density (OD) units above the mean absorbance of two negative control specimens included on each plate.

Data Analysis

The collected data were systematically recorded and analyzed using IBM SPSS Statistics, version 22.0. We used the chi-squared test or Fisher's exact test to determine the presence of Chagas disease according to several demographic variables, considering results to be significant where the $P < 0.05$.

RESULTS

The study included 300 pregnant women with a mean age of 26.7 years (standard deviation [SD] 6.4; range 13–38), mean of number of deliveries of 2.9 (SD: 1.7), and a mean gestation period of 172 days (SD: 59). Just under half (44.7%) lived in peri-urban areas, while the rest lived in the city. **Table 1** shows the sociodemographic and epidemiological characteristics of the participants in the study. **Figure 1** Health centers in Iquitos city and surrounding area.

Four participants tested positive on the Chagatest ELISA recombinant v.4.0 with titers > 0.2, but only one had a second positive serology test (Chagatest ELISA lysate) (**Table 2**). Therefore, just one participant (0.33%, 95% CI: 0.02–2.13%) was considered as a definitive positive for Chagas disease. Three other participants had inconclusive results by both ELISAs. Their infection status therefore remained unresolved, and their data were excluded from further analysis. There were no statistically significant differences in the sociodemographic conditions, knowledge of Chagas disease, or housing conditions in cases with positive and negative serology against *T. cruzi*.

The positive case was a 25-year-old woman who lived in a periurban area of Iquitos (**Figure 1**). She had been living in the same house—a wooden construction with a leaf roof—for the last 5 years. She had two other children and was familiar with the "chirimacha" (kissing bugs or triatomines) and reported seeing them at home, but she did not remember being bitten by one. She had not received any blood transfusion and did not have any symptoms of Chagas disease (chest pain, palpitation, dysphagia). Her electrocardiogram was normal, showing a repolarization disorder.

DISCUSSION

Our study demonstrates that the prevalence of asymptomatic Chagas disease in pregnant women is low (0.33%) in the Iquitos area of the Peruvian Amazon Basin. This evidence helps to fill gaps in knowledge arising from the few seroprevalence studies in pregnant women in Peru (7). In 2005, a study performed in Arequipa (southern Peru) in 3,000 pregnant women showed serological positives in 22 (0.7%) participants; only one newborn was IgM positive (9).

The prevalence of Chagas disease in pregnant women, both in our study and the one performed in Arequipa, is low compared to those performed in Bolivia, northern Argentina, or Brazil, which have reported a prevalence of 21, 12.1, and 1.1%, respectively (10–12). In the general population, the literature reports a seroprevalence of Chagas disease in the southern department of Arequipa ranging from 2 to 5.8% (4, 13, 14). In

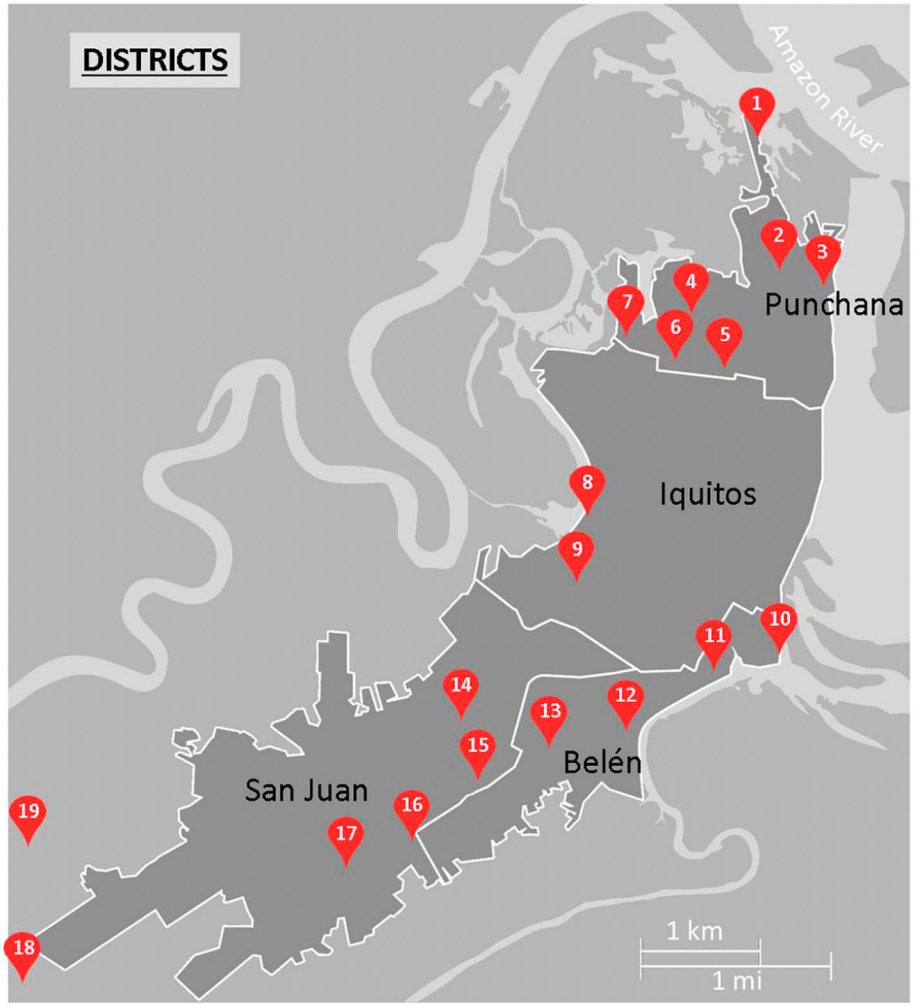

FIGURE 1 | Health centers in Iquitos city and surrounding area.

the north (La Joya and Cajamarca departments), prevalence is slightly higher, between 7.6 and 14.9% (5, 15). Our results from Iquitos are sensibly lower than in these reports from elsewhere in Peru.

However, our results are relevant because they show that Chagas disease, although uncommon, is actually present in the Iquitos area. Asymptomatic Chagas disease cases like the one detected in our survey do occur. Other acute cases have previously been reported in 2006 and 2008 in the Amazon area (6, 16, 17). Therefore, Chagas disease is present not only in the jungle of northern Peru (5) but also in the Amazon basin. That said, seroprevalence of *T. cruzi* in pregnant women appears to be lower than that reported in the endemic areas of Peru (9). There may be small pockets of vector transmission of Chagas disease in Iquitos. Performing small studies around this case could help to uncover more; this adaptive strategy could efficiently identify secondary cases (13).

Chagas disease has been studied elsewhere in the Amazon basin, including in Ecuador [prevalence 3.6% (18)] and in Brazil (seroprevalence 1.5%). Throughout all these areas, Chagas continues to pose a threat to public health, one amplified by deforestation and its associated changes in transmission vectors

(19, 20). Indeed, this region is at risk of transmission due to triatomine-contaminated food (21). Several initiatives, like the Iniciativa Andina (IA), have been launched in priority areas of Latin America to ensure the interruption of vector-borne transmission of Chagas as well as to improve surveillance and disease prevention (22).

The presence of pregnant women in the Amazon Basin who are at risk of congenital transmission to the fetus, along with other reported cases of acute Chagas Diseases (6, 16, 17) should serve as a warning of an (emerging) problem in this area of Peru. While the low prevalence does not justify screening in pregnant or puerperal women, other measures are called for. WHO/PAHO recommends interventions to disrupt vector transmission, including the elimination of the triatomines or other vectors from the study area; another possibility is assessing seroprevalence of *T. cruzi* infection in children 5 or younger (23). Several environmental and social factors may also directly or indirectly influence the biology and behavior of triatomides (24). Spatial clustering of infestation in the urban context may both challenge and inform surveillance and control of vector reemergence after insecticide intervention. These measures have been performed in several departments in Peru, such as Moquegua and Tacna, which were subsequently declared free of vector transmission by the WHO/PAHO (25).

Entomologic investigation of *T. infentans* and other triatomides is important for knowing the ecology of vector transmission (26). It is important to implement measures for controlling transmission of *T. cruzi* by triatomines in the Amazon basin, with vector surveillance and control with insecticide as has been happening in other parts of Peru (5). In light of our exploratory results and other cases reported in the area, it could be of interest to perform similar studies to those carried out in other parts of Peru (15, 17, 23). Another topic to investigate in the area is dogs, which are important reservoirs of *T. cruzi* and may play a role in reinitiating transmission in previously sprayed areas. Dogs may also serve as indicators of reemerging transmission (24).

Another potential line of research about Chagas disease in the Peruvian Amazon basin is the risk of oral transmission. In Brazil, Venezuela, Colombia, Bolivia, and French Guiana, several outbreaks of orally transmitted Chagas disease have been reported; these have been epidemiologically associated with the consumption of beverages like açai juice (the fruit from a species of palm tree) or sugar cane juice (27). Some cases of Chagas disease have also been described in young indigenous people who drank contaminated juice (17, 26). This line of research should continue in the area, as should health education, in order to prevent the contamination of juice with *T. infestans* excreta (26).

This study has some limitations. First, a complete study of congenital transmission was not performed. Secondly, there were three participants with borderline results according to both ELISAs. As this situation might reflect a window period, it would be necessary to re-test them after 6 months to confirm or rule out infection. Finally, there was no entomologic investigation about species living at the patient's home.

TABLE 1 | Epidemiological characteristics of pregnant women.

Variables	Frequency
Sociodemographic conditions	
Mean age (years)	26 (SD: 6.4)
Mean time living in the place (years)	9.9 (SD: 9.7)
Residence in rural area	134 (44.7%)
Characteristics of pregnancy	
Mean gestational age (days)	172 (SD: 59)
Primiparous	64 (21.3%)
Mean number of pregnancies	2.9 (SD: 1.7)
Mean number of children alive	1.8 (SD: 1.5)
Risk factors for Chagas disease	
History of blood transfusion	15 (5.0%)
Knowledge of Chagas disease	
Any knowledge about Chagas disease	10 (3.3%)
Contact with person with Chagas disease	0 (0.0%)
Characteristics of living houses	
Wood house	286 (95.3%)
Leaf roof	234 (78.0%)
Soil floor	89 (29.7%)

SD, standard deviation.

CONCLUSIONS

Although the prevalence of Chagas disease is low in the urban and peri-urban area of the city of Iquitos, it is relevant to advise local authorities that the disease is actually present in the Peruvian Amazon Basin in pregnant women with a risk of congenital transmission. Our results indicate a probable low rate,

TABLE 2 | Results of two serological procedures in positive cases.

Code	Age/health center	Antecedents blood transfusion	Chagatest ELISA recombinant v.4.0 titers	Chagatest ELISA lysate titers	Chagas disease
1	25 years/santa clara	No	1.118	1.416	Yes
2	19 years/san juan	No	0.267	0.011	Inconclusive
3	33 years/san juan	No	0.237	0.029	Inconclusive
4	19 years/San juan	No	0.301	0.038	Inconclusive

but it is necessary to perform more studies and monitor the prevalence of the disease.

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 studies involving human participants were reviewed and approved by The Ethics Committee of the General University Hospital of Alicante (Spain) approved the project (PI2018/113), as did the Ethics Committee of Loreto Regional Hospital in Iquitos (Peru) (027-CIEI-HRL-2019). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-MR-R: conceptualization, formal analysis, methodology, project administration, supervision, writing—original draft, writing-review and editing, designed the study, analyzed clinical data, prepared, and reviewed the manuscript. SO-M: formal analysis, investigation, project administration, software, supervision, writing—original draft, and writing-review and editing. M-EV-C: methodology and writing-review and editing. O-NG-P: data citation, software, supervision, and writing-review and editing. V-VP-C: data curation, methodology, project administration, and writing-review and editing. CR-A: investigation and writing-review and editing. MG-H-M: conceptualization, writing—original draft, and writing-review and editing. MC-M: conceptualization, project administration, writing—original draft, and writing-review and editing manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Veterinary Science

CASE REPORT
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Case Studies of Severe Microfilaremia in Four Dogs Naturally Infected With *Dirofilaria repens* as the Primary Disease or a Disease Complicating Factor

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Subcutaneous dirofilariosis in dogs, caused by *Dirofilaria repens*, is an underdiagnosed disease, now recognized for its zoonotic potential, and growing distribution and prevalence across Europe and Asia. Our understanding of the pathogenicity in human and canine host remains unclear, but case reports suggest that microfilariae (Mf) as well as adult *D. repens* may directly cause internal organs damage or may be a factor complicating the course of other ailments. The purpose of the study was to report high Mf in dogs and to discuss potential relevance with co-morbidity. Our data from a modified Knott's test performed on 62 infected dogs indicate that the median Mf count in *D. repens* infections is 675 Mf/ml and we consider microfilaremia above 10,000 Mf/ml as high intensity. This collection of case reports discusses 4 cases of high intensity *D. repens* microfilaremia in companion dogs; one presenting pathology from a very high intensity of adult *D. repens* with post-treatment complications, and 3 dogs in which high microfilaremia was detected incidentally during the management of other primary illnesses. To our knowledge this report describes the highest *D. repens* microfilaremia ever detected in a dog, at 178,000 Mf/ml. The issue of high microfilaremic infections in dogs is poorly studied and there is growing need to identify the presentation and understand the mechanisms of associated pathogenesis in the host-parasite relationship.

Keywords: dog, dirofilariosis, microfilaremia, immunology, natural Dirofilaria repens infection

INTRODUCTION

Dirofilaria repens is a zoonotic filarial nematode transmitted to dogs by a mosquito vector and is the principal agent of human dirofilariosis in the Old World (1). Humans are considered a dead-end, accidental host for *D. repens*, but a growing body of case reports suggests that humans may instead be a dual facultative host in which the parasite can achieve maturity and release microfilariae (Mf) into the bloodstream (2, 3). Recently, there has been documentation in Poland of

a human patient having microfilaremia (360 Mf/ml) (4). Furthermore, an ocular mucosa associated lymphoid tissue lymphoma (MALT) has been reported in a human patient in Poland as a possible consequence of dirofilariosis (5). The number of human subcutaneous dirofilariosis cases in Europe has increased in recent years and current estimations in some areas is roughly 10 infected out of 100,000 inhabitants (6). True infection prevalence is likely underestimated because of misdiagnosis and under sampling. Additionally, with increasing globalization and climate change (1, 7), these numbers are expected to continue to grow and, therefore, proper diagnosis and treatment in dogs and humans will be increasingly important for managing the spread and disease burden of *D. repens* infections.

Currently, the treatment recommended by the European Society for Dirofilariosis and Angiostrongylosis (ESDA) for *D. repens* infections consists of 2.5 mg/kg moxidectin (in a spot-on formulation containing also 10 mg/kg imidacloprid) given monthly[1]. However, because so few cases of high intensity *D. repens* microfilaremia have been reported (8), there are no guidelines for its management (1), or rationale for its cause.

The gold standard for diagnosis of subcutaneous dirofilariosis is the modified Knott's test which allows visual detection and morphological identification of Mf (9), and was used to evaluate the Mf burden in all cases described in this article. Our data from a modified Knott's tests performed on 62 infected dogs indicate that the median Mf count in *D. repens* infections is 675 Mf/ml (unpublished data), and the authors consider microfilaremia above 10,000 Mf/ml as being high. Dogs presented in this report all tested positive for having high microfilaremia with the highest level ever reported in *D. repens* infection at 178,000 Mf/ml. Helminths are known to suppress the host immune response in order to establish infection. It is therefore possible that patients presenting with high Mf counts may represent a population with pre-existing immunosuppression and are thus, unable to naturally control or respond to infection (9). All dogs in this study were diagnosed with terminal conditions which might be correlated with an underlying immunodeficiency.

CASE #1

Clinical Presentation

A 9-year-old entire male Shepherd dog was admitted to a veterinary clinic on 27/05/2016 due to severe weight loss and two infected purulent wounds in the scrotum area, associated with intensive licking. Physical examination revealed the presence of a chronic bilateral *otitis externa* and an inflammation of the interdigital spaces. Swabs from both ears and interdigital spaces were taken.

Diagnosis

During a blood examination on 03/09/2016 we detected severe leucocytosis [33,64 G/l (reference value 6–12 G/l)] related to the increase in band neutrophils [10% (reference value 0–6%)] with a mild increase in aspartate aminotransferase [91 U/l (reference value 0–45 U/l)], moderate increase in phosphatase kinase [1,160 U/l (reference value 25–467 U/l)], decrease in albumin [1,8 g/dl (reference value 3,3–5,6 g/dl) and the presence of Mf was observed in the blood smear. Identification of *D. repens* was obtained using multiplex PCR performed using primers designed by Genchi et al. (10). The intensity of microfilaremia was evaluated using a modified Knott's test and revealed 14,512 Mf/ml of blood. The ear swab sample tested positive for *Malassezia pachydermatis*, and the swab from interdigital space was positive for *Pseudomonas aeruginosa*.

Treatment

During surgical castration on 7/09/2016, 26 adult *D. repens* worms were found in the spermatic cord, testicles and scrotum tissue. The external canals were treated with nystatin according to the antifungal susceptibility test, and a general antibiotic was administered for the dermatitis of interdigital space according to the antibiogram.

After castration and wound excision, the dog started to gain weight. *Otitis externa* as well as the dermatitis were ameliorating after appropriate treatment. Beginning on 24/10/2016, 6 monthly treatments of topical moxidectin with imidacloprid were administered as efficacious treatment against adult *D. repens* in dogs (11). One week after the first application no Mf were detected in the peripheral blood. The body condition of the dog changed from very thin to normal within 4 months. Despite amelioration of the general condition, a necrosis and pyogranuloma of interdigital spaces associated with severe dermatitis of the abdomen and limbs developed with episodes of amelioration and deteriorations between 10/11/2016 and 28/08/2018. A course of antibiotics was administered according to the antibiogram but no improvement was observed. A generalized demodicosis, caused by *Demodex canis,* was diagnosed and, as the declining clinical state of the dog was progressing and no amelioration could be established, the owner did not wish to pursue further treatment. Finally, the patient became anorexic and expired on 15/09/2018. The cause of death reported after necropsy was a perforated stomach ulcer. Moreover, the histopathological examination of internal organs revealed a chronic cardiac insufficiency and pathological changes indicating a chronic inflammation in the kidneys, liver and spleen.

Discussion

The presence of 26 adult *D. repens* likely caused severe pain and pruritus. It is noteworthy that there were no noticeable behaviors to indicate the extremely high number of Mf circulating in the blood stream. The only obvious clinical and hematological signs were likely associated with presence of a large number of adult worms in the scrotum area. We suspect that the severe dermatitis which appeared post-treatment might have been associated with *Wolbachia* release after *Dirofilaria* spp. adulticide treatment as reported by previous studies in *D. immitis* infections (12–15). Furthermore, German Shepherds are predisposed to primary immunodeficiency (16) and it has been postulated that high microfilaremia might be associated with a state of

[1]https://www.esda.vet/wp-content/uploads/2017/11/GUIDELINES-FOR-CLINICAL-MANAGEMENT-OF-SUBCUTANEOUS-DIROFILARIOSIS-IN-DOGS-AND-CATS.pdf.

immunosuppression (17). The chronic yeast infection (usually secondary to an immune disorder in dogs) as well as the appearance of generalized demodicosis are possible indicators of immunodeficiency in this patient (18, 19).

CASE #2

Clinical Presentation

A 7-year-old male entire Yorkshire Terrier was admitted to the veterinary clinic on 06/08/2019 due to sudden anorexia, apathy and diarrhea. During the clinical examination the dog presented a hypotonic-hyporesponsiveness concomitant with exophthalmos, severe dehydration, hypothermia, bradycardia and pale mucous membranes. A treatment based on dexamethasone, caffeine, atropine, ceftriaxone and fluid therapy was initiated and a blood sample taken for analysis.

Diagnosis

Hematology revealed a severe leukocytosis [58.1 $\times$ 10^3/mm^3 (reference value 6.0–12.0 $\times$ 10^3/mm^3)] resulting from neutrophilia [54.2 $\times$ 10^3/mm^3 (reference value 3.00–10.00 10^3/mm^3)], and eosinophilia [23.42 $\times$ 10^3/mm^3 (reference value 0–0.60 10^3/mm^3)] with mild anemia [HCT = 39.5% (reference value 44.0–57.0%)], and a severe increase in alkaline phosphatase [551 U/l (reference value 20–150 U/l)], alanine aminotransferase [131 U/l (reference value 10–118 U/l)] and a decrease in albumin [1.5 g/dL (reference value 2.5–4.4 g/dL)]. The glucose level was 99 mg/dl (reference value 80–120 mg/dl) and the modified Knott's test revealed 11,560 Mf/ml. After testing and excluding Canine Coronavirus (CCV), Canine Parvovirus and *Giardia* spp. infections, and according to anamnesis, a diagnosis of poisoning with taxine alkaloid was decided. On 08/08/2019 the state of the patient continued to deteriorate despite intense care therapy, and a decision of euthanasia was made.

Discussion

It is hard to evaluate the impact of co-infection with *D. repens* in this case, but one can suppose that it may have had a negative influence on the development of the condition, as taxine alkaloids (from yew) can cause lethal poisoning, in particular due to the compound's toxic effect on the cardiovascular apparatus (20). It is certainly possible that the severe microfilaremia might create a blood flow disturbance of capillary circulation by mechanical obstruction. Additionally, it has been observed in other filarial parasitic diseases that dead Mf release toxic products that affect the capillaries (21, 22). Unfortunately, the prognosis in taxine alkaloid poisoning without immediate stomach flushing is usually fatal and the presence of subcutaneous dirofilariosis was not likely influential in the course of the intoxication (20), but may be of concern for chronic anemia.

CASE #3

Clinical Presentation

A 10-year-old entire male mix breed dog was admitted to the clinic due to constipation. During the physical examination a testicular asymmetry, perineal hernia, fecal impaction as well as an anal sac tumor of 2 $\times$ 2 cm were detected.

Diagnosis

The blood test revealed moderate anemia [RBC = 4.98 T/l (reference value 5.5–8.5 T/l), HCT = 32.6% (reference value 37.0–55.0%), HGB = 11,1 g/dl (reference value 12–18 g/dl)], severe leucocytosis [41.04 G/l (reference value 6.0–12.0 G/l)] resulted from: neutrophilia [34.06 G/l (reference value 2.9–13.6 G/l)], monocytosis [2.05 G/l (reference value 0.4–1.6 G/l)], eosinophilia [3.69 G/l (reference value 0–3.1 G/l)], thrombocytopenia [19 G/l (reference value 150–500 G/l)] and 40 150 Mf/ml. In addition, a mild increase in alanine aminotransferase [72 U/l (reference value 0–60.0 U/l)] and a decrease of albumin [3.2 g/dl (reference value 3.3–5.6 g/dl)] were detected, and ultrasonography revealed ascites, which was analyzed. The ascites contained Mf, granulocytes and atypical binucleated cells with many mitotic figures suggesting a neoplastic process. The owner did not wish to pursue any treatment nor euthanize and decided to take the patient home.

Discussion

This is the second report of finding Mf in the body fluid in a dog, the first of which was by Pazdzior-Czapula et al. (23). The issue and association between neoplastic processes and the presence of *D. repens* Mf or adults has been previously observed in cancer of the perianal gland, mast cells, epithelial cells, hemangiopericytoma, and trichoblastoma ribbon in dogs (20), as well as an ocular lymphoma from which the parasite was previously removed from a human patient (5). It has also been reported that parasitic infections or abnormalities of the immune system are at a high risk for developing cancer (5, 24), but the exact relationship between subcutaneous dirofilariosis and neoplastic process is still unknown.

CASE #4

Clinical Presentation

A 10-year-old entire Saint Bernard male was admitted to the clinic due to intolerance of any physical activity and permanent dyspnoea. Four months prior, the dog was pursuing therapy in another clinic and received a treatment of benazepril, spironolactone, theophylline, and nitroglycerin with no diagnosis, but anamnesis of increasing fatigue after physical exercise. During our physical examination, dyspnoea and cyanosis of tongue and mucous membranes (hypoxia) were noted.

Diagnosis

After sedation of the patient, additional diagnostics were performed. Thoracic radiographs revealed a dilation of the esophagus, computed tomography and electrocardiography revealed no alternations. A moderate leucocytosis [16.7 $\times$ 10^3/mm^3 (reference value 6.0–10.00 x 10^3/mm^3)], mild neutrophilia [13.90 x 10^3/mm^3 (3.0–10.00 $\times$ 10^3/mm^3)], and eosinophilia [1.5 $\times$ 10^3/mm^3 (reference value 0.0–0.60 $\times$ 10^3/mm^3)] were observed in the hematology, and the

biochemistry parameters were all in normal reference ranges. The echocardiographic measurements were within standard normal age limits. The rapid CANIV-4 commercial test was positive for *D. immitis*, but multiplex PCR revealed only the presence of *D. repens*. A modified Knott's test was performed which detected 178,000 Mf/ml and a double morphological identification of *D. repens*, not *D. immitis* was confirmed. As systolic dysfunction was suspected aspirin was administrated which resulted in mild improvement of clinical signs. A few days later echocardiographic examination was performed and no nematodes were detected in the pulmonary artery. The echocardiographic measurements were within standard normal age limits. Only immobilization brought relief to the dog. A final diagnosis of severe laryngeal paralysis was decided upon, and pharmacological treatment was performed, but with no improvement. Before any surgical interventions could be considered the patient died at home from apparent suffocation. The owners did not permit a necropsy.

Discussion

Laryngeal paralysis is a respiratory disorder that primarily affects older (> 9 years of age), large-breed and giant-breed dogs, such as the Saint Bernard in this case. In many dogs the etiology remains idiopathic, but one possible cause is suggested to involve immune-mediated neuromuscular disease or other immune disorders like systemic lupus erythematosus (25, 26) In humans, a case of a girl treated for systemic lupus erythematosus was diagnosed with concomitant non-human filarial microfilaremic infection. The authors suggested that an immunity disorder might have led to this filarial infection (27). Leucocytosis and eosinophilia are both commonly associated with helminth infections, and neutrophilia can inhibit a Th1 type response, all of which could be expected to be associated with an increased Mf count. However, these numbers alone are very unlikely to facilitate or explain such a high level of microfilaremia. Although it is not possible to come to any conclusions from this case, the incredibly high level of microfilaremia and its co-morbidity with a presentation of symptoms known to be related to compromised immune system function, is highly suggestive of a causal relationship.

FINAL CONCLUSIONS

The goal of this article was to report the details of four cases of high *D. repens* microfilaremia. Hematological and biochemical findings in dogs infected with *Dirofilaria repens* included anemia, thrombocytopenia, leucocytosis, neutrophilia, eosinophilia, monocytosis and increased values of alkaline phosphatase, aspartate transaminase, alanine aminotransferase, blood urea nitrogen, creatinine, bilirubin as well as hypoalbuminemia, which is in keeping with results described by other authors (28, 29).

In the cases presented here, it is difficult to determine how long the animals were infected for or to evaluate the contribution of subcutaneous dirofilariosis to the progression of morbidity in the presence of concomitant conditions. Due to the severity of the underlying diseases and symptoms, anthelmintic treatment was only administered to one dog seen in this collection of case studies. Even in the presence of a high burden of microfilaria, treatment with a standard dose of moxidectin was sufficient to reduce Mf count below detection 6 days after application. It should be noted that there is a 24-h microfilarial periodicity, which makes the time of sampling a factor influencing the result of a Knott test (30).

We suspect that the severe dermatitis that appeared post-treatment might have been associated with *Wolbachia* release after *Dirofilaria* spp. worm death as reported in *Dirofilaria immitis* infections (12). Recently, a case report of *D. repens* microfilaremic infection in a human showed successful treatment with only doxycycline targeting the *Wolbachia* endosymbiont (31).

All of the *D. repens* infections reported here were diagnosed incidentally while investigating concomitant conditions, suggesting that high intensity microfilaremia *per se* may go undiagnosed. This implies that there is a greater reservoir for human infection than previously suspected, and perhaps warrants increased monitoring in endemic areas, such as the regular use of a modified Knott's test. It might be discussed if high microfilaremic dogs represent an epidemiological risk of increased transmission, as most mosquitoes die when feeding on hosts with microfilaremia superior to 7,500 Mf/ml (21). However, the daily microfilarial periodicity permits the possibility that some mosquitoes can survive ingesting blood even from high microfilaremic hosts. Therefore, anthelmintic treatment, even in the absence of parasitic symptoms, should be administrated.

It remains unclear why in some patients, *D. repens* infections appear asymptomatic, and in other cases Mf are suspected to be responsible for severe renal or liver damage (28). Veterinarians should consider dirofilariosis as a primary or co-existing disease that may modify or mask the symptoms or progression of a co-morbidity. Furthermore, high intensity microfilaremia in a blood samples should be taken as a potential indictor of the presence of an underlying serious pathology.

Personal correspondence with veterinary practitioners suggest a beneficial effect of adding to the moxidectin standard treatment an anticoagulant, corticosteroids, and doxycycline, depending on the severity of the clinical state of the patient infected with *D. repens;* this is also recommended in high risk thrombosis development with heartworm disease (12). As reported in lymphatic filariasis, doxycycline treatment against *Wolbachia* provides clinical improvement in patients with lymphedema (32). It has also been noted in loiasis that high microfilarial loads (30,000–50,000 Mf/ml) can lead to capillary obstruction from damaged parasites after anthelmintic treatment, and adding corticosteroids to the anti-parasitic treatment should be taken into consideration by ESDA and veterinary practitioners in high intensity microfilaremia (22).

In human *Loa loa* infections occurring with microfilaremia >10,000 Mf/ml of blood, encephalopathy following ivermectin has been well-documented. Treatment with microfilaricidal agents such as ivermectin and diethylcarbamazine may provoke the passage of L. loa Mf into the cerebrospinal fluid and precipitate an encephalopathy (33, 34). Those reactions were not yet described in filariosis of dogs, but a case of a human *D. repens*

infection concomitant with meningoencephalitis was successfully treated by initiation of anti-helminth and anti-inflammatory medicine (35). This could be an interesting issue to follow and explain in further studies.

Adult parasite burden cannot be determined precisely in helminth infections (36), especially in *D. repens* where the worms may reside in any number of tissue locations. In Case #1 we found 26 adult worms during castration. Most case reports present findings of just one or few adult worms, however, we cannot exclude the possibility that these are under representations of total adult numbers localized in different tissues. It has been previously documented that a dog infected with over 300 adult *D. repens* showed a microfilaremia of 7,780 Mf/ml (28). This suggests that the quantity of adult worms may be only one of the factor determining Mf in the blood stream, and it is very likely that the immune system of the host plays a large role in regulating microfilariae burden, as is observed in other helminth infections (28, 37). The mechanism regulating the burden of Mf in the bloodstream is not known. It is suspected that certain aspects of host-helminth relationship may be beneficial to the host given the proper balance parasite load and humoral response in the host (37). This relationship is beginning to be investigated pertaining to autoimmune disease, and could be of future interest in the study of *D. repens* infections.

Density-dependent processes play a key role in the transmission dynamics of vector-borne diseases (36). The relationship between microfilarial density and worm burden in *Onchocerca volvulus* infections in humans has been investigated, yet not fully understood. It has been observed that the microfilariae burden increases with host age, suggesting a development of immunological tolerance, while the density of adult parasites correlating with the microfilariae burden has not been confirmed. Since a purely age-related decline in host responsiveness is not shown, it is likely that immunological responsiveness against Mf diminishes as a consequence of cumulative parasite experience, pointing to immunological habituation or tolerance to the parasite (36).

All four cases presented here feature high microfilaremia in animals with severe co-morbidity and suspected immunodeficiency-related conditions. Helminths are known to suppress the host immune response in order to establish infection. For instance, it has been recently reported that D. repens may influence the host immune response by inducing a state of chronic in the canine host (29). It is possible that patients with high Mf counts may represent a population with pre-existing immunosuppression and are unable to naturally control or respond to the parasites. An improved screening and monitoring to determine the impact of chronic immunodeficiency disease on subcutaneous dirofilariosis and its correlation with neoplastic processes should be taken into consideration by investigators and practitioners for better understanding of *D. repens* pathogenicity.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical approval was not required for the animal study, because no interventions outside routine care and diagnostics were performed (as per Resolution Number 22/2006 of the National Commission for the Ethics of Experiments on Animals, 7th November 2006 and Act of 15th January 2015 on the protection of animals used for scientific purposes). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MW analyzed the data and wrote the manuscript. MS-F performed the histopathological examination of internal organs of the first case. MK, ED, MW, and MS-F critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Seroepidemiological Study of Canine and Human Dirofilariasis in the Endemic Region of Northern Serbia

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Dirofilariasis is a vector-borne zoonotic disease caused mainly by *Dirofilaria immitis* and *Dirofilaria repens* that affect dogs and humans all over the world. Serbia is considered an endemic country to both forms of dirofilariasis, although most of the population is concentrated in the north of the country. The aims of this study were to show the prevalence of *D. immitis* and *D. repens* in dogs and the seroprevalence in humans compared to previous studies in Northern Serbia. In total, 346 dog sera samples and 265 human samples were analyzed. Dog blood samples were analyzed using the modified Knott's method to check whether there were *Dirofilaria* spp. microfilariae and serum samples were checked by a commercial *D. immitis* antigen test. Human serum samples were analyzed with a non-commercial ELISA for detection of specific anti-*D. immitis*, anti-*D. repens,* and anti-*Wolbachia* IgG antibodies, and confirmed by western blotting. The overall prevalence for *Dirofilaria* spp. in dogs was 29.19%. The overall prevalence for *D. immitis* was 26.30%. The percentages of *D. immitis* and *D. repens* microfilaremia in dogs were 25.72 and 1.45%, respectively, while *D. immitis./D. repens* microfilaremia co-infections were also 1.45%. The overall seroprevalence for *Dirofilaria* spp. in humans was 3.77%. The overall seroprevalence for *D. immitis* was 1.51, 1.13% for *D. repens*, and for *D. immitis*/*D. repens* co-infections was 1.13%. The results indicate that *D. immitis* and *D. repens* are present in dogs and humans in the province of Vojvodina, in the northern part of Serbia. It is most likely associated with the presence of many rivers, the climate, and presence of mosquitoes in the area, so there could be a real public health risk.

Keywords: ***Dirofilaria immitis***, ***Dirofilaria repens***, **Serbia, dogs, humans, prevalence, seroprevalence, Europe**

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INTRODUCTION

Dirofilariasis is a vector-borne zoonotic disease caused mainly by *Dirofilaria immitis* and *Dirofilaria repens*. *Dirofilaria immitis* causes heartworm disease in canines and pulmonary dirofilariasis in humans, whereas *D. repens* causes canine subcutaneous dirofilariasis and ocular/subcutaneous dirofilariasis in humans. Both parasites are transmitted by culicid mosquitoes, which inoculate larva 3 into definitive hosts in both animals and humans. For that reason, dirofilariasis is considered a veterinary and public health problem (1, 2).

Canine heartworm disease is a chronic, progressive, and life-threatening disease in which adult worms stay in the pulmonary artery and the heart, in the right ventricle of definitive hosts. In canine subcutaneous dirofilariasis, the adult worms are usually beneath the skin forming a subcutaneous nodule. In both cases, microfilariae circulate in the blood stream and are ingested by several species of mosquito vectors during their blood-feeding (3) and after two successive molts, during the next blood meal (4), stage-3 larvae are inoculated into the definitive host. In humans, *D. immitis* immature worms cause embolization in the pulmonary microarteries, leading to the formation of benign lung nodules (pulmonary dirofilariasis), although most cases are asymptomatic (1). On the other hand, *D. repens* worms do not usually reach the adult stage and immature worms may cause larva migrans syndrome and form subcutaneous nodules, in the ocular region and other organs (1, 3, 5–7). Pulmonary dirofilariasis usually has no clinical symptoms, so most diagnostic tools cannot be used, making it much more difficult to identify. However, subcutaneous/ocular dirofilariasis presents clinical signs that are easier to detect (1, 3). Moreover, *D. immitis* and *D. repens* harbor endosymbiotic bacteria of the genus *Wolbachia.* This bacteria participates in the parasite's life cycle and embryogenesis and plays a key role in the immune and inflammatory response of the organism to the disease (4, 8–10).

Dirofilariasis is on the rise in the European population of dogs and humans (1, 3, 11, 12). It is considered to be an endemic disease in southern European countries and in central and northern countries such as Switzerland, Germany, Netherlands, Lithuania, Slovenia, Czech Republic, Slovakia, and Russia (2, 4, 13–18). In addition, in the last decade, different epidemiological and seroepidemiological studies, alongside clinical reporting, have shown that dirofilariasis has been introduced into the countries of the Balkans peninsula (1, 3, 4, 11, 19). Serbia is considered as an endemic country to both forms of dirofilariasis in dogs (20–24). Few human cases have been reported to be caused by *D. repens* or have had specific antibodies found (25, 26). To explain the rise of dirofilariasis, studies suggest that red foxes and golden jackals may serve as reservoir hosts (27) and *Culex pipiens* and *Aedes vexans* act as vectors of both diseases in Northern Serbia (28).

The aim of this study was to show the prevalence of *D. immitis* and *D. repens* in dogs and the seroprevalence in humans compared to previous studies in Northern Serbia.

MATERIALS AND METHODS

Study Area

The northern part of Serbia (Province of Vojvodina) lies between Hungary, Croatia, and Romania. This northern part of the country is largely plains with a continental climate and a lot of rivers. Summers are hot and have lengthened over time due to climate change, so temperatures over 14°C usually last even through to the end of October. Winters are less and less cold and there has not been much snow during the last several winters. All this means that the mosquito season is prolonged from March to October. The air humidity during the warm period of the year is mostly high, meaning that the conditions for the development of mosquitoes are appropriate. During springtime there is a lot of rain and often in some parts of the country there are floods.

In the province of Vojvodina there is the Danube river, which crosses the country from east to west, while the Tisa river flows from north to south, and there are smaller rivers all around the region (**Figure 2**). There is also an artificial canal system called Danube–Tisa–Danube Canal. It covers the total area in Vojvodina of about 12,700 km² and it consists of a number of canals. This Canal is a unique hydro-engineering system for flood control and hydrotechnical management, forestry, water supply, wastewater evacuation, navigation, tourism, fishing, and hunting. Besides these purposes, it also represents a substantial amount of water, convenient for development of mosquitos.

Serum Samples

We analyzed a total of 611 sera samples: 346 dog sera samples (173 male and 173 female) were obtained from dogs analyzed by several veterinary clinics (**Table 1**) and 265 human samples (208 male and 57 female) were provided to the research laboratory from different departments of clinical centers in Northern Serbia (**Table 2**). Human samples were taken from patients with different symptoms, not only those with specific symptoms that could point to dirofilariasis. Variables considered for the analyses were gender, age, and municipality of residence. Samples were collected preserving the privacy of the patients and informing them of how the samples would be used. All samples were collected during the period of 2018 to 2019.

Methods

Dog blood samples were analyzed by applying the modified Knott's technique (29) to check whether there were *Dirofilaria* spp. microfilariae in the blood of the animals included in the study. Morphological characteristics of microfilariae (cephalic and caudal ends) were used in order to differentiate *D. immitis* and *D. repens* microfilariae (30). Dog serum samples were tested for the presence of *D. immitis* adult antigens using a commercial immunochromatographic test kit (VetLine *Dirofilaria* Antigen, NovaTec, Germany) according to the manufacturer's instructions. There is no commercial laboratory test of any kind for *D. repens* in dogs.

Human serum samples were analyzed using a non-commercial ELISA for detection of specific anti-*D. immitis*, anti-*D. repens,* and anti-*Wolbachia* IgG antibodies with some modifications (16, 17, 31, 32). *D. immitis* and *D. repens* adult worm extracts (DiSA and DrSA, respectively) and 1:100 serum dilutions were used to detect the presence of anti-DiSA and DrSA IgG antibodies. Sera samples diluted at 1:40 with a recombinant form of the *Wolbachia* Surface Protein (rWSP) were used to detect the presence of anti-rWSP IgG antibodies. In both cases, goat anti-human IgG (H+L) conjugated to horseradish peroxidase (Sigma-Aldrich, Spain) was used at a 1:4000 dilution. Easy Reader (Bio-Rad laboratories, USA) was used for measuring optical densities (OD) at 492 nm. The cut-off point (OD = 0.8 for DiSA and DrSA and 0.5 for rWSP) was determined by calculating the mean value + 3 standard deviations (3SD) of 50 serum samples obtained from dogs and clinically healthy humans (negative controls) who belonged to an area free of

TABLE 1 | Distribution of prevalence of *D. immitis*, *D. repens,* and co-infections in dogs in Northern Serbia by gender and age.

		D. immitis		*D. repens*		*D. immitis/D. repens*		*Dirofilaria* spp.
	Dog samples *n*° (%)	Test Positive (%)	Knott Positive (%)	Test Positive (%)	Knott Positive (%)	Test Positive (%)	Knott Positive (%)	TOTAL Positive (%)
Gender								
Male	173 (50.00%)	54 (31.21%)	52 (30.06%)	No test	1 (0.58%)	2 (1.16%)/No test	2 (1.16%)	57 (32.95%)
Female	173 (50.00%)	37 (21.39%)	37 (21.39%)	No test	4 (2.31%)	3 (1.73%)/No test	3 (1.73%)	44 (25.43%)
Age								
<3	162 (46.82%)	44 (27.16%)	44 (27.16%)	No test	1 (0.62%)	0 (0.00%)/No test	0 (0.00%)	45 (27.78%)
3–5	133 (38.44%)	34 (25.56%)	34 (25.56%)	No test	2 (1.50%)	2 (1.50%)/No test	2 (1.50%)	38 (28.57%)
6–8	30 (8.67%)	11(36.66%)	9 (30.00%)	No test	1 (3.33%)	2 (6.67%)/No test	2 (6.67%)	14 (46.66%)
>9	21 (6.07%)	2 (9.52%)	2 (9.52%)	No test	1 (4.76%)	1 (4.76%)/No test	1 (4.76%)	4 (19.05%)
Total	346	91 (26.30%)	89 (25.72%)	No test	5 (1.45%)	5 (1.45%)/No test	5 (1.45%)	101 (29.19%)

TABLE 2 | Distribution of seroprevalence of *D. immitis*, *D. repens,* and co-infections in humans in Northern Serbia by gender and age.

	Human samples n° (%)	*D. immitis*	*D. repens*	*D. immitis/D. repens*	*Dirofilaria* spp.
		ELISA Positive (%)	ELISA Positive (%)	ELISA Positive (%)	TOTAL Positive (%)
Gender					
Male	208 (78.49%)	4 (1.92%)	3 (1.44%)	2 (1.44%)	9 (4.33%)
Female	57 (21.51%)	0	0	1 (1.75%)	1 (1.75%)
Age					
<20	2 (0.75%)	0	0	0	0 (0.00%)
20–40	48 (18.11%)	2 (4.17%)	0	1 (2.08%)	3 (6.25%)
>40	215 (81.13%)	2 (0.93%)	3 (1,40%)	2 (0.93%)	7 (3.25%)
Total	265	4 (1.51%)	3 (1.13 %)	3 (1.13 %)	10 (3.77%)

D. immitis and *D. repens*. When both non-commercial ELISAs gave positive results for the same serum sample, that human sera were considered positive. Additionally, by using western blot analysis performed according to a previously described methodology (16, 33–36), these results were confirmed. Both antigenic extracts were subjected to SDS–PAGE in 12% gels under reduced conditions, and proteins were transferred onto nitrocellulose. Human sera were analyzed at a 1:40 dilution and anti-conjugates at a 1:500 dilution. All samples that were positive with these kits were also analyzed by western blot to determine if they recognized the specific bands for *D. immitis* (17–22 kDa) and for *D. repens* (43–70 kDa).

Geographical Information System

The ArcGIS Pro online software was used for the construction of a map of the sampling area. All layers of relevant environmental information (rivers, irrigated croplands, natural parks, among others) were included and symbolized for a better understanding of the map. All dog and human samples infected with *D. immitis*, *D. repens,* and co-infections were manually georeferenced by GPS at the point of capture. Georeferenced positive data for both hosts are shown in the map.

Statistical Analysis

The SPSS Base 18.0 software for Windows was used for the data analysis. The descriptive analysis of the considered variables was carried out studying the proportions in the qualitative variables. To compare proportions, Chi-square tests were performed. In all the cases, the significance level was established at $p < 0.05$.

RESULTS

The overall prevalence for *Dirofilaria* spp. in dogs was 29.19% (**Table 1**). The overall prevalence for *D. immitis* was 26.30%. The percentage of *D. immitis* microfilaremia in dogs was 25.72% (in all cases with a positive *D. immitis* antigen test), 1.45% for

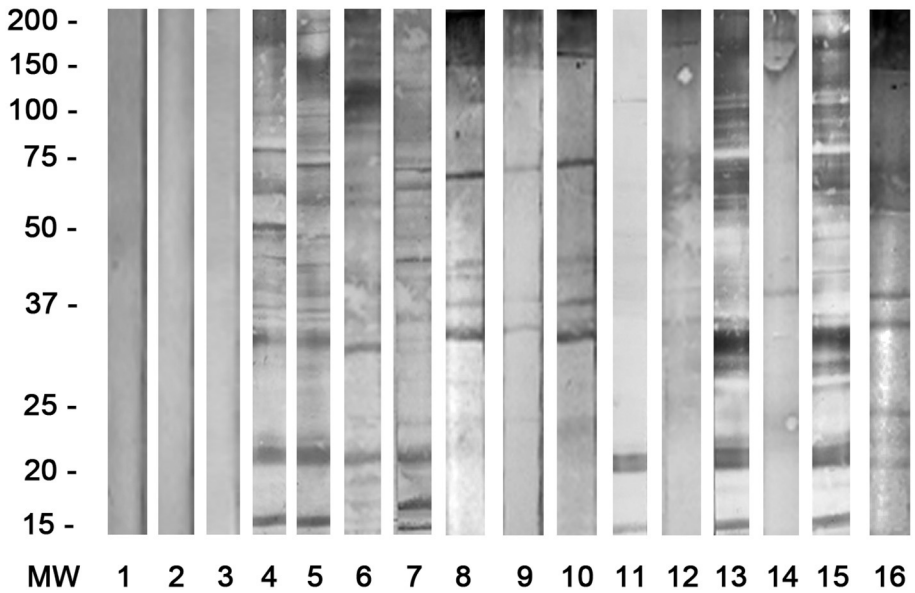

FIGURE 1 | Western blot in all human seropositive cases for *D. immitis* (4–7) with specific bands at 17–22 kDa; for *D. repens* (8–10) with bands at 43–70 kDa; for co-infections *D. immitis/D. repens* (11/12, 13/14, 15/16), and negative sera (1–3).

D. repens microfilaremia, and 1.45% for *D. immitis*/*D. repens* microfilaremia co-infections (in all cases with a positive *D. immitis* antigen test). There are significant differences between the prevalence for *D. immitis* infected male and female dogs with a higher prevalence in male dogs, whereas the prevalence of *D. repens* was higher in females ($p < 0.05$).

The overall seroprevalence for *Dirofilaria* spp. in humans was 3.77%. These results are shown in **Table 2**. The overall seroprevalence for *D. immitis* was 1.51, 1.13% for *D. repens*, and 1.13% for *D. immitis*/*D. repens* co-infections. All positive cases were detected in males with significant differences ($p < 0.05$) for *D. immitis* (1.92%) and *D. repens* (1.44%), but not in co-infection (1.44%). All positive samples via western blot analysis are shown in **Figure 1**.

By age, there are significant differences between the seroprevalences of *D. immitis* between the 20–40 range and the other ranges, the seroprevalences of *D. repens* between the over-40 age group and the other ranges and the seroprevalences of co-infections between the 20–40 age group and the other ranges ($p < 0.05$).

Regarding the geolocation of the positive samples of both dogs and people on a map (**Figure 2**), all of them were located in the vicinity of rivers, forest parks, green areas, and even within some cities in northern Serbia. There are only two cases of humans infected with *D. immitis* and *D. repens* in the northeast of the country.

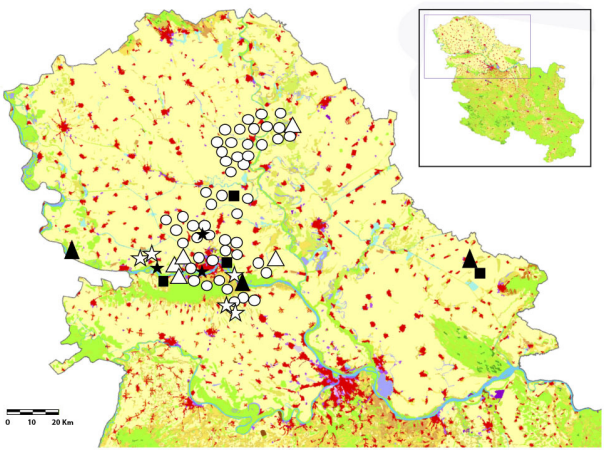

FIGURE 2 | Localization of the *D. immitis* and *D. repens* positive dogs and humans and co-infections in Northern Serbia: *D. immitis* in dogs (O), *D. repens* in dogs (Δ), *D. immitis/D. repens* in dogs (⋆), and *D. immitis* in humans (■), *D. repens* in humans (▲), and *D. immitis/D. repens* in humans (★). In green (natural grasslands, mixed forest, broad-leaved forest, moors and heathland, parks, forest parks), in red (continuous and discontinuous urban areas) and in blue (rivers, areas of stagnant water, and water bodies).

DISCUSSION

Wherever canine dirofilariasis exists, there is a risk of human infection. The general climatic conditions, local environmental factors, human interventions on the environment, and pet management are also factors that determine the distribution and incidence of illness (36). Humans and *Dirofilaria* spp. species have developed limited mutual adaptation (1). L3s inoculated by mosquitoes are usually eliminated from the host by the immune system. However, an undetermined percentage survives and continues to develop until pre-adult, and in some cases of *D. repens*, until adult, causing pulmonary and subcutaneous nodules

or sometimes in the eye area, encapsulated or non-encapsulated. In any case, contact with the infecting larvae and subsequent stages of development stimulates an immune response that can be measured with appropriate techniques (37).

Epidemiological studies of human dirofilariasis, unlike in the dog population, have followed two different approaches. One is reported retrospective reviews and the other is seroepidemiological analyses. Each of these approaches provides information on different yet complementary aspects of human infections. The information obtained through retrospective reviews of reported cases offer only a partial view, since it only includes the part of the affected population that develops some type of clinical manifestation, and regions of endemicity showing vectors with zoo-anthropophilic habits probably have higher frequencies of human infections than reported in the literature. The problem of underreporting may exist due to the fact that symptoms in dirofilariasis patients, especially in pulmonary infections, may be misdiagnosed or unnoticed (1). Seroepidemiological studies compliment this information by detecting contact through the measurement of anti-*Dirofilaria* antibodies, allowing the evaluation of the risk of dirofilariasis infection in a defined geographical region, and constituting an excellent measure of the risk of infection for the human population which resides in an endemic area. Seroepidemiological studies of residents of areas of endemicity reported higher rates of infection in a similar manner to those of canines of the same areas (1, 5, 13, 16, 17, 34).

The aim of the present study was to analyze the prevalence in dogs and the response to anti-*D. immitis* and/or anti-*D. repens* antibodies in the northern region of Serbia (Vojvodina), taking into account that this region has been considered endemic for some time. Furthermore, the aim was to identify the potential risk of infection of the human population in an endemic area.

In Serbia, humidity and temperature conditions during a large proportion of the year allow for the transmission of dirofilariasis, with seroepidemiological data revealing noteworthy prevalence rates in the country's dog populations and human clinical cases caused by both *D. immitis* and *D. repens* (1, 3, 24).

In the current study, a prevalence of 26.30% was observed in dogs infected by *D. immitis*, with 25.72% microfilaremia in the total population, and the presence of *D. repens* larvae in 1.45% of the analyzed dogs. In two regions of Vojvodina (Pancevo and Veliko Gradiste), the previously reported seroprevalence was 22.9% for *D. immitis* and the presence of *D. repens* microfilariae was 39.34% (38). In Bulgaria and Croatia, two neighboring countries, there are studies about the prevalence of *D. immitis* and *D. repens* in dogs (8.1 and 11.1%, respectively) and co-infections (3.2%) (11). Several studies point to an increase in *D. immitis* infections and a decrease of an infection with *D. repens* in recent years (21–23) which is corroborated by results in this study. In addition, infections have also been found to be prevalent in wild canids (39), which could mean there is a risk of *D. immitis* infection between the dog population and the wild canid population. Meanwhile, *D. repens* was found to circulate mostly in golden jackal and red fox populations (27).

With regards to the human population, the seroprevalence for *Dirofilaria* spp. in humans was 3.77, 1.51% for *D. immitis*, 1.13% for *D. repens*, and 1.13% for *D. immitis*/*D. repens* co-infections. This is the first time these tests have been conducted in this region of Serbia. Human cases originating from *D. repens* have only previously been reported in the region of southeastern Serbia (24, 40, 41). In addition, other studies have reported seroprevalences of 9.7% and 8.1% against *D. repens* and D. immitis polyproteins specific antibodies, respectively, and 2.3% in individuals with specific antibodies to both species (26). Similar studies in neighboring countries such as Romania and Moldova have reported seroprevalences of 10.7% for *D. immitis*, 0.2% for *D. repens*, and 0.9% for both parasites (16). In addition, in Croatia there have been human *D. repens* cases reported (3). These seroepidemiological studies are a good tool to measure the risk of infection in a population where there is a high population of infected animals, as well as the presence of vectors, which serve as a vehicle for transmission of the disease (1, 42).

Both animals and infected people were geolocated in the immediate vicinity or in a relatively close environment of potential mosquito breeding areas, which poses a risk to those areas. The two cases of humans infected by *D. immitis* and *D. repens* in the northeast of the country are close to cities where cases of dogs and humans had already been reported (38) and were located in areas near rivers and green areas where mosquitoes breed. The spatial distribution of positive cases has a clear association with different geo-environmental factors, humidity and temperature, the existence of irrigated areas, areas with abundant water, rivers in valleys protected from winds, and proximity to the coast, which are considered risk factors for the transmission of dirofilariasis (43).

In conclusion, the results indicate that *D. immitis* and *D. repens* are present in dogs and humans in the province of Vojvodina, in the northern part of Serbia. It is most probably associated with the presence of many rivers, the climate, and the presence of mosquitoes, so there is a real public health risk. Serology studies in humans can be very useful for indicating the exposure to *Dirofilaria* spp. in a healthy population in order to obtain useful data on the epidemiological scenario of human dirofilariasis in Serbia and in Europe. That exposure was confirmed in the current study. Further studies addressing the control of dirofilariasis in the dog population are needed to reduce the risk of infection in the human population.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical approval was not provided for this study on human participants because People data don't require permission, everyone has verbal consent. Written informed consent to

participate in this study was provided by the participants' legal guardian/next of kin. Ethical review and approval was not required for the animal study because the blood samples were taken during a regular blood checkup of the dogs and the consent was gained from the owners so there was no need for the ethical approval. Written informed consent for participation was not obtained from the owners because Verbal consent of client owned dogs.

AUTHOR CONTRIBUTIONS

SS and RM designed the study and wrote the manuscript. MS, DM, IH, AP, SO, and MR performed the fieldwork, collected the data, and performed the experiments. All authors participated in the discussion of the results, corrected, read, and approved the final manuscript.

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ORIGINAL RESEARCH
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Study of Zoonotic Enteric Pathogens of *Atelerix algirus* in Tenerife, Canary Islands, Spain

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Atelerix algirus is an invasive species in the Canary Islands (Spain). There are few studies about the zoonotic pathogens this species could be hosting; therefore, this study was focused on analyzing causative agents of diarrhea in humans in feces from hedgehogs. A total of 45 fecal samples obtained in Tenerife (Canary Islands) were analyzed in this study using Biofire FilmArray gastrointestinal panel with an integrated Biofire FilmArray system. Forty-two (93.33%) of the samples presented at least one of the pathogens detected by the panel. The prevalence of four bacteria stands out as for enteropathogenic *Escherichia coli* (71.11%), *Salmonella* (66.67%), *Clostridioides difficile* (33.33%), and *Campylobacter* sp. (22.22%), all of which were widely distributed along Tenerife. Besides, other pathogens were found, *Cryptosporidium* sp. and enterotoxigenic *E. coli* lt/st in 6.66% of the animals, *Shigella*/enteroinvasive *E. coli* in 4.44%, and *Norovirus* GI/GII, *Plesiomonas shigelloides*, and *Vibrio* sp. in 2.22%. Of the hedgehogs, 26.66% were hosting just one pathogen, and the others showed coinfection: 24.44% hosted two, 31.11% hosted three, and 11.11% hosted four or more. The close contact with hedgehogs may imply the transmission of not only one causative agent of diarrhea but also multiple agents, since coinfection is highly prevalent. The lack of management measurements for this animal in the Canary Islands, the common habit of adopting hedgehogs from wildlife without veterinary control, and the fact that most of the hedgehogs studied belonged to highly populated areas imply a high risk of transmission of pathogens to humans.

Keywords: *Atelerix algirus*, FilmArray gastrointestinal panel, enteropathogens, hedgehog, Canary Islands

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INTRODUCTION

The Algerian hedgehog, *Atelerix algirus* (Lereboullet, 1842), is an invasive species in the Canary Islands (Spain), located in NW Africa (13°23′-18°8′W and 27°37′-29°24′). Although it is not clear if the arrival of this mammal to the archipelago was accidental or intended, nowadays it seems to have colonized the islands of Fuerteventura, Lanzarote, Gran Canaria, Tenerife, and, most recently, La Palma (1, 2). Not many studies have been done in order to establish *A. algirus* diet or biodiversity impact on the Canary Islands, and only few studies had been carried out studying the zoonotic pathogens that this hedgehog could be hosting around the world, including *Leishmania major* and

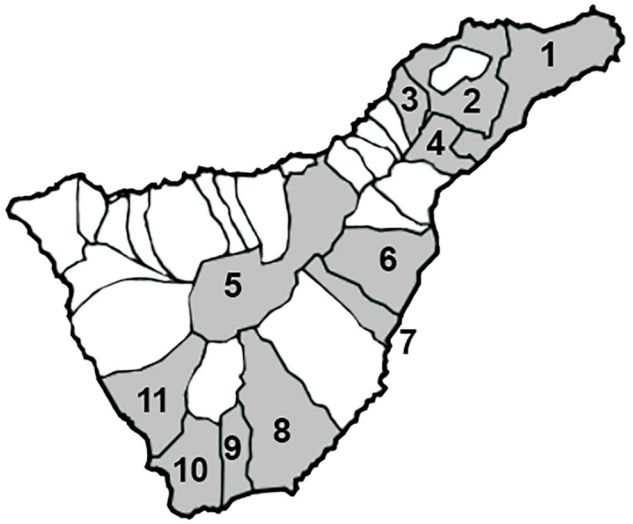

FIGURE 1 | Map showing the municipalities of Tenerife in which hedgehogs were captured (1. Santa Cruz, 2. La Laguna, 3. Tacoronte, 4. El Rosario, 5. La Orotava, 6. Güímar, 7. Fasnia, 8. Granadilla, 9. San Miguel, 10. Arona, 11. Adeje).

some species from the *Rickettsiae* family (3, 4). However, a recent study that also took place in Spain found *A. algirus* specimens hosting *Angiostrongylus cantonensis* (5), a parasite highly distributed in rats and mollusk of Northern Tenerife (6, 7).

Due to its appearance and non-aggressive behavior, wild hedgehogs are often kept as pets in the Canarian archipelago, especially by families with children (authors' personal communication). Because of the lack of knowledge about the possible pathogens that *A. algirus* of the Canary Islands could be hosting, the aim of this study was to examine feces of *A. algirus* from Tenerife island, in order to analyze the presence of causative agents of infectious diarrhea in humans.

MATERIALS AND METHODS

A total of 45 feces samples from hedgehogs collected widely along the island of Tenerife in 11 of its 31 municipalities were analyzed in this study (**Figure 1**). Samples belonged to two different categories, fresh feces from living specimens ($n = 43$) and feces from the intestine of dead animals ($n = 2$). All samples as well as data regarding their provenance were provided by La Tahonilla, a wildlife recovery center of Excmo Cabildo Insular Tenerife. For each fecal sample, no longer than 24 h elapsed from the death of the animal or the collection of the feces and its analysis, and samples were kept refrigerated until the analysis was performed. In the case of dead hedgehogs, after their dissection, feces were collected from the colon and directly analyzed.

All assays on the feces samples were performed at Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias (Universidad de La Laguna), using the Biofire FilmArray gastrointestinal panel for the detection of 22 pathogens, which are causative agents of infectious diarrhea in humans, including bacteria, *Campylobacter* (*jejuni, coli, upsaliensis*), *Clostridioides difficile* toxin A/B, *Plesiomonas shigelloides*, *Salmonella*, *Yersinia enterocolitica*, *Vibrio* (*parahaemolyticus, vulnificus, cholerae*), *Escherichia coli* O157, enteroaggregative *E.coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC) *lt/st*, Shiga-like toxin-producing *E. coli* (STEC) *stx1/stx2*, *E. coli* O157, and *Shigella*/Enteroinvasive *E. coli* (EIEC); parasites, *Cryptosporidium, Cyclospora cayetanensis*, *Entamoeba histolytica*, and *Giardia lamblia*; and viruses including Adenovirus F 40/41, Astrovirus, Norovirus GI/GII, Rotavirus A, and Sapovirus. For each sample, 200 µl was added into each panel, following the manufacturer's instructions, and then analyzed in a Biofire Filmarray integrated system (Biomerieux, France). Results are presented as prevalence and 95% confidence intervals are included.

RESULTS

The results of this study show a wide distribution of causative agents of diarrhea in humans in hedgehogs of Tenerife, as pathogens were found in every municipality tested, and nearly all of the animals tested, 42 of 45 (93.33%) were positive for at least one pathogen (**Table 1** and **Figure 1**). A total of 11 of the 22 pathogens included in the FilmArray gastrointestinal panel were recorded in the island of Tenerife including nine bacteria, EPEC, enterotoxigenic *E. coli lt/st*, *Shigella*/Enteroinvasive *E. coli*, *Salmonella*, *Campylobacter*, *Vibrio* sp. (*parahaemolyticys* or *vulnificus*), *P. shigelloides*, *Clostridioides difficile*, and one protozoa, *Cryptosporidium* sp.; and one virus, Norovirus GI/GII.

The high prevalence of four bacteria is remarkable: for EPEC, 32 of the 45 samples were positive (71.11%; CI: 55.68–83.63); for *Salmonella*, 30 of 45 were positive (66.67%; CI: 51.05–79.99); for *Clostridioides difficile* (previously known as *Clostridium difficile*), 15 of 45 were positive (33.33%; 20.00–48.95); and in the case of *Campylobacter* sp., 10 of the 45 samples were positive (22.22%; CI: 11.20–37.09). Among the other pathogens, the prevalence of *Cryptosporidium* sp. and enterotoxigenic *E. coli* lt/st was 6.67% (3 of 45; CI: 13.96–18.27); for *Shigella*/enteroinvasive *E. coli*, Norovirus GI/GII, *P. shigelloides*, and *Vibrio* sp., only 1 of the 45 samples (2.22%; CI: 0.56–11.77) were positive. Most pathogens were highly distributed along the island, although *Cryptosporidium* sp., *Shigella*/enteroinvasive *E. coli*, *Vibrio* sp., *and P. shigelloides* were only found in Northern municipalities, while Norovirus GI/GII was only detected on the South.

Regarding the infection rate, 12 hedgehogs were hosting just one pathogen (26.66%), 11 hosted two pathogens (24.44%), 14 hosted three (31.11%), and 5 hosted four or more pathogens (11.11%). The most common combination was EPEC and *Salmonella* (51.11%), being 35.55% with other pathogens. It is remarkable that *C. difficile* was always detected in coinfection; however, in no occasion was it detected only with *Salmonella*, as these two bacteria were always found in coinfection with others (**Supplementary File**).

TABLE 1 | Municipalities where *Atelerix algirus* were captured in Tenerife (Canary Islands, Spain), number of animals per area, and pathogens found.

Municipality	Number of samples analyzed	FilmArray Results (number of positive samples)
La Laguna	11	*Cryptosporidium* sp. (2), enteropathogenic *E. coli* (5), *Campylobacter* sp. (3), *Salmonella* (7), *Clostridioides difficile* (5), *Shigella*/enteroinvasive *E. coli* (1)
Santa Cruz	8	Enteropathogenic *E. coli* (8), enterotoxigenic *E. coli lt/st* (1), *Salmonella* (4), *Clostridioides difficile* (2), *Campylobacter* sp. (1)
Arona	7	*Clostridioides difficile* (2), enteropathogenic *E. coli* (4), enterotoxigenic *E. coli lt/st* (1), *Salmonella* sp. (5), *Campylobacter* sp. (2)
El Rosario	7	*Clostridioides difficile* (1), *Salmonella* sp. (6), enteropathogenic *E. coli* (5), *Campylobacter* sp. (2), enterotoxigenic *E. coli lt/st* (1), *Vibrio* sp. (1)
La Orotava	2	*Cryptosporidium* (1), *Clostridioides difficile* (2), *Salmonella* sp. (1), enteropathogenic *E. coli* (1), *Plesiomonas shigelloides* (1)
San Miguel	3	*Clostridioides difficile* (1), *Salmonella* sp. (3), enteropathogenic *E. coli* (3)
Granadilla	2	Enteropathogenic *E. coli* (1), *Campylobacter* sp. (2)
Adeje	2	*Clostridioides difficile* (1), *Salmonella* sp. (2), enteropathogenic *E. coli* (2)
Tacoronte	1	*Clostridioides difficile* (1), *Salmonella* sp. (1), enteropathogenic *E. coli.* (1)
Güímar	1	*Salmonella* sp. (1)., enteropathogenic *E. coli* (1), norovirus GI/GII (1)
Fasnia	1	Enteropathogenic *E. coli* (1)
Total	45	*Campylobacter* sp. (10), *Clostridioides difficile* (15), *Plesiomonas shigelloides* (1), *Salmonella* sp. (30), *Vibrio* sp. (1), enteropathogenic *E. coli* (32), enterotoxigenic *E. coli lt/st* (3), *Shigella*/enteroinvasive *E. coli* (1), *Cryptosporidium* (3), Norovirus GI/GII (1)

DISCUSSION

The introduction of invasive species in a delicate ecosystem like the Canary Islands not only implies a potential danger to the environment, as more than 500 endemic species inhabit the archipelago (8), but also can contribute to the dispersion of different diseases. The results of this study show a wide distribution of causative agents of diarrhea in Tenerife, as the majority of the hedgehogs included in this study were found in the most populated areas of Tenerife, since La Laguna and Santa Cruz are the biggest cities of the island and Arona, San Miguel, and Adeje are highly touristic municipalities. The finding of causative agents of human diarrhea in the hedgehogs from the wildlife of these areas could imply a risk of transmission to humans, specially taking into consideration that in the Canarian archipelago, *A. algirus* is commonly adopted from the environment without any veterinary control.

Regarding the pathogens found in this study, the finding of *Salmonella* sp. in exotic animals of Spain is not unusual, as in 2013 it was found in free-living turtles of Eastern Spain, with a prevalence of 11% (9), although this is the first time this bacterial genus is found in *A. algirus*. The high prevalence of *Salmonella* and *Campylobacter* species in *A. algirus* may be due to the fact that this hedgehog species is mainly an insectivore, although its diet may vary according to availability and weather conditions (10), and some studies have shown that insects can act as carriers of *Salmonella* and *Campylobacter* species (11). The high prevalence of both bacteria found in this study compared to other wildlife species may imply that insects of the Canary Islands are probably carriers of these pathogens and *A. algirus* feeding habits facilitates their infection. Apart from the acute gastroenteritis that both bacteria cause, campylobacteriosis can sometimes lead to long-term sequelae such as Guillain–Barré syndrome, irritable bowel syndrome, or reactive arthritis (9).

The prevalence found in this study regarding *Cryptosporidium* sp. is similar to those found in pigeons of the Canary Islands in 2009, in which 28 wild pigeons were captured in Santa Cruz and its feces were posteriorly analyzed by PCR for the presence of *Cryptosporidium*, with 5.9% of them being positive (12). Further investigations in the presence of *Cryptosporidium* in wildlife species of the Canary Islands should be done in order to prevent risk focuses of cryptosporidiosis, a disease that can cause severe enteritis and malabsorption (13).

The presence of *P. shigelloides* in a hedgehog from La Orotava is also remarkable, a bacterium capable of causing sepsis and meningitis to immunosuppressed hosts, specially babies, in which 12 cases has been described, 6 of whom died due to the infection (14). Besides, the presence of *C. difficile* may be one of the most problematic among the bacteria found in this study, as its infection is transmitted by spores resistant to acid, antibiotics, or even heat and are commonly found in the environment. Also, elderly people, cancer patients and other diseases seem to be risk factors for the susceptibility to this bacterium, which may cause colitis. It is important to note that 40% of the community acquired infections of *C. difficile* requires hospitalization (15). The finding of this bacterium in the feces of animals that can potentially be adopted constitutes a risk of transmission to its owners, which is specially concerning in the case of immunosuppressed individuals.

The finding of *Vibrio* sp. (*parahaemolyticus* or *vulnificus*) in a hedgehog of Tenerife is also interesting, as these bacteria species are usually transmitted by seafood, with *V. vulnificus* being of special concern as it is responsible for more than 95% of seafood-related death in the United States, usually in individuals with liver disease or those who are immunocompromised (16). Besides, both *V. parahaemolyticus* and *V. vulnificus* are the most common causative agents isolated when *Vibrio* spp. are isolated from skin and soft tissue infections, which range from bullous skin lessons to severe necrotizing of the tissue with secondary septicemia (17). In the Canary Islands, one case of skin and soft tissue infection due to *Vibrio* sp. was reported in a patient with cancer and diabetes mellitus type II (17); the presence of this genera of bacteria in the wildlife, especially in a species that usually lives in close contact with humans, could constitute a high risk for

immunocompromised individuals, as well as people presenting chronic diseases such as cancer and diabetes.

Regarding the *E. coli* strains found in this study, the prevalence of EPEC found in hedgehogs was considerably higher than other wildlife species studied along mainland Spain (18). That is why hedgehogs should be considered as an important wildlife reservoir of this bacterium, as nearly three quarters of the animals studied hosted EPEC.

In the particular case of Norovirus, one third of the people infected are asymptomatic; however, elderly, children, and immunocompromised people are at greater risk for severe symptoms and complications, such as acute renal failure leading to hemodialysis, cardiac complications including arrhythmias, acute graft organ rejection in transplant recipients, and death (19). The finding of Norovirus GI/GII in animals that can potentially be adopted by families may constitute a risk focus for this viral infection.

Another factor to take into consideration is that hedgehogs present a behavior named anting or anointing, which consist in moistening their spines with saliva (20). The fact that hedgehogs showed high prevalence of gastrointestinal pathogens implies a risk for humans to get in contact with them only by touching the animals, even more taking into consideration that coinfection was commonly found in these animals, so multiple pathogens can be transmitted. Also, further investigations should be done in the islands of the Canary archipelago where *A. algirus* presence has been spotted, as this is the first study on the zoonotic pathogens that this species hosts in the archipelago.

In mainland Spain, two species of hedgehogs inhabiting the country, *Atelerix albiventris* (Wagner, 1841) and *Hemiechinus auratus* (Gmelin, 1770), are included in the catalog of exotic invasive species (RD 63/2013, de 2 de Agosto) (21), which allows the competent authorities to control the population. Management strategies as well as educative measurements should be applied in order to reduce the impact that this species may be having in the Canary archipelago.

In conclusion, the lack of management measurements, the non-controlled selling, and the fact that some of the hedgehogs included in this study belonged to highly populated areas of Tenerife imply a high risk of transmission of pathogens to humans.

PERMISSION TO REUSE AND COPYRIGHT

The original image from the figure in this article was acquired from https://commons.wikimedia.org/wiki/File:Santa_Cruz_de_Tenerife_-_Mapa_municipal.svg in which the permission to copy, distribute, or modify it is established. It was later edited in photoshop CS6 by Izquierdo-Rodríguez.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because we analyzed fresh feces from living specimens and from dead animals donated by an Animal Recovery Center.

AUTHOR CONTRIBUTIONS

EI-R and NM-C were responsible for the collection of the samples as well as the analysis. PF coordinated the relationships between the wildlife recovery center and the laboratory, as well as supervised the analysis and the writing of the article. BV and PF obtained the funding for the study and supervised the work. EI-R did the main writing of the article. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.579602/full#supplementary-material

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BRIEF RESEARCH REPORT
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Survey of Zoonotic and Non-zoonotic Vector-Borne Pathogens in Military Horses in Lisbon, Portugal

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Vector-borne diseases of zoonotic and/or veterinary relevance have been increasingly reported in horses globally, although data regarding working and military horses is lacking. Portuguese military horses may constitute a risk group for these pathogens, as they frequently work outdoors in various regions of the country. This study included 101 apparently healthy horses belonging to the Portuguese National Republican Guard. Blood samples were analyzed to determine the presence and prevalence of piroplasms, *Anaplasmataceae*, *Rickettsia* spp., and filarioid helminths. Overall 32.7% of the horses gave positive results for *Theileria equi*. Two genotypes of *T. equi* were verified. No positive results were recorded for *Anaplasma* spp., *Rickettsia* spp., filarioid helminthes, and *Babesia caballi*. As equine piroplasmosis is a severe infectious tick-borne disease responsible for significant losses in equine production and with numerous impacts in the international movement of horses, adequate treatment, and preventive measures are needed to reduce exposure to vectors and future infections.

Keywords: equine piroplasmosis, military horses, *Theileria equi*, vector-borne diseases, zoonosis, Portugal

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INTRODUCTION

Vector-borne diseases (VBDs) have been increasingly reported in horses worldwide (1). Several equine VBDs are of zoonotic relevance and horses potentially serve as sentinels for human infections. The distribution and spread of vector-borne pathogens are limited by the presence of competent arthropod vectors (e.g., ticks, fleas, and mosquitoes) capable of transmitting these pathogens. Military horses may constitute a risk group for VBDs, as they frequently work outdoors in different areas and thus are exposed to vectors present there.

Equine piroplasmosis is a tick-borne disease of equids such as horses, donkeys, mules, and zebras, and is caused by the protozoan parasites *Theileria equi* and *Babesia caballi* (2). Infected animals may carry *B. caballi* for several years and *T. equi* for a whole lifetime (2).

Theileria equi (Laveran, 1901) Mehlhorn, Schein 1998 is one of the most important pathogens of horses in many parts of the world, including Southern Europe. Clinical signs range from asymptomatic to acute, subacute, and chronic cases with fever, anemia, inappetence, and spleno- and hepatomegaly. In severe cases, the infection can lead to death. Transplacental transmission from mares to fetuses can lead to abortion (3), causing significant losses in the equine

industry. Additionally, international horse trade facilitates the spread to non-endemic areas (4). Currently, more than 20 potential tick vectors of *T. equi* are known (5, 6). Species of the genera *Dermacentor*, *Rhipicephalus*, and *Hyalomma* are known as competent vectors and several *Ixodes*, *Haemaphysalis*, and *Amblyomma* species are discussed to transmit the parasite as well (2). Various tick species that may act as vectors for equine piroplasms are present in mainland Portugal, namely *Rhipicephalus sanguineus, Rhipicephalus annulatus, Rhipicephalus bursa, Dermacentor marginatus*, *Hyalomma lusitanicum*, and *Hyalomma marginatum* (7, 8). Several genotypes of *T. equi* have been reported in different populations of equids (2, 9). There is evidence that specific genotype assortment occurs within the tick vectors (6). In Portugal, previous studies show different prevalence rates for *T. equi*, which may be associated with the kind of horses/facility involved, the study area and the diagnostic tests used. In horse stud farms in the Ribatejo region, central Portugal, using Complement Fixation (CF), seroprevalence was 45.3%, while using blood smears, the infection was prevalent on 42,6% of the examined horses, with a potential transuterine transmission in 80% of positive mares (10, 11). Further South, in Alentejo region, using CF and IFAT the seroprevalence for *Theileria equi* was 85.1% and recently, using qPCR/nPCR, 56% of the horses examined from the Lisbon and Alentejo areas showed a prevalence of 56% (12, 13).

Members of the family *Anaplasmataceae* (e.g., *Anaplasma, Neoehrlichia*, and *Ehrlichia*) are gram-negative, intracellular bacteria infecting domestic and wild animals, but also humans. *Anaplasma phagocytophilum* has been documented in various animals including horses and is the causative agent of equine, canine, and human granulocytic anaplasmosis. Various strains are known and horses can harbor strains of zoonotic potential (14). In Europe, the main vector of this tick-borne pathogen is *Ixodes ricinus*, which is also present in mainland Portugal (7). *Anaplasma phagocytophilum* has previously been detected in *I. ricinus* on Madeira and in *Ixodes ventalloi* in mainland Portugal (Baixa de Palmela, Setubal district) (15, 16). *Anaplasma phagocytophilum* is regularly reported in serology-based studies on horses in Europe including Portugal [e.g., (8, 16, 17)].

Rickettsia species are obligate intracellular, gram-negative bacteria transmitted by various types of arthropods [e.g., ixodid ticks are the main vectors of spotted fever group rickettsiae (18)]. Several studies have documented *Rickettsia* spp. in horses.

Filarioid nematodes are also parasites of horses in Europe. Because of their asymptomatic to minor symptomatic effects, these parasites are neglected and understudied. Adults of *Setaria equina* are located in the peritoneal cavities of horses. Microfilariae of this worldwide distributed helminth can be found in the peripheral blood (19). Mosquitoes of the genera *Culex* and *Aedes* are the vectors of *S. equina.* Another filarioid species parasitizing medial layers or outside layers of tissues within the artery wall of horses is *Onchocerca boehmi* (syn. *Elaeophora boehmi*) (20, 21).

Little is known about the risk of military horses regarding VBDs. In fact, few studies have been conducted so far and no surveillance mechanisms are in place to assess geographical range and prevalence in the country. Considering the emergence of VBDs in Europe, as well as the lack of data on this topic, an epidemiological study was conducted, in order to identify the presence and prevalence of the most significant bacterial and parasitic VBDs of zoonotic and/or non-zoonotic relevance in Portuguese military horses using molecular analysis followed by sequence analysis of positive DNA products.

MATERIALS AND METHODS

Overall 101 military horses (Puro Sangue Lusitano breed) belonging to the Portuguese National Republican Guard (GNR), stabled at guard facilities in metropolitan Lisbon were included in this study. Horses from various job sites were included (55 GNR Lisbon, 46 GNR Lisbon Braço de Prata Expo, 2 GNR Évora, 1 GNR Faro, and 1 GNR Tomar), and information on sex and age were determined. All horses were apparently healthy with no clinical signs compatible with VBDs. Horses were dewormed with moxidectin and praziquantel administered annually (with the exception of sportive horses, which were dewormed twice a year). Horses were not treated against tick infestation. Blood samples were collected at the jugular vein from apparently healthy horses in June ($n = 55$) and July ($n = 46$) in 2017.

A total of 50 µl of blood was spotted on Whatman® filter paper (VWR International GmBH, Vienna, Austria) and dried at room temperature. Afterward, each filter paper was sealed in an envelope separately. Filter papers were shipped to the University of Veterinary Medicine Vienna for molecular analysis. Sections of ∼4 mm in diameter were cut out of the center of the blood spots with sterile blades. DNA was extracted with a modified chelex-based technique using InstaGene™ matrix (Bio-Rad Laboratories, Hercules, California) as established previously (22). Extracted DNA was stored at −20°C until further analysis. Samples were screened for the presence of DNA of various vector-borne pathogens of zoonotic and non-zoonotic relevance using specific broad-range PCR assays, under conditions reported previously [(23); **Table 1**] as follows: piroplasms (incl. *Babesia* and *Theileria*) within the *18S* rRNA gene [BTH-1F/BTH-1R; (24)]; *Anaplasmataceae* (incl. *Anaplasma*, *Ehrlichia,* and *Neoehrlichia*) within the *16S* gene [EHR16SD/EHR16SR; (26)]; *Rickettsia* spp. within the *23S*/*5S* rRNA gene [ITS-F/ITS-R; (27)] and filiarioid helminths targeting a fragment of the mitochondrial *cytochrome c oxidase subunit I gene* [*COI*; H14FilaCOIFw/H14FilaCOIRv; (28)]. Additionally, all samples were further analyzed with a *Babesia* specific PCR analysis [Babfor/Babrev; (25)]. PCR products were analyzed by gel electrophoresis on 2% agarose gels stained with Midori Green Advance DNA stain (Nippon Genetics Europe, Düren, Germany). Positive reaction products were commercially purified and sequenced at LGC Genomics GmbH (Berlin, Germany).

Phylogenetic Analysis of the *T. equi 18S* Sequences

To show the diversity of *18S* lineages of *T. equi*, Bayesian inference (BI) and Maximum likelihood (ML) trees were

TABLE 1 | Primer sequences and PCR protocols used for molecular analysis of vector-borne pathogens in military horses.

Organism	Gene/Locus	Primer sequences (5′→3′)	Amplification protocol	Product size (bp)	References
Piroplasms (*Babesia* spp., *Theileria* spp.)	*18S* rRNA	BTH-1F: CCTGAGAAACGGCTACCACATCT BTH-1R: TTGCGACCATACTCCCCCCA	94°C: 2 min, 40 cycles - 95°C: 30 s, 68°C: 1 min, 72°C: 1 min, 72°C: 10 min	∼700	(24)
Babesia spp.	*18S* rRNA	B-For: GACTAGGGATTGGAGGTC B-Rev: GAATAATTCACCGGATCACTC	94°C: 2 min, 30 cycles - 95°C: 1 min, 53°C: 1 min, 72°C: 1 min, 72°C: 7 min	∼650	(25)
Anaplasmataceae	*16S* rRNA	EHR16SD: GGTACCYACAGAAGAAGTCC EHR16SR: TAGCACTCATCGTTTACAGC	95°C: 2 min, 35 cycles - 94°C: 1 min, 54°C: 30 s, 72°C: 30 s, 72°C: 5 min	345	(26)
Rickettsia spp.	*23S/5S* rRNA	ITS-F: GATAGGTCGGGTGTGGAAG ITS-R: TCGGGATGGGATCGTGTG	96°C: 4 min, 35 cycles - 94°C: 1 min, 52°C: 1 min, 72°C: 2 min, 72°C: 3 min	350–550	(27)
Filarioid nematodes	mt *COI*	H14FilaCO1Fw: GCCTATTTTGATTGGTGGTTTTGG H14FilaCO1Rv: AGCAATAATCATAGTAGCAGCACTAA	95°C: 2 min, 30–35 cycles - 95°C: 1 min, 53°C: 1 min, 72°C: 1 min, 72°C: 5 min	724	(28)

calculated based on the sequences of the present study, and data was mined from NCBI GenBank. The GenBank sequences were retrieved by performing a BLAST search on a 530 bp *18S* section of *Theileria* spp. To identify the lineages of *T. equi*, the sequences were aligned and sorted with MAFFT v.7 applying the default settings (29). The sequences were visually inspected using BioEdit (30) and all sequences were removed which did not cover the entire length, contained ambiguity characters, and/or obvious sequencing errors, resulting in a total of 220 *T. equi* sequences. The sequences of the present study and NCBI GenBank were combined and a sequence of *Theileria ovis* (AY533144) was added for outgroup comparison. All sequences were re-aligned with MAFFT v.7 applying the option "G-INS-i" and then collapsed to haplotypes using DAMBE (31). The alignment contained 49 unique *T. equi* lineages and 535 sites, of which all 15 gap positions were removed before the phylogenetic analysis. The best-fit substitution model for the 520 bp alignment was inferred using the model search implemented in the W-IQ-TREE web server [http://iqtree.cibiv.univie.ac.at/; (32)], resulting in the model HKY+G. A ML consensus tree was calculated from 1000 bootstrap replicates using W-IQ-TREE. A BI tree was calculated with MrBayes v.3.2.7 (33). The Bayesian analysis was run for 10 million generations (2 runs each with 4 chains, one of which was heated) and every thousandth tree was sampled. The first 25% of trees were discarded as burn-in and a 50% majority-rule consensus tree was calculated from the remaining 7,500 trees.

RESULTS

In this study, 101 military horses were included of which 60 were males and 41 mares. The age ranged between 4 and 24 years (x = 12.4 years; x_{med} = 12 years). Overall 33 animals (32.7%; CI_{95}: 24.3–42.3%) were infected with a vector-borne pathogen, namely *Theileria equi*. Seventeen males and 16 mares aged between 4 and 18 years (x = 10.5) gave positive signals at the molecular analysis for piroplasms. All of the positive horses came from Lisbon (23 at GNR Lisbon Braço de Prata Expo and 10 at GNR Lisbon). Two genotypes of *T. equi* were recorded of which 24 were genotype 1 and seven genotype 2. Both genotypes were present in GNR Lisbon Braço de Prata Expo and GNR Lisbon. The phylogenetic tree of the *18S* sequences shows three main sequence groups or clades, one of which may be divided into two sub-groups (**Figure 1**). The *T. equi* lineages identified in the present study clustered in the first and second clade. Of the 33 *T. equi* positive samples, 24 featured a lineage (genotype 1, 100% match with HM229407), which clustered in the first clade. This lineage and similar ones were isolated from horses in China, Kazakhstan, Russia, Mongolia, Saudi Arabia, South Korea, Spain, Sudan, and Switzerland. Three slightly distinct lineages (KF597077, KF597078, and KF597081) in this clade were isolated from the waterbuck *Kobus ellipsiprymnus* in Kenya. Seven horses sampled in the present study featured a *T. equi* lineage, which clustered in the second clade (**Figure 1**). This lineage (genotype 2, 100% match with EU888906) and similar ones were isolated from horses in Brazil, Croatia, India, Jordan, Egypt, Israel, Saudi Arabia, South Africa, Spain, Turkey, and the USA. The phylogenetic tree (**Figure 1**) features a third clade, which can be subdivided into two subclades (3a and 3b). Subclade 3a contains lineages isolated from horses in Egypt, Israel, Palestine, Turkey, Nigeria, and Sudan. All records come from the Middle East and Northern and Eastern Africa. Two slightly deviating lineages (KF597074 and KF597076) were isolated from the waterbuck *K. ellipsiprymnus* in Kenya. Subclade 3b features lineages isolated from horses in Brazil, China, Cuba, Egypt, Iraq, Israel, South Africa, and the USA.

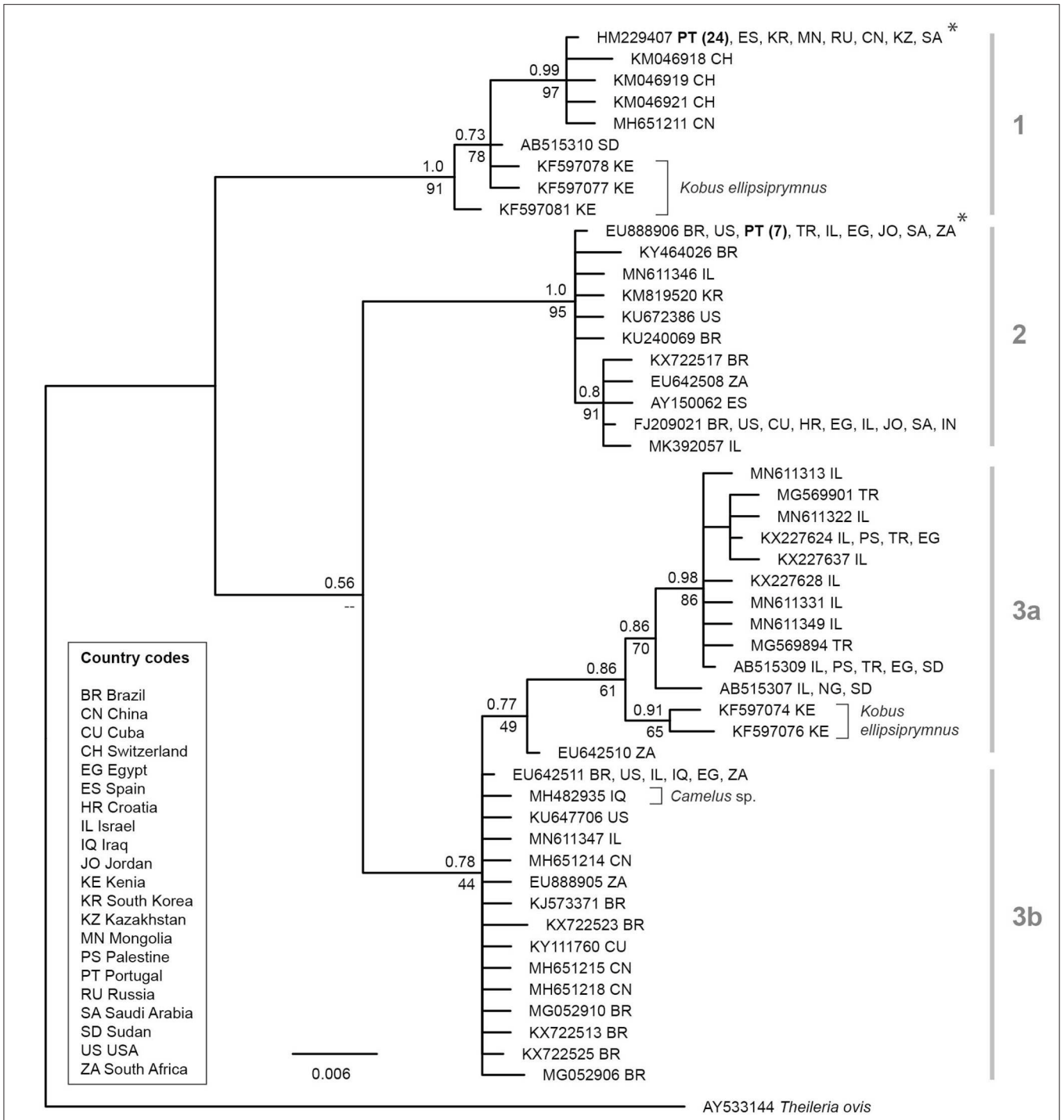

FIGURE 1 | Bayesian inference tree of *T. equi* lineages based on 530 bp sections of the *18S*. The BI posterior probabilities and ML bootstrap values are indicated at most nodes. The scale bar indicates the expected mean number of substitutions per site according to the model of sequence evolution applied. Asterisks mark the lineages isolated from horses in Portugal in the present study. As outgroup a sequence of *Theileria ovis* (AY533144) was included.

Analyses for the presence of DNA of zoonotic agents (e.g., *Anaplasma* spp. and *Rickettsia* spp.) but also other non-zoonotic pathogens (e.g., *Babesia caballi* and filarioid helminths like *Setaria equina* and *Onchocerca boehmi*) gave negative results.

DISCUSSION

Prevalence of equine piroplasmosis vary in endemic regions in Europe for *T. equi* and *B. caballi*, depending on study design

and diagnostic techniques used [IFAT, ELISA, PCR; (reviewed in 2)]. The prevalence of *T. equi* is generally higher than that for *B. caballi*. In healthy military horses included in this study, 32.7% (33/101) tested positive for the presence of DNA of *T. equi*. These data confirm a relatively high prevalence of *T. equi* in military horses in Portugal (mainly from the Lisbon area). In the Alentejo region (Southern Portugal) serological analyses with IFAT of 154 horses resulted in 85.1% positive for *T. equi* and 65.6% for *B. caballi* (12). Out of 73 "Puro Sangue Lusitano" horses from Vila Viçosa (Alentejo region, Southern Portugal) 53.4% were positive for *T. equi* using cELISA (34). Ribeiro et al. (8), combining serology and microscopy, reported *T. equi* in 19.1% and *B. caballi* in 11.7% of 162 horses in North of Portugal. Moreover, using PCR techniques, *T. equi* was detected in 56% of horses from central Southern Portugal that presented with clinical or subclinical signs (13). DNA of *T. equi* was found in two out of nine *Rhipicephalus bursa* male ticks collected from horses in the Comunidade Intermunicipal do-Ave (Northern Portugal), but because of the proof of DNA only, the vector capacity of this tick species remains unclear (35).

In the present study, horses between 4 and 18 years were infected with this parasite. It is known that the prevalence of equine piroplasmosis increases with age and that equids infected with *T. equi* remain life-long carriers of this parasite (2). Moreover, it is discussed that male and female horses have different susceptibilities to infection, but the results of various studies are contradictory. Host activity also increases the chance for horses to become infected (2). Grazing was documented to double the risk of becoming infected with *B. caballi* and *T. equi* (36). Outdoor or mixed indoor/outdoor type of housing was also reported as a risk factor for *T. equi* (8). Military horses included in this study are regularly outside, but are also stabled at military facilities. However, regular travel activity to various regions in the country increases the possibility of tick contact. Furthermore, the type of horse breed may also influence the risk of *T. equi* infections (37).

Interestingly we were not able to detect DNA of *B. caballi* in military horses from this study, although an additional *Babesia*-specific PCR was run. Previous studies have shown that horses seropositive for *B. caballi* (but also *T. equi*) are often not positive at PCR analysis (38). However, it is recommended to run cELISA, IFAT, and PCR to assure the identification of acute and chronic equine piroplasm infections (39). Recent research carried out on Lisbon horses, found a 7% prevalence for *Babesia* spp., meaning it may be a residual, but persistent agent, in this area (13).

Anaplasmataceae were not detected in the blood samples of healthy horses analyzed in this study. Using serology, *A. phagocytophilum* has previously been confirmed in nine of 302 horses from mainland Portugal (16) and 13% of 162 horses in Northern Portugal (8). However, it has been shown that seropositivity to *A. phagocytophilum* was significantly higher if compared to PCR results [e.g., (40)]. Therefore, we cannot exclude that horses in this study had previously been in contact with this pathogen. It is discussed that climate change might increase the threat of disease in European horse populations (41).

Although no horses were positive for *Rickettsia* spp. in this study, several *Rickettsia* species have been documented in horses in Europe. Spotted fever-group rickettsiae were reported in 15.03% of 479 horses in Italy using serology (17). Using IFAT, *Rickettsia helvetica* was documented in 36.5% of 63 horses in Sweden (42). In another study, *R. helvetica* and *R. monacensis* were confirmed in ticks collected from ponies in Poland using PCR techniques (43). On Corsica, the presence of *Rickettsia slovaca* and *Rickettsia aeschlimannii* in ticks collected on horses was confirmed (44).

DNA of microfilaria of filarioid helminths was not detected in the present study. However, although infections with these parasites are mainly asymptomatic and neglected, they have been reported in horses in several regions in Europe. In Hungary, microfilariae of *S. equina* were reported in 9.2% (18/195) of horses analyzed, and infection was associated with the presence of still waters nearby (45).

Although only one parasite species was documented, it can be concluded that *T. equi* is among the most important vector-borne transmitted agents infecting horses in Europe. As equine piroplasmosis is a severe infectious tick-borne disease responsible for significant losses in equine reproduction, and with numerous impacts on the international movement of horses, treatment and preventive measures are needed to reduce exposure to future infections.

DATA AVAILABILITY STATEMENT

The 18S sequences of *Theileria equi* were deposited in NCBI GenBank under the accession numbers MT767139-MT767169.

ETHICS STATEMENT

This animal study was reviewed and approved by GNR–5373/17/CDF/GAB. Whenever possible, blood sampling was performed during normal sanitary surveys for the GNR horse population. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

H-PF, AA, and LM contributed to the conception and design of the study. AA, HRos, JT, and HRoc conducted field and laboratory work. FK and BS conducted the lab work. H-PF and JH analyzed the sequences and performed the phylogenetic analysis. H-PF, AA, JH, and LM wrote and reviewed the manuscript. All authors read and approved the final manuscript.

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Donkey Fascioliasis Within a One Health Control Action: Transmission Capacity, Field Epidemiology, and Reservoir Role in a Human Hyperendemic Area

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A One Health initiative has been implemented for fascioliasis control in a human hyperendemic area for the first time. The area selected for this multidisciplinary approach is the Northern Bolivian Altiplano, where the highest prevalences and intensities in humans have been reported. Within the strategic intervention axis of control activities concerning animal reservoirs, complete experimental studies, and field surveys have been performed to assess the fascioliasis transmission capacity and epidemiological role of the donkey for the first time. Laboratory studies with altiplanic donkey-infecting *Fasciola hepatica* and altiplanic *Galba truncatula* snail vector isolates demonstrate that the donkey assures the viability of the whole fasciolid life cycle. Several aspects indicate, however, that *F. hepatica* does not reach, in the donkey, the level of adaptation it shows in sheep and cattle in this high altitude hyperendemic area. This is illustrated by a few-day delay in egg embryonation, longer prepatent period despite similar miracidial infectivity and shorter patent period in the intramolluscan development, lower cercarial production per snail, different cercarial chronobiology, shorter snail survival after shedding end, shorter longevity of shedding snails, and lower metacercarial infectivity in Wistar rats. Thus, the role of the donkey in the disease transmission should be considered secondary. Field survey results proved that liver fluke prevalence and intensity in donkeys are similar to those of the main reservoirs sheep and cattle in this area. Fasciolid egg shedding by a donkey individual contributes to the environment contamination at a rate similar to sheep and cattle. In this endemic area, the pronounced lower number of donkeys when compared to sheep and cattle indicates that the epidemiological reservoir role of the donkey is also secondary. However, the donkey plays an important epidemiological role in the disease spread because of its use by Aymara inhabitants for good transport, movements, and travel from one locality/zone to another, a repercussion to be considered

in the present geographical spread of fascioliasis in the Altiplano due to climate change. Donkey transport of parasite and vector, including movements inside the zone under control and potential introduction from outside that zone, poses a problem for the One Health initiative.

Keywords: human fascioliasis hyperendemic, One Health, donkey, *Fasciola hepatica*, *Galba truncatula* experimental transmission, field epidemiology, reservoir role, Bolivia

INTRODUCTION

Human fascioliasis is a parasitic disease to which only secondary public health importance was given until the 1990s (1). The scenario completely changed henceforth, when the number of case reports began to gradually increase in both the Old and New Worlds, but mainly with the description of human endemic areas in many developing countries of Latin America, Africa, and Asia (2), although human infection also occurs in developed countries (3). The public health importance of this emergence was early on recognized by the World Health Organization when including this disease among the group of food-borne trematodiases within the list of Neglected Tropical Diseases (NTDs) (4).

Among the many factors underlying this decision of WHO, the following stand out: (i) the worldwide distribution of this disease caused by two large-sized liver fluke species, *Fasciola hepatica* and *F. gigantica* (5); (ii) its high pathogenicity (6–8), immunological consequences in both the acute (9) and chronic phases (10) of the disease, and its increasing morbidity caused by the immunesuppression-induced very frequent coinfections with other pathogenic microorganisms (11, 12) and parasites (13, 14); (iii) the strong influences of climate change and global change on its transmission and epidemiology (15, 16) because of the lack of a premunition buffer at life cycle end at definitive host level (17, 18); and (iv) its impact on the development of rural communities of low-income countries, including severe clinical pictures (8, 19) and sequelae (8, 20).

The availability of a very efficient drug for human treatment, triclabendazole (21), became crucial in this WHO's decision and subsequent defining of the worldwide strategy of preventive chemotherapy according to different control programs. Such control approaches were adapted to the different transmission patterns and epidemiological situations in the countries presenting human endemic areas and are to be strengthened within the new WHO 2030 road map on NTDs by contributing to sustainable and resilient health systems (22).

South America is the continent presenting the highest number of human fascioliasis endemic areas reported. These areas are characterized for their location at high altitudes in altiplanos and valleys of the Andean region. The main countries affected by this disease because of presenting human endemic areas are Peru (14, 23, 24), Bolivia (13, 25, 26), Argentina (27–29), and Chile (30, 31).

Bolivia stands out because of including the hyperendemic area where the highest prevalences and intensities of fascioliasis have been reported in humans (32) and where children become infected very early in their lives (33). This area is distributed throughout the Northern Bolivian Altiplano, between Lake Titicaca and the valley of the capital city La Paz, at 3,820–4,100 m a.s.l. In 2007–2008, a wide fascioliasis control initiative by yearly mass treatment campaigns was launched by the World Health Organization (WHO) in this area (34, 35) and has been yearly implemented henceforth. Subsequent interannual monitoring assessments indicated infection and reinfection of children by ingestion of metacercariae with freshwater plants or drinking water (36), as a consequence of the high endemicity maintained by the infection rates in livestock (32, 37).

At a PAHO-WHO meeting in La Paz in 2014, it was decided to implement a One Health action to decrease the human risk of liver fluke infection. The One Health is a strategy that encourages interdisciplinary collaboration and communication on health at the human-animal-environmental interface, including multidisciplinary efforts to attain optimal health of people and animals, and the most appropriate measures for the environment (38). This type of approach has been supported by WHO, FAO, and OIE to face the control of zoonotic diseases (39, 40). For the control of fascioliasis in the Bolivian Altiplano, the initiative was planned to include long-term experimental studies, field monitoring assessments and control activities, according to modern standards already analyzed for trematode diseases (41–43).

The wide heterogeneity of transmission patterns and epidemiological scenarios of fascioliasis creates difficulties when applying control activities within a One Health action. Two factors cause these problems:

- Snail vector specificity: Fasciolid species use a wide spectrum of freshwater species of the family Lymnaeidae which present different ecological requirements. These snails are distributed worldwide, but only given species groups are used by the liver flukes, mainly species of *Galba*/*Fossaria* by *F. hepatica*, species of the *Radix* group by *F. gigantica*, and a singular species *Pseudosuccinea columella* by both fasciolids (44). In South America, *Radix* is absent and fascioliasis transmission is assured by *Galba*/*Fossaria* species which are malacologically indifferentiable and need DNA marker sequencing for specimen classification (45, 46). Fortunately, only one lymnaeid species has been proved to inhabit the Northern Bolivian Altiplano hyperendemic area, namely *Galba truncatula* imported from Europe by the Spanish "conquistadores" some time ago and which is geographically spreading along the Altiplano at present, due to climate change and human activities (47).
- Mammal host specificity: The adult stage of fasciolids is able to successfully infect and develop in many different domestic and wild species of mainly herbivorous but also

omnivorous mammals. In the Bolivian Altiplano, similarly as throughout all of the Americas, only *F. hepatica* is present, which pronouncedly simplifies the disease characteristics. Wild lagomorphs and rodents, including domestic guinea pigs (*Cavia porcellus*, locally known as "cuyes" or "quwis"), were already proved to play no role in fascioliasis transmission (48). Among domestic animals, only sheep, cattle, pigs, and donkeys have been involved as important reservoirs in that area (32). Other species such as goats, horses, llamas and alpacas, despite being present, play no transmission role due to different reasons (49).

Within the aforementioned One Health initiative, the aim of the present study is to expose the results obtained in experimental studies on *F. hepatica* transmission and field surveys on animal liver fluke infection to assess the role of the donkey in the transmission and epidemiology of the disease in the very high altitude hyperendemic area of the Northern Bolivian Altiplano. To catalog the contribution of the donkey to fascioliasis in this area, results are compared with those of similar studies previously performed on the main altiplanic reservoir species: sheep and cattle (50).

The laboratory studies included the experimental follow-up of the development stages of egg, miracidium, lymnaeid snail vector infection, intramolluscan larval development, cercarial production, chronobiology of the cercarial shedding, vector survival to infection, metacercarial infectivity of mammal host, and adult stage development of altiplanic donkey isolates through altiplanic *G. truncatula* isolates. This is the first time that such experimental fascioliasis studies focus on the donkey. The field studies included the assessment of prevalence, intensity, egg measurements, and egg shedding rates in nature. This is the first time that the epidemiological role of the donkey as animal reservoir is assessed in a human endemic area.

MATERIALS AND METHODS

Experimental Studies

Fasciolid Materials

Fecal samples from naturally infected donkey individuals from the Altiplanic locality of Ancocagua were used for the experimental study of the embryonation of the eggs of *F. hepatica* (**Figure 1**). Eggs isolated by filtration were conserved in natural water under complete darkness at 4°C until starting of the embryogenesis follow up study.

For the experimental infection of lymnaeid snails, *F. hepatica* eggs were similarly obtained from donkeys from Ancocagua (**Figure 1**). Eggs were similarly isolated by filtration and conserved until used for snail infection in the laboratory.

For the experimental infection of Wistar rats, *F. hepatica* metacercariae were obtained from the aforementioned experimentally infected lymnaeid snails. These metacercariae were stored in natural water in total darkness until required. The storage temperature was 4°C, according to the usual standards in liver fluke studies (51).

Eggs of *F. hepatica* were also collected from sheep, cattle, and pigs of the same Northern Bolivian Altiplano human hyperendemic area, although the studies of the isolates of these three definitive reservoir hosts are the focus of other articles to avoid excessive text length and reference number.

Study of the Egg Embryogenesis

The egg embryogenesis was experimentally followed at constant 20°C, at microscopic study intervals of 4 days. Egg development was made by differentiation of (i) eggs including an early developing morula (E.E.D.M.), (ii) eggs in the phase of advanced morula, showing vitelline granules and/or spheroidal cells (E.A.M.), (iii) eggs in the phase of outlined miracidium, in which a miracidial form begins to be observed (E.O.M.), and (iv) eggs in the phase of developed miracidium, in which a fully developed miracidium is observed inside (E.D.M.). For each 4-day study, a total of 33 eggs from each of four host individuals were analyzed between slip and coverslip. Counting did not only include E.E.D.M., E.A.M., E.O.M., and E.D.M., but also (i) degenerated eggs, (ii) empty eggs, and (iii) broken eggs (**Figure 2**). Degenerated, empty, and broken eggs (eggs easily break when open and empty after miracidial release) are very few at the beginning but of course increase with time and are the marked majority in the last days of the follow-up study. Egg counts were noted in percentages per observational day. Percentages of degenerated, empty and broken eggs are not included in the graph of follow-up curves because they do intervene in the transmission.

Experimental Infection of Snails

Fully embryonated eggs were put under light to force the hatching of developed miracidia which were afterwards used for the experimental infection of snails (52). Only laboratory-borne snails were used. Lymnaeid snails of a size of 4.0–5.0 mm were used to assess infection susceptibility, by exposing each snail to miracidia for 4 h in a small Petri dish containing 2 ml of fresh water. The disappearance of the miracidium was taken as verification of its successful penetration into the snail.

The donkey *F. hepatica* isolate was used for individual infection assays of lymnaeid snails under the experimental conditions of a monomiracidial dose and 20/20°C day/night temperature according to a photoperiod of 12 h light/12 h darkness in climatic chambers (HPS-1500, VB-0714, and HPS-500 models of Heraeus-Vötsch) (46). The characteristics, conditions, and number of snails in these snail infection experiments are detailed in **Table 1**. After the infection, snails were returned to 2,000 ml containers, at 90% relative humidity (r.h.), 20/20°C day/night temperature according to 12/12 h light/darkness, and dry lettuce *ad libitum*, until day 30 post-infection, in which they were again isolated in Petri dishes to allow daily monitoring of cercarial shedding by individual snails. Lettuce was provided *ad libitum* to each snail in a Petri dish during both shedding and post-shedding periods until death of the snail. The cercarial shedding was followed by daily counting of metacercariae in each Petri dish.

Laboratory Cultures of the Snail Vector

Galba truncatula has recently been proved to be the only lymnaeid species inhabiting the Northern Bolivian Altiplano

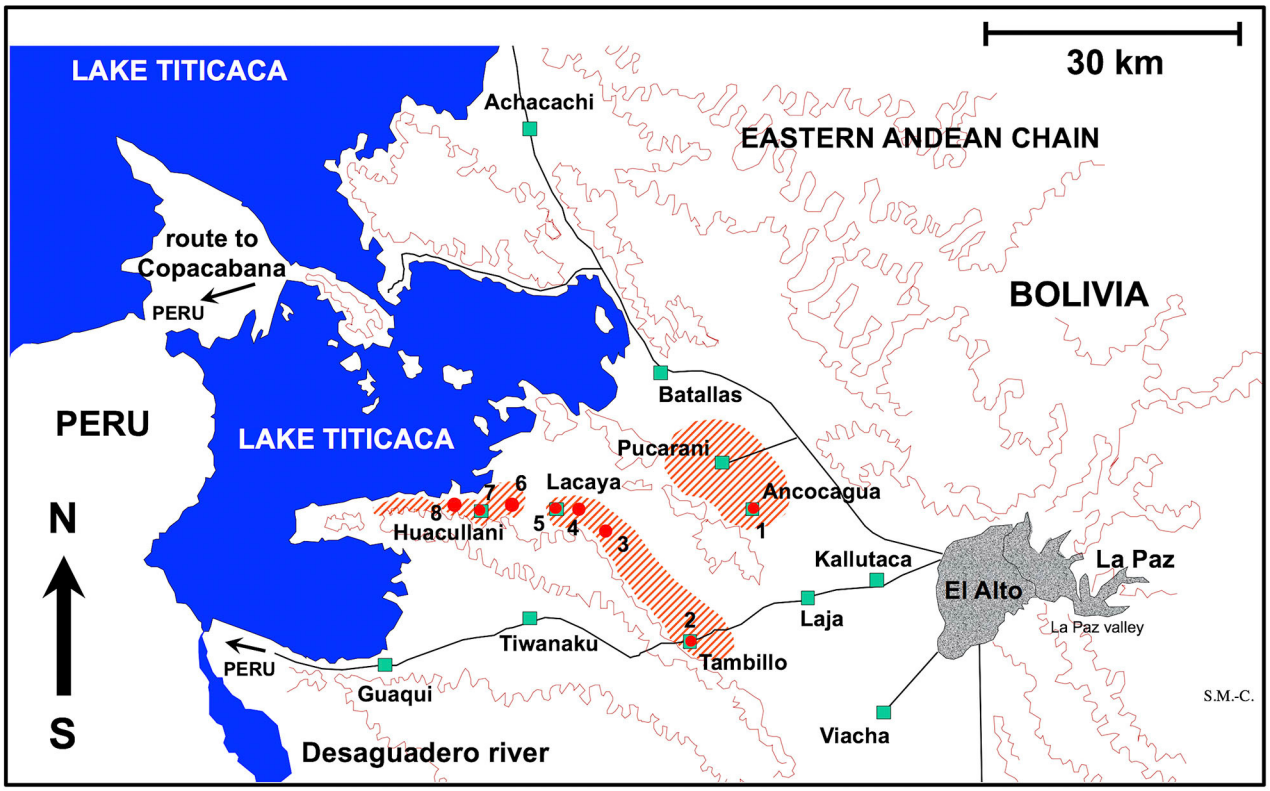

FIGURE 1 | Map showing the Northern Bolivian Altiplano human fascioliasis hyperendemic area, at 3,820–4,100 m altitude, including zones where donkeys were surveyed and localities where lymnaeid snail vector specimens of *Galba truncatula* were collected. Localities: (1) Ancocagua; (2) Tambillo; (3) Korila; (4) Chiripujo; (5) Lacaya Baja; (6) Chojasihui; (7) Huacullani; (8) Queroni.

hyperendemic area, by the sequencing of complete nuclear ribosomal DNA and mitochondrial DNA markers (47). This species is of European origin and differs from the Neotropical species of the *Galba*/*Fossaria* group of lymnaeids which also act as vectors of fascioliasis in South America (45). Living specimens of *G. truncatula* were collected in the locality of Tambillo (**Figure 1**) and transported under isothermal conditions for their laboratory adaptation to standardized controlled conditions of 20°C, 90% r.h. and a 12/12 h light/darkness photoperiod in the aforementioned precision climatic chambers. The possible natural infection by fasciolids was always individually verified prior to the launch of laboratory cultures. This was performed by keeping each lymnaeid specimen isolated in a Petri dish containing a small amount of natural water. After 24 h, the presence or absence of motionless metacercarial cysts or moving cercariae was verified in each Petri dish. Non-infected lymnaeids were arranged in standard breeding boxes containing 2,000 ml fresh water, to assure locality-pure cultures. The water was changed weekly and lettuce added *ad libitum*.

Laboratory Infections of Wistar Rats

A total of 18 male Wistar rats (Iffa Credo, Barcelona, Spain) aged 4–5 weeks were used throughout. A balanced commercial rodent diet (Panlab Chow A04) and water were provided *ad libitum*, according to standards previously reported (53).

Wistar rats were infected according to methods previously described (54). A dose of 20 *F. hepatica* metacercariae per rat was used (**Table 2**). Animal care, animal health, body condition, and well-being were assessed on a weekly basis by means of checking their body weight and the appearance of the fur. Infected animals presented a lower body weight than negative controls at the end of the experiment. No mortality occurred. Infection prevalence and intensity (number of worms successfully developed in each rat) were established by necropsy 12 weeks after infection. Metacercariae were inoculated orally by means of a gastric tube. The number of Wistar rats in each infection experiment are noted in **Table 2**. Finally, animals were euthanized with an overdose of an anesthetic (IsoFlo; Dr Esteve SA, Barcelona, Spain), and *F. hepatica* worms were collected under a dissecting microscope, according to methods already outlined before (55). The bile duct was initially examined for the presence of flukes, followed by the whole liver, although the rest of the organs were also evaluated. The thoracic and abdominal viscera and cavities were examined and thoroughly rinsed with water to assure the recovery of all worms.

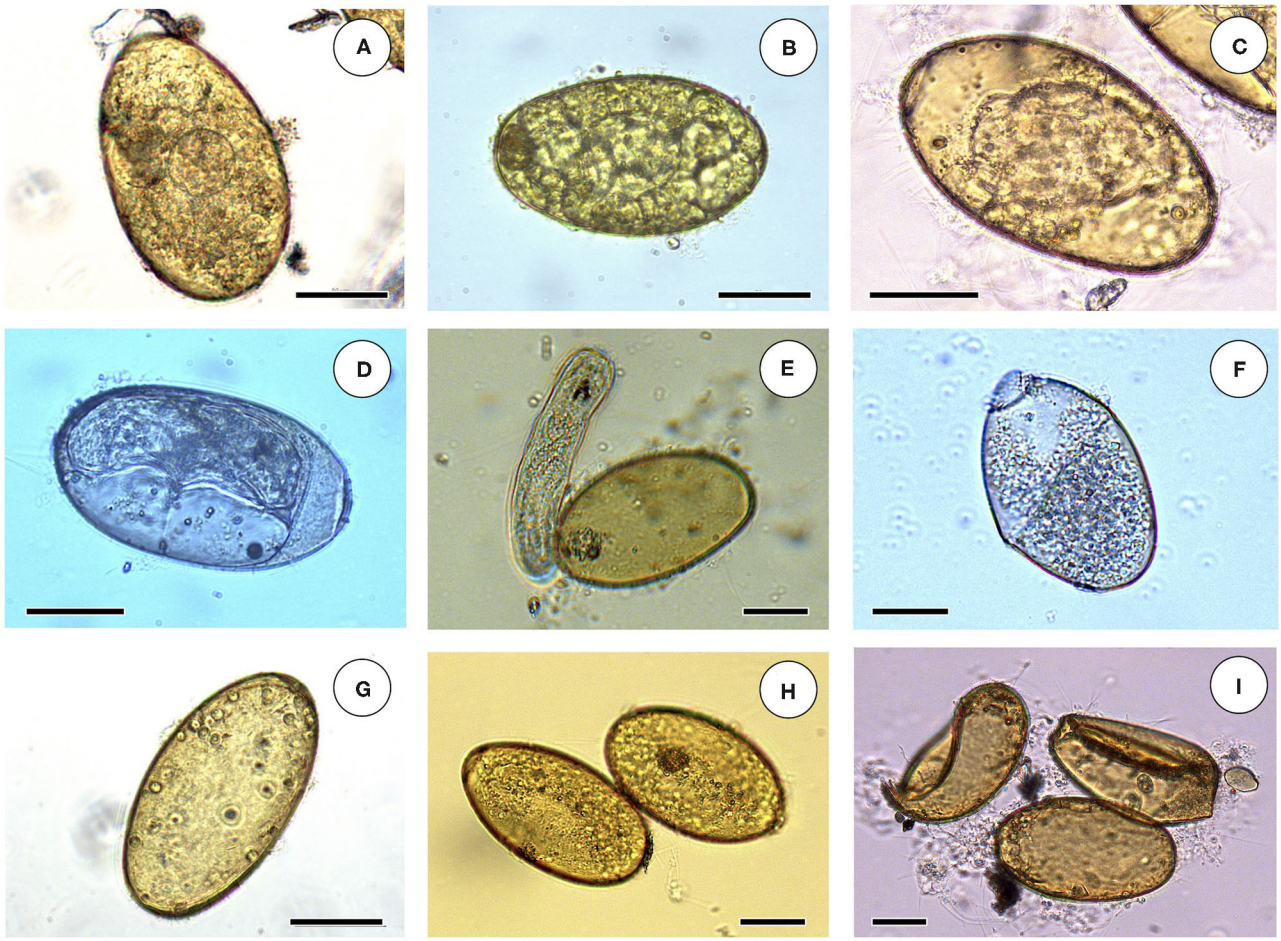

FIGURE 2 | Embryonation stages in liver fluke eggs: **(A)** egg including an early developing morula (E.E.D.M.); **(B)** egg in the phase of advanced morula, showing vitelline granules and spheroidal cells (E.A.M.); **(C)** egg in the phase of outlined miracidium, in which a miracidial form begins to be observed (E.O.M.); **(D)** egg in the phase of developed miracidium, in which a fully developed miracidium is observed inside (E.D.M.); **(E)** empty egg immediately after miracidium hatching; **(F)** broken open egg after miracidial release; **(G)** empty egg; **(H)** two degenerated eggs; **(I)** two broken eggs and an empty egg. Scale bar = 40 μm.

Field Surveys of Donkeys

Host Animals Studied

Fecal samples from a total of 84 donkeys were collected from different zones of the Northern Bolivian Altiplano hyperendemic area (**Table 3**). The geographical distribution of the localities and zones surveyed is shown in **Figure 1**.

In the Altiplano, donkeys are usually found isolated, only rarely more than one specimen accompanying grazing herds of sheep and/or cattle. The surveys were made to assure that a fecal sample of all donkeys found during the survey days could be obtained. Unfortunately, stool sample collection could not be made following the different annual seasons in a zone and, thus, no data are available to analyze the variations of prevalence throughout the different months of the year. In the Altiplano hyperendemic area, donkeys are not treated against liver fluke infection.

Stool Sample Preparation and Study

Only coprological methods for qualitative and quantitative analyses were carried out. Fecal samples were placed in numbered plastic bags, transported to the laboratory within the following 5 h, and maintained at 4°C until examination. From each stool sample a quantity of 4 g was sedimented twice, first with 50 ml of detergent solution (1 ml/1,000 cm^3) after filtration and second with 50 ml water, and stained with methyl green according to a standard method (58), before examination under light microscope for *F. hepatica* eggs. A host individual was considered negative when no eggs were found in its respective stool sample after studying 3 slides. The number of eggs shed by a donkey was used to estimate the infection intensity and was expressed in eggs per gram of stools (epg).

Egg measurements were carried out using a computer image analysis system (CIAS) on the basis of standardized measurements known to be useful for fasciolid species (59). Standardized measurements were taken using a microscope and images captured by a digital camera (3CCD color videocamera Sony DXC-930P), which were then analyzed by image analysis software (ImagePro plus version 5.0 for Windows, Media Cybernetics, Silver Spring, Maryland, USA). Egg characteristics studied were: (a) linear measurements: egg length (EL), egg width

TABLE 1 | Experimental infections of altiplanic lymnaeid snails with *Fasciola hepatica* donkey isolate from the Northern Bolivian Altiplano human hyperendemic area.

Host isolate	Donkey	Sheep[a]	Cattle[a]
F. hepatica geogr. origin	Ancocagua	Batallas	Batallas
Lymnaeid geogr. origin	Tambillo	Huacullani	Huacullani
Miracidial dose	Mono-miracidial	Mono-miracidial	Mono-miracidial
Temperature (12 day/12 h night)	20/20°C	20/20°C	20/20°C
No. lymnaeids infected	35	62	55
No. survivor snails at beginning of shedding (%)	25 (71.4%)	54 (87.1%)	48 (87.3%)
No. shedding snails (%)	7 (28.0%)	28 (51.8%)	12 (25.0%)
Prepatent period in dpi (mean)	52–69 (60.5)	48–92 (55.6)	49–76 (55.5)
Shedding end in dpi (mean)	52–117 (86.1)	52–136 (89.4)	58–135 (101.6)
Shedding length in days	1–49 (26.6)	1–88 (34.7)	1–85 (47.1)
No. total cercariae shed	333	5,542	3,672
No. cercariae/snail (mean)	2–92 (47.6)	8–562 (197.9)	8–581 (306.0)
Snail survival after shedding end in days	1–43 (14.8)	1–132 (24.5)	1–133 (42.3)
Longevity of shedding snails in dpi	77–126 (101.0)	53–192 (113.8)	76–268 (143.9)
Longevity of non-shedding snails in dpi	34–183 (60.7)	49–196 (139.1)	31–209 (105.4)

dpi, days post-infection.
[a]*Data from Mas-Coma et al. (50).*

(EW), and egg perimeter (EPe); (b) areas: egg area (EA); (c) ratios: EL/EW ratio. For each measure, minimum and maximum values, mean, and standard deviation were determined.

Statistical Analyses

To assess the transmission capacity and epidemiological role of the donkey isolate, each characteristic studied was compared with that of the sheep and cattle isolates of *F. hepatica* from the same Northern Bolivian Altiplano, which have been proved to be the main animal reservoir species in this human hyperendemic area. This comparative assessment is feasible because based on data obtained following the same methods and techniques both in the laboratory experiments and in the field work and which have been reported in another study (50).

Statistical analyses were performed using SPSS Statistics 26. Development egg data (E.E.D.M., E.A.M., E.O.M., E.D.M.) were compared by ANOVA test. Statistical comparison of categorical variables was carried out with the Chi-square test and Yates continuity corrected Chi-square test. Means obtained in data from experimental infections of lymnaeid snails and from experimental infections of Wistar rats were compared by non-parametric Kruskal-Wallis test. Fasciolid egg size measurements (EL, EW, EPe, and EL/EW) were compared by *post-hoc* tests (L.S.D., Student–Newman–Keuls and Duncan's tests). Results were considered statistically significant when $p < 0.05$.

RESULTS

Egg Embryonation

The egg embryonation of the donkey isolate of altiplanic *F. hepatica* could be experimentally followed at 20°C until complete development of all eggs (**Figure 3**). An outlined miracidium form begins to be observed inside eggs at day 24. The first fully developed miracidium appears at day 28. Eggs including a fully developed miracidium were henceforth observed in each observational day. The percentage of fully developed miracidia follows an increasing curve until a peak on day 60, after which it gradually decreases until day 127. No statistically significant differences in the average egg embryonation (E.O.M., E.D.M.) of the donkey isolate (36.63%) in the 60 days were detected when compared to the sheep isolate (38.93%) and cattle isolate (26.63%) from the Altiplano ($p > 0.05$).

Snail Infectivity and Intramolluscan Development

Snail vector infection assays performed with the altiplanic donkey isolate furnished results which are shown in **Table 1**, including their comparison with the altiplanic sheep and cattle isolates. The donkey isolate (28.0%) was even slightly more efficient than the cattle isolate (25.0%), although both at distance from the sheep isolate (51.8%). Nevertheless, no statistically significant differences in the percentage of snails successfully infected by a monomiracidial dose at 20/20°C (= the snail infectivity) between the donkey isolate and the two main reservoirs ($p > 0.05$) were detected.

The donkey isolate prepatent period (60.5 days) showed a slightly higher mean value than the sheep isolate (55.6 days) and the cattle isolate (55.5 days). However, no statistically significant differences in prepatent period between the donkey isolate and the two main reservoirs ($p > 0.05$) were detected. The donkey isolate cercarial shedding period (average: 26.6 days) showed a shorter mean value than the sheep isolate (average: 34.7 days) and the cattle isolate (average: 47.1 days). No statistically significant differences in the cercarial shedding period were anyway detected between the donkey isolate and the two main reservoirs ($p > 0.05$).

Significant differences were observed when comparing the smaller production of cercariae per infected snail of the donkey isolate (average: 47.6 cercariae/snail) with that of the sheep (average: 197.9 cercariae/snail) and cattle (average: 306 cercariae/snail) isolates ($p < 0.05$).

The assessment of the infection impact on snails was measured by the snail survival after shedding end in days, as well as by the comparison of the longevity of shedding and non-shedding snails. These three characteristics in the donkey isolate showed mean and maximum dpi values clearly shorter than in sheep and cattle isolates, all of them proving to be statistically different ($p < 0.05$).

TABLE 2 | Experimental infections of Wistar rats with experimentally obtained metacercariae from donkey isolate and comparison with sheep and cattle isolates from the Northern Bolivian Altiplano human hyperendemic area.

Host isolate	Donkey		Sheep[a]		Cattle[a]	
F. hepatica geographical origin	Ancocagua	Ancocagua	Batallas	Ancocagua	Kallutaca	Batallas
Age of metacercariae	6 weeks	10 weeks	1 week	2 weeks	6 weeks	8 weeks
No. metacercariae inoculated per rat	20	20	20	20	20	20
No. inoculated rats	8	10	14	23	4	4
No. rats infected (%)	3 (37.5%)	4 (40.0%)	11 (78.6%)	18 (78.3%)	4 (100%)	2 (50.0%)
No. flukes recovered per rat (mean)	1–6 (3.3)	2–3 (2.7)	1–8 (3.6)	1–10 (3.7)	1–2 (1.7)	1–2 (1.5)
Intensity[b]	6.2%	5.5%	14.3%	14.6%	8.8%	3.7%
Mean % flukes recovered/rat[c]	16.7%	11.2%	18.2%	18.6%	8.8%	7.5%

[a] *Data from Mas-Coma et al. (50).*
[b] *Intensity = total % of flukes recovered = (total No. of flukes recovered/total No. of metacercariae administered in all rats) × 100.*
[c] *Mean % flukes recovered/rat = Mean % of flukes recovered per infected rat = (flukes recovered/metacercariae administered per infected rat) × 100.*

TABLE 3 | Prevalence and intensity of *Fasciola hepatica* infection found by coprological analyses in donkeys of different zones of the Northern Bolivian Altiplano human hyperendemic area and comparison with local prevalences in sheep and cattle from the same zones.

Locality	Endemic zone	Donkey				Sheep[a]	Cattle[a]
		Prevalence		Intensity		Prevalence	Prevalence
		No. analyzed/ infected	%	Range (epg)	Mean (epg)	No. analyzed/infected (%)	No. analyzed/infected (%)
Ancocagua	Pucarani	35/3	8.6[b]	3–43	22.3	170/97 (57.1)	195/22 (11.3)
Tambillo	Tambillo-Lacaya	2/0	0[c]	–	–	230/113 (49.1)	50/2 (4.0)
Korila	Tambillo-Lacaya	1/0	0[c]	–	–	100/87 (87.0)	–
Chiripujo	Tambillo-Lacaya	2/0	0[c]	–	–	–	141/17 (12.0)
Lacaya Baja	Tambillo-Lacaya	10/3	30.0[b]	11–58	32.7	100/64 (64.0)	43/3 (47.0)
Chojasihui	Huacullani	6/3	50.0	14–101	50.7	100/70 (70.0)	29/9 (31.0)
Huacullani	Huacullani	21/11	52.4	4–90	74.0	120/81 (67.5)	210/95 (45.2)
Queroni	Huacullani	7/0	0[c]	–	–	100/70 (70.0)	–
Total		84/20	23.8[b]	3–101	28.1	920/582 (63.7)	768/148 (19.3)

[a] *Data from Buchon et al. (56), Grock et al. (57), Mas-Coma et al. (32, 50).*
[b] *Significantly different vs. sheep and cattle, determined by Chi-square/test Chi-square with Yates' correction ($p < 0.05$).*
[c] *Statistical comparison could not be made.*

Chronobiology of Cercarial Shedding

The chronobiological patterns of cercarial emergence in the donkey isolate are shown in **Figures 4A–D**. The shedding period analyzed according to the mean amounts of cercariae shed daily and weekly from the day of the emergence of the first cercaria by each snail is shown in **Figures 4A,B**, The total length of the shedding period lasted up to 49 days (**Figure 4A**) or 7 weeks (**Figure 4B**), with a mean of 26.6 days (**Table 1**). The daily shedding process appears as an irregular succession of waves in which up to seven peaks may be distinguished. Among them, the first peak is the highest and corresponds to the first day of shedding. The weekly analysis of the curve shows that the highest numbers of cercariae are shed during the first 2 weeks. Interestingly, a gradual increase of the weekly cercarial number from week 3 gives rise to a late peaks on week 6.

When the analysis is made from the day of the miracidial infection, the shedding period shows completely different daily and weekly curves (**Figures 4C,D**). When compared with the aforementioned chronobiological patterns from the day of the

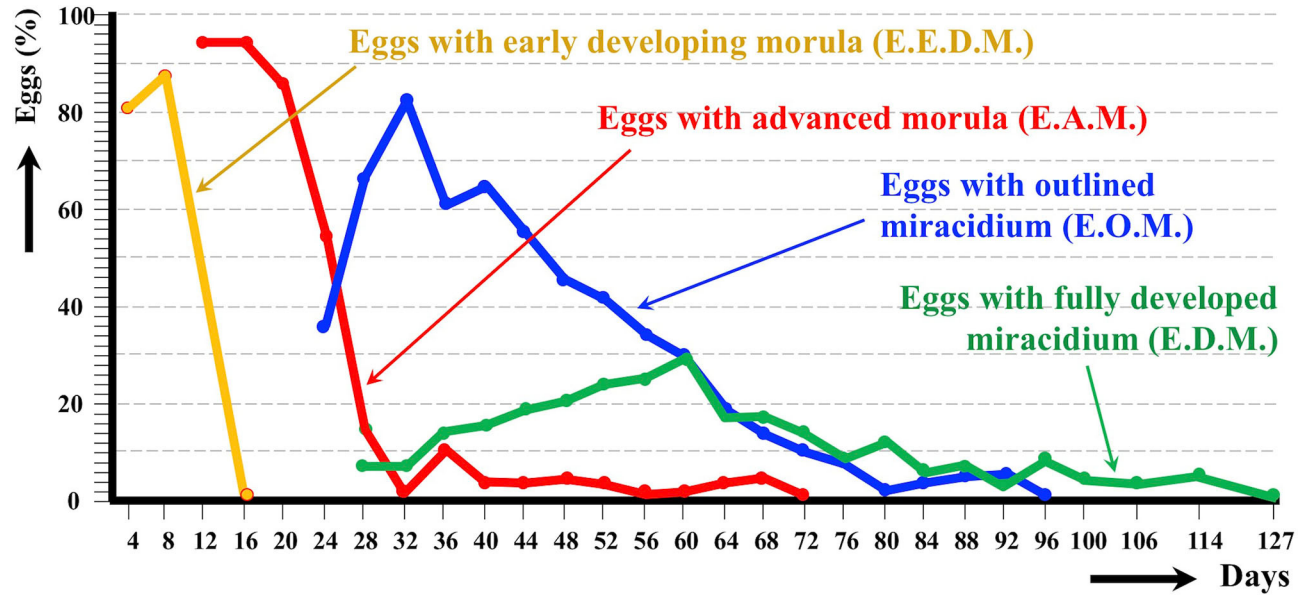

FIGURE 3 | Graph showing the results of the experimental follow-up study of the egg embryonation of the altiplanic donkey isolate of *Fasciola hepatica*, at 4-day study intervals and constant temperature of 20°C. Curves of the percentages of degenerated, empty, and broken eggs are not included.

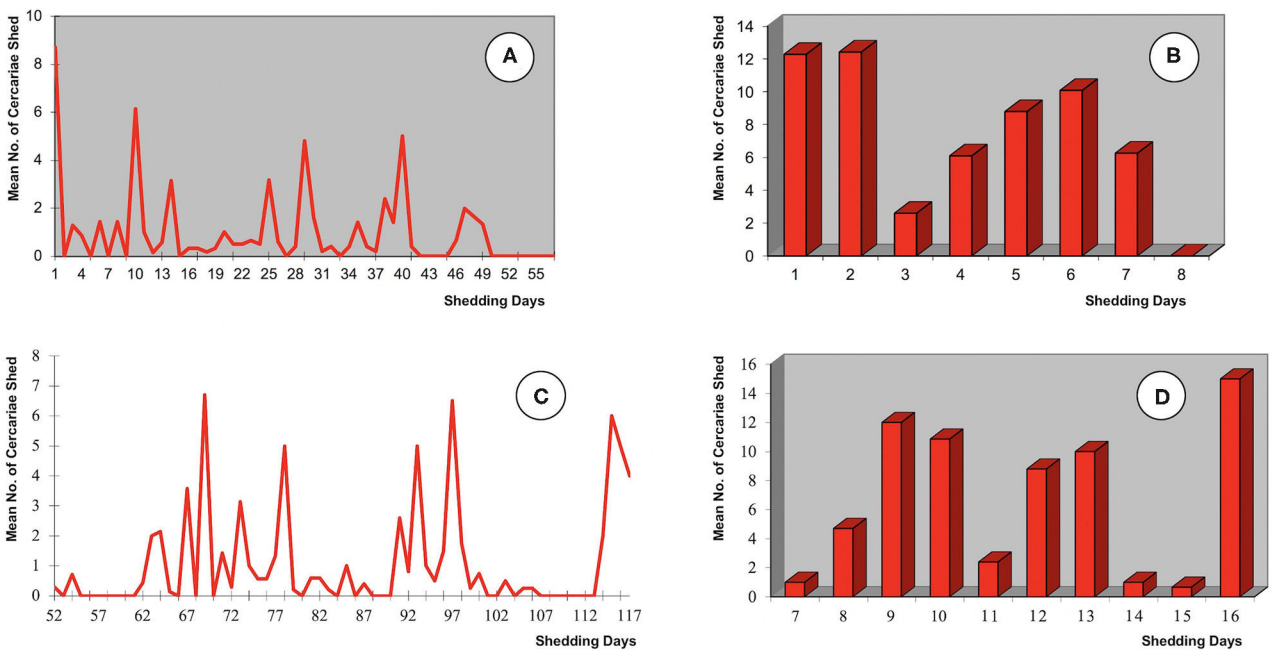

FIGURE 4 | Chronobiological patterns of cercarial emergence by altiplanic *Galba truncatula* monomiracidially infected with the donkey isolate of *Fasciola hepatica* from the Northern Bolivian Altiplano human fascioliasis hyperendemic area: **(A,B)** shedding period analyzed according to the mean amounts of cercariae shed daily and weekly from the day of the emergence of the first cercaria by each snail; **(C,D)** shedding period analyzed according to the mean amounts of cercariae shed daily and weekly from the day of the miracidial infection; prepatent period not shown.

emergence of the first cercaria by each snail (**Figures 4A,B**), several similar peaks appear but two main differences may be distinguished, namely the complete disappearance of the initial peak and the appearance of an evident final peak. These initial and final differences are well-evident in both the daily and the weekly curves. Different delays in the beginning of the cercarial shedding according to different snail specimens, due to individual differences in the prepatent period (**Table 1**), explain the longer daily and weekly curves obtained in this analysis.

Mammal Host Infectivity

Results of the experimental infection of Wistar rats with experimentally obtained metacercariae of the altiplanic donkey isolate are shown in **Table 2**. No statistically significant differences appeared in the infectivity between the donkey isolate and the sheep and cattle isolates regarding the percentage of rats successfully infected (donkey: 38.9%; sheep: 78.4%; cattle: 75.0%; $p > 0.05$). Furthermore, the intensity of infection, measured by the number of flukes recovered per metacercariae inoculated and also per rat, did not show significant differences when comparing the donkey isolate with the sheep and cattle isolates ($p > 0.05$).

Prevalence, Intensity, Egg Measurements, and Shedding Rates

Results obtained in the coprological surveys on donkeys inhabiting selected zones of the fascioliasis hyperendemic area (**Figure 1**) are shown in **Table 3**. However, in the Altiplano the distribution of donkeys is unfortunately very dispersed because of their reduced number when compared to other domestic animals as sheep, cattle, or pigs. So, this equine most frequently appears as individuals isolated throughout large tracts of land and consequently the donkey prevalence data should be considered with the appropriate caution.

A total of 161 eggs from donkey stools were measured. Results and the comparative analysis with fasciolid eggs shed by altiplanic sheep and cattle are shown in **Table 4**. The statistical analysis by *post-hoc* tests (L.S.D., Student–Newman–Keuls and Duncan's tests) showed that, in the Altiplano, EL, EW, EPe, and EL/EW of the fasciolid eggs shed by donkeys are significantly different from the same parameters in the sheep and cattle isolates ($p < 0.05$).

Intensity of infection in donkeys, measured by eggs shed by gram of feces, varied between 3 and 101 epg, with a mean of 28.1 epg (**Table 3**). When considering the amount of stools defecated by each domestic animal species per day, the total number of *F. hepatica* eggs shed with feces by each domestic animal species per day in the Bolivian Altiplano could be estimated (**Table 4**). The daily egg output per donkey per day proved to fit well with the same estimations for sheep and cattle.

DISCUSSION

Donkey Participation in Fascioliasis Transmission

In the life cycle of digenean trematodes, the infection and development of the adult stage in a definitive host species does not mean that this species participates in the transmission of the parasite. This definitive host species may only represent a dead end for the digenean life cycle. In several trematode species, there may even be differences in the viability of the same definitive host species, with geographical populations playing a transmission role whereas others being a blind alley. This occurs in trematode species which show different local adaptations which may be interpreted as long-term repetitive infection events evolving locally. *Schistosoma haematobium*, a human-specific species in nature, is an illustrative example. Although natural infections by *S. haematobium* have also been reported in non-human primates (60), this does not appear to have an epidemiological importance regarding the human disease. A genetically pure strain, i.e., not an hybrid, of this schistosomatid species can be experimentally forced to adapt to a laboratory rat model after long-term reinfection passages (61).

When dealing with a trematode species with a low definitive host species specificity, as is the case of *F. hepatica* or *F. gigantic*a, results of surveys on animals in nature may give rise to misinterpretations. The finding of natural infections of a mammal species allows to include this host species among the list of animals affected by the disease caused by the trematode, but does a priori not enable to epidemiologically consider it as a reservoir because it may indeed only be a dead end. This is the case of the donkey in fascioliasis. There have been several surveys on naturally-infected donkeys, but there is still no experimental study demonstrating that it is not a dead end.

To prove that a definitive host species participates in the transmission of a trematode, there is the need to (i) experimentally assess that the life cycle of the parasite is not blocked at any of the subsequent life cycle larval stages until the infection of another definitive host individual, and (ii) demonstrate that the rates of development in all these life cycle phases is sufficient as to estimate a successful transmission in nature. In *Fasciola*, this implies appropriate laboratory research on definitive host egg shedding, egg embryonation, miracidial hatching and snail vector infection, complete intramolluscan larval development until cercarial production, cercarial shedding and its characteristics, metacercarial production and definitive host infection, and final adult stage development until mature stage. The present study has included all these aspects.

Egg Embryonation in Altiplanic Donkey Isolate

Egg embryonation is a key aspect in the assessment of the viability of a definitive host species isolate in the life cycle of fasciolids. Even despite the maturation of the adult stage of the liver fluke, eggs shed with feces of given host species, or local geographical strain of that species, fail to reach complete embryonation of the miracidium. When successful, this process is temperature-dependent.

It should be noted that storage time can influence the development of the egg. This influence was kept to a minimum by reducing the storage period to only 1 month, that is the time from the day of collection in the field until the day after arrival to the laboratory of Valencia where the experimental study was performed taking advantage of the availability of the appropriate high accuracy climate chambers.

In ruminants, the following hatching times were found in southern Europe: 56 days at 15°C, 50 days at 18°C, 27 days at 20°C, 17 days at 25°C, 22 days at 27°C, and 17.5 days at 30°C (62). Results on first hatching times proved to be different In the southern part of Chile: 101 days at 9.1°C, 80 days at 10°C, 57 days at 12.4°C, 44 days at 12.6°C, 42 days at 13.8°C, 34 days at 15.1°C, 28 days at 16°C, 30 days at 16.4°C, and 20 days at 17°C (63). A hatching time of 19–20 days was obtained for 20°C when analyzing the linear correlation between egg development and

TABLE 4 | Comparison of egg measurements **(A)** and egg shedding **(B)** between the *Fasciola hepatica* donkey isolate and the sheep and cattle isolates in the Northern Bolivian Altiplano hyperendemic area.

Host	Donkey		Sheep		Cattle	
No. of eggs studied	**161**		**104**		**168**	
Measurements in μm	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**
(A) Egg Measurements						
EL	96.4–140.8	125.4 ± 8.3	114.8–151.2	130.8 ± 7.1	105.3–155.9	132.0 ± 10.5
EW	63.3–84.7	75.0 ± 3.7	65.5–81.4	72.6 ± 3.9	61.7–82.5	71.1 ± 4.4
EPe	272.1–350.4	318.8 ± 16.3	294.2–368.2	327.6 ± 15.0	270.6–422.9	340.0 ± 33.4
EA	5,562.6–8,686.2	7,177.4 ± 646.1	5,998.2–8,608.4	7,238.0 ± 532.8	5,286.5–9,676.8	7,170.2 ± 802.5
EL/EW	1.3–2.0	1.7 ± 0.1	1.5–2.1	1.8 ± 0.1	1.6–2.3	1.8 ± 0.2
(B) Egg Shedding						
Intensity range (epg)	3–101		3–241[a]		1–96[b]	
Stools/day (kg)	3–8[c]		1–3[d]		15–35[d]	
No.eggs/animal/day[e]	9,000–808,000		3,000–723,000		15,000–3,360,000	

EL, egg length; EW, egg width; EPe, egg perimeter; EA, egg area; EL/EW, egg length/egg width. Measurement values shown as range and mean ± standard deviation (SD).
[a] *According to Mas-Coma et al. (49).*
[b] *After Buchon et al. (56).*
[c] *Deduced for altiplanic donkeys from their weight.*
[d] *According to various sources.*
[e] *Estimations of the range of the number of F. hepatica eggs faecally shed by an animal per day in the Bolivian Altiplano.*

temperature (64). At this temperature, the first fully developed miracidium in eggs shed by sheep and cattle from the Northern Bolivian Altiplano appeared at day 24 (50), that is in agreement with the 19–27 day range from the data of the aforementioned studies. In the altiplanic donkey isolate, the first fully developed miracidium appears at day 28. A similar few-day delay is also observed in the curve peak of the percentage of fully developed miracidia, at day 60 in the donkey isolate whereas at day 56 in the altiplanic sheep isolate. However, fully developed miracidia continue to appear until day 127 in the donkey isolate, which is close to day 143 in the sheep isolate. Summing up, results indicate a slightly delayed embryonation but even so a similar complete egg embryonation in length.

Miracidial Infectivity, Intramolluscan Development, and Cercarial Chronobiology

Numerous studies on *F. hepatica*/*G. truncatula* interactions have demonstrated that the processes, including from snail infection up to cercarial shedding and related lymnaeid infection survival, are highly complex and involve many different factors which underlie a large variability (65). The monomiracidial infectivity of 28.0% at 20/20°C of 12 day/12 h night photoperiod in the altiplanic donkey isolate agrees with the range of 14.0–56.8% experimentally found in different *G. truncatula* populations in France (66, 67), as well as with that of 25.0–51.8% detected in altiplanic sheep and cattle isolates (50).

At the aforementioned miracidial dose and experimental temperature, the prepatent period (from infection up to the shedding of the first cercaria) in the altiplanic sheep and cattle isolates (50) proved to be slightly longer than in *F. hepatica*/*G. truncatula* of the lowlands (64, 68, 69). The altiplanic donkey isolate shows an even longer mean prepatent period, despite its range entering within the ranges found in the ruminants.

Under the same conditions, the patent period (length of the cercarial shedding period) in the altiplanic sheep and cattle isolates (50) agreed with the respective knowledge in ruminants of the lowlands (66, 70), but its length in the altiplanic donkey isolate proved to be shorter (**Table 1**).

The chronobiology of the cercarial shedding of the donkey isolate follows a pattern in which a gradual progressive decrease after an initial acrophase as observed in the altiplanic sheep and cattle isolates (71) is not observed. However, the donkey isolate shedding pattern fit well to the 1–14-wave pattern, with a 4–5-wave pattern folllowed by the majority, observed in *F. hepatica*/*G. truncatula* of the lowlands under constant conditions of temperature and photoperiod (72). Despite the shorter length of the shedding process in the donkey isolate, in the curve analyzed from the day of the miracidial infection (**Figure 4C**) two intervals appear in which daily shedding is completely or almost completely arrested, namely in days 80–90 and 102–113. These pauses coincide with days 90, 102, and 111 and days 84 and 121 in which cercarial shedding completely stopped by all shedding snails in the sheep and cattle isolates, respectively (71). These pauses have been linked to the redial generation replication processes.

Cercarial Production, Lymnaeid Survival, and Metacercarial Infectivity

The shorter patent period is related to the lower range and mean of the cercarial productions per snail obtained in the altiplanic donkey isolate when compared to those of not only both altiplanic sheep and cattle isolates (**Table 1**) (50), but also to the low productions found in *F. hepatica*/*G. truncatula* in given lowland populations, such as 120.0 cercariae/snail (73) or 91.7 cercariae/snail (74).

The snail survival after shedding end and the longevity of shedding snails in infections by the altiplanic donkey isolate are also shorter than in those obtained when infecting with the altiplanic sheep and cattle isolates (**Table 1**) (50), although the results confirm that this longevity is longer in infected lymnaeids from high altitude areas than the same survival period known in *F. hepatica*/*G. truncatula* in the lowlands and that this phenomenon is independent on the host isolate of *F. hepatica* (75).

The experimental infections of Wistar rats with metacercariae of the altiplanic donkey isolate allowed to verify its definitive host infectivity (**Table 2**). Although the prevalences were lower, the intensities fitted well in the results obtained with the altiplanic sheep and cattle isolates (50), and also in the present knowledge when dealing with short-aged metacercariae of ruminant origin (51, 76).

Epidemiological Role of the Donkey

The local prevalence of liver fluke infection in donkeys varied pronouncedly according to localities, with values which fit in the prevalence ranges shown by sheep and cattle in the same zones (32, 50, 56, 57). Donkeys in the more remote areas, i.e., in localities of the Tambillo-Huacullani flatland corridor distant from El Alto and La Paz, showed the highest prevalence rates. A few donkeys are usually part of the livestock of Aymara families, although their numbers vary in the different zones. Donkeys are relatively numerous in all zones of the endemic area, although in numbers markedly lower than the populations of cattle, sheep, and pigs. In the Bolivian Altiplano, donkeys are frequently found in pastures besides water collections presenting *G. truncatula* populations. The aforementioned great variability of the liver fluke infection prevalences in donkeys according to localities may be due to the local environmental conditions, mainly linked to the number of freshwater collections inhabited by lymnaeid snails.

Fasciolid infection of donkeys has been reported in many countries of Asia, Africa and the Americas, usually with low prevalence although a few exceptions of high prevalence have been published. In Asia, a local prevalence of 6.6% and an intensity of 7–17 flukes/donkey and another higher 16.6% prevalence have been reported in Iran (77). A lower prevalence of 4.1% was detected in Irak (78). A 16.13% prevalence was found in donkeys of the Central Black Sea region of Turkey (79) and another of 2.6% in Ankara city (80). In Africa, low prevalences were found in several places of Egypt, including 0.37% in Assiut, 3.03% in Gharbia, 6.7% in Al-Fayoum, and 3.08% in Giza, and mid infection rates were also found in that country, such as 14.19% in Kafr El-Sheik, 17.05% in Giza, and 17.6% in Giza and Zagazig area, as previously reviewed (81). However, markedly higher prevalences have been reported in working donkeys from Ethiopia, such as 44.4% (82) and even 80.0% (83). Anyway, low prevalences of 1.5% (84) and 5.73% (85) have also been found in this country. So, the prevalence range of 8.6–52.4% in donkeys according to local zones of the Bolivian Altiplano fit well within the aforementioned data.

In the Americas, besides Bolivia, individual donkeys have been reported to be infected by the liver fluke in Mexico (86) and Argentina (87).

Intensities, measured by individual epg amount per donkey, do not differ from the ones in altiplanic sheep and cattle. The egg outputs ranged between 3 and 145 epg (mean 44.7 epg) in altiplanic sheep (49), whereas in highly infected sheep it reached an amount of 241 epg (= 1,203 eggs per 5 g) in another previous survey performed in the same endemic area (88). In altiplanic cattle, fascioliasis intensity was between 1 and 96 epg (mean 6.8 epg) (49).

Eggs of *F. hepatica* shed by naturally infected donkeys of the Bolivian Altiplano prove to be shorter but thicker than those found in naturally infected altiplanic sheep and cattle (**Table 4**). This agrees with present knowledge about the influence of the definitive host species on the size of fasciolid eggs (89). Interestingly, *F. hepatica* eggs found in palaeoparasitological studies of old donkeys (onagers) in the former Fertile Crescent area, at present Iran, during the Sassanid period, 224-651 AD, also fit in the egg size ranges found in present day donkeys (81).

The total number of liver fluke eggs expelled with feces by a donkey per day in the Northern Bolivian Altiplano, according to the amount of stools defecated by a donkey per day, may be estimated to be between 9,000 and 808,000 eggs/donkey/day. This range overlaps with similar estimates made for sheep and cattle in the same Northern Altiplano hyperendemic area (**Table 4**).

Finally, the susceptibility of the mammal host species to liver fluke infection is an important additional aspect to be considered in a One Health control initiative. If the animal is only able to survive a given period after the infection, its contribution to the disease transmission may be only for a short time and thus negligible when compared with other mammals able to transmit for the rest of their life, as is the case of sheep. If presenting a high immunological refractory response, liver flukes will survive less time inside that host and the contribution to transmission will consequently be restricted in quantity and time, as is the case of several cattle races. Fascioliasis is known to be different in equines and ruminants from the pathogenicity point of view, with the horse as the most resistant, the donkey as the most susceptible, and the mule showing intermediate profile although closer to those of the horse (90). Indeed, *F. hepatica* infection has been reported to be fatal for donkeys (86), and the pathology caused by a *F. gigantica* infection in donkeys has already been analyzed post-mortem in Egypt (91).

The lower adaptation of *F. hepatica* to the donkey is reflected in the longer prepatent period and mainly in the significantly lower number of cercariae produced by infected snails (**Table 1**). This is a priori not surprising, considering the phylogenetic distance between equines and ruminants. Indeed, the origin of *F. hepatica* is known to be linked to mid-sized ovicaprines (5). However, similar studies would be appropriate to assess whether the liver fluke may locally increase its adaptability to the donkey in other regions of the world, mainly eastern Africa and near-eastern Asia.

In mules, it was observed that a massive infection by 97 adult flukes was highly pathogenic and finally fatal, and that the maximum daily output was 794,500 eggs/mule/day (90).

By extrapolation from mule data, the maximum of 808,000 eggs/donkey/day estimated in the Altiplano would mean a massive infection of a donkey by 99 flukes. However, amounts higher than 70 epg were only found in three donkeys, that is the 15.0% of the infected donkeys and 3.6% of the total of donkeys analyzed. Hence, massive infections representing death risk may be only sporadically reached in altiplanic donkeys and probably only in old specimens kept in a highly contaminated place for long periods.

The donkey played a crucial role in human history (92). Of African origin, it was initially domesticated in East-North Africa and the Near East Asian region (93) and was introduced into the Americas by the Spanish "conquistadores" (5). Donkeys have a range of physiological and behavioral adaptations providing them with advantages to survive extreme conditions such as those of drought and high altitude environments. They spend less energy while foraging for food which results in a lower dry matter intake requirement. Low-quality diets are digested by donkeys very efficiently because of a highly selective feeding strategy. The lower energy costs of walking, the longer foraging times per day, and their ability to tolerate thirst, allow donkeys to access more remote, under-utilized sources of forage that are inaccessible to ruminants (94). Thus, although the main introduction purpose was the *in-situ* production of mules, donkeys easily adapted to the hypoxic conditions of the high altitude environments of the Andean valleys and altiplanos, where they were increasingly used by the high altitude communities because of the lower adaptation capacity of horses (95). The biochemical and hematological parameters in mules at high altitude have recently been assessed (90), and in donkeys the respective reference intervals have also been established (96), including the Creole donkey in South America (97). However, the analysis of these parameters in high altitude for Andean donkeys is still pending. Donkeys with ligated bile ducts exhibited the biochemical, pathological, and clinical features including motor coordination impairment, constipation, oedema, dermatitis, and jaundice, together with serum increased levels of total bilirubin and ammonia and decreased levels of total protein and calcium (98).

The donkey, as equines in general, lacks a gallbladder. This means that the bile produced by the liver goes directly to the small intestine, and it is not stored. Animals lacking a gallbladder tend to eat small amounts of food several times a day. Furthermore, the natural diet of grasses that donkeys ingest while grazing is not particularly high in fat, and consequently they digest and absorb it well. Thus, from the point of view of the liver fluke transmission, the eggs cannot be stored in the gallbladder and their faecally excretion should be more regular and constant than in animals having a gallbladder.

Infection by the liver fluke may underlie severe pathogenicity in donkeys. Indeed, the high efficiency of donkeys for energy storage and mobilization has also made them prone to pathological conditions associated with negative energy balance. Pathological consequences mainly concern excessive lipolysis, excessive hepatic triglyceride synthesis, and release into systemic circulation (dyslipidemia), with severe systemic damage. Mortality rates up to 80% have been reported. Dyslipidemias in donkeys occur secondary to physiological dysfunctions and pathological processes, among which mainly liver disease (99). Donkeys are particularly susceptible to hyperlipaemia, a disease caused by too much fat in the blood. When a donkey stops eating enough it goes into a state of negative energy balance. In this state, the body begins to use energy stored as fat deposits and consequently free fatty acids are converted to glucose in the liver under a system controlled by complex hormonal events. Donkeys are not able to appropriately turn off this fat release and blood circulation soon fills up with excess fat. Large amounts of fat trigger liver and kidney failure. Subsequently, all organs may fail leading to irreversible organ damage and death (99). Detection of an increase of plasma triglyceride levels (hyperlipemia) is crucial to assess fat circulation. Measuring the levels of other serum markers indicating liver dysfunction may additionally help. The alterations of serum biochemical and hematological parameters have recently been studied in the infection by fasciolids in both the acute and chronic phase of the disease (7) and may be used for donkey control within a One Health action.

CONCLUDING REMARKS

The experimental studies performed demonstrate that the donkey is a definitive host which assures the viability of the whole life cycle of *F. hepatica* in the Northern Bolivian Altiplano human hyperendemic area. Several aspects indicate, however, that *F. hepatica* does not reach in the donkey the level of adaptation it shows in sheep and cattle in this very high altitude area. This is illustrated by a few-day delay in egg embryonation, longer prepatent period despite similar miracidial infectivity and shorter patent period in the intramolluscan development, lower cercarial production per snail, different cercarial chronobiology, shorter snail survival after shedding end, shorter longevity of shedding snails, and lower metacercarial infectivity in Wistar rats. Thus, the role of the donkey in the transmission of the disease should be considered secondary.

Results of the field surveys proved that liver fluke prevalence and intensity in donkeys are similar to those of the main reservoirs sheep and cattle in this hyperendemic area. Worth mentioning, moreover, fasciolid egg shedding by a donkey individual proved to contribute to the environment contamination at a rate similar to sheep and cattle in that area. The very low percentage of donkeys with high infection burdens suggests a negligible liver fluke pathological impact on donkey survival and hence a long-term contribution of infected donkeys to fascioliasis transmission. In this endemic area, nevertheless, the pronounced lower number of donkeys when compared to sheep and cattle populations indicate that the epidemiological reservoir role of the donkey is also secondary when compared to these ruminants.

There is, however, an aspect of the donkey related to its management by the Aymara inhabitants which has a crucial repercussion in the transmission and epidemiology of the disease in the human hyperendemic area of the Bolivian Altiplano. Aymaras use the donkeys for the transport of goods and merchandises and for movements and travel from one locality/zone to another (**Figure 5**). Thus, this equine host

FIGURE 5 | In the Northern Bolivian Altiplano human fascioliasis hyperendemic area, **(A)** donkeys are used for the transport of goods, and **(B)** for movements and travel from one locality/zone to another. Orig. S. Mas-Coma.

species plays an important epidemiological role in the spread of the disease from one zone to another inside the wide endemic area. The participation of the donkey in this disease dissemination takes place in a dual manner. On one side by releasing fasciolid eggs along the routes they follow and on another side by passively transporting the lymnaeid vectors. According to what is known, lymnaeids may remain in dried mud stuck to the feet of animals, then go into hibernation or estivation, and are able to reactivate once in a new location following contact with water or sufficient humidity (5). This becomes even more important in a period as the present one in which the geographical distribution of the disease is spreading throughout the Northern Altiplano due to climate change (47).

Additionally, this fascioliasis spreading capacity of the donkey poses a problem for the implementation of a One Health initiative, because the donkey may give rise to movements of the parasite and the vector from one part to another of the zone selected for control intervention, or the introduction of the parasite and/or the vector from outside into that zone.

This study is an example of the appropriate and complete way to assess the participation of a mammal host species

in the transmission and epidemiology of fascioliasis in an endemic area. It illustrates the long-term research complexity and difficulties involved in the needed experimental tasks and field activities in a disease of very high heterogeneity as that caused by liver flukes. To clarify the participation rate of a definitive host species in the case of parasites of low definitive host species specificity as fasciolids by comparing with the participation rates of other definitive host species in the same endemic area becomes crucial to establish correct control measure priorities within a One Health action. All in all, the extreme complexity of a One Health action against fascioliasis becomes evident, further if it is considered that in the Northern Bolivian Altiplano the simplicity is maximum because the disease is only caused by *F. hepatica* and transmitted by only genetically uniform *G. truncatula*, and that the present study only concerns the One Health intervention axis of the mammal reservoirs.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All experimental research was performed with the approval of the Evaluation of Projects concerning Animal Research at University of Valencia (Organo Habilitado para la Evaluación de Proyectos de Experimentación Animal de la Universidad de Valencia) (A1263 915389140), strictly following the institution's guidelines based on Directive 2010/63/EU. Permission for animal research was additionally obtained from the Servicio de Sanidad y Bienestar Animal, Dirección General de Producción Agraria y Ganadería, Consellería de Presidencia y Agricultura, Pesca, Alimentación y Agua, Generalitat Valenciana, Valencia, Spain (No. 2015/VSC/PEA/00001 tipo 2). Animal ethics guidelines regarding animal care were strictly adhered. The study was approved by the Comisión de Etica de la Investigación of the Comité Nacional de Bioética, La Paz (Certificate dated 10 September 2007), Comité de Etica y Bioética de la Facultad de Medicina de la Universidad Mayor de San Andres, UMSA, La Paz - COMETICA (Resolución COMETICA No. 03/2019, dated 23 July 2019), and Comité de Revisión Etica (PAHOERC) of the Pan American Health Organization, PAHO, Washington DC (Dictamen Ref. No. 2018-02-0007, dated 10 September 2019). Written informed consent for participation was not obtained from the owners because all investigations were made after permission was obtained from local Aymara community chiefs (jilakatas and malkus), and with the consent of animal owners.

AUTHOR CONTRIBUTIONS

SM-C performed One Health studies, designed the protocols, wrote the manuscript, and obtained project funding. PB performed the surveys and diagnosis of animals. IRF performed the snail cultures, experimental snail infections, and chronobiological assessments. RA coordinated local research and logistics and participated in surveys. CM-B reviewed donkey physiology and metabolism and analyzed parasite-induced pathological repercussions. PA participated in surveys and diagnosis of animals. MAV performed the experimental mammal infections, statistical analyses, and obtained project funding. MDB performed the egg development studies, designed and participated in the experimental snail infections, participated in surveys, and obtained project funding. All authors contributed to the article and approved the submitted version.

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Pet Reptiles: A Potential Source of Transmission of Multidrug-Resistant *Salmonella*

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Salmonella spp. is widely considered one of the most important zoonotic pathogens worldwide. The close contact between reptiles and their owners provides favourable conditions for the transmission of zoonotic pathogen infections, and ∼6% of human salmonellosis cases are acquired after direct or indirect contact with reptiles. Moreover, antimicrobial resistance is one of the most important health threats of the twenty-first century and has been reported in *Salmonella* strains isolated from pet reptiles, which could entail therapeutic consequences for their owners and breeders. The aim of this study was to assess *Salmonella* carriage by pet reptiles in pet shops and households, and their role in the transmission of antimicrobial resistance, to inform the owners about the possible risks factors. During the period between January 2019 and December 2019, 54 reptiles from pet shops and 69 reptiles from households were sampled in the Valencian Region (Eastern Spain). Three different sample types were collected from each reptile: oral cavity, skin, and cloacal swabs. *Salmonella* identification was based on ISO 6579-1:2017 (Annex D), serotyped in accordance with Kauffman-White-Le-Minor technique, and antibiotic susceptibility was assessed according to Decision 2013/652. The results of this study showed that 48% of the pet reptiles examined from households and pet shops carry *Salmonella* spp. All the strains isolated presented resistance to at least one antibiotic, and 72% were multidrug-resistant strains, the most frequently observed resistance patterns being gentamicin-colistin and gentamicin-colistin-ampicillin. The present study demonstrates that pet reptiles could be a source of human multidrug-resistant *Salmonella* infection. In this context, the most optimal prevention of multidrug-resistant *Salmonella* infections necessarily involves strict control of the sanitary status of reptile pet shops and hygienic handling by the individual owners at home.

Keywords: reptile-associated salmonellosis, multidrug-resistant *Salmonella*, pet reptiles, One Health, zoonosis, *Salmonella*

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INTRODUCTION

Salmonella is widely considered one of the most important zoonotic pathogens worldwide. This pathogen has become an important public health concern with a significant economic impact, which has been estimated at 3.6 billion dollars annually (1, 2). In Europe, salmonellosis was responsible for 94,203 human cases, of which 9.3% corresponded to Spain (3). The infection usually causes self-limited diarrhoeal illness, although severe illness and death may occur, especially in children, elderly or immunocompromised adults (4). However, the overall epidemiological pattern of human salmonellosis cases is related to *Salmonella*-contaminated food from animal origin, especially eggs and poultry meat, and ∼6% of human salmonellosis cases are acquired after direct or indirect contact with reptiles (3, 4).

In the last few years, exotic reptiles have risen in popularity as pets, with a population of over 7 million in European households (5). This increase in "living presents for children" is resulting in a trade of non-conventional species around the world, with Europe as the leading reptile importer (6, 7). The close contact between reptiles and their owners provides favourable conditions for the transmission of zoonotic pathogens infections, constituting a public health concern, as these pets have been considered as potential *Salmonella* carriers (7–11). Reptiles are natural reservoirs of *Salmonella*, which can hold a wide variety of serovars simultaneously without symptoms (12–14). However, reptile-associated salmonellosis seems to be responsible for more serious complications, with invasive disease and hospitalisation, especially in children (14, 15). From a public health standpoint, pet reptiles represent a persistent source of salmonellosis in households (16–19).

In addition, *Salmonella* multi-resistant strains emerge as a potential concern for public health safety, with implications of increased disease severity, longer hospitalisations and higher cost rates (20, 21). In this context, the World Health Organisation deemed antimicrobial resistance (**AMR**) one of the most important health threats, which could cause 10 million deaths a year by 2050, ahead of other diseases such as cancer (22, 23). In this sense, *Salmonella* has been included in the World Health Organisation priority list of twelve antibiotic-resistant bacteria (24). Interest in the role of reptiles as an antibiotic-resistant *Salmonella* reservoir has increased in recent years (7, 25, 26). Moreover, AMR had been reported in *Salmonella* isolated from captive reptiles, and their release could entail therapeutic consequences for their owners and breeders (16, 27). Moreover, the widespread use of antibiotics against *Salmonella* has been described in the international trade of pet reptiles, in order to prevent economical loses, as well as in animal welfare in crowded farms and long-distance transport (28–30). Therefore, more information on AMR in pet reptiles is needed in view of One Health (31).

In this context, the objective of the present study is to assess *Salmonella* carriage by pet reptiles in pet stores and households in Eastern Spain (Valencia Region) and gain more in-depth knowledge of their role in AMR transmission, in order to inform the owners about the possible risk factors.

MATERIALS AND METHODS

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (32).

Sample Collection

During the period between January 2019 and December 2019, a total of 349 samples from 123 different reptile species from households and pet shops reptiles were taken. Previously, the owners were contacted by advertising the project through the University community (Universidad Cardenal Herrera-CEU, and Universidad Politécnica de Valencia) and veterinary clinics of the Valencian Region (Eastern Spain).

A total of 37 species were identified from the 123 of the reptiles sampled (**Table 1**). From these species, 12 were classified as chelonians (order *Chelonia*), 16 as lizards (suborder *Sauria*) and 9 as snakes (suborder *Ofidia*) (**Table 1**). According to the individuals sampled from each group, 43.9% (54/123), 39.0% (48/123), and 17.1% (21/123) were chelonians, lizards and snakes, respectively (**Table 1**).

For each individual, whenever possible, samples from oral cavity ($n = 114$), skin ($n = 123$), and cloaca ($n = 112$) were taken using sterile cotton swabs (Cary Blair sterile transport swabs, DELTALAB®) (33). All individuals sampled were healthy and none of them presented clinical symptoms such as diarrhoea at the moment of sampling. In addition, an epidemiological questionnaire was filled in. The questionnaire contained information related to species, diet and the number of reptiles that cohabit in the same terrarium. The diet was classified as food from animal origin (including live prey, fresh meat and frozen meat), food of vegetable origin (including fruit and vegetables) and processed (including commercially manufactured reptile food). Moreover, the number of reptiles coexisting in the same terrarium was recorded as reptiles that inhabit alone, or reptiles that cohabit with two or more reptiles.

Detection of *Salmonella* spp.

The collected samples were analysed within 24 h of collection according to ISO 6579-1:2017 (Annex D) recommendations (34). Samples were pre-enriched in 1:10 vol/vol Buffered Peptone Water 2.5% (BPW; Scharlau, Barcelona, Spain), and then incubated at 37 ± 1°C for 18 ± 2 h. The pre-enriched samples were transferred onto Semi-Solid Modification Rappaport Vassiliadis (MSRV; Difco, Valencia, Spain), and incubated at 41.5 ± 1°C for 24–48 h. For the positive plates, the cultures obtained in MSRV were inoculated onto two specific agar plates for *Salmonella* spp. detection: Xylose Lysine Deoxycholate Agar (XLD; Liofilchem, Valencia, Spain) and a selective chromogenic agar medium specific for detection of C8-esterase activity (ASAP, bioMérieux, Marcy l'Étoile, France), then incubated at 37 ± 1°C for 24 h. After incubation, one typical colony was collected and inoculated into a pre-dried nutrient agar plate (Scharlau, Barcelona, Spain), then incubated at 37 ± 1°C for 24 h. Finally, an API (API-20-E; bioMérieux, Madrid, Spain) biochemical test was performed to confirm *Salmonella* spp. The *Salmonella* isolates were stored at −80°C for further serotyping and antimicrobial susceptibility testing.

TABLE 1 | *Salmonella* isolated from reptiles in relation to reptile species.

Category	Reptile species	Number of reptiles examined	Number of positive reptiles (%)
Suborder *Sauria*	*Zonosaurus ornatus*	1	1 (100)
	Hemitheconyx caudicinctus	2	1 (50.0)
	Correlophus ciliatus	1	1 (100)
	Eublepharis macularius	21	11 (52.4)
	Paroedura picta	2	2 (100)
	Iguana	2	2 (100)
	Tupinambis teguixin	1	1 (100)
	Physignathus cocincinus	5	5 (100)
	Petrosaurus thalassinus	2	2 (100)
	Pogona vitticeps	4	4 (100)
	Chamaleo calyptratus	2	1 (50.0)
	Varanus glauerti	1	1 (100)
	Varanus albigularis	1	1 (100)
	Phelsuma grandis	1	0
	Pseudopus apodus	1	0
	Gecko gecko	1	0
Suborder *Ofidia*	*Python regius*	5	5 (100)
	Boa constrictor imperator	1	1 (100)
	Gongylophis colubrinus	1	1 (100)
	Acrantophis madagascariensis	1	1 (100)
	Elaphe guttata	7	5 (71.4)
	Spalerosophis diadema	2	2 (100)
	Lampropeltis getula	2	1 (50.0)
	Basiliscus plumifrons	1	0
	Heterodon nasicus	1	0
Order *Chelonia*	*Graptemys pseudographica*	5	3 (60.0)
	Testudo marginata	1	1 (100)
	Testudo hermanni	11	5 (45.5)
	Testudo horsfieldii	8	1 (12.5)
	Trachemys scripta elegans	5	0
	Pelusios	1	0
	Mauremys reevesii	3	0
	Cuora flavomarginata	1	0
	Pelomedusa subrufa	1	0
	Stigmochelis pardalis	1	0
	Testudo graeca	16	0
	Pseudemys nelsoni	1	0

Serotyping and Antimicrobial Susceptibility Testing

From each individual, the serotyping was performed from a cloacal strain, and when not present, a strain from the skin or oral cavity was analysed. Thus, all the strains were unfrozen and revived (ASAP) and the selected isolates were serotyped at the National Reference Laboratory for Animal Health (Algete, Madrid, Spain). The method used for serotyping was antigenic agglutination with specific antisera according to the White-Kauffmann-Le Minor scheme (35).

From all strains, *Salmonella* antimicrobial susceptibility was tested according to the European Committee on Antimicrobial Susceptibility Testing guidelines (36). *Salmonella* strains were inoculated into Mueller-Hinton agar (Scharlab, S.L.) to form a bacterial lawn and were allowed to dry for 30 min at ambient (25°C) temperature; then, the antibiotic discs were applied and plates were incubated at 37°C for 24 h. The antimicrobial agents selected were those set out in Decision 2013/652 (37), including three b-lactams: ampicillin (AMP, 10 μg), cefotaxime (CTX, 30 μg) and ceftazidime (CAZ, 30 μg); two quinolones: ciprofloxacin (CIP, 5 μg) and nalidixic acid (NA, 30 μg); one phenicol: chloramphenicol (CHL, 5 μg); one potentiated sulfonamide: trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 μg); one polymyxin: colistin (COL, 10 μg); one macrolide: azithromycin (AZM, 15 μg); one glycylcycline: tigecycline (TGC, 15 μg); one aminoglycoside: gentamicin (GM, 10 μg); and one pyrimidine: trimethoprim (TM, 5 μg). The source for zone diameters used for interpretation of the test and plates after incubation at 37°C for 24 h was the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/clinical_breakpoints/), and where this was not possible, according to Clinical and Laboratory Standards Institute (CLSI) indications (https://clsi.org/media/2663/m100ed29_sample.pdf) (38). The isolate strains were categorised as susceptible (S) or resistant (R), based on EUCAST imperative criteria (39). Multidrug resistance (MDR) was defined as acquired resistance to at least one agent in two or more antimicrobial classes (40).

Statistical Analysis

A Generalised Linear Model, which assumed a binomial distribution for *Salmonella* shedding, AMR and MDR, was fitted to the data to determine whether there was an association with the categorical variables (species and order or suborder of reptile, the habitat of the reptile, sample type, diet and number of reptiles that cohabit in the same terrarium). A reptile was considered *Salmonella* positive if one or more samples collected (oral cavity, skin and/or cloacal) tested positive. A $P \leq 0.05$ was considered to indicate a statistically significant difference. Data are presented as least squares means ± standard error of the least squares means. In addition, a descriptive analysis has been done to assess the subespecies isolated in this study. Analyses were carried out using a commercially available software application (SPSS 24.0 software package; SPSS Inc., Chicago, IL, 2002).

RESULTS

From all samples collected during this study, 25.2 ± 2.3% (88/349) tested positive for *Salmonella*. The type of sample taken was significantly associated with *Salmonella* carriage ($P = 0.000$), with higher positive samples from cloaca (38.0 ± 4.6%, 43/112) than from skin (22.0 ± 3.7%, 27/123) and oral cavity (16.0 ± 3.4%, 18/114).

Salmonella spp. was detected in 48.0 ± 4.5% (59/123) of individuals sampled, with significant differences between snakes (76.0 ± 9.3%, 16/21) and lizards (69.0 ± 6.7%, 33/48), compared to chelonians (19.0 ± 5.3%, 10/54) ($P = 0.000$).

The reptiles sampled in this study inhabited households with private owners (56.1%, 69/123), as well as pet shops (43.9%, 54/123) in the Valencian Region (Eastern Spain). Significant differences for *Salmonella* isolation were found among the different reptile habitats (owners vs. pet shops) ($P = 0.000$), being higher in pet shop reptiles (67.0 ± 6.4%, 36/54) than in household pets (33.0 ± 5.7%, 23/69).

Moreover, the number of reptiles cohabiting the same terrarium were known for 111 of the 123 reptiles analysed, 49 for pet shops and 66 for households. In pet shops, significant differences were found between the number of reptiles present in the same terrarium and *Salmonella* shedding ($P = 0.008$). Thus, 89 ± 7.4% of reptiles that cohabit in terrariums with two or more reptiles were positive for *Salmonella* (16/18), while 58 ± 8.9% of reptiles that inhabit terrariums alone were positive for the bacterium (18/31). In contrast, for private owners' reptiles, no significant differences were observed between reptiles that cohabit in terrariums with two or more reptiles or alone and *Salmonella* shedding ($P = 0.064$), 21.0 ± 7.8% (16/38) and 42.0 ± 8.0% (6/28), respectively.

The diet was significantly associated with *Salmonella* carriage ($P = 0.000$), with higher frequency in reptiles that were fed with food from animal origin (65.0 ± 5.6%, 47/72), in contrast to reptiles that were fed with food from vegetable origin, and processed (24.0 ± 6.6%, 10/42, and 22.0 ± 13.9%, 2/9, respectively).

From the 59 strains selected for serotyping, 51 were viable after culture and were serotyped. All *Salmonella* isolates were classified as *Salmonella enterica*. The most represented subspecies were *S. enterica* (56.9%, 29/51), *S. houtenae* (19.6%, 10/51), *S. diarizonae* (11.8%,6/51), *S. salamae* (9.8%, 5/51) and *S. arizonae* (2.0%, 1/51). Fifteen different serovars of *S. enterica* subspecies were identified (**Table 2**). From all the strains serotyped, one *Salmonella enterica* serovar was indeterminate.

Seventy-five out of 88 *Salmonella* strains isolated were viable after culture and included in the antimicrobial susceptibility study. All strains analysed were resistant to at least one out of the twelve antibiotics tested ($n = 75/75$). The highest percentages of AMR were found to COL (97.3%, $n = 73$), followed by GM (84.0%, $n = 63$), AMP (46.7%, $n = 35$) and TGC (42.7%, $n = 32$), AZM (26.7%, $n = 20$), NAL (12.0%, $n = 9$), CHL (9.3%, $n = 7$), SXT and TM (8.0%, $n = 6$, both), and finally CAZ (6.7%, n =5), CTX (4.0%, $n = 3$), and CIP (1.3%, $n = 1$) ($P = 0.000$). Antimicrobial resistance of the different *Salmonella enterica* serovars was summarised in **Table 3**.

Furthermore, a total of 72.0% (54/75) *Salmonella* isolates were resistant to two or more antimicrobials. No significant differences in MDR rates were shown between lizards (78.0%, 32/41), chelonians (73.3%, 11/15) and snakes (57.9%, 11/19) ($P = 0.206$). Although the type of sample collected was not significantly associated with MDR carriage, oral cavity (75.0%, 12/16), skin (77.3%, 17/22) and cloacal samples (67,6%, 25/37) ($P = 0.692$), significant differences were found between the type of sample and the different types of reptiles, except for lizards (**Table 4**, $P < 0.05$). Moreover, no significant differences were found between the habitat (pet shop and household), diet (food from animal origin, vegetable origin, and processed) and MDR *Salmonella* carriage ($P = 0.065$ and $P = 0.432$, respectively)

Overall, 25 different resistance patterns were observed. The combination of GM-COL (18.7%, 14/75) was the most frequently observed pattern, followed by GM-COL-AMP and GM-COL-TGC (10.7%, 8/75, both), COL alone (9.3%, 7/75) and GM-COL-AMP-TGC (8.0%, 6/75).

TABLE 2 | *Salmonella* serovars isolated from private owners and pet shops.

Sample origin	Subspecies	Serovar	*n*
Private Owner	*enterica*	Albany 8,20:z4,z24	2
		Cerro 18: z4,z23	1
		Lattenkamp 45:z35:1,5	3
		Newport 6,8:e,h:1,2	1
		Paratyphi 4,12:b:1,2	1
	diarizonae	60:r:e,n,x,z15	1
		48:z53	1
		50:z52:z35	1
		47:z10:z35	1
		47:i:z53	1
	arizonae	44:z4,z23	1
	houtenae	11:z4,z23	1
	salamae	13, 22:z29:1,5	1
Pet Store	*enterica*	Cotham 28:i:1,5	2
		Fresno 9,46:z38	1
		Hadar 6,8:z10:e,n,x	3
		Hvittingfoss 16:b:e,n,x	1
		Muenster 3,15:e,h:1,5	2
		Newport 6,8:e,h:1,2	2
		Panama 9,12:l,v:1,5	1
		Pomona 28:y:1,7	3
		Sandiego 4,12:e,h:e,n,z15	1
		Vitkin 28:1,v:e,n,x	4
	houtenae	11:z4,z23	6
		16:z4,z32	2
		16:z36	1
	salamae	30:l,z28:z6	2
		21:g,s,t	1
		52:g,t	1
	diarizonae	42:k: z35	1

n: Number of strains isolated.

DISCUSSION

The present study demonstrates that 48% of the pet reptiles examined from households and pet shops carry *Salmonella* spp. All the strains isolated showed resistance to at least one antibiotic and 72% were multidrug-resistant strains. To our knowledge, this is the first study in the literature evaluating the prevalence and the antimicrobial resistance of this zoonotic pathogen from a considerable sample size in pet reptiles of Eastern Spain (Valencia Region).

TABLE 3 | Percentage of antimicrobial resistance of *Salmonella enterica* isolated from pet reptiles.

Salmonella enterica serovars	*n*	AMP	CTX	CAZ	CIP	NA	CHL	SXT	COL	AZM	TGC	GM	TM
Albany	2	100	0	0	0	100	100	100	100	0	100	100	100
Cerro	1	0	0	0	0	0	0	0	100	0	0	100	0
Cotham	2	100	0	0	0	0	0	0	100	0	100	100	0
Fresno	1	0	0	0	0	0	0	0	100	0	0	100	0
Hadar	3	66.7	0	0	0	0	0	0	100	100	66.7	100	0
Hvittingfoss	1	100	0	0	0	0	0	0	100	0	100	100	0
Lattenkamp	3	0	0	0	0	0	0	0	66.7	0	0	33.3	0
Muenster	2	100	0	0	0	0	0	0	100	50	50	100	0
Newport	3	0	0	0	0	0	0	0	100	0	33.3	66.7	0
Panama	1	0	0	0	100	0	0	0	100	0	0	100	0
Paratyphi	1	0	0	0	0	0	0	0	100	0	0	100	0
Pomona	3	100	0	33.3	0	0	0	0	100	66.7	0	100	0
Sandiego	1	100	0	0	0	0	0	0	100	100	100	100	0
Vitkin	4	50	0	25	0	0	0	0	100	0	75	75	0

n: Number of samples. The resistance was determined by disc diffusion. AMP, Ampicillin (10 μg); CTX, Cefotaxime (30 μg); CAZ, Ceftazidime (30 μg); CIP, Ciprofloxacin (5 μg); NA, Nalidixic acid (30 μg); CHL, Chloramphenicol (5 μg); SXT, Trimethoprim-sulfamethoxazole (1.25/23.75 μg); COL, Colistin (10 μg); AZM, Azithromycin (15 μg); TGC, Tigecycline (15 μg); GM, Gentamicin (10 μg); TM, Trimethoprim (5 μg).

TABLE 4 | Multidrug-resistant *Salmonella* isolated according to the type of sample collected in the different type of reptiles.

Reptile classification	Type of sample	*n*	MDR rate
Suborder *Sauria*	Oral cavity	9	89.0 ± 10.5
	Skin	13	79.0 ± 9.4
	Cloacal	19	69.0 ± 12.8
Suborder *Ofidia*	Oral cavity	3	0.0 ± 0.0^{a}
	Skin	23	100.0± 0.0^{b}
	Cloacal	13	62.0 ± 13.5^{c}
Order *Chelonia*	Oral cavity	4	100.0± 0.0^{a}
	Skin	6	83.0 ± 15.2^{ab}
	Cloacal	5	40.0 ± 21.9^{b}

Data are presented as least squares means ± standard error of the least squares means. ^{a,b,c}Different superscripts in each file means significant differences in the same reptiles' classification with a $P < 0.05$. MDR, Multidrug resistance. n, Number of samples.

Reptiles have been known to be important carriers of *Salmonella* spp. worldwide, which may pose a health hazard as a source of human infection, particularly in children (4, 16, 41–43). However, there is a lack of consensus regarding the role of reptile shops on MDR *Salmonella* strains spreading. The results of this study showed that *Salmonella* strains isolated in reptiles from shops were twice as high as those from private owners (67 vs. 33%) (44, 45). This may be due to poor hygienic management of terrariums, especially in pet shops where they are usually occupied and ensuring a proper cleaning and disinfection procedure is not easy (45). This fact could facilitate that MDR strains remain in the shop environment among different reptile batches. In addition, the reptiles' stress related to cohabiting with individuals of different ages and origins could result in an increase in the bacterial infection, shedding in the terrarium and reptile-to-reptile transmission (45, 46). Conversely, reptiles from private owners are exposed to better hygiene practises and less stressful environments, leading to lower *Salmonella* shedding (45).

In reptiles, *Salmonella* is spread by faecal-oral route with an asymptomatic natural colonisation of the enteric tract, so in this study cloacal swabs collected were more sensitive for *Salmonella* isolation than other samples collected, such as skin or oral cavity. However, it is important to highlight that because *Salmonella* is excreted through faeces, it could contaminate the reptile's skin, oral cavity and the environment, being a source of infection for humans who handle the reptile or who are exposed to the reptile's environment (11, 46–50). Moreover, the *Salmonella* serovars most frequently detected in this study have been cited previously in reptile studies (51, 52), as well as in human outbreaks (3, 51–55). In addition, it has been reported that cold-blooded animals could be the major reservoir for the subspecies *houtenae*, *diarizonae*, *salamae*, and *arizonae* (56, 57).

The results of this study showed higher *Salmonella* prevalence among snakes and lizards compared to chelonians, in accordance with previous research (11, 31, 52, 58, 59). Particular attention has recently been given to snakes and lizards, as human interaction with these reptiles has become increasingly common in domestic environments (11, 60). In this sense, it is important to highlight that these reptiles are mainly fed with food from animal origin, which represents an important source of *Salmonella* (49, 61, 62). Previous studies carried out in the United Kingdom reported the important role of commercial feeder rodents in bacterial transmission among reptiles, and even their owners (63, 64). Thus, handling *Salmonella*-contaminated feeder rodents, as well as cross-contamination in the kitchen due to the rodents being kept in the freezer and thawed in microwaves, also in

contact with food for human consumption, have been linked to human *Salmonella* outbreaks (63, 65). In this context, to avoid *Salmonella* infection of reptiles, control of food products of animal origin has to be mandatory for the food suppliers (64).

On the other hand, special attention must be given to chelonians, due to their popularity as a pet for children. In this context, several countries, such as the US, have implemented strict bans in an attempt to curtail chelonian-associated salmonellosis; however, in Europe there are not many regulations to control its prevalence (53, 66). In the present study, *Salmonella* has been isolated from 18.5% of the chelonians tested. Seasonal effects, such as hibernation or season of sampling, have been speculated by previous studies to explain the low isolation rate of *Salmonella* in chelonians compared to other reptiles (31). Moreover, the diet may also have an important role (43, 49) because, as reported above, a large proportion of the chelonians are fed with food from vegetable origin or processed, and not from animal origin, frequently related with *Salmonella* outbreaks (11, 31, 67).

The increase in MDR *Salmonella* strains is of worldwide interest because it enhances the risk of therapeutic failure in cases of life-threatening salmonellosis in human and veterinary medicine (68, 69). In fact, it has been estimated that AMR could be the main cause of human mortality in 2050 (22). One of the most relevant outcomes in this study was the level of MDR isolated from pet reptiles, the most frequently observed resistance patterns being GM-COL and GM-COL-AMP. The high resistance against GM could be explained due to the indiscriminate use of aminoglycosides in pet reptile breeders, especially in the chelonian industry (28, 70). The use of GM as prophylactic *Salmonella* treatment in eggs to ensure sanitary conditions is a common practise in the US, the main supplier country of live reptiles for the EU (28, 71). Indeed, this practise has contributed to the finding of high-level plasmid-mediated gentamicin resistance in *Salmonella* isolated in its breeder farms (28).

Polymyxins have been widely used against Gramme-negative infections in animals, especially in animal production, the origin of several products involved in reptile feeding (72, 73). Currently, polymyxins such as COL represent the last line of defence against severe resistant infections in humans (72). Thus, it is highly restricted for animal infection treatments, and it is expected that resistance to this antibiotic will decrease in the coming years (74–76).

AMP was the third most frequent resistance shown in this study of *Salmonella* reptile strains, in line with a previous study conducted on species of gecko in Italy (10). This antibiotic is the most widely used in human medicine in Spain (76) and could thus be implicated in possible transmission of resistance from humans to reptiles, as a consequence of the direct and indirect contact between reptiles and their owners (75, 77, 78).

On the other hand, the level of resistance to NAL, CHL, SXT, TM, CAZ, CTX, and CIP in reptile *Salmonella* strains was relatively low. Resistance to CAZ contrasts with a survey conducted on geckos by Russo et al. (10), who showed a high resistance of *Salmonella* species to this antimicrobial, that is used in reptiles to treat infections or prophylactically after a traumatic injury (79). Moreover, it is important to note that fluoroquinolones (e.g., CIP) are the drugs of choice for invasive salmonellosis infections in humans (adults) and cephalosporins (e.g., CTX and CAZ) in children (21), although both are implicated in a reduced effectiveness of *Salmonella* treatment (77).

Should be essential to inform pet reptile owners about the risks of wrongly handling these animals (60). Proper hygienic management measures should be taken, such as the use of gloves when cleaning the reptile, and even during cleaning and disinfection of the surfaces that come in contact with the pet reptile (13). Moreover, it is important to thorough hand washing after handling the reptiles, especially the wounds due to bites or scratch (13). Reptiles and its feed should keep away from the kitchen and areas where the owners prepare their own food (64). Besides, before introducing a new reptile in the household, microbiological exams should be carried out to avoid cross-infection (13). Finally, it highlights the importance of extreme caution with young children and immunocompromised patients, because they are especially susceptible to *Salmonella* spp. infections (60).

CONCLUSIONS

The present study clearly demonstrates that pet reptiles could be a source of human MDR *Salmonella* infection. The problem of MDR in reptiles could start with the shops, where *Salmonella* presence is extremely high, and seems to be linked with the origin of reptile food. In this context, the most optimal prevention of MDR *Salmonella* infections involves strict control of the sanitary status of reptile pet shops and hygienic handling of the individuals in the household. Nevertheless, it is important to highlight that the number of included samples is relatively small, which may restrict the interpretation of our results to Eastern Spain. Further studies are needed to validate our results in a larger study sample.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (32). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

CM, OL, and SV: conceptualisation. OL, JV-G, CM, and SV: data curation. LL-R, CM, OL, and JV-G: methodology. CM and SV: investigation, writing–review and editing, and funding acquisition. CM and LL-R: writing–original

draft preparation. SV: project administration. All authors: have read and agreed to the published version of the manuscript.

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Tackling the Threat of Rabies Reintroduction in Europe

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Rabies is one of the oldest, most important zoonoses worldwide due to its extreme and inevitably lethal nature, causing one death every 9 min worldwide. Recent reports have demonstrated that the *Lyssavirus* continues more alive than ever, despite the control carried out against the virus throughout Europe. In this context, this work reviews the main immunological implications, transmission risk factors and current prevention measures for virus control in Europe, and especially in Spain.

Keywords: rabies, *Lyssavirus*, One Health, zoonosis, dog, bat, Europe

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INTRODUCTION

Rabies is one of the oldest and most important zoonoses worldwide, due to its extreme and inevitably lethal outcomes (1, 2). Although the enzootic transmission of rabies is through Carnivora (dogs, jackals, wolves, etc.) and Chiroptera (bats), it can spill over to other mammalian species such as humans, who can end up developing the disease (2). Each year, rabies is estimated to be responsible for 59,000 human cases, mostly in Africa and Asia, and ∼99% of human rabies cases are acquired after direct contact with dogs (3–5). The infection usually causes acute progressive encephalitis, and death eventually occurs if it is not treated before symptoms appear (1, 4). Although rabies remains one of the most feared and important threats to public health in the 21st century, it is considered one of the neglected diseases (2, 3).

WHAT HAVE WE LEARNED FROM RABIES DISEASE?

Rabies, a Long Etiological History

Rabies is caused by a group of neurotropic viruses of the genus *Lyssavirus*, belonging to the family *Rhabdoviridae* and order *Mononegavirales* (1, 6). This disease has been known since at least the 23rd century BC (Before Christ) in the Eshuma Code of Babylon (1). Moreover, the Greek ancient world called the disease "lyssa" (after the Greek goddess of madness, rage, and frenzy), due to the clinical signs it presented (6, 7). Since then, rabies has been present worldwide, except in Antarctica (4). The *Lyssavirus* contains a single-stranded RNA genome of negative sense, which encodes five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the RNA-dependent RNA polymerase (L), in the order 3′-N-P-M-G-L-5′ 7 (6). Currently, the International Committee on Taxonomy of Viruses (ICTV) has delineated the genus into seventeen species, plus one related virus not yet taxonomically assessed, and segregated into three phylogroups (I, II, III-IV) (**Table 1**) (8, 9).

TABLE 1 | Classification of the species of the genus *Lyssavirus* and geographical distribution.

Phylogroup	Specie	Virus name	Distribution
I	*Aravan lyssavirus*	Aravan virus	Central Asia
I	*Australian bat lyssavirus*	Australian bat lyssavirus	Australia
I	*Bokeloh bat lyssavirus*	Bokeloh bat lyssavirus	Europe
I	*Duvenhage lyssavirus*	Duvenhage virus	Southern Africa
I	*European bat 1 lyssavirus*	European bat 1 lyssavirus	Europe
I	*European bat 2 lyssavirus*	European bat 2 lyssavirus	Europe
	Gannoruwa bat lyssavirus	Gannoruwa bat lyssavirus	Asia
III-IV	*Ikoma lyssavirus*	Ikoma lyssavirus	Africa
I	*Irkut lyssavirus*	Irkut virus	East Siberia
I	*Khujand lyssavirus*	Khujand virus	Central Asia
II	*Lagos bat lyssavirus*	Lagos bat virus	Africa
III-IV	*Lleida bat lyssavirus*	Lleida bat lyssavirus	Europe (Spain)
II	*Mokola lyssavirus*	Mokola virus	Sub-Saharan Africa
I	*Rabies lyssavirus*	Rabies virus	Worldwide (except several islands)
II	*Shimoni bat lyssavirus*	Shimoni bat virus	East Africa
I	*Taiwan bat lyssavirus*	Taiwan bat lyssavirus	–
III-IV	*West Caucasian bat lyssavirus*	West Caucasian bat virus	Caucasian region
I	–	Kotalahti bat lyssavirus	–
Unclassified viruses	–	Taiwan bat lyssavirus	–

Fifteen of the seventeen lyssavirus species are hosted by bats (10). Indeed, bats are considered to be the ancestral hosts of lyssaviruses, and although the risk of human exposure is low, sporadic human rabies cases infected through a bat bite have been reported (10–13). Thus, from an epidemiological point of view, there are two epidemiological cycles: terrestrial rabies, maintained by domestic and wild carnivores, and rabies in chiropterans, where the virus is maintained in colonies of bats, both blood-sucking and insectivores or frugivores (14). Furthermore, it is difficult to differentiate the symptoms of the disease caused by any *Lyssavirus* species (10, 11).

Rabies, a Hazardous Disease

The disease has a marked neurotropic character and its action on the nervous system gives rise to a characteristic manifestation of disease with excitatory signs, hallucinations and hydrophobia (furious rabies), or signs of generalised paralysis and coma (paralytic rabies), as a consequence of generally fatal encephalomyelitis (1, 4). As there is no clinical treatment, post-exposure prophylaxis has been demonstrated to be the most effective method to control and prevent human rabies cases worldwide (4). Based on the importance of prevention, the European Union has been focused on the need for the elimination of wildlife rabies in European countries by 2020 (15). Moreover, rabies control under a One Health approach is a top priority for the World Health Organisation (WHO), Food and Agriculture Organisation (FAO), World Organisation for Animal Health (OIE), and Global Alliance for Rabies Control (GARC). These agencies have set the goal of the elimination of dog-mediated human rabies by 2030 (4).

Diagnostic Tools

Rabies diagnosis has been historically based on histopathological methods, such as cytoplasmic inclusion bodies detection (Negri bodies) (16, 17). At present, this methodology has been replaced by immunofluorescence. Also, when virus isolation has been necessary, rabies tissue culture infection or the mouse inoculation test has been used (16, 17). Currently, new techniques are being used for the diagnosis of the virus, such as flow cytometry (18), mass spectrometry (19), immunochromatographic tests and the polymerase chain reaction (PCR) (17).

HISTORICAL OVERVIEW OF RABIES IN EUROPE

Since the earliest descriptions of the disease, the presence of the virus in dogs and wildlife has been reported in European countries (20). In dogs, rabies was endemic until the enforcement of regulations in the keeping of dogs and development of the vaccination progressively eliminated rabies in dogs during the 20th century (20, 21). Although Pasteur developed the earliest effective vaccine against rabies in dogs in 1885, the development and implementation of domestic animal vaccination was carried out from the 1920s (1). With the development of these prevention measures, rabies in dogs was eliminated from European countries, except for the European part of Turkey (22).

However, during the 1940s and thereafter, the disease emerged and became established in the red fox (*Vulpes Vulpes*) population between Russia and Poland, spreading southwards and westwards, and at its maximum extension in the early 1990s

reached Southern France (2, 23). At this point, several European governments, with financial support from the European Union, rolled out oral rabies vaccination (**ORV**) campaigns in foxes, laying out the vaccines in baits, which managed to control vulpine rabies (22, 24). The development of ORV was a breakthrough for fox rabies control, and has resulted in the elimination of fox rabies from western Europe, with rapid progress also being made toward elimination in eastern Europe (22). Nevertheless, another rabies wildlife epidemiological cycle, maintained by the racoon dog (*Nyctereutes procyonides*), has been developed in the Baltic countries, many parts of eastern Europe, and Finland (25, 26). The racoon dog was introduced into the European part of Russia by the fur industry from eastern Asia in the first half of the 20th century, and has expanded throughout Europe (25, 26). The potential role of the raccoon dog in rabies transmission has been proven in rabies-free European areas, where the virus re-emerged due to this infected host (27). Therefore, the blueprint for eliminating wildlife-mediated rabies has also been extended to raccoon dogs (21).

Currently, most European countries are considered free of classical rabies, with confirmed rabies cases restricted to bats (1). The first evidence of infected European bat was reported in 1954 in Germany (28). From then on, the cases in bats were regarded as scientific curiosities, until 1985, when the intensive surveillance established after the first human case revealed more than a thousand bat rabies cases in Europe (28, 29).

Six lyssaviruses are reported to circulate in Europe: *European bat 1 lyssavirus* (EBLV-1), *European bat 2 lyssavirus* (EBLV-2), *Bokeloh bat lyssavirus* (BBLV), *West Caucasian bat lyssavirus* (WCBV), *Lleida bat lyssavirus* (LLEBV) and *Kotalahti bat lyssavirus* (KBLV) (30). Four of the viruses, EBLV-1, EBLV-2, BBLV, and KBLV, are associated with European bats of the family *Vespertilionidae* (30). Moreover, the other two lyssaviruses, WCBV and LLEBV, are linked to bats of the family *Miniopteridae* (30). Of these, only EBLV-1 and EBLV-2 have been reported to have caused rabies in humans, and more than 90% of the bat rabies cases have been associated with the bat species *Eptesicus serotinus (**E. serotinus**)* infected by EBLV-1 (12). The last European Union One Health 2018 Zoonoses Report confirmed bats as a reservoir for rabies in Europe, as it was present in 2% of the bats sampled (31). In this sense, taking samples from bats is the cornerstone to the knowledge of the dynamics of rabies within their hosts (32). To this end, guidelines on passive and active surveillance programmes were established by a European research consortium, Med-Vet-Net (33). Passive surveillance could include the investigation of sick or dead bats of all indigenous bat species, for testing lyssavirus infections using the detection methods. Moreover, active surveillance comprises the monitoring of free-living indigenous bats for the detection of viral RNA or virus-neutralising antibodies (33, 34). However, in Europe all bat species have been protected, which should be considered in the design and the undertaking of the surveillance initiatives (32). These studies showed that the principal ecological factors for viral persistence have been cross-species mixing and host migration patterns (10). Thus, the public health hazard of bat rabies must not be underestimated in Europe (31).

The Border Between Europe and the African Continent: The Special Situation of Rabies in Spain

Spain is a country located in South-Western Europe on the Iberian Peninsula. At the southern extreme, it is separated from Morocco in Africa by the Strait of Gibraltar sea channel. The two continents are separated by just fourteen kilometres. On the north-eastern border, the Iberian Peninsula is linked to the rest of Europe through the Pyrenees mountain range, which forms a natural border between Spain and France. The Canary Islands at the Atlantic Ocean and the cities of Ceuta and Melilla in the Northern African coast are also part of Spain.

The first evidence of rabies in Spain was reported in the first century AD (*Anno Domini*), in Roman times (35). Over the centuries, attempts to control rabies had been developing through measures such as the control of the movement of dogs and cats in 1786, or washing of bite wounds with soap in an attempt to prevent the disease in 1863 (35). During the 20th century, Spain focused all its efforts on the elimination of rabies with an anti-rabies programme, until 1966, when rabies was eliminated in dogs, and the country was declared free of rabies for the first time (2, 36). The European vulpine rabies never crossed the Pyrenees due to the French ORV programmes (2, 35). However, in 1975, a new outbreak was declared in Malaga, in southern Spain, which might been related to the geographical proximity with Africa and the intense traffic of people, and the most accepted hypothesis was an introduction from Morocco (35). During the epidemic, the disease spread to 133 animals, 76 dogs, 30 cats, two foxes and six of other species with one human fatality (37). To avoid dog-mediated transmission, health measures were established, which included a dogs census, vaccination of susceptible animals, physical confinement and quarantine measures for suspect animals, control and reduction in the number of stray dogs, and the monitoring of dog bite wounds (35). The outbreak continued until 1978, when Spain was considered rabies-free in terrestrial carnivores (36). Nevertheless, every year, the autonomous cities of Ceuta and Melilla report rabies cases, mostly imported from Morocco (36). Infected animals have been also imported into mainland Spanish territories disregarding the official border controls and eventually spreading to other European countries (38).

Since the control of rabies in non-flying mammals, there has been increasing attention to lyssaviruses associated with European bats (39). The first case of rabies in bats was recorded in 1987 in Valencia (eastern Spain), and some sporadic cases in bats have been reported since then (9). A study carried out from 1992 to 2000 showed that EBLV-1, or even other lyssaviruses with cross-reactive antibodies, could have been circulating among bats of different species in several areas of Spain (40). *Lyssavirus* antibodies were detected in four bat species: *Myotis myotis* (*M. myotis*), *Miniopterus schreibersii* (*M. schreibersii*), *Tadarida teniotis* (*T. tenioti*) and *Rhinolophus ferrumequinum* (*R. ferrumequinum*) (40). Moreover, these findings raised the possibility of *M. schreibersii* as a dispersion vector of the disease in southern Europe due to

its seasonal migrations, and the possible African origin of the EBLV-1 present in Spain due to the migrations of *T. teniotis* and *M. schreibersii* (40). During 1998–2003, a study of EBLV-1 viruses from *E. isabellinus* in southern Spain revealed the close communities in which EBLV-1 independently circulates, and yet distinct from other EBLV-1 strains circulating in the serotine bats (41). In addition, recent studies have demonstrated the geographical expansion of EBLV-1 in *E. serotinus* and *E. isabellinus* across Iberian Peninsula from Europe (12). In 2011, a new lyssavirus was confirmed in a *M. schreibersii* in the City of Lleida (north-eastern Spain), analysed as part of the rabies surveillance programme in Spain (42). The novel *Lyssavirus* was named LLEBV, and was classified into phylogroup III (42).

According to the previous premises, the emergence of sporadic human cases due to a bat or dog bite would be potentially feasible. Moreover, the dog is the main source responsible for a possible outbreak in Spain, as there is a high risk of importing an infected dog (36, 43). Spain has been officially free of rabies in non-flying mammals since 1978, although two imported cases of human rabies have been recorded in the last 30 years (13). Rabies surveillance data for Spain for the period of 1978–2020 reported 113 cases of rabies in dogs, of which 18 were stray dogs, and two both in cats and horses. However, these cases, except one, were limited to the cities of Ceuta and Melilla, which are located in North Africa (13). Likewise, rabies in wildlife was confirmed in 39 bats, as well as one fox in Ceuta, during this period (13). Thus, rabies remains a public health concern in Spain, and the main risk factors should be considered.

CROSS-IMMUNITY BETWEEN DIFFERENT LYSSAVIRUSES

A certain degree of cross-immunity between different lyssaviruses that can produce rabies has been described. Studies so far seem to show that such cross-immunity is possible between antigenic and genotypically "close" viruses, but not between more "distant" lyssaviruses belonging to different phylogroups (44). Thus, vaccination against classical canine rabies (phylogroup I) appears to confer protection against infection by lyssaviruses belonging to the same phylogroup, but not against European and African lyssaviruses belonging to phylogroups II and III.

New lyssaviruses are being discovered frequently, and although the risk is low, many of these viruses cannot be neutralised by antibodies from the traditional vaccine administered for the prevention of this disease. In view of the goal to end human deaths from dog-mediated rabies from the world by 2030, considering the elimination of dog-mediated rabies from a great part of Europe, consideration should be given to these other viruses that can produce rabies in humans and animals, for which the current vaccine is not protective. Therefore, some experts consider it essential to produce a universal vaccine that covers a broad spectrum of these lyssaviruses (45).

PRINCIPAL RISK FACTORS OF THE DISEASE IN EUROPE

Rabies is widely distributed worldwide, with only a few countries considered free of the disease (1). Since 1977, for rabies information exchange in Europe, the WHO Collaborating Centre for Rabies Surveillance and Research, Friedrich Loeffler Institute in Wusterhausen, Germany, has developed the Rabies Bulletin Europe (46). To date, several European countries have become rabies-free of terrestrial rabies, but rabies remains present in others (38). European rabies risk is based on distinct epidemiological situations: illegal importation of animals, travel to endemic regions, differences in dog vaccination programmes and wildlife rabies.

Illegal Importation of Animals

Although the successful elimination of dog-mediated rabies has been witnessed in the majority of European countries, it remains in certain European countries, as well as on the borders of Europe (47). In this sense, to ensure a sufficient level of safety in rabies, a European regulation establish strict measures on the movement of pet animals into a Member State from another Member State (48). However, illegal importation of pets through failure of border controls remains one of the main risks for rabies in Europe, leading to sporadic cases of rabies in free regions from endemic countries (13, 47, 49). From 2012 to 2020, five illegally imported dogs infected with rabies have been reported in France (2012, 2015, and 2020), Spain (2013) and the Netherlands (2013) from endemic areas (13, 36, 50, 51). These situations have occurred mainly because of importation of animals as pets from North Africa, disregarding the legislation on the movement of pet animals into Europe, and without disclosing the imported animals to the veterinary border control staff and customs officials (38, 43, 52, 53).

Spain presents geographical proximity and a commercial relationship with North Africa, with two cities in North Africa bordering with Morocco (Ceuta and Melilla), and a long-standing historical commercial traffic, fishing activities and labour migration between the Spanish-Moroccan border (54). Currently, rabies is endemic throughout Morocco, with human death reported yearly, remaining a serious public health problem (53). The domestic dog is considered the main reservoir and vector of the virus, and for this reason mass annual vaccination campaigns are conducted (53). However, the concept of responsible dog ownership does not feature in the Moroccan legislation, and vaccination coverage has not exceeded 6% of the total dog population (53). This situation affects Moroccan citizens, and the country has also become a source of rabies for neighbouring European states (43, 53). Every year, imported cases from Morocco are identified in Ceuta and Melilla (36), so compulsory and free of charge rabies vaccination programmes are carried out in these cities, in an attempt to reduce the risk (55). Imported cases pose a threat of rabies reintroduction into rabies-free areas, highlighting the need for reinforcement of border surveillance (38, 56).

Travel to Endemic Regions

Although the risk of a traveller contracting rabies is considered low, no prophylaxis or specific rabies vaccinations are needed for travel to endemic areas (52, 56). In 2019, four imported human rabies case were reported in Latvia, Spain, Italy, and Norway after having contracted the disease through a dog or cat bite while travelling in endemic areas (India, Morocco, Tanzania, and Philippines, respectively) (57). The imported cases of human rabies reflect lack of awareness by travellers visiting rabies-endemic countries, especially in Africa and Asia (43, 52, 57). Travellers to endemic areas should be aware of this, acting with caution and avoiding touching all animals, including puppies, to prevent animal bites (9, 57, 58). Moreover, the recommendations include local wound care, the vaccination, and if indicated, passive immunisation (9).

In addition, the risk increases when travelling with animals. In this sense, recent reports related lack of awareness by travellers of the risk posed by taking their non-vaccinated dogs abroad to an endemic region, or by adopting animals from these areas and taking them back home with them (43, 47).

Differences in Dog Vaccination Programmes

As there is no clinical treatment for this zoonotic disease, and dogs represent a main source of human infection, prevention by vaccination is the mainstay approach to avoid the spread of rabies (1). According to the WHO, vaccination coverage should reach 70% of the dog population to prevent rabies transmission (4). Regular application of the vaccines provides a more cost-effective basic instrument than the post-bite treatment of rabies cases, both short-term and longer-term (1). Indeed, the cost of a post-bite treatment in humans is around US$ 100, while dog vaccination costs are around US$ 0.50 per dog (59).

The WHO Expert Committee on Rabies considered mass vaccination programmes the basis of canine rabies control (46). Moreover, it was recommended that these mass vaccination programmes, which included the primary immunisation of all dogs between 3 months and 1 year old, should be carried out annually, and also emphasised the importance of including cats in these programmes (46).

The threat of rabies requires a constant state of alert, as an immunisation rate of <70% poses a risk to herd immunity in Europe (60). Furthermore, the geographical proximity to territories that are not free of rabies should be of special consideration, as it will pose a risk to the whole of Europe.

In addition to the lack of a coordinated vaccination programme, vaccine failures may occur, due to causes such as failure to administer the vaccine, poor quality rabies vaccine, and poor immune, health and nutritional status of the vaccinated animal (46). In addition, a single shot of vaccine is not enough to achieve long-lasting optimal immunity against rabies, leading to insecure rabies protection rates in a biting animal, despite a history of rabies vaccination (1).

Circulation of the Virus Among Wildlife population

Insectivorous bat species have often been employed as a mosquito biocontrol strategy, and it has been observed that a single bat is able to consume up to 600 mosquitoes per hour (61). Mosquitoes may serve as vectors of mosquito-borne diseases such as Zika virus, West Nile virus, malaria, or dengue (62). This highlights the important role of insectivorous bats in the reduction of mosquito populations and their implication in the protection against mosquito-related disease (63). In fact, in Spain bats are used as a control measure for mosquito populations, although some of the species used have been found positive for the presence of lyssaviruses (30, 64). Even though the risk of possible transmission of bat lyssaviruses to terrestrial mammals are very low in European countries, human and animal rabies cases following a bat bite have been reported (13, 65). Besides, exposure to bats should be regarded as a potential rabies risk in Europe, especially for spelunkers or bat biologists, who have a high risk of contact with rabid bats (38, 66). Recently, Italy has reported a case of WCBV in a cat, which lived near to bat colonies, that could represent the source of the virus (67). Ecological factors such as cross-species mixing and migrations of bat populations could be responsible for the viral persistence in European bats (10).

In Europe, the chiropteran rabies cycle is independent from the terrestrial rabies cycle (24). In 2019, the surveillance data confirmed five cases in animals in Europe, the highest proportion of the cases being in red foxes (two in Romania and one in Poland), followed by one case in a cow and in a wild boar, also in Romania (68). In the early 1960s, red fox rabies emerged in many European countries (13, 25). Over the past three decades, European fox-mediated rabies has been successfully controlled and eliminated in response to the effective implementation of the ORV programmes (13, 69). Countries can be officially declared free of terrestrial rabies when no cases have been detected for a 2-year period (15), a status achieved by European countries such as Finland and The Netherlands in 1991, Italy in 1997, Switzerland in 1998, France in 2000, Belgium and Luxembourg in 2001, the Czech Republic in 2004 and Germany and Austria in 2008 (13). Despite the success achieved, in Italy fox rabies re-emerged in 2009 (13), and a recent emergence and spread of fox rabies into previously rabies-free areas, due to insufficient cross-border cooperation and a false sense of security, highlights the need to stay alert (15, 49).

WILL RABIES EVER BE PREVENTED IN EUROPE?

Although rabies is 100% preventable, one person dies from it every 9 min worldwide (4). Although most of the cases are in Africa and Asia, the public health hazard of rabies must not be underestimated in Europe, because of all the risks their countries present (4, 31). Rabies prevention is based on three cornerstones: (1) improving education and public awareness; (2) access to mass dog vaccination; and (3) increasing the access to treatment medicines and vaccines (4).

Improving Education and Public Awareness

Despite 3 billion people continuing to be at risk of rabies worldwide, rabies continues to be a largely neglected disease (4, 70). In this sense, the European population often overstates the health security of rabies and surveillance should continue (71). Awareness and understanding of how to prevent the disease in animals, when there are reasons to suspect rabies, and what to do in the event of a bite, are crucial to save people (4), not only in endemic areas, but also in rabies-free countries.

In Europe, legislative measures for the control of this zoonosis have been implemented (72). Nevertheless, stricter laws should be enforced to raise awareness of the potential risks of rabies disease and prevent the introduction of rabies into rabies-free European countries (1, 73). These measures should be focused on raising awareness mainly on travel to endemic areas, travelling with pets, and importing and trading in animals from endemic areas (1).

Access to Mass Dog Vaccination

Vaccination of dogs is the key to curtailing rabies transmission between dogs, and from dogs to humans. For ethical, ecological and economic reasons, the slaughtering of rabies vectors should not be considered as a main control and eradication method (74). Prevention of the transmission of rabies at its source through dog vaccination is the most cost-effective strategy to save lives (4). Although rabies control programmes involve a high cost in many countries, the cost of pre-exposure dog vaccination is much lower than the current cost of the emergency post-exposure treatment (74). In fact, just 10% of the financial resources used in post-exposure treatment would able to significantly reduce, or even eliminate, the disease in the canine population, and consequently, the number of human cases (74).

In wildlife, ORV baits have proven to be an effective sophisticated strategy to control and even eliminate fox rabies in European countries, mitigating rabies risks to humans (75). Nevertheless, few eastern European countries and countries bordering Europe are still trying to control it, posing a risk to free-rabies wildlife countries (22). For this reason, the European oral vaccination financing programmes continue to be a necessary strategy, as they are the basis of rabies control in wildlife (22).

Increased Access to Treatment Medicines and Vaccines

The best method of rabies prevention is to avoid bites from mammals, especially high-risk rabies reservoir species (1). However, this is difficult to achieve, as accidental exposures are very common (1). Worldwide, post-bite vaccination incidence has been estimated at 29 million people in a year (9). Appropriate wound management and immediate and adequate post-exposure treatment is almost 100% effective in preventing human rabies deaths (4).

In any case, the severity of the process will mainly depend on the place and type of bite and the speed of the post-exposure prophylaxis (76). Therefore, the importance of knowing the risk to which the population are subjected and the ability to react is the basis for being able to save lives, and because of this, rabies is the only disease where there is a post-exposure immunoprophylaxis protocol in humans (77). Treatment consists of thoroughly washing the bite wound with soap, and if possible with viricidal antiseptic (e.g., povidone iodine or ethanol) for at least 15 min, followed by the administration of passive immunisation with immunoglobulins, and vaccination for active immunity (46).

FINAL COMMENTS AND FUTURE DIRECTIONS

The risk of importing dog rabies cases from North Africa is in increasingly important evidence. Given this situation, the health authorities must increase surveillance, especially at the entry points to the Iberian Peninsula, as motor vehicles entering can illegally transport animals that are sick or in incubation period from North Africa.

Although the risk of importing rabies cases from other territories within Europe is considered to be lower than in the case of North Africa, it should be considered that (1) the free movement of persons and goods in EU countries easily allows the entry of illegally-transported animals from countries that present cases of rabies in domestic animals (mainly dog and cat), and even in wild species (mainly red fox); (2) the lack of specific measures and some relaxation of controls concerning vulpine populations may facilitate outbreaks in countries declared free of this type of rabies; (3) it should not be forgotten that the spread of rabies from sick foxes to dogs is well established, and *vice versa*, as seems to have been in the case of Spain in 1977, when two cases of rabies in foxes were described in the province of Malaga during the 1975 rabies outbreak. These were fortunately isolated cases, unrelated to each other, probably due to the single infection from sick dogs or the consumption of carrion from carcasses.

It is considered necessary to maintain a single criterion throughout Europe regarding compulsory vaccination, which must be annual (depending on the authorised product used for immunisation, which must be applied by authorised veterinarians) to guarantee sufficient protective immunity against rabies, in any case systematically, and which must cover all dogs and cats without exception, as in the case of ferrets or raccoon dogs, which are also particularly susceptible to the rabies virus.

Given the recent cases of bat rabies described in Europe and the considerations set out above concerning the possibility of transmission to terrestrial species, as well as the arrival of animals from other latitudes, whatever the cause, a continuous state of alert of the competent authorities is required. In the same vein, the authorities responsible should encourage and provide adequate funding for specialised research groups to constantly improve the resources available for diagnosis and vaccination, and to search for new vaccine products capable of protecting humans and animal populations from possible exposure to these viruses, which sometimes have insufficient links with classical rabies virus to ensure adequate protection.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript and performed all the necessary literature searches and data compilation. All authors approved the submitted version.

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frontiers in Veterinary Science

BRIEF RESEARCH REPORT
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Virulence Plasmids of *Rhodococcus equi* Isolates From Cuban Patients With AIDS

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Rhodococcus equi is an animal pathogen and zoonotic human opportunistic pathogen associated with immunosuppressive conditions. The pathogenicity of *R. equi* is linked to three animal host-associated virulence plasmids encoding a family of "Virulence Associated Proteins" (VAPs). Here, the PCR-based TRAVAP molecular typing system for the *R. equi* virulence plasmids was applied to 26 *R. equi* strains isolated between 2010 and 2016 at the Institute of Tropical Medicine "Pedro Kourí," Cuba, from individuals living with HIV/AIDS. TRAVAP detects 4 gene markers, *traA* common to the three virulence plasmids, and *vapA*, *vapB*, and *vapN* specific to each of the host-associated plasmid types (equine pVAPA, porcine pVAPB, and ruminant pVAPN). Of the 26 isolates, six were positive to the *vapB* (porcine-type) marker, 4 (15.4%) to the *vapA* (equine-type) marker, and 1 (3.8%) to the *vapN* (ruminant-type) marker. Most of the isolates 14 (53.8%) were negative to all TRAVAP markers, suggesting they lacked a virulence plasmid. To our knowledge, this work is the first to report the molecular characterization of *R. equi* isolates from Cuba. Our findings provide insight into the zoonotic origin of *R. equi* infections in people and the potential dispensability of the virulence plasmid in immunosuppressed patients.

Keywords: *Rhodococcus equi*, aids, human immunodeficiency virus, Cuba, animal host-associated virulence plasmids, TRAVAP virulence plasmid typing, *R. equi* zoonotic infection

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INTRODUCTION

Rhodococcus equi is a well-known horse pathogen first described in 1923 by Magnusson as the causative agent of pyogranulomatous pneumonia and lung abscesses in foals aged <6 months (1). *R. equi* also infects other mammals including pigs, cattle, goats, cats, dogs, and humans, in which the infection is believed to be zoonotic (2). The first case of human infection was reported in an adult receiving immunosuppressive therapy in 1966. In the 1980–90's, *R. equi* emerged as an opportunistic pathogen associated with AIDS and other immunosuppresive conditions such as organ transplant chemotherapy and steroid therapy. In humans, *R. equi* causes purulent bronchopneumonia and occasional extrapulmonary pyogenic infections (3, 4).

R. equi is widelspread in nature, inhabits soil and colonizes the intestine of grazing animals and omnivores. The most likely transmission mechanism is inhalation of the bacteria in aerosolized dust particles. In addition, the accidental inoculation of the microorganism in injuries, mucous membranes or ingestion of contaminated food are other possible routes of infection (5). The pathogen is a facultative intracellular parasite capable of replicating within macrophages, thus evading host defense mechanisms (2, 6, 7).

The pathogenicity of *R. equi* is associated with the presence of a virulence plasmid encoding a family of "Virulence Associated Proteins" (VAPs) (2, 6–8). Two different *R. equi* circular virulence plasmid variants were initially characterized: the VapA-encoding pVAPA, found in virulent isolates of equine origin, and the VapB-encoding pVAPB, found in isolates showing intermediate virulence in mice, recovered from submaxillary lymph nodes of slaughtered pigs and human clinical specimens (2, 9–11). Recently, the VapN-encoding linear virulence plasmid, pVAPN, was identified in isolates from bovids and human clinical specimens (12). These *R. equi* virulence plasmids are animal host-specific, with pVAPA being associated with horses, pVAPB with pigs and pVAPN with cattle (ruminants) (2, 8, 11–13). Human isolates, in contrast, can carry any of the three animal host-associated plasmid types. This finding suggested that humans were accidental hosts for *R. equi* and that human infections had a zoonotic origin (2, 8, 13).

Molecular typing of *R. equi* is insufficiently developed and little is known about the epidemiology and transmission of this multihost pathogen (13). Thus far, there has only been one case report of a human *R. equi* infection in Cuba (14). In this study, we used the PCR-based virulence plasmid typing system developed by Ocampo-Sosa et al. (13), complemented with the novel vapN marker for the ruminant-associated virulence plasmid pVAPN (12), to analyze the virulence plasmid carriage of a series of human *R. equi* strains recently isolated from people living with HIV in Cuba.

MATERIALS AND METHODS

A case series study of 26 isolates of *R. equi* from 26 people living with HIV/AIDS was conducted during the period between January 2010 and December 2016. These isolates were obtained from all the patients with *R. equi* / HIV coinfection (one isolate/patient) admitted to the Hospital Center of Institute of Tropical Medicine "Pedro Kourí" (IPK) during this 7-year period. The isolates belong to the collection of the Department of Clinical Microbiology Diagnostic of the Hospital Center of IPK, Havana, Cuba. No data were available in the clinical records of previous contact of patients with farm animals or farm environments.

R. equi isolates were stored at −70°C and revived by incubation in Muller-Hilton broth at 37°C for 24 h. The colonies from the isolates were used for DNA purification using QIAmp kit (Qiagene, Hilden, Germany) following manufacturer's instructions.

The DNA extracted from each isolate (50 ng) was PCR-amplified in a reaction volume of 50 μL, using the *traA*-, *vapA*-, and *vapB*-specific primers of the TRAVAP typing system (13) updated with additional primers for the *vapN* gene marker for the novel pVAPN plasmid (12). The sequences of the primers are shown in **Supplementary Table 1**, PCR reactions were performed using the modifications reported elsewhere (15). Previously, the isolates were confirmed as *R. equi* based on the amplification of a fragment of the *choE* gene encoding cholesterol oxidase (16).

For the detection of each PCR product, 15 μL of the resulting mixture was run on a 1.6% agarose gel containing ethidium bromide. The visualization of the amplicons was carried out by exposing the gel to ultraviolet light in a transilluminator equipment (Macrovue 2011, LKB, Sweden).

The study was approved by the Ethic Committee of Institute of Tropical Medicine "Pedro Kourí" (CEI-IPK 51-18). It was conducted in accordance with national regulations and the Declaration of Helsinki. All participants signed a written informed consent.

RESULTS

The analysis of the 26 isolates of *R. equi* showed that 4 (15.4%) were positive for the *vapA* gene of the equine-associated pVAPA virulence plasmid. The porcine-associated pVAPB plasmid was identified in six isolates (23.1%), as determined by a positive reaction for the *vapB* gene marker. One isolate gave a positive PCR with the *vapN* marker, suggesting it carried the ruminant-associated pVAPN plasmid. The *traA* gene was detected in a total 11 of the 26 cases analyzed (42.3%). Most isolates in this investigation (53.8%) were TRAVAP negative (i.e., negative to the *traA*, *vapA*, *vapB*, and *vapN* PCR markers). The classification of the isolates according to the TRAVAP system is shown in **Supplementary Table 2** and the percentage distribution of each virulence plasmid type in the analyzed sample in **Supplementary Table 3**.

DISCUSSION

In this study, we provide the first molecular characterization of *R. equi* isolates in people living with HIV/AIDS in Cuba, with a focus on the molecular analysis of the animal host-adapted virulence plasmids using the PCR-based TRAVAP typing method (13). These plasmids, designated pVAPA, pVAPB, and pVAPN (2, 11, 12), enable *R. equi* to, respectively, colonize horses, pigs, and ruminants, while they can at the same time be indistinctly found in isolates recovered from non-adapted animal species (i.e., the case of humans) (2, 8). Because these plasmids are associated with specific animal hosts, it is in principle possible to infer the source of zoonotic transmission of human *R. equi* isolates by determining the type of virulence plasmid they carry. The molecular typing of the *R. equi* virulence plasmids has therefore recently emerged as a tool of great epidemiological importance that can provide valuable insight into the possible sources of human rhodococcal infections (2, 12, 13).

A previous survey involving a global collection of *R. equi* isolates showed that about half of human strains carried a pVAPB (porcine type) plasmid (13), suggesting that exposure to pig farms is a major risk factor. This is consistent with our finding that most of the virulence plasmid-positive Cuban isolates carred the pVAPB plasmid type. Our results are comparable to those of previous studies by Takai et al. in Thailand (10), and Ribeiro et al. in Brazil (17, 18), also involving human isolates from HIV-infected patients, which found that the most abundant plasmid type in these isolates was the porcine ($vapB^+$) plasmid type, pVAPB. This indicates that the infection by *R. equi* in the sample of Cuban people living with HIV/AIDS involves, like in other countries, the pig as the probable main source of transmission of the microorganism. Pork is one of the main sources of protein intake by Cuban people and many individuals are in close contact with pigs for breeding and meat trade.

The first of the Vap antigen-encoding virulence plasmids to be identified, by Takai et al. in 1991, was the equine-associated pVAPA (9, 19). This plasmid is as an essential virulence determinant of the pathogen in foals and mice (20). In the present work, four human isolates carrying the pVAPA plasmid were identified. This fact suggests that contact with horses might be another significant source of *R. equi* infection in humans in Cuba (transportation by horse-drawn vehicles is a common practice in rural areas), although of comparatively lesser importance than pigs or pig farm environments.

The *traA* gene, used in the TRAVAP typing scheme as an indicator of "presence of a virulence plasmid" (13), encodes a putative conjugative relaxase that is conserved in the three host-associated virulence plasmid types (11, 12). Ocampo et al. demonstrated that of 89 *vapA/B*-negative strains, 40 (44.9%) tested positive for the *traA* gene, indicating that they could also contain a plasmid (13). This $traA^+/vapAB^-$ genotype was later shown to correspond to strains carrying the bovine (ruminant)-associated pVAPN (12). In the study by Ocampo-Sosa et al., as many as 26% of the human isolates analyzed carried the bovine-associated $traA^+/vapAB^-$ (pVAPN) plasmid, suggesting that cattle farm environments are a significant source of human *R. equi* infections. This does not seem to be the case in Cuba, because in the series of 26 isolates analyzed here, only one (3.8%) was positive to the *vapN* marker. Our data are comparable to those of Ribeiro et al. in Brazil, who found that only two out of 74 *R. equi* strains isolated from the lungs of HIV/AIDS patients in the period 1997–2016 carried the $vapN^+$ (pVAPN) plasmid (17).

It must be noted that before the introduction of the *traA* marker in 2006 (13), many of the $traA^+/vapAB^-$ (pVAPN) strains—mostly bovine and human clinical isolates—were initially considered to be devoid of a virulence plasmid. This means that all the *R. equi* literature prior to the discovery of the *traA* (13) and *vapN* (12) gene markers, particularly those studies reporting "avirulent/plasmidless" human isolates, must be interpreted with caution. Nevertheless, a significant proportion of *R. equi* human isolates (23 to 43%) actually appear to be devoid of a virulence plasmid, as judged by their negative reaction to the TRAVAP markers (13, 15). This seems to be the case for most of the human strains analyzed in our study, in which the TRAVAP-negative genotype clearly predominated (53.8% of isolates). This high percentage of "avirulent/plasmidless" isolates could be explained by the increased susceptibility of people living with HIV, which may render the virulence plasmid dispensable for *R. equi* to cause an infection. About half of environmental soil isolates of *R. equi* lack a virulence plasmid (13), presumably because it imposes a fitness cost during saprophytic growth in the absence of host selection (2, 8). It is therefore possible to speculate that the *R. equi* strains found in human patients could primarily be non-virulent environmental isolates not directly associated with an animal host. Alternatively, the strains could have lost the plasmid during subculturing in the laboratory. Indeed, spontaneous virulence plasmid curing can be observed during *in vitro* growth of *R. equi* at 37°C (21, 22). However, it cannot be excluded that some of the 14 TRAVAP-negative isolates might harbor some unknown plasmid encoding novel virulent determinants.

Finally, one isolate was positive to *traA* but negative to each of the plasmid type-specific markers *vapA*, *vapB*, and *vapN*. A similar situation was previously observed in a human isolate in a study performed in the United State of America (15), suggesting that there might be microvariability in some of the virulence plasmids' PCR target sequences. An alternative and more interesting possibility is that this may reflect the existence of an additional virulence plasmid type(s) yet to be characterized. It is also worth noting that the $vapN^+$ isolate was negative for *traA*, suggesting the existence of microvariability in the *traA* sequence (the *traA* gene is actually a pseudogene in the pVAPN plasmid and is thus theoretically prone to genetic drift) (12). The possibility of TRAVAP gene marker variability may also in part account for the lack of detection of the virulence plasmid in a number of the "avirulent/plasmidless" *R. equi* isolates.

Our study has three limitations: (i) small sample size ($n = 26$), although the isolates were obtained in a period of 7 years and included all cases of *R equi* / HIV coinfection admitted at IPK hospital; (ii) we did not isolate/sequence the virulence plasmids, which would have provided insight into their genetic makeup or the variability underlying the unusual $traA^+/vapABN^-$ and $traA^-/vapAB^-/vapN^+$ TRAVAP genotypes; and (iii) a history of contact with farm animals, manure or occupational exposure to farm environments was not available from the clinical records. In any case, this study has value in that it is the first, to our knowledge, in reporting the molecular characterization of *R. equi* isolates in people living with HIV/AIDS in Cuba, and indeed in the Caribbean islands.

In summary, our study provides interesting insight into possible animal sources of *R. equi* infection in AIDS patients from Cuba, of value to public health authorities and, more generally, in the interpretation of the epidemiology of *R. equi* infections in people.

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 studies involving human participants were reviewed and approved by Comité de Ética del Instituto Pedro Kouri. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YA, EC, and JV-B: conceptualization and supervision. DS-R, YA-S, EI, JV-B, and YA: methodology. AP-H and HP-G: formal analysis. YA, EI, EC, and JV-B: data curation and writing—review and editing. DS-R, YA-S, AP-H, and HP-G: writing—original draft preparation. DS-R and YA-S: visualization. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.628239/full#supplementary-material

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BRIEF RESEARCH REPORT
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A Novel Sampling Model to Study the Epidemiology of Canine Leishmaniasis in an Urban Environment

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Background: Visceral leishmaniasis (VL) is one of the most important parasitic diseases in the world. The domestic dog is the main reservoir of zoonotic VL and a high prevalence of canine leishmaniasis (CanL) is associated with transmission of infection to humans. Here we describe the methodology used to obtain a rapid and representative sample of domestic dogs in the city of Posadas, Misiones, and compare the prevalence of *Leishmania* infection with a sample of shelter dogs.

Methodology: We used the city land registry to make a random selection of homes and systematically recruited 349 domestic dogs from the selected properties. We also included all dogs from the main canine shelter within the city. Dogs were examined by two experienced veterinarians who recorded the presence of clinical signs common in CanL using a standardized protocol. We extracted a blood sample from each dog and performed four different serological tests to reveal the presence of anti-*Leishmania* antibodies.

Results: After clinical examination, 145 domestic dogs (41.5%) and 63 (90%) shelter dogs had clinical signs compatible with CanL ($p < 0.001$). The seroprevalence among domestic dogs was 20.1% (95% CI 16.1–24.6) which was significantly lower than among the abandoned dogs (38.6%, 95% CI 27.7–50.6, $p < 0.001$). The spatial distribution of infected dogs was fairly homogenous throughout the city. Among domestic dogs, we observed a positive association between where the dog slept and presence of anti-*Leishmania* antibodies ($p = 0.034$). Of the seropositive domestic dogs 38 (54.4%) were asymptomatic.

Conclusions: Our findings demonstrate how seroprevalence results can be highly influenced by sampling methodology. We demonstrate how the land registry can be

used to estimate the prevalence of CanL in representative sample of domestic dogs in an urban setting, allowing decision makers to deepen their understanding the epidemiology of CanL in a timely and efficient manner for the development of plans to address both human and canine disease.

Keywords: visceral leishmaniasis, canine leishmaniasis, sampling, epidemiology, canine population

INTRODUCTION

Visceral leishmaniasis (VL) is one of the most important parasitic diseases in the world. The domestic dog is the main reservoir of zoonotic VL (ZVL) and a high prevalence of canine leishmaniasis (CanL) is associated with transmission of infection to humans (1, 2). ZVL is widespread in Latin America (3), where it is caused by *Leishmania infantum* (syn. *L. chagasi*) transmitted by phlebotomine sand flies of the genus *Lutzomyia* (Psychodidae) (4), and is an increasing public health problem (5).

It has a strong complex link with poverty mainly in rural and suburban areas but has been growing among urban populations in recent years (6). Emerging focuses of disease can be difficult to manage and have been associated with widespread culling of infected dogs although evidence supporting this approach is lacking (7). Many factors are thought to contribute to the expansion and urbanization of ZVL, such as environmental changes, changes in the ecology and biology of *Lutzomyia longipalpis* and population migration from rural to urban areas (8).

In Argentina, leishmaniasis is an emerging disease, with a growing number of human and canine clinical cases (9). In Posadas, in the province of Misiones, Argentina, the first human transmission of ZVL associated with dogs and *Lu. longipalpis* was reported in 2006 (10). The presence of *L. infantum* was further described in *Lu. longipalpis* sandflies and dogs using molecular methods (11, 12). In 2009, after human cases of Leishmania had been identified within the city, local health authorities were keen to understand the spread of canine infection within the city in a non-biased manner. Together with local health authorities, we set out to estimate the prevalence of *Leishmania* infection among the canine population in Posadas, by designing a rapid but robust sampling method to understand the epidemiology of the infection within the city using the available but limited resources. This current report shares the methods we used and demonstrate the importance of the adequate selection of the sample and its impact on the epidemiological interpretation of the disease.

In order to provide a valid estimation of disease prevalence it is necessary to include a representative sample of the total population. Ideally, estimates should be drawn through a simple random sample where all members of the population have an equal opportunity to be selected in the sample, but when it comes to dog populations it can be logistically difficult to determine the total population size let alone the probability of the inclusion of each animal in the sample (13). When recent canine censuses are available, prevalence studies are able to obtain a representative sample fairly easily by extracting a random sample from the census or performing the serosurvey alongside the census. Surveys of this type may be carried out due to the known presence of human or canine cases of VL (14). Even when canine censuses are present, limitations remain because they are rarely up to date and do not include free roaming dogs. Other options include the recruitment of animals through the local veterinary practices (15), or taking advantage of local rabies vaccination campaigns (16).

In Posadas, like many of the places in Latin America where CanL is widespread, there was no official census of canines that could be used to extract a random sample. Furthermore, there was a significant population of free-roaming dogs with no owner, and registration of the domestic dogs with local veterinary practices is far from comprehensive. For this reason, we needed to develop a novel strategy to extract a random sample of domestic dogs within the city, working with the information available. Furthermore, Misiones is one of the poorest provinces in Argentina and given the competing health problems in this area, we wanted to make the estimation as efficiently as possible, i.e., using the minimum number of dogs to make an accurate estimation with adequate precision.

In this paper, we describe the methodology used to obtain a representative sample of domestic dogs in the city of Posadas. We describe the presence of anti-*Leishmania* antibodies in domestic dogs and a systematic sample of dogs housed in a private dog shelter in the same region in the same time period. We identify variables related to infection in both populations. The main objective of this report is to share the methodology used to obtain a rapid and representative sample of domestic dogs in this urban setting. Furthermore, we aim to reveal how the sampling strategy can have major implications on the validity of epidemiological indicators for the development and monitoring of activities to address canine disease.

METHODS

Study Area

The surveys were conducted between 1st of October and 15th of November 2009 in the city of Posadas (27° 23′ S, 55° 53′ W), located in the southwest of Misiones province, northeast Argentina. In 2008, Posadas had an estimated population of 297,499 inhabitants. The surface area of the city is 324 Km^2, and it is characterized by a subtropical humid climate with an annual rainfall of 1,700 mm and an average temperature of 21.5°C.

Sample Size

To estimate the prevalence of *Leishmania* infection in domestic dogs in Posadas, we attempted to obtain a random sample of all domestic dogs in the city. We calculated a priori that we would need between 322 and 368 dogs to provide a reliable estimate with an error margin of ± 5%, at a 95% confidence level, using Epidat

3.1 software [Jan 2006; Servizo de Epidemioloxía da Dirección Xeral de Innovación e Xestión da Saúde Pública a Consellería de Sanidade (Xunta de Galicia) and the Pan American Health Organization (PAHO), http://dxsp.sergas.es]. This calculation required the following assumptions: (i) an estimated total dog population of 100,000 and (ii) an estimated prevalence of 30–40%. These assumptions were made after consultation with local government as to the expected size of the domestic dog population and observation for the prevalence obtained in similar settings in the region (3, 12).

Sampling Strategy

Given that it is unfeasible to carry out simple random sampling of the Posadas city domestic dogs, we used the City Land Registry Census to define our primary sampling unit. In this census, the city of Posadas is divided into almost 90,000 separate registries which mostly refer to individual properties. To allow for registry errors, non-urbanized entries on the land registry (e.g., parks, wasteland, recently flooded areas, etc.), dog owner's refusal to participate in the survey and ineligibility of the property (e.g., commercial properties or properties without a dog) we selected a total of 600 properties from the City Land Registry by simple random sampling. To minimize losses, it was decided a priori that if the initially selected property was ineligible (due to being commercial or not having a dog) the field workers would go to the residence located directly to the right. Furthermore, when more than one dog lived in the property all dogs were offered diagnosis but only one was selected at random to be included in the prevalence survey. Finally, 349 dogs were included in the survey. A detailed description of the selection process can be found in **Figure 1**. The location of the sampled dogs was plotted on the map of the city using a GPS point taken in the residence.

Finally, we also recruited 70 abandoned dogs from the "El Refugio" dog shelter, located in the outskirts of Posadas city. The sample constituted of all dogs present at the shelter when the study team visited. Repellent was not used at the dog shelter.

Clinical Observation and Data Collection

Each dog was examined by two experienced veterinarians who recorded clinical signs using a standardized protocol. Dogs were considered symptomatic if they had one or more of the following signs, common in canine leishmaniasis: alopecia, desquamations, skin ulcers, alterations in oral and nasal mucosa, apathy, weight loss, cachexia, bleeding, onychogryphosis, ocular signs (conjunctivitis, uveitis or any other), lymphadenopathy, hepatomegaly and splenomegaly. Other parameters recorded were age, sex, breed, the number of dogs in the residence, and whether the dog slept inside or outside the house. Dogs were grouped according to their age in four different groups: younger than 1 year, from 1 to 4 years, from 5 to 9 years and 10 years or over.

Biological Samples

After clinical examination 0.6 mL of peripheral blood was collected in a Multivette® 600 EDTA tube (Sarstedt AG & Co., Nümbrecht, Germany). Plasma was separated by centrifugation (5 min to 3,000 r.p.m. in a bench top micro centrifuge). Blood samples were analyzed at extraction time and the rest of the samples were stored at 4°C (Universidad Nacional de Misiones, UNaM) until shipment to the WHO Collaborating Centre for Leishmaniasis (WHO-CCL) in Madrid (Spain), where they were stored at −20°C until later analysis. All analysis were performed within 4 months of sample collection.

Detection of Anti-*Leishmania* Antibodies

Four different serological tests were performed to reveal the presence of anti-*Leishmania* antibodies, two of them based on the recombinant antigen rK39 (Kalazar Detect® immunochromatographic test, rK39-ICT, and an *in-house* enzyme linked immunosorbent assay, rK39-ELISA), and the other two based on whole antigen (a commercial direct agglutination test, DAT, and *in-house* immunofluorescence antibody test, IFAT) (17). A more detailed description of these methods can be found in the **Supplementary Data 1**. A dog was considered seropositive when it yielded a positive result for at least one serological method using total antigen (IFAT and/or DAT) as well as one using recombinant antigen (ICT whole blood and/or plasma and/or ELISA).

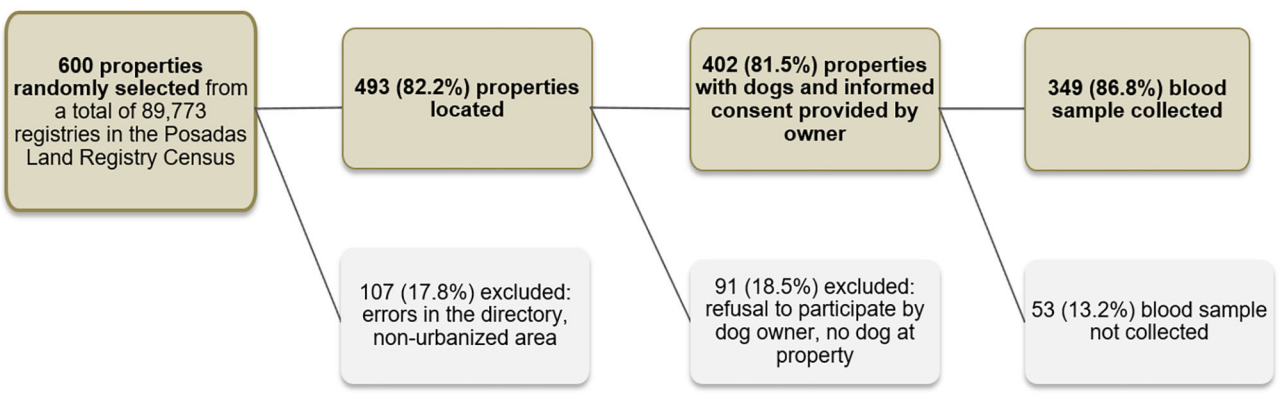

FIGURE 1 | Sampling strategy used to obtain a representative sample of domestic dogs in Posadas, Argentina, 2009.

Statistical Analysis

Data recorded from the surveys were entered into an Excel (Microsoft, Redmond, WA, USA) spread sheet and imported into Stata SE version 15.0 (StataCorp LLC, USA). The proportion of seropositive dogs was calculated with a 95% confidence interval. The relationship between the characteristics of the dog and seroprevalence was evaluated using Pearson's chi-square test and differences were considered statistically significant when the p-value was <0.05.

RESULTS

Of 600 randomly selected land registries, 107 (17.8%) were excluded because of errors in the registry or because they were non-urbanized plots of land. Of 493 registries with built properties, we further excluded 91 (18.5%) because there was no dog residing at the property, or because the dog owner refused to participate in the study. Finally, of 402 dog owners who agreed to participate and signed the informed consent, 52 (13.2%) were not included in the sero-survey because they did not attend the appointment with the veterinarians where biological samples were extracted and clinical signs recorded.

Finally, 349 domestic dogs were included aged between 4 months and 16 years, with a mean age of 5 years. The 70 dogs from the shelter were older, aged between 3 months and 10 years, with a mean age of 7.3 years ($p < 0.001$). **Table 1** shows the characteristics of the dogs from both survey populations. There were no significant differences in sex between the two populations, but the domestic dogs were more likely to be pure breed. After clinical examination, 145 domestic dogs (41.5%) and 63 (90%) shelter dogs had clinical signs suggestive of CanL ($p < 0.001$). The most common clinical sign observed in the dogs was lymphadenopathy, present in 124 (35.5%) of domestic dogs, and 52 (74.3%) of the shelter dogs. Other common signs observed in the dogs were onychogryphosis (51, 14.6% of domestic dogs and 43, 61.4% shelter dogs), desquamations (13.7 and 30%, respectively) and alopecia (12.6 and 52.9%, respectively).

The spatial distribution of the population in the study area are shown in **Figure 2**, along with the seroprevalence. We can observe a greater concentration of dogs, both with and without anti-*Leishmania* antibodies, in the north-east part of the city corresponding to the more densely populated city center. In less populated areas the selected dogs are more scattered.

The seroprevalence among domestic dogs was 20.1% (95% CI 16.1–24.6) which was significantly lower than among the abandoned dogs (38.6, 95% CI 27.7–50.6, $p = 0.001$). A detailed description of seroprevalence with regards to the sex, age group, breed, where dog slept, number of dogs in the house and clinical status of the dogs can be found in **Table 2**. We did not observe an association between seroprevalence and sex, age group and clinical status in either of the dog populations. Among domestic dogs, we observed a positive association between where the dog slept and presence of anti-*Leishmania* antibodies ($p = 0.034$). Among the dogs from the canine shelter, although there were few pure breed dogs, we observed that they were more likely to have anti-*Leishmania* antibodies ($p = 0.048$). Of the seropositive domestic dogs 38 (54.4%) were asymptomatic. Of the 204 asymptomatic domestic dogs, 38 (18.6%) were seropositive.

TABLE 1 | Characteristics of the dogs included in the sero-surveys according to sampling strategy.

Symptoms	Representative sample of domestic dogs *N* (%)	Systematic sample of shelter dogs *N* (%)	*P*-value[a]
Sex			0.756
Male	191 (54.9)	37 (52.8)	
Female	157 (45.1)	33 (47.1)	
Age group			<0.001
<1 year	21 (6.1)	2 (2.9)	
1–4 years	161 (46.5)	11 (15.7)	
5–9 years	118 (34.1)	29 (41.4)	
10+ years	46 (13.3)	28 (40.0)	
Breed			<0.001
Pure bred	94 (26.9)	5 (7.1)	
Mongrel	255 (73.1)	65 (92.9)	
Clinical status			<0.001
Asymptomatic	204 (58.4)	7 (10.0)	
Symptomatic	145 (41.5)	63 (90.0)	
Lymphadenopathy	124 (35.5)	52 (74.3)	<0.001
Onychogryphosis	51 (14.6)	43 (61.4)	<0.001
Desquamations	48 (13.7)	21 (30.0)	0.001
Alopecia	44 (12.6)	37 (52.9)	<0.001
Weight loss	39 (11.2)	22 (31.4)	<0.001
Hepatomegaly	32 (9.2)	22 (31.4)	<0.001
Splenomegaly	31 (8.9)	21 (30.0)	<0.001
Ocular signs	31 (8.9)	20 (28.6)	<0.001
Pale oral mucosa	28 (8.1)	14 (20.0)	0.002
Ulcers	25 (7.2)	13 (18.6)	0.002
Bleeding	11 (3.2)	4 (5.7)	0.292
Apathy	9 (2.6)	4 (5.7)	0.167
Alterations in nose mucosa	9 (2.6)	5 (7.1)	0.052
Cachexia	4 (1.2)	4 (5.7)	0.011
Total	349 (100)	70 (100)	

[a] *P-values are comparing the shelter dogs with the random sample of domestic dogs.*

DISCUSSION

The prevalence of antibodies for *Leishmania* in domestic dogs from the city of Posadas was 20.1% (95% CI 16.1–24.6), suggesting that at least one in every five domestic dogs in the city is infected or has been exposed to CanL. In the sample of shelter dogs, the observed seroprevalence of CanL was nearly double at 38.6% (95% CI 27.7–50.6). Although our sampling strategy was designed to obtain a random sample of domestic dogs, and not to make geographical comparisons between areas, it was noticeable that seropositive dogs show a fairly homogeneous distribution pattern throughout the city. Because the sampling unit we used was household, we can observe a greater concentration of dogs,

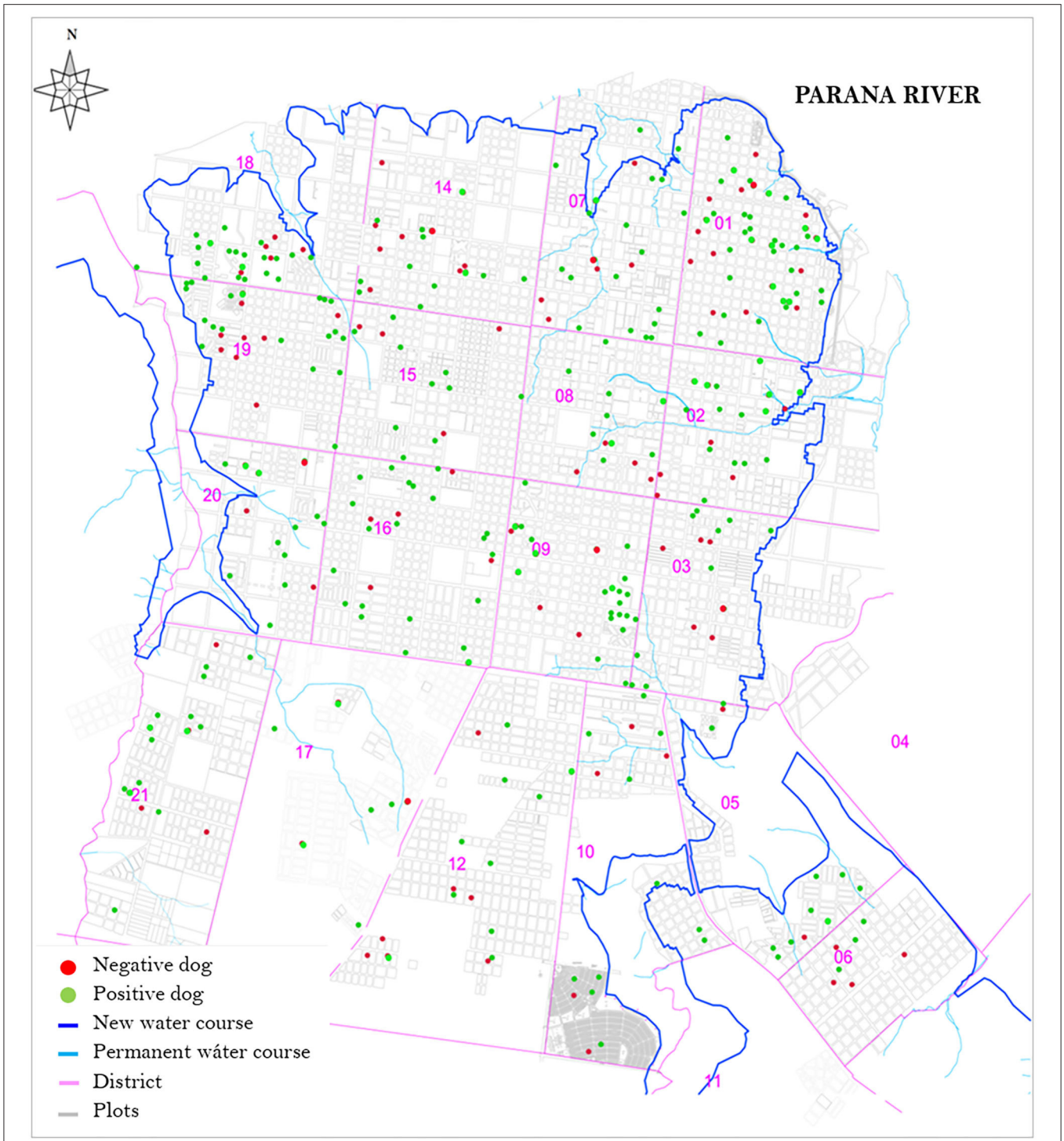

FIGURE 2 | Spatial distribution of the sample of domestic dogs included in the study, according to presence of anti-*Leishmania* antibodies. New water course: New permanent water course due to construction of the Yacyretá- Apipé hydroelectric power station downstream.

both with and without anti-*Leishmania* antibodies, in densely populated areas, while in less populated areas the selected dogs are more scattered. There is a stark contrast with suggestions of a relationship between the focal distribution of potential vectors in the presence of human clusters of infection in the city of Posadas (18). Even if the vector is clustered, the homogeneous distribution of the disease reservoir, perhaps associated with the high mobility of domestic dogs could have important

TABLE 2 | Seroprevalence of *Leishmania* infection according to sampling strategy and characteristics of the dogs, in Posadas, Argentina 2009.

	Representative sample of domestic dogs			Systematic sample of shelter dogs		
	Seropositivity *n* (%)	Total *n*	*P*-value	Seropositivity *n* (%)	Total *n* (%)	*P*-value
Sex			0.910			0.532
Male	38 (19.9)	191		13 (35.1)	37	
Female	32 (20.4)	157		14 (42.4)	33	
Age group			0.988			0.603
<1 year	4 (19.0)	21		0 (0)	2	
1–4 years	33 (20.5)	161		4 (36.4)	11	
5–9 years	23 (19.5)	118		13 (44.8)	29	
10+ years	10 (21.7)	46		10 (38.6)	28	
Breed			0.518			0.048
Pure bred	21 (22.3)	94		4 (80.0)	5	
Mongrel	49 (19.2)	255		23 (35.4)	65	
Where the dog slept			0.034			NA
Inside the house	7 (9.6)	73		–	–	
Outside the house	61 (23.2)	263		–	–	
Both	2 (15.4)	13		–	–	
Number of dogs in residence			0.075			NA
1	27 (15.7)	172		–	–	
2	32 (27.8)	115		–	–	
3	7 (15.9)	44		–	–	
>3	4 (22.2)	18		–	–	
Clinical status			0.429			0.567
Asymptomatic	38 (18.6)	204		2 (28.6)	7	
Symptomatic	32 (22.1)	145		25 (39.7)	63	
Total	70 (20.1)	349 (100)		27 (38.6)	70 (100)	

implications for control and may play an important role in the persistence of the disease in urban settings. Other studies have shown similar high prevalence of CanL is the surrounding area which has shown a decrease in recent years (19).

While seroepidemiological surveys are commonly used to measure the prevalence of leishmaniasis, there is a lot of variation in the methods used to recruit the canine study participants, and the details of how the sampling procedures are carried out is often lacking. For example, a recent study in Colombia (20) described how the sample being carried out in municipalities with increasing numbers of clinical cases reported by health authorities, but the procedures used to access the dog population is not provided. Some studies report using veterinary practices or samples extracted during a vaccination campaign (15, 16), but it can be difficult to determine if the sample if truly representative, Confounding factors could be that dogs participating in a vaccination campaign are less likely to be infected because of the behavioral factors associated with their care. Estimating the prevalence using a canine census can derive valid estimated of domestic dog infection but canine censuses are not always available, and it can be resource heavy ensuring they are up to date. In this study we used the land registry and simple random sample to obtain a representative sample of domestic dogs.

Our study is not without limitations. On the one hand the fact that we did not attempt to include free roaming dogs should be considered a limitation as it could lead to an underestimation of the true prevalence of CanL in the city. A higher prevalence of CanL among stray dogs compared to domestic dogs has been observed in other studies (21). We can expect free roaming dogs to be more similar to shelter dogs than domestic dogs, especially if we consider the higher prevalence in dogs sleeping outside the house, which has been observed in other studies (8). We observed a markedly higher prevalence among animals recruited from the shelter. A pilot study 3 years earlier with a convenience sample had observed a prevalence of 43.6% (12). Another limitation is the incomplete sampling of shelter dogs. We recruited all dogs from "El Refugio" animal shelter, which is one of two shelters in the city, and by far the largest shelter in the city. Although it is possible that the prevalence of CanL varies between the shelters, inclusion of this shelter was made due to interest and collaboration of the shelter owner, and the disease status of the dogs was not considered when deciding which shelter to include. The dogs from the canine shelter were significantly older, and more likely to be in poor health than the domestic dogs.

Many studies have pointed out the importance of identifying asymptomatic carriers in endemic areas (22–26). Some studies

show that asymptomatic dogs may comprise several between 50 and 60% of all infected dogs in the area (27–29). What is striking in this study is that despite having randomly selected the domestic dogs from a normal population a total of 54.2% of seropositive dogs were asymptomatic and only the 7.4% on canine shelter. Otherwise, 22.1% of symptomatic domestic dogs were seropositive. Therefore, a diagnosis based only on the appearance of clinical signs related to the disease could overestimate the number of cases infected by *Leishmania* in this area.

We must also acknowledge that the study was carried out in 2009, which is a major limitation if one objective is to know the prevalence of infection in the city, but the purpose of this report here is to describe our methods and demonstrate the importance of the adequate sample and its impact on the epidemiological interpretation of the disease. We did not collect information about the use of insecticide. Some studies have shown how this can influence disease presence and it should be considered in future studied (30, 31).

We did not use a multi-stage or stratified sampling strategy because we were interested in observing the geographical spread of the disease in the entire city. We could have improved the efficiency of our strategy by undertaking a multistage sampling strategy where we take a first stage random sample of larger geographical units (sectors or parishes), and then sample a specific number of land registries within this sample. This would be logistically simpler and reduce the number of movements of the team within the city, which can be important especially when the geographical area is large. In this study we used the land registry census as our primary sampling unit, and there was a significant proportion of errors in the registry. Another attractive option would be to carry out geospatial sampling and obtain a the random selection of properties or animals using randomly generated GPS points or grid-squares (19, 32, 33). Such methods are highly valid but require users to be competent in using Geographical Information Systems (GIS) which may be a challenge in many low-resource settings. Here we propose a simpler strategy using available registries which can allow local stakeholders to obtain a valid estimation of disease prevalence with limited technology.

In response to the results from the study and the model used for epidemiological investigation of the disease, preventative and curative services for canine disease were strengthened throughout the municipality of Posadas. The local government implemented a control program where dog owners from low-income families were offered a free screening service for canine leishmaniasis. It is estimated that 40% of the dog owner population took part in the scheme. More recently, the city established a Centre for Vectorial and Zoonotic Diseases, with the support of the Municipality and the Ministry of Health. Tackling CanL in the city is a key line of work within the center.

CONCLUSIONS

Overall, the two prevalence estimations observed and the spatial distribution of the domestic dogs with anti-*Leishmania* antibodies suggest that this focus of disease was well-established in 2009. Our novel sampling strategy allowed us to obtain a representative sample of domestic dogs in an urban setting. The prevalence of CanL obtained was much lower than the prevalence among dogs from the animal shelter. Although this difference is not surprising, it is important to acknowledge how seroprevalence results are influenced by sampling methodology, and the fact that samples using convenience sampling may under or overestimate disease prevalence and can lead to misinformed decisions about control and management. We demonstrate how a representative sample can be obtained in a timely and efficient manner, allowing decision makers to deepen their understanding the epidemiology of CanL for the development of plans to address both human and canine disease, while protecting animal welfare.

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 study was reviewed and approved by the bioethics committee of the Ministry of Health in Misiones, Argentina. (Expediente: 6106-135-08); Comité de Bioética, División de Zoonosis de la Subsecretaría de Atención Primaria y Salud Ambiental Salud del Ministerio de Salud de Misiones; Resolución Ministerial No.: 2332/2008). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

IC, LA, and FB-L conceived the study. LA, LP, FB-L, and IC designed the study. LA and LP drafted the original draft, analyzed data and realized formal analysis and interpretation of data. IC and FB-L critically reviewed it and contributed to its design. FB-L and ED supervised and coordinated the study. LA, MG, and ED contacted dog owners to enroll the study. LA, MG, JN, FB-L, and IC carried out clinical examination and obtained biological samples from the dogs. JN and MG designed the protocol for clinical scoring of the dogs. IC, LA, and JN performed serological diagnosis. All authors revised and edited the different draft versions, and read and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.642287/full#supplementary-material

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Effectiveness of Fenbendazole and Metronidazole Against *Giardia* Infection in Dogs Monitored for 50-Days in Home-Conditions

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A field trial performed in-home conditions was conducted on 24 dogs naturally infected with *Giardia*, in order to compare the efficacy of fenbendazole and metronidazole. Animals were allocated in groups randomly in order to obtain two groups of 12 dogs each with similar parasitic loads of *Giardia* cysts: dogs in Group A were treated with fenbendazole (Panacur®, Intervet Italia Srl) administered at the dose of 50 mg/kg orally once a day for 5 consecutive days, dogs in Group B were treated with metronidazole (Flagyl®, Zambon Italia Srl) administered orally at the dose of 50 mg/kg, once a day for 5 consecutive days. All the dogs that were shedding *Giardia* cysts after the first treatment (Day 0) were retreated (either at Day 7 or at Day 14 or at Day 21) until a negative result was obtained with the same treatment. Additionally, all the dogs were re-examined at Day 50. All the dogs were tested for the presence of *Giardia* cysts using a fecal flotation method (FLOTAC). The percent efficacy of the treatments (A and B) was calculated at each sampling point (Days 7, 14, 21, and 50) as reduction in mean *Giardia* cysts. After the first therapy, on day 7, 4/12 (33.3%) dogs tested positive for *Giardia* cysts in the Group A and 5/12 (41.7%) in the Group B. Efficacies at (Days 7, 14, 21, and 50) of the treatments against *Giardia* infection were 80.9, 94, 100, and 97% in the Group A and 70.8, 99, 100, and 97.1% in the Group B. Statistically significant differences were not observed between the efficacy of Fenbendazole and Metronidazole against infection by *G. duodenalis* ($P = 0.686$). Molecular analysis revealed full homology (i.e., 100% with JN416550) with the canine specific assemblage D in six positive dogs. Different hypotheses might explain the re-appearance of the *Giardia* cysts in some dogs after treatment, e.g., re-infection from the home environment, the correct medication given by the owners, the diet, as well as treatment failure, but also biological issues related to the intermittent excretion of *Giardia* cysts.

Keywords: *Giardia*, dogs, 50 days post-treatment, fenbendazole, metronidazole, assemblage

INTRODUCTION

Giardia duodenalis (syn. *Giardia lamblia*, *Giardia intestinalis*) is the causative agent of giardiosis and one of the most commonly found parasite in dogs throughout the world (1, 2). Molecular studies have shown that *G. duodenalis* comprises at least eight distinct genetic assemblages (A–H), of which assemblages C and D are found exclusively in dogs, while *Giardia* assemblages A and B have zoonotic potential (3–5).

Infection by *G. duodenalis* in dogs has a worldwide distribution with prevalence rates that vary according to the test population, the diagnostic method used and the geographical area (6, 7). In Italy, prevalence values range between 11.1 and 28.9% in northern (8–10), between 6.4 and 21.4% in central (11–14) and between 7.7 and 14.2% in southern regions (15, 16).

The diagnosis of *Giardia* infection in dogs may pose a challenge, due to a low infectious dose and marked persistence of cysts in the environment as well as a fluctuating excretion of the cysts in the feces that makes the monitoring of this parasite problematic (17, 18). Traditional tools for identification of *Giardia* cysts include fecal smear, zinc sulfate flotation technique (17, 19), immunofluorescence assay (IFA; the gold standard), immunochromatography, enzyme-linked immunosorbent assays (ELISA), and molecular analyses that permit the different *G. duodenalis* assemblages to be distinguished (17, 20, 21). Among the copromicroscopic techniques, a recent study reported a high sensitivity and specificity of FLOTAC technique with zinc sulfate for diagnosis of *Giardia* cysts infection in dogs (19, 22).

Also the control of *Giardia* infection in dogs is prone to a number of issues. Several compounds have been tested against *Giardia* infections in dogs as benzimidazoles, in particular fenbendazole, and metronidazole that poses activity against the parasite (23–28). Other studies reported the efficacy of ronidazole, nitazoxanide, azithromycin, tinidazole, and ipronidazole and other drugs such as quinacrine, furazolidone, in reducing *Giardia* cysts shed by infected dogs (17, 29–32). Another option recommended by the European Scientific Counsel Companion Animal Parasites (ESCAAP) is the combination of febantel (a prodrug metabolized *in vivo* to fenbendazole)/pyrantel/praziquantel at the standard deworming dose (15.0 mg/kg of febantel, 14.4 mg/kg pyrantel, 5.0 mg/kg praziquantel) repeated once daily for 3 days (33). Recently, secnidazole, a molecule that is used for the treatment of giardiosis in humans, has been reported as an effective drug for the treatment of clinical canine giardiosis (34). However, fenbendazole and metronidazole are used routinely to treat giardiosis in dogs and are the only compounds registered in most European countries.

Resistance to antiparasitic drugs has been often suggested to explain treatment failure due to the incomplete parasite removal following treatment or other underlying diseases (e.g., inflammatory bowel diseases, bacterial overgrowth, coinfection with other organisms). Shampooing of dogs (e.g., with a product containing chlorhexidine digluconate) at the beginning and at the end of antiprotozoal treatment is also recommended to reduce re-infections through fecal material on the fur (30). Despite treatment recommendation with fenbendazole for eliminating *Giardia* cysts in dogs, currently, unpublished data from veterinary practices are showing a low efficacy of this drug in eliminating the infection. Taking into account that the ESCCAP Guidelines recommends fenbendazole (1x/d, 50 mg/kg for 3 days) and metronidazole (2x/d, 25 mg/kg for 5-7 days) for the treatment of *Giardia* infections in dogs (33), the purpose of the present study was to reassess the efficacy of these two specific drugs for the treatment of giardiosis in owned dogs, monitored for 50 days in home-conditions.

MATERIALS AND METHODS

Animal Testing and Study Design

A field trial performed in-home conditions was conducted in 2018–2019 on 24 owned dogs naturally infected by *Giardia* spp. and living in the Campania region, southern Italy. The dogs included in the study were referred to the Laboratory of Parasitology and Parasitic Diseases at the Department of Veterinary Medicine and Animal Production, University of Naples Federico II, for coproparasitological analysis. Dog owners were informed about the study protocol and they gave their consent to inclusion of their pets. Animals were allocated in groups randomly in order to obtain two groups of 12 animals each with similar parasitic loads of *Giardia* cysts: group A-fenbendazole (mean cyst per gram of feces-CPG = 20,995) and group B-metronidazole (CPG = 18,580). The cysts were enumerated using a fecal flotation method (Flotation, Translation and Centrifugation) FLOTAC technique (see Laboratory Analysis). In addition, the dogs had the following characteristics: 11 males (five long-haired and six short-haired dogs) and 13 females (six long-haired and seven short-haired dogs), aged between 2 months and 5 years, living indoor but with access to outdoors. All the dogs included in the study did not live in the same household with other animals. Exclusion criteria were animals treated with any antiparasitic drug in the 2 weeks before, animals with any aggressive behavior and animals showing clinical signs of any other diseases. For each animal included in the study, data regarding age, fur length, clinical signs and consistency of fecal samples were recorded and analyzed. For ethical reasons, no untreated control-group of animals was available. Dogs in Group A were treated with fenbendazole tablets (Panacur®, Intervet Italia Srl) administered at the dose of 50 mg/kg orally once a day for 5 consecutive days. Dogs in Group B were treated with metronidazole tablets (Flagyl®, Zambon Italia Srl) administered orally at the dose of 25 mg/kg, orally, twice a day for 5 consecutive days. All the dogs started the first treatment at Day 0. All the dogs that were shedding *Giardia* cysts after the first treatment were retreated (either at Day 7 or at Day 14 or at Day 21) until a negative result was obtained with the same treatment. Additionally, all the dogs were re-examined at Day 50. Treatment efficacy was evaluated based on the mean CPG found in the fecal samples on Day−5 (pre-treatment) and on Days 7, 14, 21, and 50 (post-treatment). Additionally, each dog was subjected to shampooing with chlorhexidine digluconate at the beginning and at the end of each treatment applied. Furthermore, each owner was suggested to follow some basic rules to avoid the

reinfection by cleaning and drying the environment, the use of clean utensils for feed and water and proper disposal of feces (33).

Laboratory Analysis

Dogs were initially identified and verified as infected with *Giardia* spp. and then subsequently analyzed at Day−5 (pre-treatment) and at Days 7, 14, 21, and 50 after the first treatment applied (Day 0). The fecal samples analyzed at each sampling point were represented by pools collected on a daily basis for 2 days (prior to the treatment date). Analyses were performed within 24 h of sampling. At each sampling day the feces for all dogs were tested for the presence of *Giardia* cysts using the FLOTAC technique with an analytic sensitivity of 1 cyst per gram (CPG) of feces (19). Each sample was analyzed using zinc sulfate (specific gravity = 1.350).

Fifteen samples collected at Day−5 were fixed in 2.5% potassium bichromate (dilution 1:4) and processed for molecular studies. Briefly, genomic DNA was extracted using the FastDNA SPIN Kit for feces (MP Biomedicals, Solon, Ohio, USA). A nested PCR assay was used to amplify a 511 bp fragment of the beta-giardin gene according to a published protocol (35). DNA from axenic cultures of *Giardia duodenalis* strains of assemblage A (WB, genotype AI) and assemblage B (GS, genotype BIV) was used as positive controls in PCR assays. PCR products were purified using spin columns (QiaQuick PCR Purification kit –Qiagen) and sequenced from both strands.

Treatment Efficacy

The percent efficacy of the treatments (A and B) was calculated at each sampling point from the trial (Days 7, 14, 21, and 50) according to the following formula [adapted from (36)].

$$\%\text{Efficacy} = \frac{\text{Mean CPG day} - 5 - \text{Mean CPG day } 7}{\text{Mean CPG day } - 5} \times 100$$

$$\text{CPG} = \text{cysts per gram of feces.}$$

Statistical Analysis

Statistical analysis was performed using Windows SPSS® (version 17.0). The non-parametric Mann-Whitney U test was used to determine the level of significant difference between groups of treatment (A, B).

Assessment Criteria and Clinical Outcome

An arbitrary scoring system (from 1 to 4) was used and a score assigned to each dog, based on fecal consistency and clinical symptoms (lethargic attitude, growth retardation, weight loss, vomiting and flatulence) as follows: (1) formed feces and no clinical symptoms, (2) formed feces and clinical symptoms, (3) soft stools and clinical symptoms, and (4) diarrhea and clinical symptoms.

RESULTS

Table 1 shows dog's data (gender, age, fur length, clinical score), *Giardia* cysts per gram (CPG) of feces (mean, standard deviation) and efficacy (%) of treatment with fenbendazole (Group A) and metronidazole (Group B) at the different study Days (Day−5, Day 7, Day 14, Day 21, and Day 50).

Parasitological results at Day−5 showed mean values of 20,995 *Giardia* CPG (minimum 2,780 CPG, maximum 48,000 CPG) in the Group A (fenbendazole) and 18,580 *Giardia* CPG (minimum 10,848 CPG, maximum 32,178 CPG) in the Group B (metronidazole). On Day 7, cysts of *Giardia* spp. were found in 33.3% (4/12; 95% Confidence Interval (CI) = 11.3–64.5) of the dogs in the Group A and in 41.7% (5/12; 95% CI = 16.5–71.4) of the dogs in the Group B.

The efficacy of fenbendazole was 80.9% at Day 7, 100% at day 14, 97.0% at day 21, and 95.0% at day 50. The efficacy of metronizazole was 70.8% at Day 7, 99.0% at Day 14, 100% at Day 21, and 97.1% at Day 50. Overall, the efficacies of Fenbendazole and Metronidazole against the infection by *G. duodenalis* were not significantly different ($P = 0.686$).

No other co-infections with intestinal parasites were found at copromicroscopic examinations. Briefly, 6 dogs from Group A and 5 dogs from Group B remained negative until Day 50 after they received the first treatment at Day 0. Eight dogs (four from each group), received the second treatment at Day 7 (after the first therapy at Day 0), and two other dogs (one from each group) were retreated for the second time at Day 21 from the Group A and Day 14 from the Group B. Moreover, in the Group B, there was one dog that received three treatments during the study at Days 0, 7 and 14. Finally, two dogs (one from each group), that had received the first treatment at Day 0, were still shedding *Giardia* cysts at the last study Day (Day 50).

Assessment Criteria and Clinical Outcome

At Day−5, feces were unformed to diarrhoeic in dogs belonging to both treatment groups but no adverse responses due to the medications were observed. 33.3% of the dogs had score 1, 25% score 2, 25% score 3, and 16.7% score 4. Regarding the dogs that tested positive for *Giardia* after the treatment (13/24), five belonged to the first category (38.6%), four to the second category (30.8%), one to the third category (7.7%), and three to the fourth (23%).

Molecular Characterization

Giardia DNA could be amplified only from six of the 15 fecal samples tested, three from the Group A (dog's ID numbers = F1, F6, F8) and three from the Group B (dog's ID numbers = M4, M7, M11). The beta-giardin amplification products were sequenced and compared, using BLAST, with all available sequences in the GenBank database. This revealed full homology (e.g., 100% with JN416550) with the canine specific assemblage D in the six positive dogs. The lack of amplification in the remaining samples is likely due to cyst damage resulting from storage conditions.

DISCUSSION

During the last 60 years, a number of chemotherapeutic agents have been introduced and are still in use in the therapy for giardiosis (37). Unfortunately, most drugs used have considerable

TABLE 1 | Groups A-Fenbendazole and B-Metronidazole: dog's data (gender, age, fur length, clinical score), *Giardia* cysts per gram (CPG) of feces (mean, standard deviation), and efficacy (%) of the treatment at the different study days.

Dog ID	Gender (Male/Female)	Age (months/years)	Fur length (Long/Short)	Clinical score	*Giardia* cysts per gram (CPG) of feces				
					Day-5 (Pre-T)	Day 7 (T1)	Day 14 (T2)	Day 21 (T3)	Day 50
GROUP A (FENBENDAZOLE)									
F1	M	2 mos	L	4	22,300	0	0	8,360	0
F2	F	4 yrs	S	3	15,600	0	0	0	0
F3	M	8 mos	L	1	19,200	21,600	0	0	0
F4	M	2 yrs	S	1	7,830	1,230	0	0	0
F5	F	1 yr	S	3	18,894	0	0	0	0
F6	F	3 mos	L	4	24,600	16,828	0	0	0
F7	F	1 yr	L	2	21,080	0	0	0	0
F8	M	9 mos	S	3	31,984	0	0	0	0
F9	F	2 yrs	S	2	22,824	0	0	0	12,576
F10	M	3 yrs	L	1	16,854	0	0	0	0
F11	F	7 mos	S	3	48,000	0	0	0	0
F12	M	3 yrs	L	1	2,780	8,400	0	0	0
				Mean CPG	20,995.5	4,004.8	0.0	696.7	1,048.0
				SD	11390.1	7564.0	0.0	2413.3	3630.4
				%Efficacy	**NA**	**80.9%**	**100%**	**97.0%**	**95.0%**
GROUP B (METRONIDAZOLE)									
M1	F	4 yrs	S	4	19,654	0	0	0	0
M2	F	4 yrs	S	3	11,900	0	0	0	0
M3	F	8 mos	L	1	10,900	0	0	0	0
M4	M	2 yrs	S	1	21,234	0	0	0	0
M5	F	2 yrs	L	2	10,848	0	0	0	0
M6	M	9 mos	S	1	21,280	0	0	0	6,546
M7	M	3 yrs	S	1	18,732	560	156	0	0
M8	F	1 yr	L	2	15,832	17,560	0	0	0
M9	M	6 mos	S	3	17,459	18,981	0	0	0
M10	F	5 yrs	L	2	17,178	17,562	0	0	0
M11	M	5 mos	L	4	32,178	10,400	0	0	0
M12	F	7 mos	S	2	25,765	0	1,987	0	0
				Mean CPG	18,580.0	5,421.9	178.6	0.0	545.5
				SD	6224.6	8161.8	571.3	0.0	1889.7
				%Efficacy	**NA**	**70.8%**	**99.0%**	**100%**	**97.1%**

Pre-T (before treatment); T1 (first treatment); T2 (second treatment) T3 (third treatment). Clinical score: (1) formed feces and no clinical symptoms, (2) formed feces and clinical symptoms, (3) soft stools and clinical symptoms, and (4) diarrhea and clinical symptoms.

adverse effects such as vomiting, bloody diarrhea, abortion, neurological dysfunctions and so they are contraindicated (38). In this context, studies on chemotherapeutic agents play a fundamental role in the rationale for the treatment of giardiosis.

Several benzimidazoles (39, 40), and in particular fenbendazole (23), or the combinations of febantel/fenbendazole with other compounds proved to be effective (24, 25, 41). Metronidazole, a nitroimidazole, and fenbendazole are used routinely to treat giardiosis in dogs (30). Ronidazole showed a good antiprotozoal effect against *Giardia* in dogs (30) and oxfendazole showed a significant decrease in the number of cysts (40).

The findings of the present study did not reveal a significant difference of efficacy between the drugs used (fenbendazole 80.9% vs. metronidazole 70.8% at Day 7). Moreover, after applying the first treatment, both drugs failed to eliminate the *Giardia* cysts in all the dogs. Re-appearance of the cysts in the feces could be attributed to a re-infection, treatment failure, the correct medication given by the owners, the

diet, as well as biological issues related to the intermittent excretion of *Giardia* cysts. However, there are no specific information of the dog's environment at home if any attempt was made by the owner to clean up contaminated areas. Faure et al. (42), reported a significant difference of efficacy between fenbendazole and metronidazole (30.3% vs. 91.9%). In our study, fenbendazole showed lower efficacy than that reported by Zajac et al. (26), who obtained 90% efficacy (26). However, it is important to note that in our present study both drugs showed 100% efficacy after two consecutive cycles of treatments, i.e., at Days 14 in the Group A and at Day 21 in the Group B.

Fifteen (62.5%) out of 24 dogs treated from both groups (eight dogs from Group A and seven dogs from Group B) were tested negative for *Giardia* cysts after the first therapy at Day 7. Moreover, there were dogs from both groups A and B, thus showing a persistent infection that has been cleared either at Day 14 or at Day 21 after two or three treatments of 5 days each. It could be possible, that some *Giardia* remained at low abundance in the intestine of these dogs that were still positive after the first treatment applied, or for the dogs that needed to be treated three times until a negative result was obtained, or perhaps we were facing with a treatment failure due to drug resistance. However, future investigations are needed, in order to establish the availability of the drugs in the blood and the capacity of these to eliminate the *Giardia* cysts by *in vitro* methods.

In addition, two dogs from each group (A and B) remained negative after the first therapy (Day 0), until Day 50, when turned positive. In these cases, it was suggested that reinfections from the environment are the most common cause for treatment failure (25, 30) considering that the prepatent period can be as short as 4 days (33). Also, the presence of cysts on the animal's fur, associated with the resistance of the parasite to disinfectants (chlorhexidine gluconate in our study) and environmental conditions and with stress, may explain reinfections (30). Since this is a field trial performed in-home conditions we were not sure about the failure of cleaning and bathing due to the lack of compliance by the owner, thus, the use of hygiene measures such as chlorhexidine digluconate shampoo before and after the treatment might have a considerable influence on our results representing a major issue of this study. Moron-Soto et al. also reported that dogs re-shed cysts following a brief period after antiparasitic treatment when no disinfection or cleaning of their enclosures was performed (31).

Some experimental studies have evaluated also the efficacy of the oral administration of probiotics together with albendazole (38), therefore during the treatment the veterinarian can recommend the administration of probiotics that play a synergistic role with the drug regenerating the intestinal flora. However, in the present study, none of the dogs received a special diet, or probiotics supplements during and after the therapy. Moreover, there are few recent studies that showed that the microbial diversity was not altered by fenbendazole administration, which is in contrast to metronidazole which significantly altered microbial structure and diversity (43–47). Administration of metronidazole to healthy dogs caused significant changes in the microbiome, some of which persist following discontinuation of the drug with unknown clinical effects. Furthermore, some dogs on metronidazole can get neurotoxicity (48, 49). However, in the present study, no adverse responses due to the medications were observed including transient neurological signs due to metronidazole.

There have been two reports examining the effects of secnidazole on *Giardia* in dogs. One, reported 100% efficacy of cyst reduction in two groups of six hospitalized dogs (50). The other one, showed that at 43 days after the first treatment, all 9 dogs were considered to have normal stools, but 4 of the 9 puppies were positive for *Giardia* antigen (34). Bearing these facts in mind, perhaps secnidazole applied twice, 2–3 weeks apart, could be an alternative and easier treatment, with a better compliance as well for treating *Giardia* infection. Further data are needed to prove its efficacy both in field and experimental studies.

Finally, genotyping of *Giardia* demonstrated the host-specific assemblage D in all positive dogs, in agreement with other studies in Italy (12, 35). However, our study presented limitations due to the sample size and due to the poor and inappropriate storage conditions to ensure the effective isolation of DNA from fecal samples. Therefore, further research on distinguish *G. duodenalis* strains, are needed, in order to perform a realistic estimation of zoonotic risk (51).

Based on the findings, as expected in a trial with a small number of individuals in-home conditions, and especially for a parasite with the characteristics of *Giardia* (i.e., having a direct infection and short prepatent period), the authors could only speculate that treatment alone is not sufficient for controlling *Giardia* infection.

A main limitation of our study is based on the small sample size (no. = 12 dogs), the absence of a control group and the allocation of the animals in the two treatment Groups based only on individual *Giardia* CPG assessed at Day−5. However, the limited number of dogs in our study was mainly due to type of study (field trial in-home conditions) that requires a full compliance of both vet practitioners and dog owners. Ethical issues were the main reasons for not including a control group in such a kind of field trial performed in-home conditions. In a similar study, Mirò et al. (52) assessed the efficacy of fenbendazole and mebendazole using 10 per group. As a consequence, in the allocation phase we could not consider other key epidemiological parameters as age, fur length and style of living. Different hypotheses might explain the re-appearance of the *Giardia* cysts in some dogs after treatment, e.g., re-infection from the home environment, the correct medication given by the owners, the diet, as well as treatment failure, but also biological issues related to the intermittent excretion of *Giardia* cysts. Therefore, due to the uncontrolled parameters in this field trial performed in-home conditions, future studies are warranted to produce conclusive evidence for the evaluation of integrated approaches needed for the treatment of dogs naturally infected by *Giardia*.

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 animals used in the present study were sampled following approval by the animal ethics and welfare committee of the University of Naples Federico II (in Italian, Comitato Etico-scientifico per la Sperimentazione Animale dell' Università di Napoli Federico II; protocol number PG/20170055343). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MG, LR, LC, LK, SMC, ARS, and GC contributed to the conception and design of the study. PP, AB, AV, and MPM organized the database and performed the statistical analysis. MG, LR, and LC wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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BRIEF RESEARCH REPORT
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Evidence for Transmission of *Taenia solium* Taeniasis/Cysticercosis in a Rural Area of Northern Rwanda

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Cysticercosis is a parasitic infection caused by the metacestode larval stage (cysticercus) of *Taenia solium*. In humans, cysticercosis may infect the central nervous system and cause neurocysticercosis, which is responsible for over 50,000 deaths per year worldwide and is the major cause of preventable epilepsy cases, especially in low-income countries. Cysticercosis infection is endemic in many less developed countries where poor hygiene conditions and free-range pig management favor their transmission. A cross-sectional study was conducted in 680 children from a rural primary school in Gakenke district (Northern province of Rwanda). Stool samples were collected from participants and analyzed using the Kato-Katz method (KK), formol-ether concentration (FEC), and/or copro-antigen enzyme-linked immunosorbent assay (CoAg-ELISA) to detect taeniasis. Blood samples were collected and analyzed using enzyme-linked immunoelectrotransfer blot (EITB) and antigen enzyme-linked immunosorbent assay (Ag-ELISA) to detect human cysticercosis. The overall proportion of taeniasis positivity was 0.3% (2/680), and both cases were also confirmed by CoAg-ELISA. A total of 13.3% (76/572) of the children studied were positive to cysticercosis (*T. solium*-specific serum antibodies detected by EITB), of whom 38.0% (27/71) had viable cysticercus (*T. solium* antigens by Ag-ELISA). This study provides evidence of the highest cysticercosis prevalence reported in Rwanda in children to date. Systematic investigations into porcine and human cysticercosis as well as health education and hygiene measures for *T. solium* control are needed in Gakenke district.

Keywords: ***Taenia solium*, taeniasis, cystcercosis, children, Gakenke, Rwanda**

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INTRODUCTION

In *Taenia solium* parasitic infections, humans are the only natural definitive host (taeniasis) while pigs are the intermediate hosts of larval stage (swine cysticercosis) (1). Human cysticercosis results when people become intermediate hosts after ingestion of microscopic viable eggs *via* the fecal–oral route from *T. solium* tapeworm carriers (2). The embryo is released (oncosphere), and it traverses the intestinal mucosa after ingestion. Later, it is transported by the circulatory system and dispersed by the organism producing cysts (cysticerci). The most common locations of cysts are the striated muscle, eyes, or heart tissue and central nervous system (3, 4). The clinical manifestations of cysticercosis are dependent on the number and location of cysticerci within the body (4). Some individuals with cysticercosis will exhibit or develop no symptoms (asymptomatic) or very mild symptoms. Many individuals with cysticercosis have central nervous system involvement (neurocysticercosis) resulting in headache, epileptic seizures, blindness, mental disturbance, and even death (3, 5). Neurocisticercosis (NCC) is the most common parasitic disease in the human nervous system and the most common cause of epilepsy in low-income countries (6).

Currently, cysticercosis is one of the 17 major Neglected Tropical Diseases (NTDs) identified by the WHO as a focus for research and control (7). It is widely prevalent where humans and domestic pig raising coexist. In many developing countries in Central and South America, Africa, and Asia, cysticercosis has major public health implications in humans and pigs (5, 8–11). It is in these places where poverty, poor education, lack of access to diagnosis, and limited management capacity, together with the absence of appropriate prevention measures and control strategies, make it highly endemic (11–13).

The distribution of *T. solium* taeniosis/cysticercosis in Africa is unclear but porcine and human cysticercosis are considered (hyper)-endemic in Central Africa (Rwanda, Burundi, the Democratic Republic of Congo, and Cameroon) (14, 15). In the last 20 years, pig production has increased significantly in the Eastern and Southern Africa (ESA) region, especially in rural, resource-poor, smallholder communities (11, 14). Several studies show a high prevalence of porcine cysticercosis in countries bordering Rwanda. In Uganda, prevalences ranging from 7.1 to 45% have been observed in urban areas in contrast to low percentages of 0.12–10.8% found in rural areas with an observed increment in recent years (9, 16–18). A prevalence of 41.2% has recently been reported in the Democratic Republic of Congo, where the overall prevalence of pigs with active cysticercosis did not significantly differ between the market and the village study sites but was much higher than previously observed by Chartier et al., in 1990 (19, 20). There are no current data for Burundi, but prevalence ranges from 2 to 39% were observed 20 years ago (21). In Tanzania, farm prevalence of porcine cysticercosis was between 17.4 and 18.2% in lingual examination or slaughter-slab prevalence by routine meat inspection respectively, while a maximum of 33.3% has been reported by cysticercal antigens by ELISA (Ag-ELISA) (22–29).

In contrast, Taeniasis has been poorly studied in humans from these countries (30). Eggs of *Taenia* sp. in feces have been reported in Uganda (31). Taeniasis prevalence ranges between 0 and 1.0% have been observed in schoolchildren of Burundi (21). However, prevalence of taeniasis ranging from 0.4 to 5.2% by the Kato-Katz technique or 2.3–5.2% by copro-Ag-ELISA has been estimated in Tanzania (32, 33).

Regarding human cysticercosis, one study estimated 21.6% prevalence of circulating antigen in the Democratic Republic of Congo (34). Several studies in human cysticercosis reveal the strong association between neurocysticercosis and epilepsy in these countries (35, 36). In Burundi, cysticercosis has been observed in 4.9–31.5% of epileptic patients, compared to 4.2% in controls (21, 37–39). On the other hand, a seroprevalence of up to 11.7% has been observed in epileptic patients and in 2.8% of controls (21). Cysticercosis was the cause of seizures in 25% of epileptic patients (38, 40). In Tanzania, cysticercosis prevalence of 16–17% was estimated and it was demonstrated that NCC contributed significantly to epilepsy in adults (32, 41–43). Given these data, it is likely that the situation in Rwanda is similar.

Rwanda has long since been known as a hyperendemic country for Taeniasis/cysticercosis. However, there are few research studies carried out in this country. Already in 1959, 20% of pigs were found to be infected with cysticercosis (44). From 2000 to 2011, pork production in Rwanda increased by 7.8% (45); however, an overall swine cysticercosis prevalence of 3.9% was found in farms, 9.2% in markets, and 4% in butchers (46). In 1956, the first case of human cysticercosis was reported in Rwanda (47). Since then, initial reports have reported isolated cases of disseminated ocular (48) and cutaneous cysticercosis (49, 50). In 1964, the presence of eggs of *Taenia* spp. in populations of the Batwa and Hutu tribes in the Northern and Southern regions of the country was detected (51). Later, *T. solium* cysticerci was detected in 7% of 300 autopsies in Butare (52). In Kigali and Butare, 21 and 21.8% of people with epilepsy are estimated to be seropositive for cysticercosis, respectively (53, 54). To date, there have been no studies in children.

During a coproparasitological study in the school population of Northern Rwanda, eggs from tapeworms were detected in two schoolchildren. After collecting the strobila, the morphology of mature proglottids allowed us to identify the species as *T. solium* in both cases. This observation motivated us to investigate the presence of cysticercosis in the school population of Nemba, with the prospect of introducing control activities.

METHODS

Study Area

Rwanda is a small landlocked country in Central Africa, bordering Uganda to the North, Burundi to the South, Democratic Republic of the Congo to the West, and Tanzania to the East. The estimated population in 2012 was 10.4 million people, thus supporting the densest population in continental Africa, with most of the population engaged in subsistence agriculture (55). A verdant country of fertile and hilly terrain with altitudes varying from 950 m to 4,519 m, the small republic bears the title "Land of a Thousand Hills." The District of Gakenke is one of the five districts of the Northern Province of Rwanda. This

FIGURE 1 | Study location map. District of Gakenke, Northern Province, Rwanda.

district is divided into 19 administrative sectors made of 97 cells, 617 villages, and 345,487 inhabitants living in a total area of 104 km^2 with a population density of 473/km^2 (56). **Figure 1** shows a schematic map of the study area.

Gakenke district is characterized, in general, by high inclined hills separated by rivers and marshlands. The climate in Gakenke district is generally the type of humid climate with the average annual temperature varying between 16.0 and 29.0°C. The rainfalls are relatively abundant with a scale between 1,100 and 1,500 mm per year (56).

Population

Between July and September 2011, the Parasitology Area of University Miguel Hernández de Elche (UMH; Spain) conducted a cross-sectional study in Nemba school I, Gakenke district of Rwanda, as part of an initial phase of a school health program. A total of 771 schoolchildren (371 girls and 400 boys) attended primary education (2nd to 6th grade) and were eligible for inclusion in the study. A total of 708 students (357 boys and 351 girls aged between 6 and 18 years of age; mean ± SD = 11.00 ± 2.33 years) were eligible for the study after their parents or guardians provided informed consent. A total of 680 answered the questionnaire and provided stool sample, while 572 provided blood sample (see **Figure 2**).

Sampling and Parasitological Tests

After collection, fresh feces samples were processed and quantified *in situ* (UMH headquarters in Nemba, Rwanda) by the method described by Kato-Katz following WHO recommendations (57). A 41.7-mg sample of fresh feces was used as template according to the Helm teste® manufacturer's recommendations (Bio-Manguinhos/Fiocruz, Brazil). If taeniid eggs were observed, 2 g of Niclosamide and 1 ml of Duphalac® oral solution (667 mg/ml of lactulose) was administered as a laxative to recover the adult where possible.

Additionally, part of each stool sample was preserved in 10% buffered formalin (1:3) (40-ml Falcon tube) and another part was preserved in ethanol 70% (2-ml tube). Sera samples were obtained from whole blood collected of every child by centrifugation and stored at 4°C in Nemba's Hospital until sent to UMH, Spain. Later, all samples were sent to UMH and stored at −20°C until processing. Formalin-embedded samples were sent to the Department of Parasitology of University of Valencia (Spain) where a formol-ether concentration was realized (58). Finally, tree drops of sediment with Lugol's iodine solution were analyzed under a microscope. Sera and fecal samples preserved in ethanol 70% were subsequently sent to the diagnostic laboratory of the Center for Global Health of the Universidad Peruana Cayetano Heredia (UPCH, Lima, Peru) for processing by *T. solium*-specific tests [Coproantigen ELISA, serum antigen detection (Ag-ELISA), and serum antibody detection by enzyme-linked immunoelectrotransfer blot (EITB) assay using lentil lectin-purified parasitic glycoprotein antigens].

Taeniasis: Coproantigen Detection by ELISA (CoAg-ELISA)

In an attempt to find additional carriers of adult *T. solium* worms, a subgroup of 144 fecal samples (∼21% of the total) were also screened by coproantigen ELISA (59, 60). Aliquots

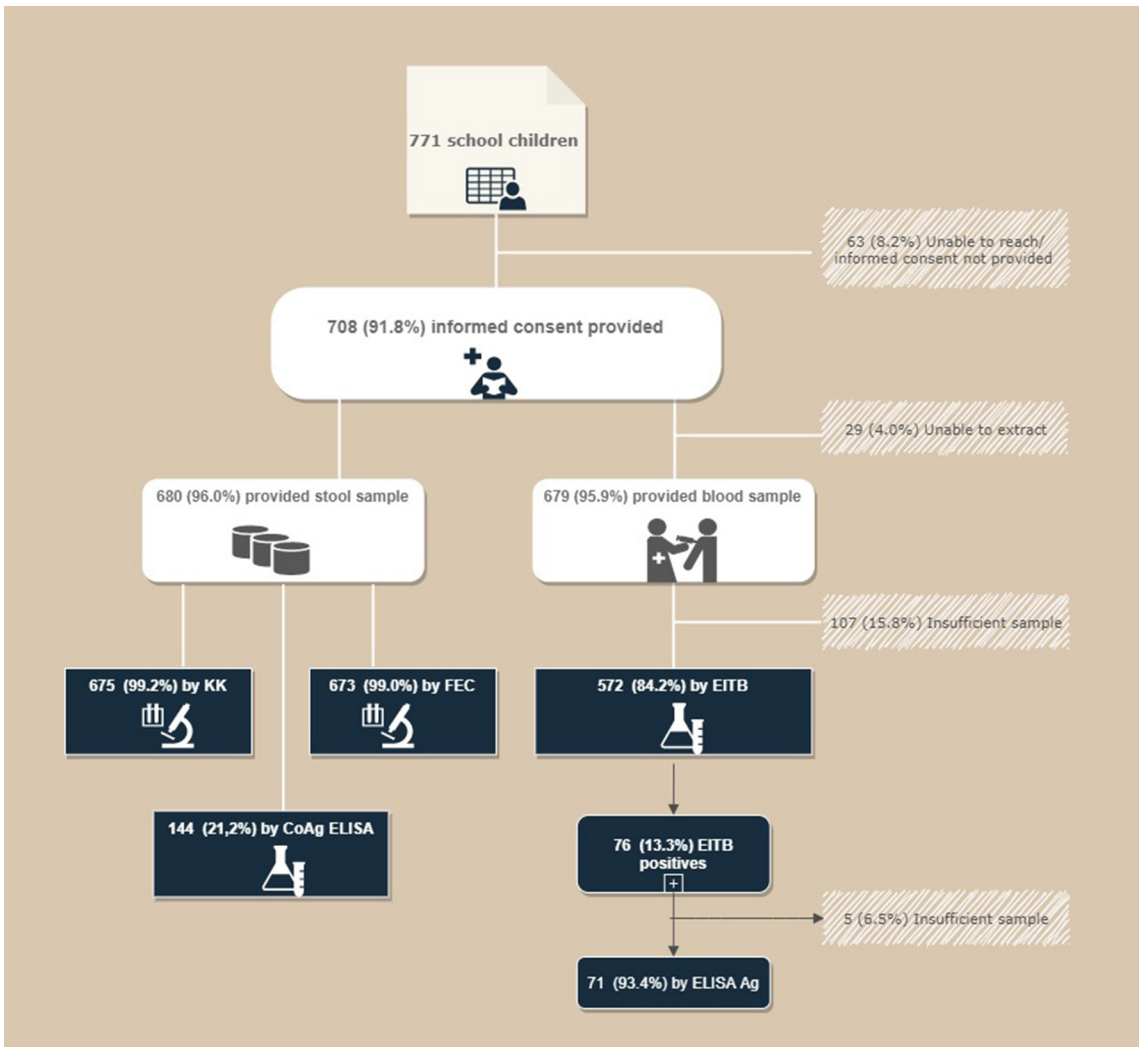

FIGURE 2 | Flow chart for the recruitment of patients.

(1.5 ml) of the stool supernatant were used for CoAg-ELISA after centrifugation at room temperature at 3,200 g for 10 min. The CoAg-ELISA technique was performed as described by Allan et al., by using hyperimmune rabbit anti-*T. solium* IgG as the capture antibody and peroxidase-labeled goat anti-*T. solium* IgG as a conjugate. Processed samples were read with a spectrophotometer (Molecular Devices Inc., Sunnyvale, CA) at 650 nm. Using a known positive pool (P1), we calculated the percentage of positivity (PP) as [optical density (OD) of the sample]/(OD of P1) 100, in order to increase the comparability of the results between plates. A cutoff was determined using a receiver operating characteristic (ROC) curve (61).

Cysticercosis-Specific Tests

Anti-*T. solium* Antibody Detection

A total of 572 sera samples were processed by EITB. The methodology used to perform the test was the same described by Tsang et al. (62) and González et al. (63). An immunoblot of seven cysticercus glycoproteins (GP50, GP42-39, GP24, GP21, GP18, GP14, and GP13), purified by lentil lectin-purified chromatography, gives close to 100% specificity and a sensitivity varying from 70 to 90% (62). This EITB has been widely used for the diagnosis of cysticercosis in human and pig serum samples (64).

T. solium Antigen Detection by ELISA

In order to demonstrate the presence of active infections, an anti-*T. solium* antibody-positive subgroup of children (71 EITB positive samples) were processed in quest by ELISA, to detect circulating cysticercal antigen in the serum (65, 66). A mAb-based ELISA for the detection of circulating antigens was used to detect circulating parasite antigen as described by Brandt et al. in 1992 (67) and later adapted by Van Kerckhoven et al. and Dorny et al. (68, 69). The assay uses Nunc MaxiSorp plates sensitized with a trapping mAb (B158C11A10) in bicarbonate buffer at 5 μg/ml. After blocking, serum samples

(pretreated with 5% trichloroacetic acid to break existing immune complexes) are added, followed by the second mAb (B60H8A4-BIOT), streptavidin, o-phenylenediamine (OPD/H2O2) as substrate/chromogen, and incubated in the dark for 15 min. The reaction is stopped with H_2SO_4 and plates are read at 490/650 nm. To minimize inter-plate variation, we used antigen ratio instead of a raw value of optic density (OD). The antigen ratio is estimated by dividing the OD of the tested sample with the mean of eight negative samples plus three standard deviations (70).

Questionnaire and Anthropometric Measurements

A questionnaire was concurrently conducted while blood and stool samples were taken. The children were interviewed, and basic socio-demographic data (age, sex, residence) were recorded as well as specific questions about their living conditions and behaviors. Each child was assigned a unique ID code and removed their shoes so that we could measure their height (in cm) and weight (in kg).

Statistical Analyses

The data were identified and entered into a Microsoft Office Excel 2010 spreadsheet (Microsoft). Statistical analysis was performed using Stata SE 15.0 (StataCorp LLC, USA). We calculated the frequencies of categorical variables and made comparisons using Pearson's Chi-squared-test. Missing values were excluded for the comparisons. Continuous variables were described with means and standard deviations.

RESULTS

Taeniasis Intestinal and Species Identification

The coproparasitological analysis revealed an overall parasitism of 94.9% (639/673) by formol-ether concentration and a helminth parasitism percentage of 31.2% (211/675) by the Kato-Katz technique. In two children [2/680 (0.3%)], eggs of taeniid tapeworm were identified by both methods: Kato-Katz technique [2/675 (0.3%)] and formol-ether concentration [2/673 (0.31%)].

After parental consent, 2 g of Niclosamide and 1 ml of Duphalac® oral solution (667 mg/ml of lactulose) as a laxative were administered and the feces were collected. Two strobiles were recovered from both children and the morphologic study under a magnifying glass after injection with India ink revealed <12 uterine branches compatible with *T. solium*-proglottid demonstrating current tapeworm infection in both children.

Moreover, 144 samples were tested by coproantigen ELISA detection and 2/144 (1.4%) samples had positive results. The positive samples obtained matched the positive samples obtained by Kato-Katz and formol-ether concentration techniques.

Cysticercosis

EITB analysis of a total of 572 sera samples revealed that 13.3% of the students (76/572) presented reactivity to one or more of the specific glycoproteins of *T. solium*, of whom 46.0% (35/76) showed reactivity to three or more bands. A total of 7 of the 76 positive samples showed a single GP 50 band reaction, whereas in 9 of the positive samples, no GP 50 band was observed.

Exposure to *T. solium* was more frequent among girls than boys ($p = 0.014$, **Table 1**) and was especially prevalent in the Mucaca area of Nemba where 21.3% of the children had anti-*T. solium* antibodies ($p < 0.001$). Children of all ages and in all classes had positive EITB results, and there was no obvious relationship with domestic animal rearing. The majority of the children came from homes that used a water pump as their main water source and had a latrine. Consumption of meat was significantly associated with *T. solium* exposure. The highest levels of exposure were among children that reported consuming meat once a month (15.6%) or once a year (19.2%); 13.1% of children who reported that they always washed their hands after going to the toilet had anti-*T. solium* antibodies, and this was not statistically different from children that reported they sometimes or never washed their hands (12.8 and 14.3%, respectively).

Taenia solium antigens were found in 38% (27/71) of children with anti-*T. solium* antibodies suggesting active infection. In addition, 14 of the 27 positive samples for antigen presented more than three bands by EIBT-test. None of the variables considered were associated with antigen presence among seropositive children (**Table 1**).

DISCUSSION

The present study demonstrates a substantial seroprevalence of cysticercosis in children of the Northern Province of Rwanda. The results suggest that more than of 13% of this group of children had been exposed to *T solium*. In addition, among the reactive individuals, 46% had three or more reactive bands by EITB, which is highly suggestive of established cysticercosis infection. Furthermore, of the children with antibodies to cysticercosis, 38% were reactive to the detection of circulating antigen by ELISA, indicating the presence of active cases of neurocysticercosis, as previously demonstrated (65).

In 1982, Fain et al. said that *T. solium* was the most prevalent tapeworm in Rwanda (47). In previous studies in Rwanda, soil-transmitted helminths and schistosomiasis but no tapeworms were found. Since 1964, human taeniosis has not been detected in the country (51). However, analysis based on both microscopy or coproantigen detection of human fecal material cannot identify the species level, and the possibility of *T. saginata* presence cannot be ruled out. Other studies showed high parasitic burden strongly associated with drinking any kind of water (71–73).

The finding of eggs of *Taenia* spp. in two schoolchildren motivated us to deepen the search for associated human cysticercosis. Both children were positive based on microscopy (KK and FEC) and coproantigen detection (copro-Ag-ELISA).

In spite of false positives reported by coproantigen test (74), a high positivity value is highly predictive of a true positive (61). No cross-reactions were observed with other intestinal parasites (protozoa and helminths) present in the children. In this study, the microscopy and coproantigen detection assays had the same sensitivity as has been reported by other authors (59, 75). Our work showed some limitations because we were

TABLE 1 | Results of the cysticercosis-specific tests conducted according to characteristics of the children.

Characteristics	EITB				Ag-ELISA	
	Positive		3 or more bands			
	n/total (%)	*p*-value[a]	*n*/positive EITB (%)	*p*-value[a]	*n*/positive EITB (%)	*p*-value[a]
Sex		0.019		0.598		0.948
Female	48/289 (16.6)		21/48 (43.7)		18/47 (38.3)	
Male	28/282 (9.9)		14/28 (50.0)		9/24 (37.5)	
No data	0/1 (0.0)		0/1 (0.0)		0/0 (0.0)	
Age		0.509		0.606		0.674
6–9 years	20/155 (13.0)		11/20 (55.0)		8/19 (42.1)	
10–14 years	46/363 (12.7)		20/46 (43.5)		15/44 (34.1)	
15–18 years	8/42 (19.0)		3/8 (37.5)		3/6 (50.0)	
No data	2/12 (16.7)		1/2 (50.0)		0/2	
Sector		<0.001		0.711		0.401
Nemba						
Buranga	3/46 (6.5)		2/3 (66.7)		0/3 (0.0)	
Gahinga	7/70 (10.0)		3/7 (42.8)		3/6 (50.0)	
Gisozi	13/202 (6.4)		5/13 (38.5)		3/12 (25.0)	
Mucaca	46/216 (21.3)		24/46 (52.2)		19/43 (45.2)	
Gakenke						
Rusagara	4/25 (16.0)		1/4 (25.0)		11/4 (27.5)	
No data	3/13 (23.1)		0/3 (0.0)		1/3 (33.3)	
School class		0.204		0.545		0.650
P2	15/150 (10.0)		7/15 (46.7)		7/14 (50.0)	
P3	17/114 (14.9)		10/17 (58.8)		6/16 (37.5)	
P4	24/131 (18.2)		8/24 (33.3)		7/23 (30.4)	
P5	15/116 (12.9)		7/15 (46.7)		6/13 (46.1)	
P6	5/61 (8.2)		3/5 (60.0)		1/5 (20.0)	
Domestic animals		0.270		0.299		0.415
Pigs	1/28 (3.6)		0/1 (0.0)		1/1 (100.0)	
Other animals[b]	64/459 (13.9)		31/64 (48.4)		21/59 (35.6)	
None	8/68 (11.8)		2/8 (25.0)		3/8 (37.5)	
Don't know/no answer provided	1/5 (20.0)		1/1 (100.0)		1/1 (100.0)	
No data	2/12 (16.7)		1/2 (50.0)		1/2 (50.0)	
Water source		0.515		0.809		0.077
Piped water	5/58 (8.6)		2/5 (40.0)		0/5 (0.0)	
Water pump/fountain	68/496 (13.7)		31/68 (45.6)		25/63 (39.7)	
Rivers/puddles	0/1 (0.0)		0/0 (0.0)		0/0 (0.0)	
Don't know/no answer provided	1/5 (20.0)		1/1 (100.0)		1/1 (100.0)	
No data	2/12 (16.7)		1/2 (50.0)		1/2 (50.0)	
Toilet		0.432		NA		NA
WC	0/10 (0.0)		0/0 (0.0)		0/0 (0.0)	
Private latrine	72/542 (13.3)		32/72 (44.4)		25/67 (37.3)	
Shared latrine	0/1 (0.0)		0/1 (0.0)		0/0 (0.0)	
Don't know/no answer provided	2/7 (28.6)		2/2 (100.0)		1/2 (50.0)	
No data	2/12 (16.7)		1/2 (50.0)		1/2 (50.0)	
How often do you eat meat?		0.003		0.070		0.818
Once a week	10/159 (6.3)		7/10 (70.0)		2/9 (22.2)	
Once a month	32/205 (15.6)		12/32 (37.5)		11 /30 (36.7)	
Once a year	27/141 (19.2)		11/27 (40.7)		10/25 (40.0)	
Never	3/44 (6.8)		3/3 (100.0)		1/3 (33.3)	
Don't know/no answer provided	2/11 (18.2)		1/2 (50.0)		2/2 (100.0)	

(Continued)

TABLE 1 | Continued

Characteristics	EITB				Ag-ELISA	
	Positive		3 or more bands			
	n/total (%)	*p*-value[a]	*n*/positive EITB (%)	*p*-value[a]	*n*/positive EITB (%)	*p*-value[a]
No data	2/12 (16.7)		1/2 (50.0)		1/2 (50.0)	
How often do you wash your hands after going to the toilet?		0.935		0.367		0.788
Always	26/198 (13.1)		9/26 (34.6)		10/24 (41.7)	
Sometimes	34/266 (12.8)		18/34 (52.9)		12/34 (35.3)	
Never	13/91 (14.3)		6/13 (46.1)		3/10 (30.0)	
Don't know/no answer provided	1/5 (20.0)		1/1 (100.0)		1/1 (100.0)	
No data	2/12 (16.7)		1/2 (50.0)		1/2 (50.0)	
Total	**76/572 (13.3)**		**35/76 (46.0)**		**27/71 (38.0)**	

EITB, Anti-T. solium antibodies detection by enzyme-linked immunoelectrotransfer blot; Ag-ELISA, T. solium antigen detection by ELISA.
[a]*p-value from a Chi squared-test comparing answer categories, excluding missing or unknown values.*
[b]*Other animals: Cows, sheep, goats, rabbits, and chicken.*

unable to carry out the coproantigen study on all samples, and the sample of children included came from a single school in the Gakenke district. However, participation was high, and with this study, we can confirm the presence of tapeworm carriers in this area. Additionally, we identified morphologically both adult specimens. Identification to species level in this country or bordering countries except in Tanzania has never been done (33).

To our knowledge, this is the first report of cysticercosis in schoolchildren in Rwanda. In cysticercosis surveys and immunodiagnostic tools applied on humans, serum samples are useful in estimating the prevalence and identifying of *T. solium* infection in the field. *T. solium* antigen in serum could predict the presence of viable brain parasites in patients with apparently calcified cysticercosis only (70). Antibody response in *T. solium* infection in field conditions was found to be a major contributor to the overestimation of the prevalence of cysticercosis in endemic areas (4, 76). The frequency found in these children was higher than prior literature reports in Butare, where 7% of autopsies were positive for cysticercosis (52). Furthermore, 21 and 21.8% of patients with epilepsy in Kigali and southern Rwanda, respectively, are positive for cysticercosis (50, 53, 54). None of the previous studies were performed in children. In our study, it is important to highlight the early age of the population studied, since it is known that the prevalence of cysticercosis in a region increases with the age range studied. We can deduce that the seroprevalence in the adult population is likely to be higher than observed here.

The highest proportion of positive schoolchildren was observed in the same sector where tapeworm carriers were located. We observed small-scale swine production system in Mucaca (personal observation).

The financial losses due to human cysticercosis are very difficult to estimate but are certainly exceeded by the social impact of the disease, especially because of the particular perception of epilepsy in many African communities (14). Conditions like neurocysticercosis are better prevented than treated, because it has the potential to make a healthy person invalid. If the treatment is delayed, there can be irreversible brain damage.

In our study, no association was observed in frequency of hand washing after going to the toilet. However, in our study, close contact with pigs and use of latrines as possible risk factors for cysticercosis were not significantly associated with positive cases as observed in Tanzania (43). The absence of association may be explained by the level of widespread poverty in this area. Interestingly, meat consumption showed a significant association with cysticercosis exposure, but it was not a dose response. It is possible that people with limited resources cannot afford quality meat in these rural areas. Children who reported consuming meat once a month or once a year had higher exposure rates than those who reported consuming meat weekly, but it is possible that here we are seeing the impact of socio-economic status of the children rather than meat consumption itself. It has been shown that vegetarians and other people who do not eat pork can acquire cysticercosis through fecal–oral contamination with *T. solium* eggs from tapeworm carriers (77). Unhygienic sanitary conditions such as limited use or absence of latrines are prevalent in rural areas of Africa where pigs are raised (14, 78, 79). Informally marketed foods, clandestine slaughtering of pigs, lack of trained and qualified meat inspectors, lack of detection and treatment of *T. solium* carriers, and consumption of undercooked or insufficiently cooked pork could be risk factors for taeniosis/cysticercosis among consumers (22, 26, 35).

A nutritional survey and coproparasitological examination was being carried out at the Nemba 1 School, Gakenke district, Rwanda, when two samples harboring *Taenia* spp. eggs were found (unpublished results). It was impossible to obtain more samples or additional information that would allow the analysis of other risk factors or possible variables related to clinical manifestations of the infection. For the reasons stated, we decided to carry out the present study with the sole objective of investigating the presence of infection and transmission of

T. solium in the community. The results obtained confirm the presence and transmission of *T. solium* and support the need to design more extensive future studies.

CONCLUSIONS

Overall, our study shows the presence and transmission of *T. solium* in Gakenke district. The high seroprevalence found in this area allows it to be considered hyperendemic for cysticercosis. To avoid neurological consequences, systematic investigations into porcine and human cysticercosis as well as health education and hygiene measures for *T. solium* control are needed. It is necessary to evaluate the clinical status of schoolchildren involved, carry out a study of contacts, and implement actions for the control and prevention of the disease.

DATA AVAILABILITY STATEMENT

All data analyzed during this study are included in this published article. The raw data or any further information supporting the conclusions of this article will be made available by the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Experimental Research Commission on Ethics from University Miguel Hernández de Elche (Spain) (Ref: DF-MPA-001-11). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FB-L, HG, and JGE contributed to conception and design of the study. LA, LP, and FB-L supervised and coordinated the field study. LA, MI-G, and FB-L carried out the coproparasitological analysis in the field. CM-A and JGE coordinated the coproparasitological procedures. JB, YC, and EP coordinated and performed the serological and coproantigen tests. LA and LP organized the database-analyzed data, realized formal analysis, and interpretation of data. LA, LP, and HG wrote the first draft of the manuscript. FB-L critically reviewed it and contributed to draft design. All authors contributed to manuscript revision, read, and approved the submitted version.

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Trends in the Epidemiology of Leishmaniasis in the City of Barcelona (1996–2019)

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Background: Leishmaniasis is a neglected zoonosis produced by 20 different flagellated parasites of the *Leishmania* genus, a protozoan transmitted to humans and other vertebrates by the bite of dipteran insects of the *Phlebotominae* subfamily. It is endemic in Mediterranean countries and the number of cases is expected to increase due to climate change and migration. Prioritizing public health interventions for prevention and control is essential. The objective was to characterize the epidemiology and temporal trends in the incidence of human leishmaniasis in the city of Barcelona, between the years 1996 and 2019.

Methods: A population-based, analytical observational study among residents in the city of Barcelona was conducted of all the cases of leishmaniasis reported between 1996 and 2019 to the Public Health Agency. The epidemiological survey contains clinical, diagnostic, and epidemiological data, including contact with suspicious mammals or insects. Annual incidence-rates were calculated by sex, age, and country of origin. Chi-square tests were used to assess association between studied risk factors, periods of time and type of leishmaniasis.

Results: During the study period a total of 177 cases of leishmaniasis were reported in Barcelona, being 74.6% ($n = 132$) of the total cases in Spanish born, although within the foreign-born population the incidence was higher. Median age was 34 years (IQR = 10–48) and 121 (66.8%) were male. The main type was cutaneous (46%) followed by visceral (35.1%). The cumulative incidence was 0.47 per 100,000 inhabitants, with the highest incidence found in 2017 (1.60 per 100,000 inhabitants). A higher incidence was observed in the 0–4-year-old group (1.73 per 100,000 inhabitants), but increased during the study period for all age groups. There was an increase of foreign origin cases, and a decrease in the number of cases associated to any immunosuppression.

Conclusion: In Barcelona, leishmaniasis incidence continues to be higher in people under 5 years of age, and 25–64 years old males, but it has also increased in population from foreign country of birth. There is an increase of the cases since 2016, probably

due to the changes in the notification system, increasing the diagnosis of cutaneous leishmaniasis. Improvements in the current surveillance system are needed. Notification of the disease, vector, and reservoir control activities are also essential for the control of the disease.

Keywords: parasitology, leishmania, zoonosis, *Phlebotomus*, surveillance, One Health, public health surveillance, infectious disease

INTRODUCTION

Leishmaniasis is a zoonosis produced by 20 different flagellated parasites of the *Leishmania* genus, a protozoan transmitted to humans and other vertebrates by the bite of dipteran insects of *Phlebotominae* subfamily (1, 2). The parasites that cause leishmaniasis are present in 98 countries around the world, causing between 700,000 and 1 million new cases annually, with an estimated 350 million people at risk of infection worldwide (1–4). There are three main clinical forms visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (ML) (1, 4). VL is the most serious form of leishmaniasis and causes a systemic disease characterized by irregular episodes of fever, spleen and liver enlargement, weight loss and anemia. CL is the most frequent presentation and causes skin lesions that evolve from nodules to painless ulcers, which can leave long lasting scars. ML is the most uncommon form of leishmaniasis, and leads to the destruction of nasooropharyngeal mucosa, usually after years of the initial skin lesions. Diagnosis is established through direct observation or molecular detection of *Leishmania* parasites in tissue specimens, mainly from skin lesions, for cutaneous leishmaniasis, or from bone marrow, for visceral leishmaniasis, although serological tests could also be used in different situations (2). The wide diversity of *Leishmania* species is associated with a difference in the clinical presentation, routes of transmission (zoonotic vs. anthroponotic) and treatment resistance (2, 5). In this sense, VL is usually caused by *L. donovani* or *L. infantum*, while CL can be caused by *L. infanfum*, *L braziliensis*, or *L. tropica*, between others (2).

In urban, periurban and domestic environments, dogs (*Canis lupus familiaris*) are usually considered main reservoirs of the parasite, although synanthropic rodents (e.g., *Rattus norvegicus*), wild rabbits (*Oryctolagus cuniculus*), hares (*Lepus granatensis*), and cats (*Felis catus domesticus*) can also play a relevant role as reservoir host (6–12).

In endemic developing countries, leishmaniasis mainly affects people of lower socioeconomic status, in rural areas with poor housing conditions, with little or no access to health services (1, 13). Within the risk groups, visceral leishmaniasis can present non-specific symptoms in children, and can be fatal without adequate treatment (14). Although coinfection with HIV is considered a public health problem in various parts of the world (15), the introduction of highly effective antiretroviral treatment in developed countries since 1997 has helped to decrease the HIV coinfection (16), so nowadays it is a pathology more common in immunosuppression non-HIV-related (17).

Incidence of Leishmaniasis in Mediterranean countries and across Europe has been described on the rise (1). Autochthonous cases have been recently described in non-endemic areas, like Southern Germany (18), and the number of imported cases has increased in areas such as Sweden or London (19, 20). In Sweden, an increase in diagnoses has been observed since 2013, especially in those under 18 years of age and migrants, mainly from Syria and Afghanistan (19). In London, most diagnoses occurred in tourists returning from the Mediterranean region and soldiers returning from other territories where the pathogen is endemic (20).

Climate change is expected to extend the geographical distribution and seasonality of phlebotomine vectors (7, 21). In Morocco, the increase in minimum temperatures has increased the survival of the sandfly larvae through the winters, extending their activity season. This combined with the increase in human circulation and tourism is thought to be one of the main causes for dissemination of cutaneous leishmaniasis (22). Spain has also experienced an increase in cases, though this phenomenon has been attributed to multiple factors, including improvements in notification, the seasonality of sandflies (16) and a growing urbanization that favors the contact between reservoirs and humans (23).

Studies in different areas of Spain have reported seroprevalence varying from 4 to 35% in dogs, with a high proportion of them not showing any clinical signs of leishmaniasis (24, 25). *Phlebotomus perniciosus* and *Phlebotomus ariasi* are the main vectors of *Leishmania infantum* in the Iberian Peninsula (23, 26). Moreover, in Spain, leishmaniasis is a hypoendemic, mandatory notifiable disease since 1995 (27), but reporting is compulsory for every Autonomous Region (CCAA) through the National Surveillance Network since 2015 (28). During the 2005–2017 period, Spain had an incidence rate of 0.62 cases per 100,000 inhabitants (16, 25), with a heterogeneous distribution, mainly affecting the Mediterranean region (the Valencian Community, Catalonia, the Balearic Islands and Andalusia), except for the Community of Madrid, where a large community outbreak was identified in 2009 and 2010 linked to a newly identified reservoir (26). The objective of this study is to characterize the epidemiology and temporal trends in the incidence of human leishmaniasis in the city of Barcelona, between the years 1996 and 2019.

METHODOLOGY

Study Design and Population

A population-based, analytical observational study was conducted with all reported leishmaniasis cases between

1996 and 2019. Data were drawn from the registry of notifiable diseases of the Epidemiology Service of the Public Health Agency of Barcelona (ASPB). The registry includes cases individually notified to the Epidemiology Service by healthcare professionals and, since 2016, it also includes laboratory notifications to the Catalan Microbiologic Notification System (SNMC) (29).

Variables

The registry of notifiable diseases contains the information included in the epidemiological surveys of cases carried out by public health nurses from the Epidemiology Service.

The epidemiological survey contains clinical, diagnostic, and epidemiological data, among others (**Annex 1**). The variables of interest included: age, sex, country of birth, date of onset of symptoms (onset, diagnosis, and notification data), type of leishmaniasis, history of immunosuppressive disease or immunosuppressive treatment, as well as history of contact with an infected dog or another animal suspected of infection and travel to an endemic country according to WHO's current guidelines at the time of the survey (30).

Inclusion and Exclusion Criteria

All cases of leishmaniasis from patients who resided in the city of Barcelona and who met the case definition according to local guidelines were included (**Annex 2**). Cases were excluded if the patients resided in another city than Barcelona.

Analysis

Annual incidence-rates and its confidence interval (95%) were calculated by sex, age, and country of birth, in relation to the municipal registry of the city of Barcelona (31). The microbiological confirmation and national surveillance system were modified in 2016 (28). Consequently, to test for temporal changes in the influence of these variables on the incidence of leishmaniasis, the study period was divided into three periods: 1996–2005 ($n = 55$), 2006–2015 ($n = 52$), and 2016–2019 ($n = 70$).

The risk factors obtained from the survey were analyzed by the three proposed periods and the clinical presentation of the leishmaniasis. Cases were classified according to the Available Family Income Index of the municipal district of residence (IRFD for "Index renda familiar disponible"), into disadvantaged (IRFD < 1) or favored (IRFD > 1) neighborhood (32). In 2019, 62.2% of Barcelona's population lived in disadvantaged neighborhoods (31). A chi-squared or a Fisher's exact test were performed, as appropriate, and a $p < 0.05$ was considered significant. All the analyses were done using the program STATA version 15.

RESULTS

Between 1996 and 2019, 177 cases of leishmaniasis were reported in Barcelona (**Table 1**). The median age was 34 years [interquartile range (IQR): 10–48]. 66.8% ($n = 121$) of the diagnoses occurred in men. According to origin, most cases occurred in native population (**Table 1**), however, a sustained increase in cases from foreign-born population were observed since 2003 (**Figure 1**). The main type of leishmaniasis was cutaneous (45.2%, $n = 80$), followed by visceral (34.5%, $n = 61$), and only 2.3% ($n = 4$) was mucocutaneous. 61.6% ($n = 109$) of the cases occurred in people who lived in disadvantaged municipal districts. 68.4% ($n = 121$) of the cases progressed to healing without sequel, 6.21% ($n = 11$) progressed with sequel, and 1.63% ($n = 3$) died (**Table 1**). The diagnosis were made by biopsy in 61% of the cases ($n = 108$), by a serological test in 21.5% ($n = 38$) and by culture in 19.4% ($n = 21$).

TABLE 1 | Descriptive statistics of the leishmaniasis cases reported in Barcelona between 1996 and 2019 ($n = 177$).

	n	%
Sex		
Men	121	68.4%
Women	55	31.1%
Country of origin		
Native	132	74.6%
Foreign	45	25.4%
Type of leishmaniasis		
Visceral	61	34.5%
Cutanous	80	45.2%
Mucocutaneous	4	2.3%
Missing	32	18.1%
Neighborhood's available family income index (AFII)		
Disadvantaged (AFII <1)	109	61.6%
Favored (AFII > 1)	57	32.2%
Missing	11	6.2%
Evolution		
Cured without sequelae (a)	121	68.4%
Cured with sequelae (a)	11	6.2%
Dead	3	1.7%
Lost to follow up (b)	42	23.7%

(a) Sequelae defined by the surveyor's criteria.
(b) Of the 42 lost cases of "Evolution," 9 cases were identified as "lost" by the person who carried out the survey, while the rest of the cases had no information.
In all the missing cases of the Type of leishmaniasis and Neighborhood, the value was not recorded in the epidemiological survey.

The cumulative incidence during the study period was 0.47 per 100,000 inhabitants (**Table 2**), with the highest incidence found in 2017 (1.6 per 100,000 inhabitants) and the lowest in 2012 (**Figure 2**). Incidence was generally higher in men than women (0.68 vs. 0.27 per 100,000 inhabitants) and in foreign-born over Spanish-born population (0.74 vs. 0.41 per 100,000 inhabitants). Slight changes in incidence were observed between the first and second period (0.36–0.32 per 100,000), increasing significantly to 1.08 per 100,000 after 2016. An increase of the incidence was observed in all age groups, especially in the third period, with the higher incidence found in the 0–4-year-old group (1.73 per 100,000 inhabitants) (**Table 2**).

During the three studied periods there was an increase of the cutaneous leishmaniasis in the second and third period (0–51.9 to 75.7%, $p < 0.001$), and a decrease of the visceral leishmaniasis in the final period (54.6 to 46.2% to a 10%, $p < 0.001$) (**Table 3**). Male sex accounted for over 80% of the cases in ages 25–64,

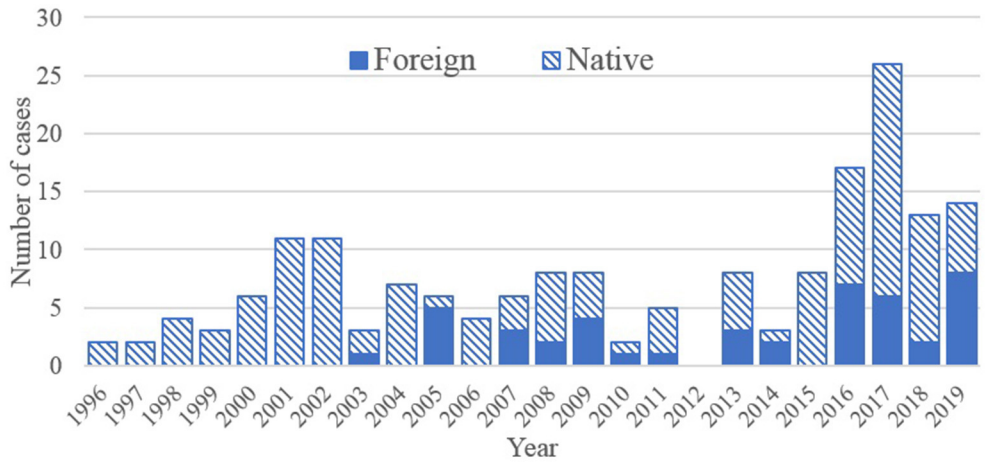

FIGURE 1 | Total number of cases of leishmaniasis, in Spanish born population (native) and foreigner country of birth, in Barcelona between 1996 and 2019 ($n = 177$ cases).

TABLE 2 | Incidence of leishmaniasis per 100,000 inhabitants and confidence interval (95%), by complete study time and periods 1996–2005 ($n = 55$), 2006–2015 ($n = 52$), and 2016–2019 ($n = 70$), Barcelona.

		1996–2005	2006–2015	2016–2019	Total
Sex	Female	0.14 [0.06–0.22]	0.22 [0.12–0.32]	0.73 [0.44–1.02]	0.27 [0.19–0.34]
	Male	0.61 [0.43–0.79]	0.41 [0.27–0.55]	1.47 [1.04–1.89]	0.68 [0.56–0.80]
Age	0–4	2.00 [0.87–3.13]	1.15 [0.35–1.94]	2.59 [0.67–4.51]	1.73 [1.52–1.93]
	5–14	0.17 [0.09–0.24]	0.00	1.64 [0.56–2.71]	0.37 [0.15–0.59]
	15–24	0.16 [0.10–0.22]	0.14 [0.08–0.20]	1.03 [0.21–1.85]	0.28 [0.11–0.45]
	25–44	0.71 [0.47–0.95]	0.44 [0.26–0.62]	0.91 [0.49–1.33]	0.62 [0.48–0.76]
	45–64	0.11 [0.00–0.22]	0.35 [0.17–0.53]	0.70 [0.30–1.09]	0.31 [0.19–0.42]
	65 or more	0.03 [0.01–0.05]	0.15 [0.02–0.28]	1.23 [0.65–1.81]	0.30 [0.18–0.42]
Country of origin	Native	0.34 [0.24–0.44]	0.28 [0.19–0.37]	0.95 [0.68–1.22]	0.41 [0.34–0.48]
	Foreign	0.50 [0.09–0.90]	0.48 [0.24–0.72]	1.48 [0.88–2.08]	0.74 [0.52–0.96]
Total		0.36 [0.27–0.45]	0.32 [0.23–0.41]	1.08 [0.83–1.33]	0.47 [0.40–0.54]

without greater differences during the studied periods. Over 60% of cases occurred in disadvantaged neighborhoods during the three periods ($p = 0.261$). Leishmaniasis in foreign subjects increased over time, but only accounted for a third of the total cases in the last period. A significant decrease in cases related to any immunosuppression (due to disease or treatment) was observed ($p < 0.001$).

All types of leishmaniasis occurred mainly in Spanish born, men, and people from disadvantaged neighborhoods (**Table 4**). Visceral leishmaniasis was present in 40.9% of the cases that referred contact with an animal and 26% of the cases that referred intravenous drug use. A 33.8% of the cases who referred travel to an endemic country presented a cutaneous type of leishmaniasis (endemic country according to WHO's current guidelines at the time of the survey). Most of the visceral leishmaniasis required a hospital admission and it was the only leishmaniasis type related to a fatal outcome.

Even if the current epidemiological survey does not systematically collect information on type of comorbidities, homelessness status, species of *Leishmania* or the type of animals that could have acted as a reservoir, it was possible to extract some of this information from the comments included by interviewers. Regarding comorbidities, it was observed that an 18.4% ($n = 35$) of the studied population had a diagnosis of HIV and two cases were described as Hepatitis C positive. Four cases were informed to be homeless. Seven cases were characterized as *Leishmania donovani*, and one case was described as *Leishmania braziliensis*.

DISCUSSION

The results of this study have shown an increase in the incidence of leishmaniasis in the city of Barcelona from 1996 to 2019. This increase only appears as statistically significant for the period 2016-2019. The establishment of the *Catalan Microbiologic*

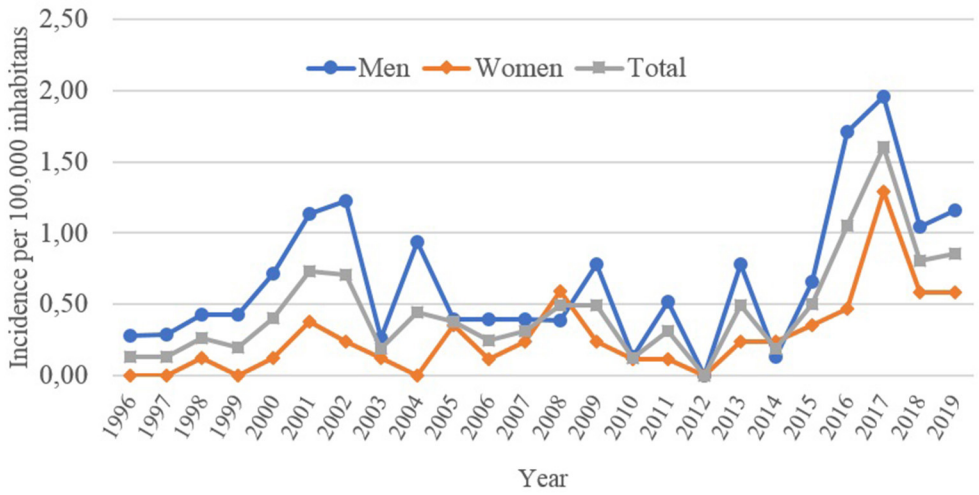

FIGURE 2 | Annual incidence of leishmaniasis per 100,000 inhabitants, by complete sample and sexes. Barcelona, 1996–2019. (n = 177 cases).

TABLE 3 | Cases of leishmaniasis according to type of disease and risk factors, during the three studied periods.

	1996–2005 *N* (%)	2006–2015 *N* (%)	2016–2019 *N* (%)	Total *N* (%)	*p*-value
Type of leishmania					<0.001
Visceral	30 (54.6%)	24 (46.2%)	7 (10%)	61 (34.5%)	
Cutaneous	0	27 (51.9%)	53 (75.7%)	80 (45.2%)	
Mucocutaneus	1 (1.82)	0	3 (4.3%)	4 (2.3%)	
Risk factors					
Male sex by ages					
Male (0–4)	7 (58.3%)	4 (50%)	2 (28.6%)	13 (48.3%)	0.545 *f*
Male (5–14)	2 (100%)	0	5 (55.6%)	7 (63.6%)	0.491 *f*
Male (15–24)	1 (33.3%)	1 (50%)	3 (50%)	5 (45.5%)	1 *f*
Male (25–44)	30 (90.9%)	16 (72.7%)	14 (77.8%)	60 (82.2%)	0.202 *f*
Male (45–64)	4 (100%)	9 (64.3%)	11 (91.7%)	24 (80%)	0.149 *f*
Male (65 or more)	0	2 (40%)	10 (56.6%)	12 (50%)	0.640 *f*
Disadvantaged neighborhood	31 (63.3%)	36 (75%)	42 (60.9%)	109 (65.7%)	0.261
Foreign origin	6 (10.9%)	16 (30.8%)	23 (32.9%)	45 (25.4%)	<0.001
Immunosuppression	25 (45.5%)	18 (34.6%)	7 (10%)	50 (28.5%)	<0.001
Due to disease	24 (61.5%)	17 (32.7%)	6 (8.6%)	47 (29.2%)	<0.001
Due to treatment	12 (30.8%)	6 (11.5%)	5 (7.1%)	23 (14.3%)	0.009
Total cases	55 (100%)	52 (100%)	70 (100%)	177 (100%)	

P-values presented with an italic f (f) when Fisher's exact test is performed.

Notification System as a consequence of the decree 203/2015 (29), which requires mandatory notification of all identified cases by laboratories, is likely to have contributed greatly to the observed trend, together with other factors such as the improvement in diagnostic tools and possibly a real change in disease burden. The increase is observed in all ages, being greater in the younger population and male adults. The number of foreign-born cases has increased since 2003 while the number of cases associated to immunosuppression has decreased. There is also a change in the type of leishmaniasis in the three studied periods, with an increase of cutaneous leishmaniasis.

The cumulative incidence during these 24 years of observation was higher than previously reported in analysis of the same database (0.33 per 100,000 inhabitants between 1997 and 2014) (33) but lower than the reported incidence of Spain (16, 25). After the modification of the confirmation and surveillance system (28, 29), there was a significant increase of the total incidence probably due to an improved reporting, and because a possible increase in the clinical suspicion at primary care level.

Our study has shown that men between 25 and 64 years old present a higher incidence of leishmaniasis in all the studied periods. Previous studies have associated this correlation with the incidence of HIV in this group (33). However, the reasons why men of working age have a higher incidence of leishmaniasis are diverse, and cannot be exclusively related to this comorbidity, even when the data obtained through the epidemiological survey does not allow us to test for further causality. In this population, men accounted for 91.9% of the cases related to alcoholism, 81.3% of cases with a history of intravenous drug use and 70% of traveling to endemic countries. Studies have described a male bias in certain infectious diseases (34), including leishmaniasis (35), proposing physiological and behavioral theories. However, the evidence is not conclusive, and we cannot exclude that man are more exposed to infected vectors because of their social or working activities.

By age groups, there was a consistent high incidence in cases under 5 years of age, but also a rise in the cases over 65 years since 2001. In the younger population, the main causes are associated to a lack of effective immune response (14, 33). In our sample, an 88.9% of the age group 0-4 was from native origin, a 66.7% presented a visceral type of leishmaniasis, and

TABLE 4 | Type of Leishmaniasis according to risk factors and evolution.

	Visceral *N* (%)	Cutaneous *N* (%)	Muco cutaneous *N* (%)	Total *N* (%)	*P*-value (a)
Risk factors					
Foreign origin	12 (19.7%)	26 (32.5%)	0	45 (27.9%)	0.092 *f*
Male sex	43 (71.7%)	52 (65%)	4 (100%)	109 (68.1%)	0.491
Disadvantaged neighborhood	39 (69.6%)	51 (64.6%)	3 (75%)	102 (75%)	0.911
Immunosuppression	31 (50.8%)	8 (10%)	1 (25%)	50 (31.1%)	<0.001 *f*
Contact with animal	25 (40.9%)	9 (11.3%)	1 (25%)	35 (21.8%)	<0.001 *f*
Travel to endemic country	8 (13.1%)	27 (33.8%)	0	35 (20.1%)	<0.001 *f*
History of transplant	1 (1.6%)	1 (1.3%)	0	2 (1.2%)	*
History of alcoholism	8 (13.1%)	3 (3.8%)	1 (25%)	12 (6.9%)	<0.001 *f*
History of transfusion	5 (8.2%)	0	0	5 (2.9%)	*
Intravenous drug use	16 (26.2%)	0	0	16 (9.2%)	*
Evolution					
Hospital admission	57 (93.4%)	1 (1.3%)	1 (25%)	62 (32.6%)	
Cured without sequels	51 (83.6%)	67 (83.8%)	3 (75%)	121 (68.4%)	
Cured with sequels	4 (6.6%)	6 (7.5%)	1 (25%)	11 (6.2%)	
Death	2 (3.3%)	0	0	3 (1.7%)	
Total	61 (34.5%)	80 (45.2%)	4 (2.3%)	177 (100%)	

Barcelona, 1996–2019 (missing values = 32; 18.08%). P-values presented with an italic f (f) when Fisher's exact test is performed, and with an asterisk (*) when not performed due to cases with very low frequency.

85.19% evolved to healing without sequel. Is important to notice that there was no differences between native or foreign-born population in this age group during the three studied periods (Fisher's $p = 0.142$). Moreover, cases in the 0-4 age group during the third studied period (2016–2019), only occurred in Spanish-born population. In the elderly group, there was a rise in the diagnosis of cutaneous leishmaniasis, being 64% of the total cases over 65 years of age. This group only presented a 20% history of immunosuppression and a 24% foreign origin. A 76% of the cases evolved to healing without sequel. The reasons for this increase are unclear and may be related to a change in the diagnosis system and access to dermatological services from primary care, but further investigation is required.

Among population from foreign country of birth, there is a persistent increase of incidence since 2003, consistent with a rise in foreign population migrating to Barcelona (3.8% of the total population in 1996 to 25.7% in 2019) (31), but also to international traveling to endemic areas, due to tourism, visiting relatives and work (22, 36). The difference between autochtonous and imported cases is difficult to establish due to the long incubation period of leishmaniasis (2 weeks to several years), and inconsistencies in the reporting of identified *Leishmania* species. Thus, our data might be overestimating the burden of locally acquired *Leishmania*. The introduction risk supposed by *Leishmania braziliensis* imported cases is thought to be limited due to the reported absence of competent vectors in Europe (7), although a possible competence of *P. perniciosus* to *L. tropica* should be accounted (37–39). Improvements in the registry should allow for the systematic report of the species of *Leishmania* involved in the clinical cases.

In relation to vulnerable populations, there was a decrease in cases related to immunosuppression, due to disease or treatment. This decrease could correspond to the improve of the antiretroviral treatments for HIV population (16), as well as improvements in the care of immunosuppressed patients, like transplanted, who presents a very low incidence of cases. In this study, most of the cases occurred in disadvantaged districts of Barcelona, without changes in time. Homeless cases are at a higher risk to get lost to follow up, facing a challenge to the prevention and surveillance strategy for years to come. Even when we did not have access to information of the individual's socio-economic status, the use of the Available Family Income Index is a good proxy to evaluate inequalities within the city (32).

Leishmaniasis is the most relevant endemic zoonosis in the last years in the Mediterranean basin, due to the coexistence of *Leishmania* protozoa; reservoirs such as hare (11, 40), rabbits (10), rodents and dogs (9, 41), and competent vectors (sandflies from the genus *Phlebotomus)*. As a Mediterranean city, Barcelona has the appropriate climatic characteristics for local transmission of *Leishmania* sp. The presence of the two main vectors, *Phlebotomus perniciosus* and *Phlebotomus ariasi*, are known due to the monitoring of the city of Barcelona by the ASPB, the institution responsible for the vector surveillance and control in the city (42). Furthermore, recent research indicates that Norway rat, a synanthropic murid that shows high abundance and deeply distribution in the sewer system of the city (6), could play a relevant role as host reservoir of Leishmaniasis (43). At this point, it is important to emphasize the sewers have been also recorded as suitable breeding site for sandflies in Barcelona (unpublished data).

Climate change is affecting the geographical distribution and seasonality of many species, including sandflies, and increased connectivity has facilitated the expansion of different insect vectors into new geographical areas (7, 21). A climate change induced expansion of vector competent species will at the same time lead to an expansion of the risk area for leishmaniasis. The expansion of the green areas across the city due to the Climate change action plan (44) should consider the potential risk of increase in competent vector distribution and reservoirs. Overall, there is a need to enforce the surveillance system of leishmaniasis. Therefore, we recommend undertaking surveys in the city to characterize the distribution and abundance of different vectors, as a strategy to establish an effective surveillance and control program to reduce the risk of transmission.

Our results show that that autochthonous transmission is occurring in the city of Barcelona and consequently a more in-depth study of the vectors present in the city should be carried out, in order to know their distribution, abundance, and blood feeding behavior and thus have a diagnosis of the risk of transmission and spillover into humans. Epidemiological, vectorial and health surveillance must have in consideration the high risk groups, like homeless and exposed workers, as those who work in sewer systems, together with vector

control programs associated to renovation works affecting the underground structure that may facilitate the movement of *Phlebotomus* into inhabited areas (3).

The main limitations of this study are the loss of follow up of some cases, and the lack of information regarding significant variables. There is a regular loss of cases each year, which may be due to cases that occurred in the homeless, or people that were in transit and the clinical history is incomplete. There is a clear need to review the current epidemiological survey to systematically collect information on the detected leishmania species, the type of treatment used and its efficacy, as well as homelessness status, to better characterize the impact of leishmaniasis on vulnerable populations. Identifying the domestic animals that act as reservoirs and ensuring they receive the necessary treatments to reduce the risk of new transmissions to humans should also be a priority to reduce the risk of local outbreaks (45, 46). It is also important to quantify the role that non-domestic animal species may play as reservoirs associated to human infection cases in the city (5, 47). Collaboration at an epidemiological and entomological level is one of the pillars to reduce the risks of transmission, thus trying to work on a One Health concept that integrates the different fields involved in the transmission processes.

Even though leishmaniasis is an endemic disease in Spain, the city of Barcelona will continue to be a highly transited city, with a higher migration rate and tourism, which may favor the occurrence of imported cases. This constitutes an entomological, clinical, and epidemiological challenge, due to the variety of clinical presentations and adaptation to vectors or local reservoirs.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

TM, J-PM, and CR worked in the conception and first design of the work. LM, PS, and EM were in charge of the acquisition of the data, and organization of the database. DP performed the statistical analysis and wrote the first draft of the manuscript. DP, LM, and JF worked in the interpretation of data for the work. DP and LM were in charge of drafting the work. RB-M, JF, PS, EM, CR, and TM critically revised the manuscript. All authors provide their final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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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.2021.653999/full#supplementary-material

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frontiers in Veterinary Science

REVIEW
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Epidemiology of Trypanosomiasis in Wildlife—Implications for Humans at the Wildlife Interface in Africa

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While both human and animal trypanosomiasis continue to present as major human and animal public health constraints globally, detailed analyses of trypanosome wildlife reservoir hosts remain sparse. African animal trypanosomiasis (AAT) affects both livestock and wildlife carrying a significant risk of spillover and cross-transmission of species and strains between populations. Increased human activity together with pressure on land resources is increasing wildlife–livestock–human infections. Increasing proximity between human settlements and grazing lands to wildlife reserves and game parks only serves to exacerbate zoonotic risk. Communities living and maintaining livestock on the fringes of wildlife-rich ecosystems require to have in place methods of vector control for prevention of AAT transmission and for the treatment of their livestock. Major *Trypanosoma* spp. include *Trypanosoma brucei rhodesiense*, *Trypanosoma brucei gambiense*, *and Trypanosoma cruzi*, *pathogenic for humans*, *and Trypanosoma vivax*, *Trypanosoma congolense*, *Trypanosoma evansi*, *Trypanosoma brucei brucei*, *Trypanosoma dionisii*, *Trypanosoma thomasbancrofti*, *Trypanosma elephantis*, *Trypanosoma vegrandis*, *Trypanosoma copemani*, *Trypanosoma irwini*, *Trypanosoma copemani*, *Trypanosoma gilletti*, *Trypanosoma theileri*, *Trypanosoma godfreyi*, *Trypansoma simiae*, and *Trypanosoma (Megatrypanum) pestanai*. Wildlife hosts for the trypansomatidae include subfamilies of Bovinae, Suidae, Pantherinae, Equidae, Alcephinae, Cercopithecinae, Crocodilinae, Pteropodidae, Peramelidae, Sigmodontidae,

and Meliphagidae. Wildlife species are generally considered tolerant to trypanosome infection following centuries of coexistence of vectors and wildlife hosts. Tolerance is influenced by age, sex, species, and physiological condition and parasite challenge. Cyclic transmission through *Glossina* species occurs for *T. congolense*, *T. simiae*, *T. vivax*, *T. brucei*, and *T. b. rhodesiense*, *T. b. gambiense*, and within *Reduviid* bugs for *T. cruzi. T. evansi* is mechanically transmitted, and *T. vixax* is also commonly transmitted by biting flies including tsetse. Wildlife animal species serve as long-term reservoirs of infection, but the delicate acquired balance between trypanotolerance and trypanosome challenge can be disrupted by an increase in challenge and/or the introduction of new more virulent species into the ecosystem. There is a need to protect wildlife, animal, and human populations from the infectious consequences of encroachment to preserve and protect these populations. In this review, we explore the ecology and epidemiology of *Trypanosoma* spp. in wildlife.

Keywords: trypanosomes in wildlife, human-wildlife interactions, wildlife-livestock interactions, human African trypanosomiasis, sleeping sickness, *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*

INTRODUCTION

The African and American trypanosomiases present significant global health challenge in human, domesticated animal, and wildlife communities. Spillover of parasites from wildlife to domestic livestock and humans and from domestic animal species to wildlife compromises health (1, 2). Most trypanosome infections in wildlife do not cause obvious damage to their host (3, 4), but some wildlife species are highly susceptible to trypanosome infections, including *Rattus nativitatis* and Macleay's rats (*Rattus macleari*) (5).

Trypanosoma are primarily transmitted by vectors (6) within which they undergo complex development cycles. Trypanosomes, which develop in the posterior section of the digestive tract in insects, are called stercorarian, for example, *Trypanosoma cruzi* the causal agent of Chagas disease, common in Latin America (7). Salivarian trypanosomes develop in the anterior part of the insect gut tract and include the causal agents of African animal trypanosomiasis (AAT) or nagana and for human African trypanosomiasis (HAT) caused by *Trypansoma brucei rhodesiense* and *Trypansoma brucei gambiense* that are present across Sub-Saharan Africa (8).

Animal trypanosomiasis is endemic in tropical regions of Africa, parts of Asia, and South America (9). *T. brucei s.l.*, *Trypanosoma congolense*, *Trypanosoma simiae*, and *Trypanosoma uniforme* are transmitted within the tsetse belts of Africa and cannot be transmitted by mechanical vectors (9). *T. vivax* and *Trypansoma evansi* can be transmitted mechanically and occur within and outside tsetse fly-infested zones (10).

EPIDEMIOLOGY OF ANIMAL TRYPANOSOMIASIS

Trypanosomiasis is one of the most important diseases affecting livestock, equines, and dogs within the Sub-Saharan region (11, 12). Cross transmission of parasites between livestock and wildlife hosts has been reported, especially in areas in close proximity to game parks and wildlife reserves. Wildlife species can survive within the tsetse belts across the Sub-Saharan region, despite being reservoir hosts for multiple species of trypanosome. The high prevalence of trypanosomiasis within protected areas traditionally has rendered these areas unattractive for livestock keeping and agricultural production (13).

Phylogenetic analysis shows a remarkable complexity of trypanosome species, subspecies, and strains in tsetse flies, human, domestic, and wildlife hosts. Examining the trypanosome species circulating within an ecosystem is a key to identifying the wildlife reservoirs of infection and transmission parameters to other animal hosts, including livestock within the ecosystem (3). Understanding the various trypanosome species harbored in wildlife hosts can guide preventive and control measures of trypanosomiasis in communities living at the livestock–wildlife interface. A wide variety of trypanosome species are circulating among wildlife hosts including *T. brucei s.l.*, *T. congolense*, *T simiae*, *T. godfreyi*, and *T. theileri* (3).

Hosts and Species of Trypanosomes

Apart from *T. cruzi*, present in South America, and *T. theileri*, present worldwide, trypanosomes infect a large number of wild and domestic ungulate species (6). Infection in the wildlife is influenced by species and habitat (12). Wildlife hosts for trypanosomiasis are numerous and include antelope species, warthogs (*Paecocherus aethiopicus*), elephants (*Loxodanta africana*), hippopotamus (*Hippopotamus amphibius*), lions, hyenas, jackals, caracals, and wild ruminants (14–16). Trypanosome species commonly found in wildlife species include *T. vivax*, *T. brucei s.l.*, *T. congolense*, and *T. evansi* (14). *T. vivax*, a pathogen affecting cattle, has been identified in waterbucks and giraffes, but the strains of *T. vivax* in these two host species were unique (3). Multiple wildlife hosts carry the human-infective zoonotic trypanosome strain *T. b. rhodesiense*, including bushbucks (*Tragelaphus scriptus*), impala (*Aepyceros melampus*), lion (*Panthera leo*), zebra (*Equus*

quagga boehma), warthog (*Phacocoerus africanus*), and duiker (*Sylvicapra grimmia*) (12). *Trypanosoma conglense*, *Trypanosoma simiae*, and *Trypanosoma godfreyi* were identified in Rhinocerus posttranslocation (16).

Infection with trypanosomes can predispose infected animals to other infections (17), and concurrent and opportunistic bacterial infections in wildlife can hasten the onset of clinical trypanosomosis (17).

There are significant associations between taxonomic groups of wildlife hosts and the prevalence of trypanosomiasis. Wildlife hosts from the bovinae group show a high prevalence of trypanosomiasis, especially *T. vivax* and *T. congolense* as well as human infective *T. brucei* (**Table 1**). Infection is attributed to their grazing habits, taking them into contact with tsetse and other biting flies. Wildlife hosts from the Pantherinae group show a very high prevalence of mixed trypanosome infections, as do those from the Suidae (12). A summary of trypanosome species in wildlife hosts and host taxonomy is shown in **Table 1**.

Transmission to Wildlife

Transmission of trypanosome species is generally by biting vectors including *Tsetse flies*, *Tabanids*, *Stomoxys*, *Heamatopota*, and *Hippobosca* (15, 30, 31). Infection in carnivores is additionally from consumption of infected meat as documented in the Felidae and Canidae (31, 32). *Desmodus rotundus* (Vampire bats) also transmit trypanosomiasis (32).

Trypanosomes engage in two patterns of transmissions: Cyclical transmission in which trypanosomes undergo active multiplication within the vectors (tsetse flies) as is common for *T. congolense*, *T. simiae*, *T. vivax*, *T. brucei*, and the human infective trypanosome species (*T. rhodesiense and T. gambiense*); and mechanical transmission through tsetse and alternative vectors including biting flies of the Tabanidae family (horse flies) as well as Stomoxys species. *T. evansi* and *T. vivax* can be mechanically transmitted (33).

Distribution of Reservoir Hosts

Preservation of natural resources including game parks and game reserves has led to an expansion of wildlife populations that serve as reservoirs for AAT and HAT (34). Human encroachment on the game parks and forests has increased AAT transmission between wildlife and domestic livestock, due to increased tsetse–human and tsetse–livestock contacts (34). The distribution of host populations within these areas determine vector and parasite survival. Wildlife hosts including monkeys and warthogs live in less restrictive habitats, unattractive to poachers with limited trophy hunting leading to increased reservoir host multiplication rates. They are widely distributed in the ecological environment and are favorable reservoirs for multiple trypanosome species due to their availability to vectors. Crocodiles and hippopotamus are limited to specific habitats, limiting access of vectors.

Distribution of Tsetse Flies

Trypanosomiasis affects one-third of Africa's land mass (35–37). Tsetse are found across most of West, Eastern, Central, and Southern Africa (38). The different species and subspecies of tsetse are shown in **Table 2**. Tsetse populations require moderate temperatures (23–25°C), high relative humidity (75–90%) with weak saturation deficit and shade (47–49). Temperatures above 34.1°C limit survival of tsetse and trypanosomes (35).

Food Source—Activity and Migration

Differences in wildlife food sources, particularly for wild bovidae, influence their exposure to trypanosomiasis. Among ruminant wildlife hosts, browsers are more at risk of trypanosomiasis compared with grazers; semi-browsers are moderately susceptible. Eland, Waterbuck, Kudu, and Bushbuck are more heavily infected, associated with their preference for inhabiting thickets during tsetse feeding hours, predisposing them to more bites (13, 18).

Diurnal wildlife hosts are more susceptible to trypanosome infection compared with the nocturnal species. Warthogs are most active in the morning and late afternoon and show high infection rates for trypanosomiasis in correlation to vector feeding hours (20). Lions are more infected in areas of high tsetse challenge than low challenge (50). The movement of large numbers of animals within wildlife ecosystems also influences infection. Animals will migrate in the dry season toward water sources that are also tsetse habitats (20).

Pathogenesis of Trypanosomosis

Trypanosome infection leads to erythrophagocytosis and heme catabolism resulting in iron accumulation in tissues and hyperbilirubinemia, liver dysfunction, and multiple organ failure (51). At necropsy, atrophy of body fats, pulmonary edema, hepatomegaly, lymphadenopathy, and hemorrhages are observed. Trypanosomes are found in tissues and body organs, and enlarged periarteriolar sheaths have been observed in wildlife (52).

Clinical Signs

Trypanosomiasis is a chronic progressive disease, and clinical signs may become obvious in advanced stages of the disease (53). Bovines affected by *T. vivax* present with severe anemia, lethargy, photophobia, lacrimation, and inappetence (17, 54, 55); pyrexia fluctuates with the fluctuating parasitemia. Leukopenia, thrombocytopenia, and degenerative and inflammatory lesions are observed in most organs (56). Body condition scores deteriorates gradually, and animals are dehydrated and debilitated before death. Superficial lymph nodes are enlarged and conspicuous. Corneal opacity may be observed with lacrimation (57). Young animals are stunted even with proper feeding, and productivity is impaired by abortions (32, 38). Animals may show localized or generalized edema (58). Except for *T. equiperdum*, other *Trypanosoma* species include similar clinical signs, but variations in intensity may happen in the various species. *Trypanosoma brucei* s.l. infection shows limited clinical signs in bovines of indigenous species but is highly pathogenic in exotic species (59).

Diagnosis of Trypanosomiasis

Trypanosomiasis is characterized by the intermittent presence of parasites in the blood and intermittent fever (54). Parasitological examinations are relatively sensitive during the acute phase

TABLE 1 | Trypanosome species and taxonomy of wildlife hosts.

Taxonomic group	Wildlife host (scientific name)	Trypanosome species	References
Bovidae	Waterbuck *(Kobus ellipsiprymnus)*	*T. vivax*, *T. congolense*, *T. brucei*, *T. evansi*	(12)
Girrafidae	Girraffe (*Giraffa camelopardalis*)	*T. vivax*, *T. evansi*, *T. brucei*	(12)
Bovidae	African buffalo (*Syncerus caffer)*	*T. b. rhodesiense*, *T. congolense*, *T. brucei*	(12)
	Bushbuck (*Tragelaphus scriptus*)	*T. b. rhodesiense*, *T. congolense*, *T. vivax*, *T. evansi*	(12)
	Greater kudu (*Tragelaphus strepsiceros*)	*T. congolense*, *T. vivax*	(12)
	Red lechwe (*Kobus leche*)	*T. theileri*	(18)
	Hartebeest (*Alcelaphus buselaphus*)	*T. godfreyi, T. brucei, T. godfreyi*	(18)
	Sable antelope (*Hippotragus niger*)	*T. brucei, T. theileri*	(18)
	African buffalo (*Syncerus caffer*)	*T. theileri, T. godfreyi*	(18)
	Eland (*Taurotragus derbianus)*	*T. vivax*, *T. congolense*, *T. brucei*	(12)
	Impala (*Impala impala*)	*T. godfreyi, T. brucei*	(18)
Elephantidae	Elephant (*Loxodanta africana*)	*T. vivax*, *T. congolense*, *T. evansi*, *T. elephantis*	(19)
Hippopotamidae	Hippopotamus (*Hippopotamus amphibius*)	*T. vivax*, *T. brucei*, *T. evansi*, *T. congolense*	(20)
Suidae	Warthog (*Phacocoerus africanus)*	*T. b. rhodesiense*, *T. vivax*, *T. congolense*, *T. evansi*	(20)
	Warthog (*Phacochoerus africanus*)	*T. godfreyi, T. brucei, T. simiae*	(18)
	Feral pig (*Sus scrofa*)	*T. evansi and T. cruzi*	(21)
Pantherinae	Lion (*Panthera leo*)	*T. brucei*, *T. evansi*, *T. congolense*, *T. congolense*	(12, 18)
	Leopard (*Panthera pardus)*	*T. brucei, T. congolense, T. evansi*	(12)
Equidae	Zebra (*Equus quagga boehma*)	*T. b. rhodesiense*	(12)
Cephalophinae	Common duiker (*Sylvicapra grimmia*)	*T. b. rhodesiense*, *T. vivax*, *T. congolense*	(12)
Aepycerotinae	Impala (*Aepyceros melampus*)	*T. b. rhodesiense*, *T. congolense*, *T. evansi*	(20)
Rhinocerotidae	Rhino (*Diceros bicornis)*	*T. brucei*	(20)
Alcephinae	Wildebeest (*Connochaetes taurinus cooksoni)*	*T. brucei*, *T. congolense*, *T. vivax*	(20)
	Hartebeest (*Alcephalus buselaphus*)	*T. evansi, T. brucei*	(20)
Hyaenidae	Hyena (*Hyaena hyaena)*	*T. evansi, T. congolense*	(12)
Cercopithecinae	Vervet monkey *(Cercopithecus species)*	*T. gambiense*	(12)
	Baboon (*Papio cynocephalus)*	*T. congolense*	(12)
Crocodilinae	Crocodile (*Crocodylus niloticus*)	*T. vivax*	(12)
Hippotraginae	Roan antelope (*Hippotraggus equinus)*	*T. vivax*, *T. congolense*	(12)
Pteropodidae	Megabat/fruit bat (*Chiroptera*)	*Trypanosoma dionisii*, *T. cruzi*	(22, 23)
Phalangeridae	Brushtail possum (*Trichosurus vulpecula*)	*Trypanosoma* spp.	(17)
Muridae	Brush-tailed rabbit-rat (*Conilurus penicillatus*)	*Trypanosoma* spp.	(5)
Potoroidae	Brush-tailed bettong (*Bettongia penicillata*)	*T. vegrandis*, *T. copemani*	(24)
Dasyuridae	Northern quoll (*Dasyurus hallucatus*)	*Trypanosoma* spp.	(5)
Peramelidae	Northern brown bandicoot (*Isoodon macrourus*)	*Trypanosoma* spp.	(5)
Phascolarctidae	Koalas (*Phascolarctos cinereus*)	*Trypanosoma irwini, T. copemani*	(25)
	Koalas (*Phascolarctos cinereus*)	*T. gilletti*	(25)
Cervidae	Marsh deer (*Blastocerus dichotomus*)	*Trypanosoma theileri*, *T. evansi*	(26)
Canidae	African wild dog (*Lycaon pictus*)	*T. godfreyi*	(18)
Potoroidae	Boodie (*Bettongia lesueur*)	*Trypanosoma spp.*	(27)
Tayassuidae	White-lipped peccary (*Tayassu pecari*)	*Trypanosoma evansi and Trypanosoma cruzi*	(21)
Mustelidae	Wild European badger (*Meles meles*)	*Trypanosoma (Megatrypanum) pestanai*	(28)
Meliphagidae	Regent honeyeater (*Anthochaera phrygia*)	*Trypanosoma thomasbancrofti*	(29)

of the disease. Wet blood film examination is used to detect the presence of motile trypomastigotes but has low sensitivity. Blood is taken from the tail or ear veins (55). Fluorescence microscopy can improve sensitivity for thin and thick smears (60). Parasitic concentration by centrifugation and examination of the buffy coat is more sensitive than the wet and thick smears (61). Dark ground or phase-contrast microscopy increases sensitivity at low parasitemia (62). Anion exchange chromatography is also sometimes deployed for detecting low parasitemia (54, 63, 64).

Molecular tests and serological tests are more sensitive than the usual parasitological tests for *Trypanosoma* (3, 63–68). Common serological tests include CFT, ELISA, and IFAT, while the common molecular tests are PCR, LOOP/LAMP, and LFA.

TABLE 2 | Tsetse species, geographical distribution, and wildlife spp. affected by trypanosomiasis.

Subgenus	Glossina species	Glossina subspecies	Country	Wildlife animal spp.	References
Nemorrhina (Palpalis)	*Glossina palpalis*	*G. p. palpalis*	Nigeria, Angola Cameroon,	Bushbuck, primates, warthogs	(36)
	G. tachinoides		Nigeria		(37)
		G. p. gambiesis	Gambia, Senegal, Republic of Guinea	Baboons, monkeys, chimps	(39, 40)
	G. fuscipes	*G. f. fuscipes*	Uganda, Sudan, Ethiopia, Kenya, DRC	Buffaloes, antelopes	(41)
		G. f. martini	Uganda, Tanzania, DRC	Buffaloes	(42)
		G. f. quanzensis	Uganda, Tanzania	Buffaloes, antelopes	(42)
	G. pallicera	*G. p. pallicera*	Cameroon, Ivory coast, Liberia	Antelopes	(43–45)
		G. p. newsteadi	DRC	Lions, leopards	(46)
	G. tachinoides		Nigeria, Ghana, Cameroon	Buffaloes, lions, buffaloes	(46)
	G. caliginea		Nigeria, Congo Brazaville	Cheetah, lions, leopards	(46)
Glossina (morsitans)	*G. morsitans*	*G. m. morsitans*	Nigeria, Uganda, Tanzania, Zambia	Buffaloes, rhinoceros, antelopes	(46)
		G. m. submorsitans	Uganda, Tanzania	Buffaloes, bushbuck, antelopes	(46)
		G. m. centralis	Uganda, Tanzania	Buffaloes, bushbucks, antelopes	(46)
	G. swynnertoni		Nigeria, Congo Brazaville	Lions, cheetahs	(46)
	G. longipalpis		Ivory Coast, Senegal, Mali	Buffaloes, lions	(46)
	G. pallipides		Ethiopia, DRC, Uganda, Kenya, Zambia, Tanzania	Buffaloes, lions, antelopes	(46)
	G. austeni		Kenya, Tanzania, Mozambique	Bushbucks, antelopes, lions	(46)
	G. vanhoofi		DRC	Lions	(46)
	G. tabanformis		Nigeria, DRC	Buffaloes, lions	(46)
	G. severini		DRC	Lions, bushbucks	(46)
	G. schwetzi		Togo. Congo Brazaville	Wild ruminants	(46)
	G. nigrofusca		Ivory Coast, Nigeria, CAR, DRC	Elephants, lions, monkeys	(46)
	G. nashi		Cameroon, Nigeria, Togo	Monkeys, baboons, wild cats	(46)
	G. medicorum		Ghana, Gambia, Nigeria	Lions, buffaloes	(46)
	G. longipennis		Tanzania, Sudan, Kenya	Antelopes, bushbucks, lions	(46)
	G. hanningtoni		Nigeria, Cameroon, Gambia	Bushbucks, buffaloes	(46)
	G. fuscipleuris		CAR, DRC Cameroon	Lions, bushbucks	(46)
	G. brevipalpis		Kenya, DRC, Tanzania	Buffaloes, antelopes	(46)

Low-flow assay (LFA) is cheaper with 96.3% sensitivity and 93.9% specificity (69). Approved tests for AAT are shown in **Table 3**.

TRYPANOSOMES IN WILDLIFE

The majority of trypanosome species require multiple obligatory hosts to complete their life cycles (heteroxenous), and the transmission of the parasites is mainly via hematophagous invertebrate vectors (2, 88). Trypanosomes are found in blood and tissues; blood-borne protozoan trypanosomes (*Trypanosoma vegrandis*) have been identified in wild animals including, but not limited to, the northern brown bandicoot (*Isoodon macrourus*), common brushtail possum (*Trichosurus vulpecula*), northern quoll (*Dasyurus hallucatus*), and brush-tailed rabbit-rat (*Conilurus penicillatus*) (5). *Trypanosoma cruzi*, *Trypanosoma dionisii*, and an insect trypanosome (*Blastocrithidia*) have been found to infect bats and other mammalian wildlife in Europe and South America (22). Bats, possums, and rats act as reservoirs of trypanosomes for domestic and wild animals, as well as humans (5, 22). *T. cruzi* (Chagas) has been identified in kidney tissue, heart muscle, and blood, urine, and peritoneal fluid of wild spp. including foxes, opossums, raccoons, and striped skunks (89, 90), and parasites can be transmitted from animal-to-animal by contamination of animal wounds with blood, urine, and peritoneal fluid (89, 90).

Trypanosome–Host Relationships

Hosts are classified according to their role as a definitive host [if the mature sexual stage(s) of the parasite occurs within them] or intermediate hosts when the more mature sexual stages of the parasite only aid part of the life cycle (91). Transfer (paratenic) hosts are not vital for the completion of parasitic development cycles but maintain the parasite before it reaches the obligatory/definitive host (92, 93). Invertebrates (vectors) acting as hosts and carriers of parasites facilitate the completion of parasitic life cycles by transmitting parasites (94–96).

Trypanosomes can infect many hosts, transmitted by hematophagous insect vectors mainly the tsetse fly and triatomid kissing bugs (subfamily: Reduviidae) (13, 97). Salivarian trypanosomes, *Trypanosoma brucei*, *T. rhodesiense*,

TABLE 3 | Approved laboratory tests for trypanosomiasis according to OIE (70).

Test criteria	Objective	Methods	References
Clinical signs	Categorize presentation	Observations	(32, 53, 71)
Microscopy	Direct examination	Wet blood films	(72)
		Thick blood films	(60, 61)
		Thin blood smear films	(72)
	Parasitic concentration	Microhematocrit centrifugation technique	(61)
		Dark ground or phase-contrast buffy coat technique	(62)
		Anion exchange technique	(73–75)
	Cultivation technique	Animal inoculation	(76–78)
Molecular detection	Antigen assays	Trypanosome antigen detection assays	(79)
	Trypanosome DNA	Monospecific PCR	(80)
		Multi-specific PCR	(81, 82)
		LOOP	(68)
		LFA	(69, 83)
Serology	Antibodies	Indirect immunofluorescence test	(84)
		IgG antibody ELISA	(85)
		IgG antibody detection	(86, 87)

T. equiperdum, *T. vivax*, and *T. congolense* are transmitted in tsetse fly (*Glossina* spp.) saliva to the host spp. Hosts are as follows: *T. brucei* s.l. (domestic mammals and humans), *T. vivax* (ruminants, horses, and camels), *T. equiperdum* (equines), *T. simiae* (pigs), and *T. congolense* (dogs and cats) causing *T. evansi* (domestic mammals), and other numerous wildlife hosts such as monkeys, guinea pigs, rabbits, rats, etc., are also affected (91, 98). Stercorian trypanosomes are transmitted through the fecal matter of the insects (*Triatominae*, e.g., *Triatoma infestans*) to host skin where they gain access to tissues. Other vectors of transmission for stercorians include Tabanid flies, stable flies, ticks, and mosquitoes. Stercorians include *Trypanosoma cruzi*, *T. lewisi*, *T. melophagium*, *T. nabiasi*, *T. rangeli*, *T. theileri*, *T. theodori*, *T. lewisi*, *T. cruzi*, bat spp. (*Trypanosoma cruzi marinkellei*, *Trypanosoma dionisii*, *Trypanosoma erneyi*, *Trypanosoma livingstonei*, and *Trypanosoma wauwau*), and others: *Trypanosoma conorhini* and *Trypanosoma rangeli* (94, 95, 98, 99). Among wildlife, *T. cruzi* is found in armadillos, dogs, possums, foxes, bats, raccoons, and striped skunks (5, 22, 89, 90). In addition, *T. melophagum* and *T. theileri* are found in Europe infecting cattle, buffaloes, and antelopes (98).

Trypanotolerance

Trypanotolerant animals show a few clinical signs (96, 97), and trypanosomes are able to evade the host immune responses (100). Trypomastigotes penetrate a variety of host cell types and multiply intracellularly as amastigotes—which eventually infect host cells and differentiate into BFT, which eventually invade the lymphatic and circulatory systems (50, 101). *Trypanosoma* cell membranes are covered with dense variable surface glycoprotein (VSG) homodimers—immunodominant antigens that trigger infected host B- and Th-cell-mediated immune responses. Over 1,000 different VSG genes and pseudogenes are present in the trypanosome genome that can undergo segmental gene recombination to encode an estimated 10,000 different VSG surface coats. High antigenic variability in VSG molecules hinders vaccine development (102).

Wildlife, while generally immunotolerant to trypanosomes (103) can, however, develop clinical trypanosomiasis (104) and show varying levels of trypanotolerance among species (105, 106). Trypanotolerance is influenced by multiple host intrinsic and extrinsic factors and can develop from previous exposure (106). Intrinsic factors include age, sex, species, physiological state, and state of nutrition, while extrinsic factors include temperature, humidity, nature of vegetation, and the nature of wildlife communities (107).

A study in the Serengeti National Park, Tanzania, showed age-dependent infection with *T. congolense* in lions (*Panthera leo*); however, the same animals appeared to have developed cross-immunity following infections to multiple trypanosome species including *T. brucei rhodesiense* (101). Lions are exposed to high challenge from trypanosomes, both from tsetse and also from infected prey, becoming exposed to extremely high numbers of VSGs. This work suggests that animals within a closed exosystem can control infections and, in this case, eliminate the human infective parasite *T. b. rhodesiense* from the infection pool.

Development of Resistance

Trypanotolerance can enable the regulation of parasite levels in the blood (parasitemia) and body tissues. Resistance in wildlife species has been associated with serum xanthine oxidase and catalase and other trypanolytic molecules (108, 109). Stress can affect trypanotolerance in wildlife (71). Trypanotolerance has been investigated in mice and cattle, although these have differences in immune response, i.e., more pronounced B-cell activation in mice than in cattle (50). In cattle, antibody (IgG and IgM) and complement activity against parasitic VSGs leads to protection (110), through antigenic neutralization and IL-4 production (111) (**Figure 1**).

In Cape buffalo, resistance has been associated with non-specific trypanocidal activity in serum, which helps to lower the parasitemia following action of xanthine oxidase (XO), which generates reactive oxygen species (ROS). Since trypanosomes cannot metabolize XO, this cripples parasitic binding and endocytosis (111), trypanosomes are starved of ATP, and death follows (**Figure 1**). An increase in catalase activity is associated with increased parasitemia (112). Some wildlife spp. including the black rhinoceros have significant deficiencies for ATP, catalase, and glycolysis enzymes (conditions that favor trypanosome parasitemia) leading to adaptive evolutionary changes, which help to protect the animals against the parasites (113).

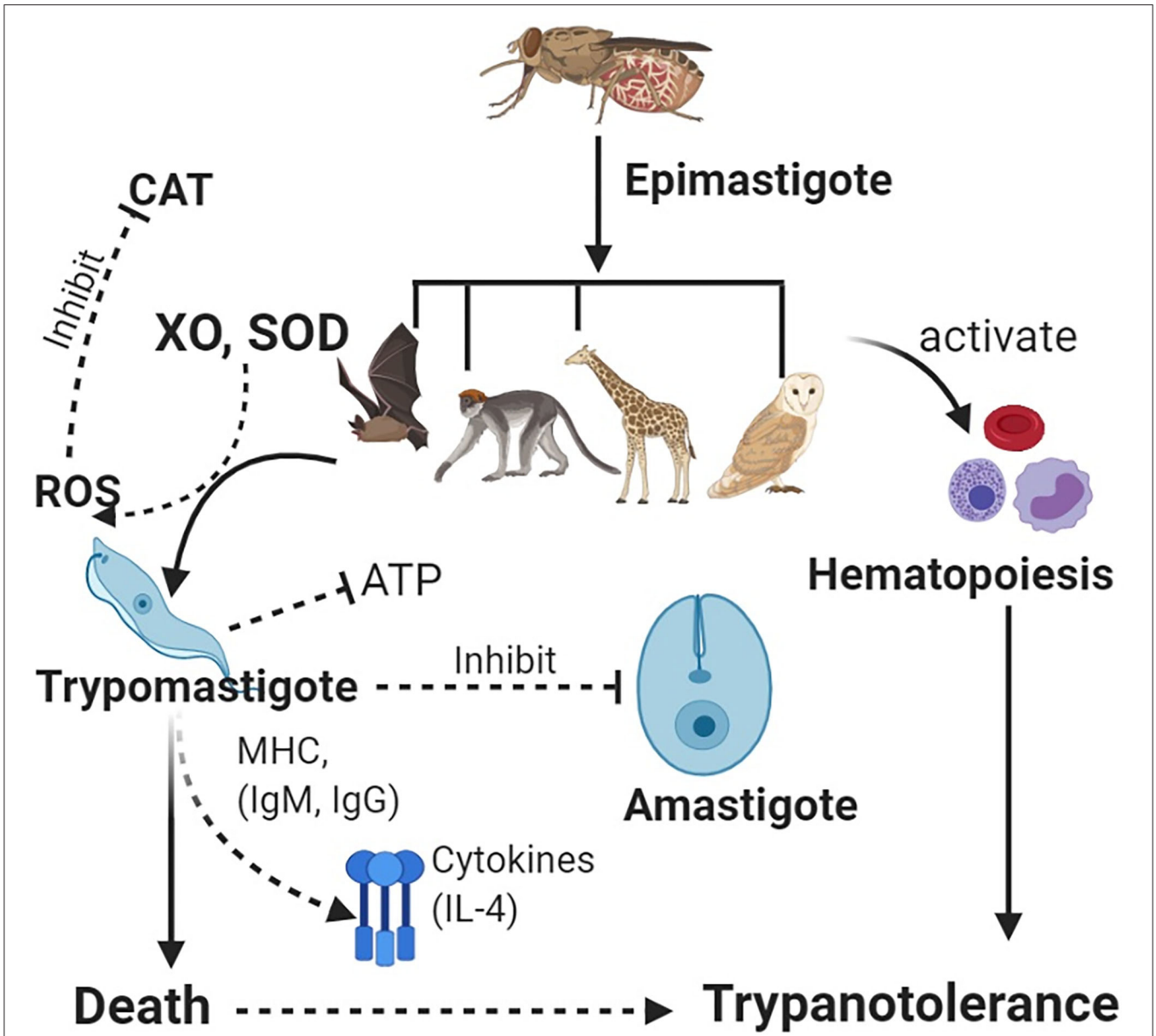

FIGURE 1 | Vectors carrying epimastigotes infect wildlife host species. Epimastigotes transform into trypomastigotes in blood. Host enzymes [e.g., xanthine oxidase (XO) and superoxide dismutase (SOD)] increase production of reactive oxygen species (ROS) such as hydrogen peroxide, which helps clear the parasites, while antioxidant enzymes such as catalase (CAT) lead to increased parasitemia. Immune system activation leads to production of antibodies and cytokines, which neutralize trypanosomes. Inhibition of glycolysis (ATP) leads to deprivation of energy in the parasite for cellular activities. Furthermore, host defenses activate the innate immunity leading to hematopoiesis and an increase in blood cells, thus, leading to trypanotolerance. Wildlife host species subsequently get infected with trypanosomes but rarely succumb to infection.

Wild animals show varying levels of trypanotolerance; the Thomson's gazelle, dikdik, blue forest duiker, jackal, bat-eared fox, ant bear, hyrax, serval, and monkey are all susceptible to *T. rhodesiense* and *T. brucei*, whereas the common duiker, eland, bohor reedbuck, spotted hyena, oribi, bushbuck, impala, warthog, bushpig, porcupine, and baboon are considered resistant (or less susceptible) to *T. b. rhodesiense* and *T. brucei* infection (114, 115). Animals most susceptible are usually found in areas of high tsetse challenge, while those least susceptible (resistant) animals within the population may have acquired resistance through low exposure and challenge over time (116).

The clinical course of trypanosomiasis has been examined in native African buffalo (*Syncerus caffer*), oryxes (*Oryx beisa*), eland (*Taurotragus oryx*), and waterbuck (*Kobus defassa*) following infection with *T. congolense*, *T. vivax*, *or T. brucei*. These animals showed resistance in the form of negligible parasitemia

and minimal anemia (115). Wild and domestic animals have been observed to develop resistance to trypanosomiasis after being subjected to prolonged continuous trypanosome infections (117, 118), and as previously mentioned, indigenous bovines are resistant to *T. brucei* within endemic foci in Uganda (114, 115).

TRYPANOSOMIASIS AT THE WILDLIFE, DOMESTIC ANIMAL INTERFACE

Diversity of Trypanosomes in Wildlife

Multiple *Trypanosoma* species and genotypes contribute to a large reservoir of parasite diversity. This presents major problems in the management of trypanosomiasis at the wildlife–domestic animal interface, with the risk of virulent strains emerging that impact both wildlife and domestic populations (5). A review of Australian animal trypanosomes found a huge variety of parasites: *T. pteropi*, *T. thylacis*, *T. hipposideri*, *T. binneyi*, *T. irwini*, *T. copemani*, *T. gilletti*, *T. vegrandis*, *T. lewisi*, *T. melophagium*, *T. theileri*, *T. nabiasi*, *T. evansi*, *T. cruzi*, *T. pteropi*, *T. hipposideri*, *T. binneyi*, *T. thylacis*, *T. copemani*, *T. Irwin*, and *T. gilleti.* Such biodiversity may have negative impacts on the wildlife and national parks, and is associated with biosecurity concerns (88, 119). Newly identified genotypes of wildlife animals include *Trypanosoma vegrandis G6* and *T. vegrandis G3* (5). Furthermore, a study in bats found three *Trypanosoma* spp. (*T. cruzi*, *T. dionisii*, and *Blastocrithidia* spp.) (22) demonstrating the great diversity in several wildlife species.

Infection at the Wildlife, Domestic Animal Interface

Climate change, population pressure, and incentives to end poverty through farming have forced humans to encroach into land previously occupied by wildlife (108, 120). Human encroachment in protected zones runs the risk of parasite transmission from wildlife to domestic animals and zoonotic transmission (108, 109, 121). Synanthropic zoonotic infections are spread from livestock to humans, and exoanthropic infections are spread by wildlife and feral animals to humans—contributing to the increasing gene pool of anthroponoses (98). The cross-species (interspecies) transmission, also known as host jump or spillover, means the potential of an external parasite to invade a new host and infect them to ultimately spread to the whole population of the new host. About 63% of host jumps are responsible for interspecies diseases (109, 122, 123).

Endemic zones are created by encroaching on places of game parks. This has caused a wildlife and livestock interface, and development of the trypanosome parasite reservoirs (117). Wildlife is often implicated as the reservoir of parasites especially trypanosomes (37, 124, 125). It is common for the high incidences of trypanosomiasis in the wildlife to spillover to the domestic cycle in the tsetse fly-infested zones (71). Domestic animals pose a risk to wildlife, particularly the great apes, especially if the domestic carriers are present, for example, cattle and dogs (32).

Infection with *T. b. rhodesiense* is common in communities living proximal to, or that are dependent on, wildlife or eco-tourism (126, 127). In the Luangwa Valley, Zambia, considerable efforts are made to keep domestic animals away from the national park, for biosecurity of livestock keepers, the national parks, and game conservancies (128). In Zambia, HAT infections have been associated with young children attending school and older women demonstrating in the homestead (129, 130).

A study in Australia failed to find *T. cruzi* and *T. evansi* in native domestic and wildlife animals; however, a spillover for exotic *Trypanosoma* spp. was expected that would affect humans, domestic, and wild animals (88). Wildlife (*Clyomys laticeps*, *Thrichomys pachyurus*, and *Oecomys mamorae)* can be reservoir hosts for *Trypanosoma* spp., e.g., *T. evansi* and *T. cruzi* that could infect humans and other wildlife populations without affecting the rodent spp. (131).

Factors That Could Influence Trypanosomiasis Epidemiology in Wildlife Areas

Host species that are widely distributed and with fewer restrictions on habitat have proved to be of more importance to trypanosome diversity due to their unlimited breeding potential and less risk for poaching and trophy hunting. Such hosts include warthogs, bushbucks, kudus, buffaloes, and giraffes (20). More habitat-restricted host species have minimal contribution to the epidemiology of trypanosomiasis in wildlife communities due to their relative safety from trypanosome vectors like Glossina and Tabanids, e.g., crocodiles and hippopotamus.

Differences in feeding patterns of the trypanosome hosts influence the distribution of trypanosomiasis in wildlife areas. Certain preferences maintained by certain hosts like bovines (bushbuck, waterbuck, eland, kudu, etc.) to bushy areas and thickets have predisposed them to higher exposure rates to tsetse and other biting flies, intensifying the spread of trypanosomes (20). Trypanosome diversity among the host species has facilitated the cross-transmission of various trypanosome strains and variants among the hosts, increasing infection rates among wildlife communities at both clinical and subclinical levels. For example, the discovery of three different variants of *T. vivax* in three different host species including a buffalo, a waterbuck, and a giraffe not similar to any published strain, demonstrating genetic diversity, provides insights on pathogen epidemiology (3).

Furthermore, the introduction of new host species from a different geographical location into a wildlife reserve can greatly influence the trypanosome species diversity in a wildlife community. This way, new variants and species of trypanosomes are spread from one host to another by tsetse and other biting flies, resulting in devastating effects on wildlife health and livestock health in the area, for example, the discovery of *T. melophagium*, *T. nabasi*, *T. theieri*, and *T. evansi* in Australia following the introduction of various mammals into their wildlife populations, which had a great impact on the native marsupials (9).

Wildlife Encroachment and the Epidemiology of Trypanosomiasis

Encroachment to wildlife and the increasing human and livestock density as well as the altered patterns in land use are key parameters that govern the transmission of trypanosomes (132). It is expected that understanding how the encroachment to wildlife affects the epidemiology of trypanosomes will inspire practical approaches to improve the understanding of epidemiological characteristics of trypanosome transmission in the context of ecological factors. Encroachment to wilderness areas of Africa increases the epidemiology of trypanosomes, hence, increasing the transmission of HAT. Primarily, wildlife are trypanosome reservoirs; however, growing human and livestock numbers around or in wildlife areas increase the significance of livestock in the transmission cycles thereby increasing the epidemiology of trypanosomes pertinent to human health (132).

Understanding undercurrents associated with the transmission of trypanosomes and in relationship with the encroachment of livestock and humans to wildlife areas are vital to developing robust control measures. This would help identify important parameters on host distributions, tsetse populations, epidemiology of trypanosomes, infection and mortality rates, the significance of livestock, humans, and livestock as hosts in wildlife areas, hence, promoting the progress of models to help in the evaluation and application of control measures (132). Parameters that determine the dynamics in encroachment levels to wildlife areas such as protected area, wildlife density, livestock density, human density, and location according to space and time, would help determine the foci of HAT. This is important since increased human and livestock populations and their distribution may lead to land-use pattern changes in fragmented tsetse habitats, and this inevitably affects the distribution of wildlife species. These ultimately result in increased tsetse abundance and distribution, host selection, and tsetse mortality, which are indicators of increased vector competence (132). Host population density is a key factor in determining the dynamics of tsetse populations, i.e., a decline in host density through encroachment to wildlife areas influences tsetse population changes in space and time. This, in turn, influences the transmission of trypanosomes, and although low host density decreases trypanosome transmission as a consequence of tsetse mortality, the low host density may also be associated with an increased level of trypanosome transmission arising from the hungrier flies that bite humans through increased host-seeking efficiency of tsetse flies (133).

INFECTION CONTROL AT THE WILDLIFE–LIVESTOCK–HUMAN INTERFACE

While communities fail to understand the value of wildlife ecosystems, continued wildlife–human conflict presents an increased risk of infection spillover to humans (18, 118, 134, 135). Game parks, the natural habitats for tsetse species, pose risks to livestock and human populations (136, 137), and parasitic infestations among the livestock and humans equally pose a risk to wildlife. Wildlife reservoirs make approaches of trypanosomosis control at the wildlife–livestock–human interface more complicated (30, 31). There is a need to limit the interaction between livestock and wildlife by preventing encroachment into wildlife-protected zones. Mitigation of risk to prevent trypanosome (and other) infections circulating among livestock and wildlife demands a holistic One Health approach (108, 138, 139). Top–down, approaches, shooting games and radical bush clearing, and insecticide spraying in protected zones are neither practical nor acceptable (140). Stakeholder and community-derived solutions are likely to be sustainable options to explore. Approaches to infection control require to be nuanced in these zones, with communication, education, and interventions embedded within the affected communities. Integrated insect control approaches including the use of insecticide-impregnated targets can protect livestock and game (141). Application of insecticides to cattle, using livestock as live baits, can offer sustainable solutions (138, 142, 143); however, challenges remain on the sustainability of this approach especially in low–middle income countries (LMICs). Insecticides are a reliable method for tsetse control and can be improved by deploying an integrated insecticide approach (139, 141). Routine prophylaxis among livestock can protect livestock and offer collateral benefits for humans and wildlife (144, 145). There is also a need to limit the interaction between livestock and wildlife by stopping encroaching on gazetted wildlife zones to lower the trypanosome prevalence in domesticated livestock (108).

Animal and Human Health for the Environment and Development (AHEAD), launched in 2003, comprises a One Health team of socioeconomic scientists, ecobiologists, veterinarians, agriculturalists, wildlife, and public health specialists that address issues at the wildlife, human, and domestic livestock interface. This includes efforts to monitor parasitic diversity in wildlife species to assist in the strengthening of disease surveillance in LMICs (146). Management and communication with regard to wildlife is key to the One Health approach; in pastoral communities, retaliatory persecution through poisonings of predatory wildlife continues to challenge conservation efforts (147). Conflicts associated with competition for natural resources between livestock-keeping communities and wildlife can be mitigated by a combination of communication and control strategies to promote peaceful coexistence of wildlife and humans as promoted by AHEAD.

Management of Spillover of Trypanosomiasis Among the Human–Wildlife Areas

Communities in the wildlife zones sometimes agree on coexistence with wildlife and the creation of buffer zones (148). However, the coexistence of human communities and wildlife poses risks of outbreaks of various zoonoses (149). In the gazetted wildlife zones, there should be no mixing of domestic animals with wild trypanosomiasis reservoirs. Proper fencing can be used to control the spillover in wildlife borders (150) as part of the integrated trypanosomiasis control strategy. Restrictive models need to be developed by engaging the communities

such that they understand these objectives (151). There is a need for legislation on fencing to have restricted movement of livestock and wildlife. The laws need to address the challenges of wildlife biodiversity (152) and penalize encroachers and poachers. This requires an appropriate land tenure system and robust enforcement teams.

Renowned trypanosome hosts like bats need to be removed from urban centers and human dwellings. Bats also live in human dwellings in ceilings and other dark points. African bats are hosts of trypanosomes (153). It is not known whether the African bats are associated with virulent chronic and acute human African trypanosomiasis. However, bats are associated with trypanosomes in central and South America (22, 154, 155).

Research on possible vaccine candidates has not broken through despite emphasis on the VSG pathway (102, 156). This, however, does not translate that research on trypanosome vaccines has reached a dead end since in all the failures, better innovations can transform science to improve understanding in this field (157, 158). This is important since drugs that are used to treat domestic animals have been used to treat wildlife with success (159, 160), although this has not been done against infections with the zoonotic trypanosomes.

Wildlife are usually in contact with insects other than the tsetse flies. It is known that lice can transmit *T. cruzi* (161). The possibility of lice transmitting the zoonotic trypanosomes is not known. Zoonotic trypanosomes have been found in fleas (162), and it is speculated that fleas may transmit trypanosomes among wildlife (163–165). The possible transmission of trypanosomes by other biting arthropods among wildlife needs further investigation. The possibility of transmission along the food chains for carnivores needs further investigation. Wild Canidae that feed on fresh blood from trypanosomiasis reservoirs may acquire infections from the fresh blood. No studies have ever been proposed among at-risk carnivores.

There is a need to study the interactions of trypanosomes with other blood parasites. Trypanosomes interact with babesia especially to worsen the stress conditions of translocation (166–168). The effect of trypanosomes on the immune system likely predisposes the animals to opportunistic infections and tick-borne diseases. The presence of other parasites is possibly a contributing factor to the trypanosomiasis spillover.

CONCLUSION

Trypanosomiasis continues to be a major global challenge, particularly so at the wildlife–domestic livestock interface. Multiple wildlife species serve as maintenance hosts promoting infections at the livestock–wildlife interface. There is a high risk of infection spillover from game parks and conservation areas where parasites and vectors are concentrated in high numbers, and domestic livestock pose risks to wildlife-protected species. The basis of trypanotolerance in wildlife species is not well-understood. The wide genetic diversity exhibited by *Trypanosoma* spp. is a challenge, both exacerbating the risk of increased virulence and making the development of a vaccine unlikely. One Health strategies that are community and environmentally friendly are needed to support stakeholders to mitigate risk. There is a need to strengthen trypanosomiasis research, particularly in LMICs, especially at the human–domestic–wildlife interface to prevent cross-species infection.

AUTHOR CONTRIBUTIONS

KIK and SCW conceptualized the study, designed the study, and analyzed and interpreted the data. KIK, GZ, FS, BB, KJA, KM, HNN, LA, DO, PB, JJO, SI, WM, DPN, GESB, LOO, TS, MA, LO, and SCW collected the data. KIK wrote initial draft while SCW critically reviewed it for intellectual content. All authors approved the manuscript for publication and remain in agreement on all aspects of the work.

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Serological Survey of Canine Vector-Borne Infections in North-Center Spain

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Various factors are currently causing an increase in vector-borne parasitic diseases at a global scale; among them, some stand out, such as climatic disturbances derived from global change, the increase in movements of reservoir animals, or changes in land made by human activity. In the European continent, there have been an increasing number of epidemiological studies focused on the detection of these diseases, especially in dogs. In Spain, there are few epidemiological studies focused on the evaluation of the biotic and abiotic factors that may influence the distribution, such as climatic zones, orography, or presence of water reservoirs. The aim of this study was to analyze the prevalence and distribution of several canine vector-borne diseases caused by *Dirofilaria immitis*, *Leishmania infantum*, *Anaplasma platys*, and *Ehrlichia canis* in the autonomous community of Castilla y León, the largest region of the Iberian Peninsula, providing a geospatial approach based on a geographic information system (GIS) analysis. Blood from a total of 1,475 domestic dogs from the nine provinces of Castilla y León were analyzed. Also, a GIS analysis of the sample locations was carried out, taking into account the most important predictor variables. The prevalence in dogs infected by *D. immitis* was 7.19%, and the seroprevalence by *L. infantum* was 4.61 and 1.56% for *A. platys* and *E. canis*. Most of the infected animals were located in areas with stagnant water, irrigated agriculture, or riverbanks, always close to forest and woodland vegetation. These results indicate that dogs living in Castilla y León should take prophylactic measures to avoid infections.

Keywords: canine vector borne disease, *D. immitis*, *L. infantum*, GIS, Spain, epidemiology, *E. canis*, *A. platys*.

INTRODUCTION

Canine vector-borne diseases (CVBDs) are caused by several infectious agents, which are transmitted by a wide variety of arthropods, mainly fleas, ticks, mosquitoes, and sandflies. Many of them are zoonotic diseases and are among the most important health problems affecting both domestic dogs and humans worldwide (1). Temperature and humidity are two determining factors

that act directly in the establishment and distribution of the vectors and the diseases, as well as other factors derived from human activity such as the increase in the mobility of people and infected animals, or modifications in the landscape, mainly caused by increased irrigated crops or intensive urbanization of new areas. These processes favor the development of some vectors and the infection of the hosts (2–4).

Many of the CVBDs are clinically important, even be fatal, and frequently show unspecific symptoms, which make them difficult to diagnose. These include several zoonotic diseases such as heartworm disease, canine leishmaniasis, and canine anaplasmosis (3–5). In addition, canine ehrlichiosis, although not classified as zoonotic, has been sporadically described in several clinical cases in humans (6, 7).

Nematode *Dirofilaria immitis*, transmitted by culicid mosquitoes, is the causative agent of canine heartworm disease, a chronic disease which can lead to the death of the animal, although many of these animals are asymptomatic and become uncontrolled reservoirs (8). It is a zoonotic parasite which can cause pulmonary dirofilariasis, an asymptomatic infection, but that can be mistaken for lung cancer (2, 9–11). Protozooan *Leishmania infantum*, transmitted by *Phlebotomus* spp., causes canine leishmaniosis and induces a severe disease with serious clinical signs which, if not treated, can lead to the death of the animal. In addition, *L. infantum* can cause cutaneous lesions in humans or visceral leishmaniasis, the latter being a serious condition especially in immunocompromised patients (12). *Anaplasma platys* is the causative agent of canine cyclic thrombocytopenia, while *Ehrlichia canis* is the causative agent of canine monocytic ehrlichiosis. These gram-negative bacteria are mainly transmitted by ticks, and the diseases are characterized by non-specific signs, making diagnosis difficult (13–16). In humans, *E. canis* and *A. platys* have been reported in clinically ill human patients causing from mild, self-limiting febrile illness to fatal infections (6, 7, 17).

In Europe, many epidemiological studies have been published showing a wide variability in the prevalence of the mentioned CVBDs. These differences may be due to the influence exerted by the biotic and abiotic variables of each region as well as the different diagnostic tools used (5, 18). In Spain, few studies have been published on the epidemiological status of these diseases mostly reporting data at a global level without evaluating the influence of environmental factors (i.e., orography, vegetation, climate) of each of the areas studied, especially those focused on *Anaplasma spp.* and *E. canis* (3, 5, 19).

These are CVBDs that are spreading throughout Europe and are considered emerging diseases. Therefore, it is important to evaluate the influence of the possible factors that contribute to this expansion; in this way, more accurate prediction models and measures aimed at stopping this expansion can be studied. The present study is focused on Castilla y León, representing a significant extension of territory, with different orographic and climatic zones depending on the province analyzed, to study the prevalence of the mentioned CVBDs as well as the influence of several biotic and abiotic factors. Also, the implementation of global positioning systems for geolocation of data together with the analysis of the environmental variables of a territory through GIS and spatial correlation models has shown to have a great capacity to understand spatial patterns of distribution of biological events in a territory (20). This ecoinformatic methodology allows the elaboration of predictive models focused on the determination of potential risks of parasitic diseases on a global scope [i.e., (21)] or in specific areas (22, 23).

FIGURE 1 | Location of Autonomous Community of Castilla y León, Spain.

Therefore, the aim of this study was to deepen the prevalence as well as the geographic distribution patterns of selected causative agents of CVBDs in the autonomous community of Castilla y León (Spain) by using GIS, to understand the causal relationship between the environmental variables and the prevalence of the infections.

METHODS

Study Area

The Autonomous Community of Castilla y León is located in the northwestern quadrant of the Iberian Peninsula (**Figure 1**). With an area of 94,224 km^2, it is the largest region in Spain and one of the largest of Europe, being greater than seven state members of the European Union (Austria, Belgium, Denmark, Holland, Ireland, Luxembourg, and Portugal). The orography of Castilla y León is mainly formed by a plateau with an average altitude of around 800 m above sea level, surrounded by a belt of mountainous reliefs to the north, east, and south, and bordering the west with Portugal. Administratively, it is divided into nine provinces (León, Zamora, Salamanca, Valladolid, Palencia, Burgos, Soria, Segovia, and Ávila) (**Figure 2**), with León being the largest (15,851 km^2) and Segovia the smallest (6,921 km^2) (24, 25).

According to Köppen's climate classification, Castilla y León falls within the continental's Mediterranean climate, presenting

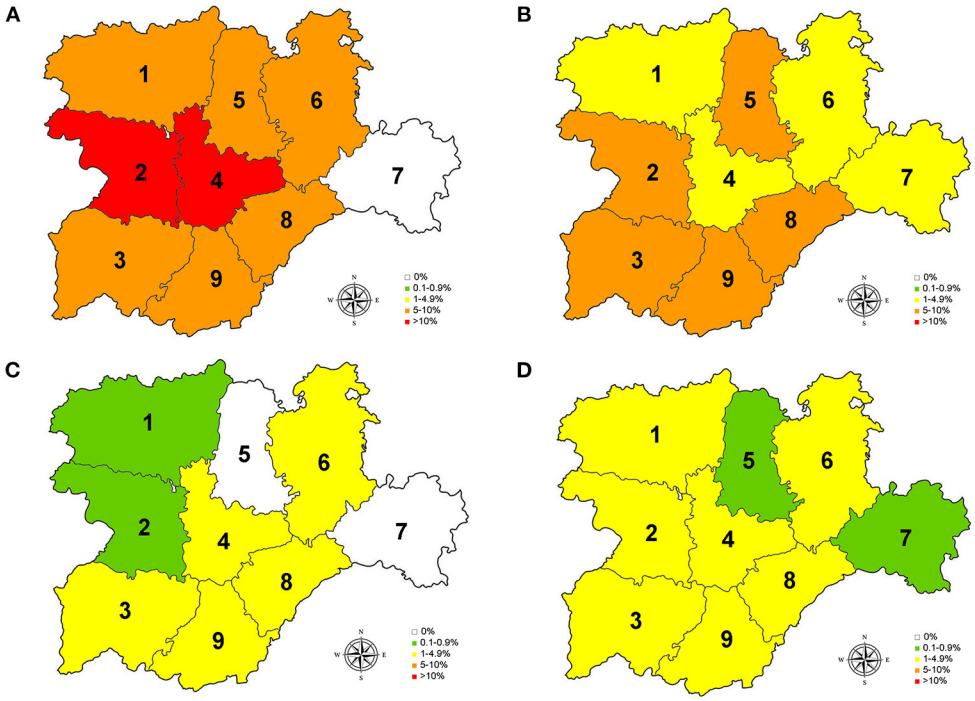

FIGURE 2 | Prevalence for *D. immitis* **(A)**, and seroprevalences for *L. infantum* **(B)**, *A. platys* **(C)**, and *E. canis* **(D)** in the nine provinces of Castilla y León, Spain. 0% (•); 0.1–0.9% (■); 1–4.9% (■); 5–10% (■); >10 (■).1, León; 2, Zamora; 3, Salamanca; 4, Valladolid; 5, Palencia; 6, Burgos; 7, Soria; 8, Segovia; 9, Ávila.

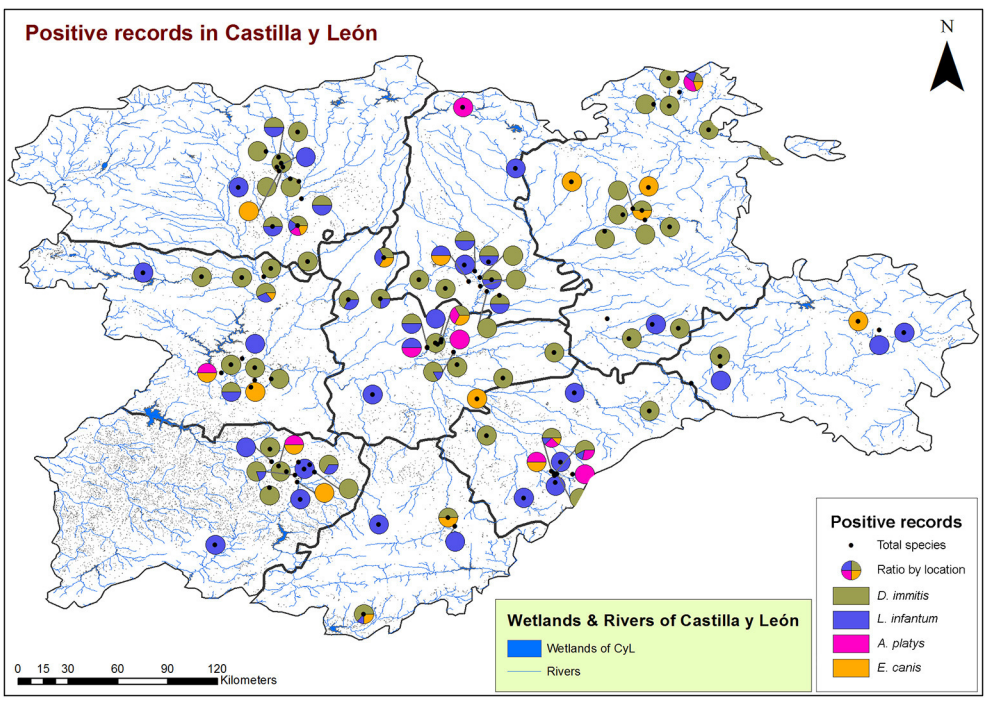

FIGURE 3 | Location of wetlands and rivers and geolocation of dogs infected by *D. immitis* (■), and seroprevalences for *L. infantum* (■), *A. platys* (■), and *E. canis* (■) in the nine provinces of Castilla y León, Spain.

long and cold winters, with average temperatures between 3 and 6°C in January, as well as short, hot summers (average temperatures from 19 to 22°C). The average annual rainfall is about 450–500 mm, accentuated in mountain ranges, but with hardly any rainfall during the summer months. Furthermore, due to the great extension and the orographic diversity of this territory, different sub-climates can be distinguished. A large part falls within the temperate with dry or temperate summer (Csb) or temperate with a dry season and temperate summer (Cfb) sub-climates, the average of the warmest month being below 22°C but above 10°C for ≥5 months. In several areas of the central plateau, the sub-climate is classified as temperate with dry or hot summer (Csa) as it exceeds 22°C during the summer, or cold steppe (BSk), with average annual temperatures below 18°C. At high altitudes in the mountain areas, the climate present is cold temperate with average temperatures below 3°C in the coldest months and dry summers (Dsb or Dsc) (26).

According to the map of phytoclimatic series by Rivas Martínez (27), the vegetation of Castilla y León is mainly included in the Mediterranean region, supra-Mediterranean floor, Carpetano-Iberico-Leonesa biogeographic province. The potential forest formations of quercines are predominant in it: holm oaks (*Quercus ilex* subsp. *ballota*), pyrenean oaks (*Q. pyrenaica*), cork oaks (*Q. suber*), and quejigo oaks (*Q. faginea*), which are planted, managed, and regularly pruned, configuring a manmade ecosystem characterized by a savannah-like physiognomy, locally known as "dehesa systems." These forests cover most of the plains and middle slopes, but some beech (*Fagus sylvatica*) and chestnut forests (*Castanea sativa*) are also present in the mountainous foothills. Sabinas and juniper trees (*Juniperus* sp.) practically complete most of this forest landscape, together with the riverside communities associated with the main rivers and streams that cross the territory.

Sample Collection

The study included a total of 1,475 blood samples from domestic dogs, collected between September 2019 and December 2020 (**Table 1**). Samples were collected from dogs undergoing medical examination in 44 veterinary clinics and hospitals, with at least four veterinary clinics for each province of Castilla y León. The participation of clinics and hospitals was voluntary, and samples were collected throughout the duration of the study. Samples came from both owned and shelter dogs. The criteria for inclusion were (a) no previous history of infection, (b) not receiving regular chemoprophylaxis for the studied vector-borne diseases, and (c) owner consent to participate in the survey. Epidemiological data, such as sex, age at presentation to the clinics, and habitat (indoor, outdoor, or indoor/outdoor: at least 1–50% of the time spent outdoors), were recorded.

Blood samples were collected from the cephalic or jugular vein, placed in 3-ml serum tubes, and centrifuged. Serum samples were kept at −20°C until tests were performed. All samples were tested for the detection of *D. immitis* antigens and for the detection of antibodies against *L. infantum*, *E. canis*, and *A. platys* by using Uranotest Quattro (Uranovet, Barcelona Spain) following the manufacturer's instructions.

Statistical Analysis

Data were analyzed by using SPSS Base 20.0 software (SPSS Inc./IBM, Chicago, IL, USA). A descriptive analysis of the variables considered was carried out considering the proportions of the qualitative variables. Chi-square and Fisher exact tests to compare proportions were performed. Sex, age, and habitat were considered as variables in the analysis for the autonomous community of Castilla y León and for each province. For the statistical analysis, dogs were grouped into five age groups (<1 year, from 1 to 5 years, from 5 to 10 years, from 10 to 15 years,

TABLE 1 | Prevalence and seroprevalences of the studied vector-borne infections by sex, age, and habitat in Castilla y León, Spain.

		D. immitis		*L. infantum*		*A. platys*		*E. canis*	
	n	+	%	+	%	+	%	+	%
SEX									
Male	748	57	7.62	36	4.81	11	1.47	9	1.20
Female	727	49	6.74	32	4.40	12	1.65	14	1.93
AGE									
<1 year	68	8	11.76	4	5.88	1	1.47	1	1.47
1–5 years	563	34	6.04	28	4.97	8	1.42	7	1.24
>5–10 years	545	44	8.07	23	4.22	10	1.83	13	2.39
>10-15 years	281	19	6.76	11	3.91	4	1.42	2	0.71
>15 years	18	1	5.56	2	11.11	0	0.00	0	0.00
HABITAT									
Indoor	546	24	4.40	22	4.03	6	1.10	2	0.37
Outdoor	646	55	8.51	39	6.04	9	1.39	14	2.17
Indoor/Outdoor	283	27	9.54	7	2.47	8	2.83	7	2.47
TOTAL	1,475	106	7.19	68	4.61	23	1.56	23	1.56

n, number of dogs sampled; +, positive animals; %, percentage of positive animals.

TABLE 2 | Numbers of dogs sampled and prevalence of *D. immitis* by sex, age, and habitat in the nine provinces of Castilla y León, Spain.

	1-León			2-Zamora			3-Salamanca			4-Valladolid			5-Palencia			6-Burgos			7-Soria			8-Segovia			9-Ávila		
	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%
SEX																											
Male	9	121	7.44	10	65	15.38	6	68	8.82	10	88	11.36	4	50	8.00	7	128	5.47	0	61	0.00	5	110	4.55	6	57	10.53
Female	7	101	6.93	3	44	6.82	4	69	5.80	7	78	8.97	4	62	6.45	17	156	10.90	0	57	0.00	6	114	5.26	1	46	2.17
AGE																											
<1 year	2	8	25.00	2	8	25.00	1	9	11.11	0	5	0.00	0	2	0.00	3	22	13.64	0	5	0.00	0	5	0.00	0	4	0.00
1–5 years	5	64	7.81	8	47	17.02	2	41	4.88	4	63	6.35	6	40	15.00	4	97	4.12	0	65	0.00	3	88	3.41	2	58	3.45
>5–10 years	4	82	4.88	3	37	8.11	4	65	6.15	10	55	18.18	1	43	2.33	13	105	12.38	0	37	0.00	4	85	4.71	5	36	13.89
>10–15 years	5	62	8.06	0	16	0.00	3	21	14.29	3	43	6.98	1	25	4.00	4	53	7.55	0	11	0.00	3	45	6.67	0	5	0.00
>15 years	0	6	0.00	0	1	0.00	0	1	0.00	0	0	0.00	0	2	0.00	0	7	0.00	0	0	0.00	1	1	100.00	0	0	0.00
HABITAT																											
Indoor	8	65	12.31	4	50	8.00	1	17	5.88	2	63	3.17	1	21	4.76	4	102	3.92	0	83	0.00	2	117	1.71	2	28	7.14
Outdoor	8	154	5.19	6	46	13.04	7	100	7.00	12	60	20.00	3	65	4.62	14	117	11.97	0	21	0.00	1	20	5.00	4	63	6.35
Indoor/Outdoor	0	3	0.00	3	13	23.08	2	20	10.00	3	43	6.98	4	26	15.38	6	65	9.23	0	14	0.00	8	87	9.20	1	12	8.33
TOTAL	16	222	7.21	13	109	11.93	10	137	7.30	17	166	10.24	8	112	7.14	24	284	8.45	0	118	0.00	11	224	4.91	7	103	6.80

n, number of dogs sampled; +, positive animals; %, percentage of positive animals.

TABLE 3 | Numbers of dogs sampled and seroprevalence of *L. infantum* by sex, age, and habitat in the nine provinces of Castilla y León, Spain.

	1-León			2-Zamora			3-Salamanca			4-Valladolid			5-Palencia			6-Burgos			7-Soria			8-Segovia			9-Ávila		
	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%
SEX																											
Male	5	121	4.13	4	65	6.15	6	68	8.82	5	88	5.68	3	50	6.00	5	128	3.91	1	61	1.64	3	110	2.73	4	57	7.02
Female	4	101	3.96	4	44	9.09	1	69	1.45	2	78	2.56	7	62	11.29	0	156	0.00	2	57	3.51	8	114	7.02	4	46	8.70
AGE																											
<1 year	2	8	25.00	2	8	25.00	0	9	0.00	0	5	0.00	0	2	0.00	0	22	0.00	0	5	0.00	0	5	0.00	0	4	0.00
1–5 years	3	64	4.69	2	47	4.26	2	41	4.88	4	63	6.35	3	40	7.50	2	97	2.06	2	65	3.08	3	88	3.41	7	58	12.07
>5–10 years	1	82	1.22	4	37	10.81	3	65	4.62	3	55	5.45	3	43	6.98	2	105	1.90	1	37	2.70	5	85	5.88	1	36	2.78
>10–15 years	3	62	4.84	0	16	0.00	2	21	9.52	0	43	0.00	3	25	12.00	0	53	0.00	0	11	0.00	3	45	6.67	0	5	0.00
>15 years	0	6	0.00	0	1	0.00	0	1	0.00	0	0	0.00	1	2	50.00	1	7	14.29	0	0	0.00	0	1	0.00	0	0	0.00
HABITAT																											
Indoor	4	65	6.15	2	50	4.00	0	17	0.00	3	63	4.76	3	21	14.29	1	102	0.98	0	83	0.00	8	117	6.84	1	28	3.57
Outdoor	5	154	3.25	6	46	13.04	7	100	7.00	3	60	5.00	5	65	7.69	3	117	2.56	2	21	9.52	1	20	5.00	7	63	11.11
Indoor/Outdoor	0	3	0.00	0	13	0.00	0	20	0.00	1	43	2.33	2	26	7.69	1	65	1.54	1	14	7.14	2	87	2.30	0	12	0.00
TOTAL	0	222	4.05	8	109	7.34	7	137	5.11	7	166	4.22	10	112	8.93	5	284	1.76	3	118	2.54	11	224	4.91	8	103	7.77

n, number of dogs sampled; +, positive animals; %, percentage of positive animals.

TABLE 4 | Numbers of dogs sampled and seroprevalence of *A. platys* by sex, age, and habitat in the nine provinces of Castilla y León, Spain.

	1-León			2-Zamora			3-Salamanca			4-Valladolid			5-Palencia			6-Burgos			7-Soria			8-Segovia			9-Ávila		
	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%
SEX																											
Male	2	121	1.65	1	65	1.54	1	68	1.47	0	88	0.00	0	50	0.00	2	128	1.56	0	61	0.00	2	110	1.82	3	57	5.26
Female	0	101	0.00	0	44	0.00	1	69	1.45	4	78	5.13	0	62	0.00	2	156	1.28	0	57	0.00	4	114	3.51	1	46	2.17
AGE																											
<1 year	0	8	0.00	0	8	0.00	0	9	0.00	0	5	0.00	0	2	0.00	0	22	0.00	0	5	0.00	0	5	0.00	1	4	25.00
1–5 years	1	64	1.56	1	47	2.13	0	41	0.00	1	63	1.59	0	40	0.00	1	97	1.03	0	65	0.00	2	88	2.27	2	58	3.45
>5–10 years	0	82	0.00	0	37	0.00	2	65	3.08	2	55	3.64	0	43	0.00	3	105	2.86	0	37	0.00	2	85	2.35	1	36	2.78
>10–15 years	1	62	1.61	0	16	0.00	0	21	0.00	1	43	2.33	0	25	0.00	0	53	0.00	0	11	0.00	2	45	4.44	0	5	0.00
>15 years	0	6	0.00	0	1	0.00	0	1	0.00	0	0	0.00	0	2	0.00	0	7	0.00	0	0	0.00	0	1	0.00	0	0	0.00
HABITAT																											
Indoor	0	65	0.00	0	50	0.00	0	17	0.00	2	63	3.17	0	21	0.00	0	102	0.00	0	83	0.00	3	117	2.56	1	28	3.57
Outdoor	1	154	0.65	1	46	2.17	0	100	0.00	1	60	1.67	0	65	0.00	3	117	2.56	0	21	0.00	0	20	0.00	3	63	4.76
Indoor/Outdoor	1	3	33.33	0	13	0.00	2	20	10.00	1	43	2.33	0	26	0.00	1	65	1.54	0	14	0.00	3	87	3.45	0	12	0.00
TOTAL	2	222	0.90	1	109	0.92	2	137	1.46	4	166	2.41	0	112	0.00	4	284	1.41	0	118	0.00	6	224	2.68	4	103	3.88

n, number of dogs sampled; +, positive animals; %, percentage of positive animals.

TABLE 5 | Numbers of dogs sampled and seroprevalence of *E. canis* by sex, age, and habitat in the nine provinces of Castilla y León, Spain.

	1-León			2-Zamora			3-Salamanca			4-Valladolid			5-Palencia			6-Burgos			7-Soria			8-Segovia			9-Ávila		
	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%	+	*n*	%
SEX																											
Male	2	121	1.65	2	65	3.08	1	68	1.47	0	88	0.00	0	50	0.00	0	128	0.00	1	61	1.64	2	110	1.82	1	57	1.75
Female	1	101	0.99	1	44	2.27	1	69	1.45	3	78	3.85	1	62	1.61	4	156	2.56	0	57	0.00	1	114	0.88	2	46	4.35
AGE																											
<1 year	1	8	12.50	0	8	0.00	0	9	0.00	0	5	0.00	0	2	0.00	0	22	0.00	0	5	0.00	0	5	0.00	0	4	0.00
1–5 years	0	64	0.00	1	47	2.13	0	41	0.00	1	63	1.59	0	40	0.00	2	97	2.06	1	65	1.54	1	88	1.14	1	58	1.72
>5–10 years	1	82	1.22	2	37	5.41	2	65	3.08	1	56	1.79	1	43	2.33	2	105	1.90	0	37	0.00	2	85	2.35	2	36	5.56
>10–15 years	1	62	1.61	0	16	0.00	0	21	0.00	1	42	2.38	0	25	0.00	0	53	0.00	0	11	0.00	0	45	0.00	0	5	0.00
>15 years	0	6	0.00	0	1	0.00	0	1	0.00	0	0	0.00	0	2	0.00	0	7	0.00	0	0	#¡DIV/0!	0	1	0.00	0	0	0.00
HABITAT																											
Indoor	0	65	0.00	1	50	2.00	0	17	0.00	0	85	0.00	0	21	0.00	0	102	0.00	0	83	0.00	1	117	0.85	0	28	0.00
Outdoor	2	154	1.30	2	46	4.35	1	100	1.00	2	61	3.28	1	65	1.54	3	117	2.56	0	21	0.00	0	20	0.00	3	63	4.76
Indoor/Outdoor	1	3	33.33	0	13	0.00	1	20	5.00	1	20	5.00	0	26	0.00	1	65	1.54	1	14	7.14	2	87	2.30	0	12	0.00
TOTAL	3	222	1.35	3	109	2.75	2	137	1.46	3	166	1.81	1	112	0.89	4	284	1.41	1	118	0.85	3	224	1.34	3	103	2.91

n, number of dogs sampled; +, positive animals; %, percentage of positive animals.

and >15 years). In all cases, the significance level was established at $p < 0.05$.

GIS Mapping

A map of the sampling area was constructed using ArcMap v.10.8 (ESRI, 2020 Redlands, CA, USA), including the following layers of relevant environmental information that have been considered to be relevant for the dynamics of the analyzed organisms and their transmission vectors: climate, potential vegetation, and surface waters and surface and edaphic humidity (rivers, lakes, lagoons, irrigated croplands, and parks) (28, 29). Thematic symbols were added for easier interpretation of the map. The canine samples were georeferenced by the location of health centers where the veterinary consultations occurred. Therefore, the points shown on the maps correspond to the centroids of the polygons of the postal codes where the analyses of the dogs were carried out. Inferences were drawn from a proximity analysis between the presence points and the environmental characteristics of the layers used. The coordinate system used was gcs_ETRS_1989.

RESULTS

Of the studied dogs, 7.72% were positive for one or several causative agents of CVBDs. The overall prevalence of *D. immitis* was 7.19%, and the seroprevalences of *L. infantum*, *A. platys*, and *E. canis* were 4.61, 1.56, and 1.56%, respectively. Results by sex, age groups, and habitat are shown in **Table 1**. Results for each of the nine provinces of Castilla y León are shown in **Figure 2** and **Tables 2**–**5**.

No significant differences were found by sex or age in any of the studied causative agents of CVBDs (**Table 1**). However, when habitat was assessed, significant differences were observed in the prevalence of *D. immitis* and seroprevalence of *E. canis* between indoor and outdoor dogs ($p < 0.05$) and between indoor and indoor/outdoor dogs ($p < 0.05$). Significant differences between outdoor and indoor/outdoor were observed in results for *L. infantum* ($p < 0.05$). By provinces, only significant differences were observed in the prevalence of *D. immitis* between indoor and outdoor dogs in Valladolid and between indoor and indoor/outdoor dogs in Segovia ($p < 0.05$) (**Table 2**).

Coinfections were observed in 0.95% of the infected dogs. Of them, 0.34% were infected by *D. immitis* + *L. infantum*, 0.14% by *D. immitis* + *A. platys*, 0.14% by *D. immitis* + *E. canis*, 0.14% by *L. infantum* + *A. platys*, 0.14% by *L. infantum* + *E. canis*, and 0.07% by *A. platys* + *E. canis*. By provinces, coinfections were found in 2.25% of the dogs from León, with 0.9% for *D. immitis* + *L. infantum*, 0.45% for *L. infantum* + *A. platys*, and 0.9% for *L. infantum* + *E. canis*. In Zamora, coinfections were found in 0.73% of the samples, corresponding to *D. immitis* + *A. platys* coinfections. In Valladolid, 0.6% tested positive for *D. immitis* + *E. canis* and 0.6% for *L. infantum* + *A. platys*. In Salamanca, only one dog was infected by *D. immitis* + *A. platys* (0.73%). In Palencia, there were two dogs infected by *D. immitis* + *L. infantum* (1.79%) and in Burgos 0.35% that tested positive for *D. immitis* + *A. platys*, *D. immitis* + *E. canis*, and *A. platys* + *E. canis*. In Soria, Segovia, and Avila, there were no coinfections in any of the samples analyzed.

Regarding *D. immitis*, the highest prevalences were located in the center and west of Castilla y León, where Csb and Cfb climates were present, while no infected dogs were found in Soria (east). Regarding *L. infantum*, the provinces with the highest seroprevalences were located in the south. On the other hand, seroprevalences for *A. platys* and *E. canis* were low but present in the whole region, except for the absence of *A. platys* in Palencia and Soria. According to climate, positive animals for *L. infantum*, *A. platys*, and *E. canis* were found in BSk, Cfb, Csa, and Csf sub-climates.

From a geospatial point of view (**Figures 3**, **4**), 94.55% of infected animals were located in areas with high edaphic availability of water, as either stagnant water, irrigated agriculture, or river banks, as well as close to forest and groves vegetation, near holm oak, pyrenean oak groves, quejigo oak forest, or riparian forest, all of them mainly found in wet locations with a great abundance of irrigated arable land in the surrounding area. The remaining 5.45% of the infected animals (12/220) did not usually live in these locations but had been in the same or nearby areas with high soil availability of water and similar vegetation. In fact, 6.6% (7/106) were infected by *D.* immitis, 5.88% (4/68) by *L. infantum*, 0% (0/23) by *A. platys*, and 4.34% (1/23) by *E.* canis.

DISCUSSION

This manuscript shows the prevalence and seroprevalence of four causative agents of CVBDs in Castilla y León, the largest region of the Iberian Peninsula and one of the largest territories of the European Union. The highest positivity detected was that of *D. immitis* (7.19%), followed by *L. infantum* (4.61%), *A. platys*, and *E. canis* (1.56%), although these seroprevalences varied according to the geographical location of the provinces and the distribution of the samples tested.

A slight increase in the global prevalence of *D. immitis* was found when compared to a previous study, which reported a prevalence of 6.23% for Castilla y León (3). Although the data should be considered carefully due to the short time between one study and another, it is necessary to keep a constant record of variations in epidemiology to determine if there is an increasing trend in prevalence, which could confirm the expansion of this parasitosis in Spain (8). In Castilla y León, data at the provincial level only had been previously published for Salamanca, where prevalence decreased from 12.3 to 5.8% within 30 years (30–32); furthermore, a hyperendemic zone was previously reported in Salamanca, characterized by irrigated lands near the riverbank and presence of stagnant water, which showed a decreasing prevalence from 33.3 to 16.7% (8, 30–32). The results of the present study showed a slight increase in Salamanca, which may be due to the spread of the disease as well. The occurrence of canine cardiopulmonary dirofilariasis depends mostly on climatic factors such as temperature and humidity, although changes in land use derived from the human activity (e.g., the increase of irrigated crops), and the management of domestic

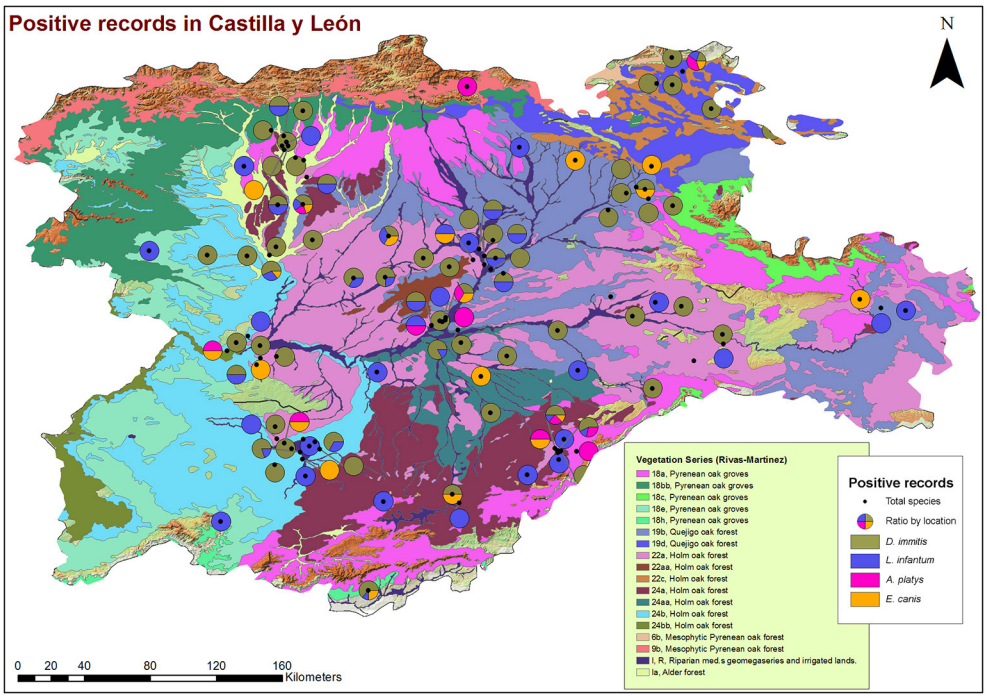

FIGURE 4 | Location of phytoclimatic series and geolocation of dogs infected by *D. immitis* (), and seroprevalences for *L. infantum* (), *A. platys* (), and *E. canis* () in the nine provinces of Castilla y León, Spain.

animals also influences on the presence of the parasite and vectors (33). Although initially relegated to south and east of Spain, studies have confirmed the expansion toward northern and colder areas (3, 8). In addition, the presence of *Cx. pipiens*, widely present in Salamanca, has also been demonstrated to transmit the disease in this province (34). The results of this study confirm the risk of transmission specially associated with the presence of stagnant water, irrigated agriculture, crops, riverbanks, forests, and wet vegetation, as was predicted by a geo-environmental model (35), since infected dogs were located in areas with high or very high risk of infection in all provinces of Castilla y León.

Canine leishmaniosis has traditionally been considered as a disease limited to the Mediterranean basin (36), but several studies have reported an incipient increase in the number of cases in some areas of the north and central regions of Spain (3, 5, 37, 38), although the number of published studies is still low (39). Previous studies carried out in Castilla y León are sporadic and with a low number of samples analyzed. One of them showed an overall seroprevalence of 5.74% (3), and at the provincial level, another study carried out in Valladolid showed a seroprevalence of 5.3% (40). Both showed slightly higher prevalences than those reported in the present study, which could be due to the difference in the sampling process (with lower number of samples) rather than a real decrease in the prevalence, since an increase of this disease is being reported in Spain (3, 5, 39). Although from a geographical and climatic point of view, conditions of Castilla y León are very different and less favorable for the development of sandfly vectors from those in Mediterranean regions considered as endemic for *Leishmania*, the disease is well-established since seropositive dogs were found in irrigated areas, with a range of vegetation prone to vector establishment, and, moreover, cases of human leishmaniosis have been reported in Castilla y León (39), so control measures are necessary. It is possible that this expansion found in heartworm and in leishmaniosis is due to the fact that both diseases are considered not present in Castilla y León and, therefore, are not subject to strict prophylactic measures between veterinarians and owners.

Regarding *A. platys* and *E. canis*, few epidemiological data are available in Spain, but those published report the presence of both infectious agents mainly distributed in the provinces along the Mediterranean, although prevalences between 1 and 4.9% have also been reported in inland and isolated areas of the Iberian Peninsula (3, 4). It seems that the presence in the studied territory has decreased, since previous studies reported global prevalences of 19.2% in 1996 and 2% in 2020 for *E. canis* (3, 40, 41). Similar findings have been seen for *Anaplasma spp.*, which previous surveys have reported a global prevalence of 2.74% and prevalence of 19% in Valladolid (3, 40). This decrease is confirmed by the present results. *Ixodes* spp. and *Rhipicephalus sanguineus*, vectors of these diseases, are widely distributed throughout Castilla y León, the Iberian Peninsula, and Europe (42), being mainly located in areas with presence of grass, bushes, or trees. Furthermore, wild animals can act as reservoirs (43, 44), and in those provinces with higher seroprevalences there is a large population of wild animals (wolves, foxes) which in some

cases live near houses and villages. In areas bordering the north of the region, where climatic and geographical conditions are much more favorable to vector development, previously reported seroprevalences were notably higher (5.01 and 3.13% to *A. platys* and *E. canis*, respectively) (45). It seems contradictory since climate changes favor the proliferation of ticks, but it could be due to the use of doxycycline for the treatment of several canine infections, as it is known that the dogs in this study did not receive adequate prophylaxis.

No significant differences were found by sex in this study, similar to other previous studies focused on these diseases such as other studies (3, 11, 19, 46). When age was assessed, no significant differences were observed between age groups, although differences have been observed in other epidemiological studies (4, 5, 11, 47–50). Regarding habitat of the animals, significantly higher prevalences were found in outdoor and indoor/outdoor dogs, as described by other authors (3, 5, 11, 51), since outdoor animals are more exposed to vectors. However, the study showed infected dogs living indoors as well. This is because some arthropods, such as mosquitoes, have access to indoors, and because animals living indoors are not fully enclosed and have a certain amount of access to the outdoors. Therefore, prophylactic measures should be applied to all dogs equally.

The preliminary GIS analysis suggests the existence of patterns of appearance of the infections with certain bioclimatic and environmental variables. The presence of areas with stagnant water, irrigation systems, irrigated agriculture, river banks, and different types of climatologies and vegetation favorable for the development of the vectors detected in the same places where positive dogs have been recorded suggests the existence of patterns of occurrence of the reported diseases with certain bioclimatic and environmental variables (35, 52, 53). The establishment of causal circumstances that can serve to predict risk areas requires a more in-depth study both in the resolution of these variables and in the incorporation of others that can explain in a more precise way the interactions between the parasite, its hosts, and the dispersal vectors. Expanding the methodology through the incorporation of spatial correlation analysis is one of the needs in this epidemiological field.

In conclusion, the results of this epidemiological study show a wide distribution of the evaluated causative agents of CVBDs in Castilla y León, which is very significant given the great geographical extension of the territory. The data obtained reveal the influence of the climate, orography, and presence of water, which will allow to comprehend their evolution in Castilla y León. Given the risk of infection or exposure to pathogens, as their presence in humans in Spain has been described, a close relationship between veterinarians, physicians, and public health administrations under the concept of One *Health* is needed. This would allow effective control measures to be carried out on infected animals and vectors, mainly focused on prophylactic measures to be applied routinely on dogs.

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 study was reviewed and approved by University of Las Palmas de Gran Canaria. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

EC, RM, and IR-E wrote the manuscript. EC, RM, and JM-A designed the study and obtained funding. PP, IR-E, and JS performed the fieldwork and collected the data. EC, RM, JM-A, and JL-M participated in the revision of the manuscript. All authors participated in the design and production of the figures and have read and agreed to the published version of the manuscript.

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