ZOONOTIC PARASITIC DISEASES IN A CHANGING WORLD

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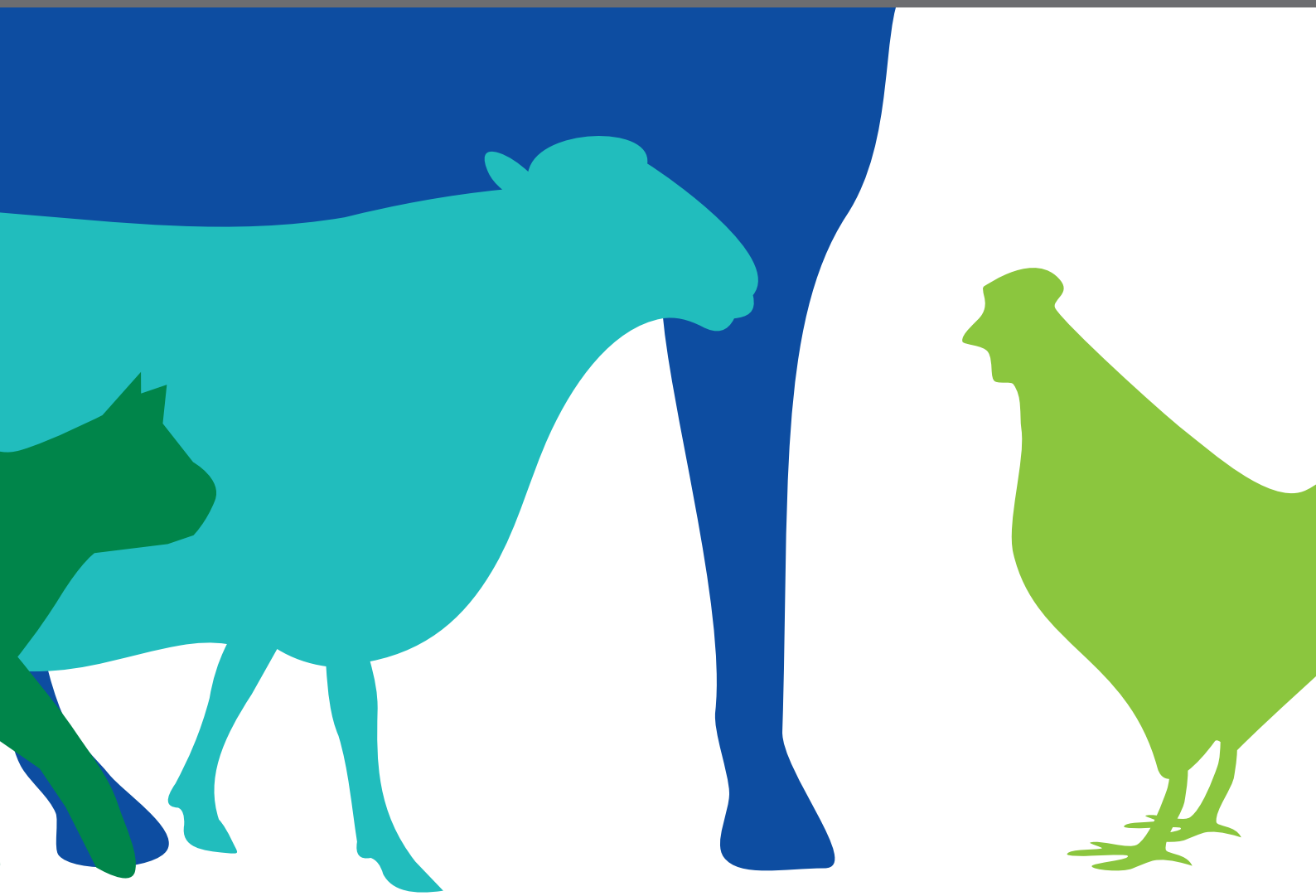

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ZOONOTIC PARASITIC DISEASES IN A CHANGING WORLD

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EDITORIAL
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Editorial: Zoonotic Parasitic Diseases in a Changing World

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Editorial on the Research Topic

Zoonotic Parasitic Diseases in a Changing World

Parasites are diverse and challenging group of eukaryotes, including zoonotic pathogens naturally occurring in the environment, already significantly changed by globalization and anthropogenic impact. Climate changes can further modify fundamental features and transmission dynamics of zoonosis (e.g., parasites' host preference, infectivity, areal). The proximity of humans and animals in several settings, such as in the rural landscapes, as well as in fragmented sylvatic habitats, in the natural environment close to urban areas and for the companion relationship between human and animals, may represent additional risk factors.

The Research Topic aims to gather the most updated studies on zoonotic neglected and foodborne parasites, taking into account two pivotal aspects: (i) challenging scenarios represented by climate change and anthropogenic impact and (ii) the "One-Health" concept.

The Research Topic collected 11 contributions including 3 reviews, 1 minireview, 1 brief research report and 6 original research articles, ranging from protozoans (*Toxoplasma, Leishmania, Cryptosporidium*) to metazoans (*Echinococcus, Taenia, Dirofilaria, Toxocara, Trichuris*).

EVIDENCES FROM PROTOZOANS

Vector-borne diseases are particularly interesting in the global change scenario, given the susceptibility of vectors to climatic variations and their large plasticity and adaptive features to several ecological contexts. In the review of Springer et al., the authors called for filling the gap in knowledge to determine if and how global changes impact, in terms of climate, land use, agricultural practices and human behavior, the frequency of zoonotic tick-borne pathogens in domestic animals. Similarly, the presence of *Leishmania infantum* was investigated in a poorly studied host, i.e., cats, in the Mediterranean basin, a key area for leishmaniosis particularly for the growing tendency of tourists to travel with pets (Morelli et al.). Apart from the Mediterranean basin, the East African region remains one of the most globally impacted for leishmaniosis: Jones and Welburn underlined the necessity to implement surveillance and disease management measures, especially in low- and middle-income countries, expected to be most impacted by climate change (3).

Another protozoan disease affecting both humans and livestock, and having a huge impact in low-income countries, is cryptosporidiosis. Utaaker et al. highlighted the need to investigate epidemiological scenarios of cryptosporidiosis in goats, one of the most frequently bred species, especially in low-income countries. Despite the resulting negligible concern for zoonotic transmission in these areas, scant information is available for developing countries so far.

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Finally, the role of two thioredoxin proteins from *Toxoplasma gondii* was studied in host-parasite interplay by the means of CRISPR-Cas9 (Zhang et al.). The study excluded their involvement as virulence factors *in vitro* and *in vivo* experiments. This study evidenced the application of an innovative approach such as the CRISPR-Cas9 system to investigate biological functions of parasitic proteins and host-parasite interactions.

EVIDENCES FROM METAZOANS

Several metazoans are included in the Neglected Tropical Diseases (NTDs) list and the World Health Organization (WHO) has recently launched its road map "Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021–2030," as highlighted in the review about neurocisticercosis, a disease that represents a significant health burden to affected communities (Butala et al.).

Cystic and alveolar echinococcosis are two of the most important zoonotic parasites with public health and economic relevance, ranked in highest positions among the food-borne zoonoses according to the international food safety agencies. Their presence in wild and domesticated animals still pose a challenge for the disease control. In the study of Dehghani et al., the genetic diversity of systematically relevant genomic region of *E. granulosus* was evaluated in samples from camels, which play a significant role in the parasite transmission cycle, especially in the Middle East and North Africa (7). By comparing the haplotypes obtained in the sample with homologous sequences from all over the world, the researchers observed a moderate genetic variation occurring in the sample, showing *E. intermedius* (G6) as the most commonly represented genotype in camels, followed by *E. granulosus* sensu stricto (G1). In addition, the Middle East isolates were more variable than the North/sub-Saharan isolates, suggesting an ancestrality for *E. granulosus* originating from the former region. The study offers a global outlook of the importance of camels in the molecular epidemiology of cystic echinococcosis in the main camel breeding areas of the world. Genetic diversity of the related species *E. multilocularis*, in which four distinct clades corresponding approximately to their continents of origin are described, was recently investigated (Umhang et al.). With molecular epidemiology, it is possible also to trace various records of genetic variants in atypical regions, which may be the result of introduction or natural migration of host animals. In this scenario, red foxes from Eastern Europe (Poland) harbor haplotypes of the typical European *E. multilocularis*, but also of the Asian cluster. The study presented the first large-scale investigation showing a zone of apparent overlap between European and Asian variants of *E. multilocularis*. Because the resolution from microsatellite EmsB analyzes and mitochondrial (mt) sequencing did not completely agree on allocation to Asian and European clusters, the authors suggested that cross-fertilization between worms of Asian origin and worms from the European Polish population might explain these conflicting results.

Regarding the phylum Nematoda, the role of microRNAs (miRNAs) was investigated in the infection progression caused by *Toxocara canis*, a neglected zoonotic parasite, which threatens the health of dogs and humans worldwide (Zou et al.). Considering the understudied and fascinating role of miRNas in host-parasite interplay, particularly in chronic infections induced by nematodes, this study is of great relevance for a better understanding of the adaptive and innate immune responses mechanisms associated with dog infections by *T. canis*.

Riveiro et al. examined the current knowledge of *Trichuris trichiura* and its relationship to whipworm of other primates. This study contributes to the debate on the systematics of *T. trichiura,* in updating data supporting the existence of several taxonomic entities with different degree of host affiliation circulating in primates.

The epidemiological status of the cardiopulmonary dirofilariosis caused by *Dirofilaria immitis* in dogs and humans is updated for the Colombian region (Esteban-Mendoza et al.) reporting a considerable seropositivity in both dogs (around 11%) and humans (around 7%), indicating comparable transmission rates also in atypical hosts as humans.

Thanks to a broader and more comprehensive view of interacting factors characterizing a zoonotic disease, the scientific community could better understand major elements that contribute to the fundamental aspects of parasite transmission and how they are closely linked to the distribution and persistence of these diseases. This article collection emphasize how interdisciplinary collaboration is becoming an essential feature of epidemiological surveys, being of paramount importance in developing effective strategies for the control of zoonotic diseases.

AUTHOR CONTRIBUTIONS

This editorial was authored by SC, with review and input by SG, VS, AG, and MP. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Current Situation of the Presence of *Dirofilaria immitis* in Dogs and Humans in Bucaramanga, Colombia

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The cardiopulmonary dirofilariosis caused by *Dirofilaria immitis*, is a vector-borne infection, which can be transmitted to humans. The main hosts are both domestic and wild canids. This species mainly occurs in tropical and subtropical climates, and temperature and humidity are the main factors that favor the presence and proliferation of culicid mosquitoes as vectors of the disease. There are few reports of cardiopulmonary dirofilariosis in dogs and humans in Colombia, a region with favorable climatic conditions which favors the presence of mosquitoes that act as vectors of the disease. Therefore, this study aimed to examine its current prevalence in dogs and the risk of human exposure to the disease in Bucaramanga, one of the most populated areas in Colombia located at the center of the country. Furthermore, its demographic and environmental characteristics could be useful as a study model for other similar locations and neighboring countries. Serum samples from 351 dogs and 506 humans from the Bucaramanga Metropolitan area were analyzed. All dog samples were analyzed by Knott's technique and tested with a commercial immunochromatographic to detect the presence of circulating antigens of *D. immitis*. Human samples were analyzed using a non-commercial ELISA test kit to detect IgG against the somatic antigens of adult *D. immitis* and *Wolbachia*. Positive results were further confirmed using western blot analysis. Thirty-eight dogs tested positive with a overall prevalence of 10.82%. Of these dogs, 18 showed *D. immitis* microfilariae, being 5.12% of the total population. The overall seroprevalence in humans was 6.71%; seroprevalence was significantly higher in individuals aged 16–34 years-old and in women than in men. To our knowledge, this study describes seropositivity to *D. immitis* for the first time in a Colombian human population located in the same area as that of dogs infected with *D. immitis*, which represents a potential threat to public health. In humans, age and gender can be considered risk factors for exposure to *D. immitis.*

Keywords: ***Dirofilaria immitis*, dog, heartworm, human dirofilariosis, prevalence, seroepidemiology, *Wolbachia pipientis*, Colombia**

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INTRODUCTION

Cardiopulmonary dirofilariosis, caused by *Dirofilaria immitis,* is a worldwide vector-borne disease in which the definitive hosts are both domestic and wild canines mainly (1). Several species of the genera *Culex* spp., *Aedes* spp., and *Anopheles* spp. are involved in the transmission of this parasite. These species represent a constant risk of infection because they feed on both animal and human hosts (2). Seroprevalence studies in humans have been conducted in regions where infected dogs have been found, which indicated previous contact with the parasite and cases with pulmonary nodules. For this reason, dirofilariosis is considered an emerging public health problem because of its zoonotic potential (3, 4).

Canine cardiopulmonary dirofilariosis is a chronic, progressive, and life-threatening disease. Adult worms are lodged in the pulmonary artery and the right ventricle of a dog's heart. Female mosquitoes ingest the microfilariae, inside which they make two successive molts until third-stage larvae (L3) are inoculated into the definitive host during the next blood meal (2). In humans, immature worms are embolized in the pulmonary microarteries, leading to the formation of benign lung nodules (pulmonary dirofilariosis), of which, most cases are asymptomatic (1, 2). Moreover, *D. immitis* harbors endosymbiotic bacteria of the genus *Wolbachia*, which participate in parasite molting and embryogenesis and play a key role in the immune and inflammatory response to the disease (5, 6).

Dirofilaria immitis is primarily distributed in tropical and subtropical climates and depends mainly on environmental factors, including temperature and humidity, in addition to human behavior, such as installation of irrigation systems, taking pets on trips, and new urban developments, that favor the presence and proliferation of its transmission vectors. However, the number of reports in areas with cooler climates has increased, which indicates that the disease is expanding (1, 2, 7, 8).

The South American continent is one of the most biodiverse areas on the planet, with a combination of factors, such as intensification of agricultural practices, landscape modification, poor ecosystem protection, and potentially unstable economies, which lead to the spread of the disease and its vectors (9). The disease has been reported in Argentina, Costa Rica, Venezuela, Peru and Brazil in domestic dogs between others, in where sporadic cases of pulmonary dirofilariosis have been described (1, 10–12). In Colombia, the disease has been reported in dogs from different areas, even in high-altitude areas with cold weather, with mean prevalence values of 0.91–16.12% according to different methodologies (10, 13). However, only one case of human pulmonary dirofilariosis has been described (14), and two seroepidemiological studies were conducted in an area within the Colombian Amazon where infected dogs were also found (15, 16).

The aim of this study was to determine the presence of *D. immitis* in dogs and their possible contact with the human population in the metropolitan area of Bucaramanga, Colombia.

MATERIALS AND METHODS

Sampling Area

Bucaramanga Metropolitan area, which belongs to the capital city of the Department of Santander in Colombia, includes the municipalities of Bucaramanga: Floridablanca, Piedecuesta, and Girón (**Figure 1**), located near the capital of Colombia, Bogotá. It extends to an area of 1,479 km^2, and the municipal area occupies 165 km^2; it is located at 959 m above sea level. The area includes two sectors of different geographical conformations: one formed by a plateau and the other by a valley. Its climate is tropical, with a mean annual temperature of 23.4°C and significant precipitation levels, with an approximate mean annual rainfall of 1,159 mm (17). It has an estimated population of 1.2 million people, and there are 32,000 censused dogs in the city of Bucaramanga alone. In addition, there are numerous uncensored vagrant dogs throughout this area (18).

Samples Used

This study included samples from 351 dogs and 506 humans collected during February-June 2018. All data collected is shown in **Tables 1**, **2**. The dog and human samples were collected by members of the veterinary staff of different clinics and associations and the Higuera Escalante Laboratory's health care staff, respectively. For the canine population, signed informed consent from the owners was considered as an inclusion criterion. Variables considered for the analysis were gender, age, municipality of residence, socioeconomic status, and whether dogs lived inside or outside of the house. For the human population, being of legal age and signing the informed consent forms were the inclusion criteria. Variables for the analysis were gender, age, municipality of residence, socioeconomic status, living with pets, type of pet (dog or other), and presence of water sources at <200 m. Confidentiality of patient information was always maintained, and all study participants gave their written consent. Socioeconomic stratification was carried out considering six strata: 1, misery; 2, poverty; 3, poverty with some economic resource; 4, middle class; 5, upper middle class; and 6, upper class (19).

Knott's Technique

Dogs blood samples were collected in 1 ml K2 EDTA plastic microtubes by applying the modified Knott technique (20) to check whether there were microfilariae in the blood of the animals included in the study.

Immunological Tests

Dogs and human blood samples were collected in 3 ml vacutainer plastic tubes and centrifuged. The resulting serum was stored at −20°C until further processing. The number of samples analyzed by the different variables and municipality are collected in **Tables 1**, **2**. Dog serum samples were tested for the presence of *D. immitis* antigens using a commercial immunochromatographic test kit (Uranotest Dirofilaria®, Urano Vet SL, Barcelona, Spain; sensitivity: 94.4%, specificity: 100%) according to the manufacturer's instructions. Human samples were analyzed for the presence of *D. immitis* and *Wolbachia* IgG antibodies using a non-commercial ELISA

FIGURE 1 | Location of Bucaramanga Metropolitan area, Colombia. **(A)** Colombia; **(B)** Department of Santander (Colombia); **(C)** Bucaramanga Metropolitan area.

with some modifications (4, 7, 21, 22). 1:100 and 1:40 serum dilutions were used to detect anti-*D. immitis* and anti-*Wolbachia* IgG antibodies, respectively. Goat anti-human IgG (H + L) conjugated to horseradish peroxidase (Sigma-Aldrich, Madrid, Spain) was used at a 1:4,000 dilutions in both cases. Optical densities (OD) were measured at 492 nm. The cut-off point (OD = 0.8 for DiSA and OD = 0.5 for rWSP) was established by calculating the mean value + 3 standard deviations (3SD) of 30 serum samples obtained from dogs and clinically healthy humans (negative controls) who belonged to an area free of *D. immitis*. Human sera were considered positive when both non-commercial ELISAs were positive for the same serum sample. These results were additionally confirmed using western blot analysis performed according to a previously described methodology (23, 24). 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 dilutions.

Geospatial Analysis

A geospatial analysis was performed on the population of dogs and humans from the metropolitan area of Bucaramanga through a spatial overlay of positive cases using the SatScan software v.9.6. and the Bernoulli's model with a 95% significance level (999 replications with $P < 0.05$) based on the Monte Carlo statistical significance test. Further, we established clusters, which are areas with a relative risk of infection in dogs and humans, with a maximum size of 50% of the exposed population, based on population census and positive cases. Clusters were imported into the QGIS software version 3.8.0 to be visualized on the study area map.

Statistical Analysis

Data were analyzed using SPSS 20.0 software for Windows (SPSS Inc./IBM, Chicago, IL, USA). This is a descriptive study applying univariate analysis for the determination of frequencies and a bivariate analysis through Chi-square and odds ratio (OR) estimation, based on which a statistical analysis was performed for the determination of the association between variables. In all cases, the level of significance was established with a *P*-value of <0.05.

RESULTS

The overall prevalence of *D. immitis* in dogs was 10.82% (38/351). Of these positive dogs, 18 showed *D. immitis* microfilariae, being 5.12% of the total population. The prevalence of *D. immitis* and of microfilariae broken down by gender, age, municipality (Bucaramanga, Floridablanca, Girón and Piedecuesta), socioeconomic status and place of permanence are provided in **Table 1**. No statistically significant differences

TABLE 1 | Prevalence in dogs in the Bucaramanga Metropolitan area in terms of gender, age, municipality, socioeconomic status, and their place of permanence.

Variable	No. of samples	No. of positive	Prevalence (%)	95% CI	OR	No. of mf dogs	Prevalence (%)	95% CI	OR
Gender									
Male	132	14	10.60	0.0474–0.1511	0.97	7	5.30	0.0144–0.925	1.03
Female	219	24	10.95	0.0679–0.1513	1.01	11	5.02	0.0211–0.0794	0.97
Age (years)									
<1	31	2	6.45	−0.0271–0.1561	0.97	0	0.00	0.000–0.000	0.00
1–3.9	109	11	10.09	0.0435–0.1584	1.05	5	4.59	0.0060–0.0858	0.94
4–6.9	119	11	9.24	0.0338–0.1357	0.72	7	5.88	0.0106–0.0911	1.04
7–10.9	67	12	17.91	0.0849–0.2733	1.21	5	7.46	−0.0061–0.0956	0.56
11–15	25	2	8.00	−0.0343–0.1943	0.48	1	4.00	0.0056–0.3144	2.31
Municipality									
Bucaramanga	144	11	7.60	0.0276–0.1122	0.85	2	1.38	−0.0055–0.0335	0.80
Floridablanca	73	10	13.70	0.0562–0.2178	1.30	2	2.74	−0.0110–0.0657	1.40
Girón	70	9	12.85	0.0482–0.2090	0.45	8	11.42	0.0379–0.1907	0.75
Piedecuesta	64	8	12.50	0.0417–0.2083	1.66	6	9.37	0.0204–0.1671	1.26
Socioeconomic status									
Stratum 1	139	16	11.51	0.0614–0.1688	1.02	12	8.63	0.0391–0.1336	1.33
Stratum 2	57	6	10.50	0.0231–0.1874	0.93	1	1.75	−0.0176–0.0527	0.25
Stratum 3	79	10	12.60	0.0516–0.2015	1.25	2	2.53	−0.0101–0.0607	0.37
Stratum 4	75	6	8.00	0.0090–0.1261	0.75	3	4.00	−0.0066–0.0865	1.24
Stratum 5	1	0	0.00	0.000–0.000	0.00	0	0.00	0.000–0.000	0.00
Stratum 6	0	0	0.00	0.000–0.000	0.00	0	0.00	0.000–0.000	0.00
Place of permanence									
Indoors	187	17	9.09	0.0493–0.1325	0.82	7	3.74	0.0100–0.0649	0.71
Outdoors	164	21	12.80	0.0718–0.1736	1.21	11	6.70	0.0286–0.1064	1.33
Total	351	38	10.82			18	5.12		

were observed for the variables gender, age, municipality, socioeconomic status, and place of permanence (indoors and outdoors).

The overall seroprevalence in humans was 6.71% (34/506). The seroprevalence broken down by gender, age, municipality (Bucaramanga, Floridablanca, Girón and Piedecuesta), socioeconomic status, water sources located at <200 m from the house and living with pets or not are described in **Table 2**. All samples positive for western blot analysis are shown in **Figure 2**.

In the spatial exploration of dogs positive for *D. immitis* within the study area (**Figure 3**), 4 significant clusters were detected taking into consideration the magnitude and distribution: one for positive dogs and 3 for seropositive humans ($P < 0.01$).

DISCUSSION

In this study, we analyzed the presence of *D. immitis* in dogs and humans in the metropolitan area of Bucaramanga, Colombia, analyzing the presence of circulating antigens of *D. immitis* within the canine population, and the response of anti-*D. immitis* and anti-*Wolbachia* IgG antibodies in the human population as a study model for other areas in South America. This first study, to our knowledge of heartworm disease in Bucaramanga, Colombia, revealed an overall prevalence in dogs of 10.82%. Bucaramanga is surrounded by vegetation and is characterized by an average annual temperature of 24°C, high humidity levels, several gullies in its proximity or even in the central areas that accumulate water during the rainy season, and the presence of two rivers (River Oro and River Surata in the areas of Girón and Bucaramanga, respectively). In addition, there are vector species described in the both area, such as *Aedes aegypti*, and other species that could be involved in disease transmission (10, 25). These conditions could promote breeding of these mosquitoes and disease transmission in Bucaramanga. Furthermore, the overall prevalence of microfilaremic dogs was 5.12%, which was heterogeneous. The same situation we can observe in other endemic areas where the disease has been reported (2–4, 7, 8, 10, 26, 27).

Regarding human infections, the overall seroprevalence was 6.71%; to our knowledge, this is the first time that a seroprevalence study for *D. immitis* was carried out in South America. The greatest seroprevalences were detected in Piedecuesta (11.68%) and Bucaramanga (6.87%), where the prevalences in dogs were 12.5 and 7.6%, respectively. We observed slightly lower seroprevalence values of 5.68% in Floridablanca and 3.12% in Girón, where the prevalence observed in dogs was 13.7 and 12.85%, respectively. The occurrence of dirofilariosis in humans depends mainly on the presence of infected dogs and vectors for transmission within a given area. At the same time, other factors, such as an

TABLE 2 | Seroprevalence of human dirofilariasis in Bucaramanga Metropolitan area, considering seropositivity is defined by the simultaneous positivity of anti-*D. immitis* and anti-*Wolbachia* antibody response.

Variable	No. samples	No. of seropositive	Seroprevalence (%)	95% CI	OR
Gender					
Male	159	9	5.66	0.3192–0.8808	0.77
Female	347	25	7.20	0.3939–0.6930	1.29
Age (years)					
18–35.9	294	17	5.78	0.2695–0.6905	0.46
36–50.9	118	10	8.47	0.2248–0.7752	1.50
51–65.9	73	7	9.58	0.2823–1.0510	1.05
66–90	21	0	0.00	−5.8531–6.8531	0.94
Municipality					
Bucaramanga	189	13	6.87	0.4540–0.9144	1.09
Floridablanca	176	10	5.68	0.2286–0.6805	0.89
Girón	64	2	3.12	−0.4187–1.4187	0.38
Piedecuesta	77	9	11.68	0.3192–0.8808	1.56
Socioeconomic status					
Stratum 1	30	6	20.00	0.3016–0.8010	2.14
Stratum 2	144	12	8.33	0.3406–0.8023	2.03
Stratum 3	193	11	5.69	0.2093–0.6055	1.12
Stratum 4	121	5	4.13	0.2630–1.1656	0.80
Stratum 5	16	0	0.00	0.000–0.000	0.00
Stratum 6	2	0	0.00	0.000–0.000	0.00
Water sources located at <200 m from the house					
Yes	232	15	6.46	0.3734–0.7804	0.93
No	274	19	6.93	0.3692–0.7165	1.06
Living with pets or not					
Yes	377	28	7.42	0.4622–0.7552	1.64
No	129	6	4.65	0.1192–0.6808	0.60
Canines	335	26	7.76	0.3760–0.7668	0.58
Other species	171	8	4.67	0.3889–0.6808	1.10
Total	506	34	6.71		

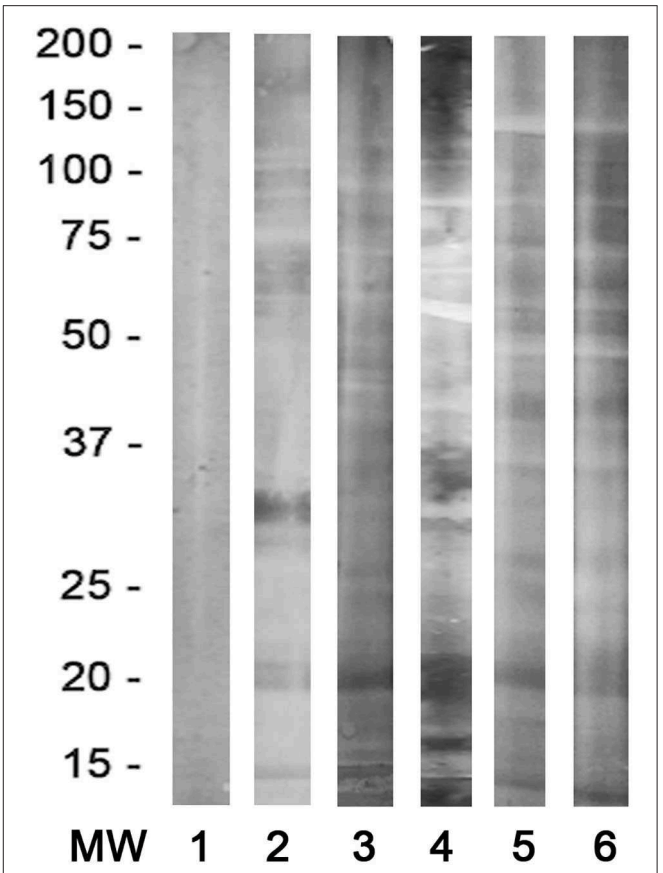

FIGURE 2 | Representative immunoblot analysis of the human sera. Lane 1: negative control; Lanes 2–6: *Dirofilaria immitis*-positive specific bands between 17 and 22 kDa.

increase in temperature and humidity owing to climate change; emergence of new disease-transmitting species; transportation of infected hosts; modification of the environment owing to human activities; agricultural practices and irrigation areas; deficiency and economic instability; and adverse meteorological events, such as hurricanes or tropical rains in the area, affect the development of the disease (1, 4, 28). Further, it is important to note that although dirofilariosis is a vector-borne disease, not all L3 that come into contact with the host develop into adults, neither in dogs nor in wild hosts (9). Most of the information regarding humans comes from clinical cases and retrospective reviews. In these cases, there are only data from the affected population that showed some type of clinical manifestation, excluding the infected population that does not have symptoms related to the disease or any clinical manifestation, making its study even more difficult. Seroepidemiological studies show the complementary part of the problem, they detect contact with the parasite by analyzing the anti-*Dirofilaria* and anti-*Wolbachia* immune response and are excellent tools to analyze the risk of infection among the human population residing in an endemic area (1, 3, 4, 7, 22, 26, 27).

In Colombia, there has been only one clinical case of a patient from whom an adult worm identified as *Dirofilaria* sp. was extracted from the lung (14), and there are two studies that warn of the existence of human infections caused by *D. immitis* in communities from the Colombian Amazon where infected dogs have been found (15, 16).

Data points related to the geographical location of the samples, and humans with positive serology have been reported in the same location as infected dogs. In addition, spatial clusters in these areas with a relative risk of <1 were detected for humans, suggesting a positive association between the variables studied and a higher frequency of contact with the parasite. These data may suggest a relationship between the presence of *D. immitis*-infected dogs and the seroprevalence detected in humans. This is similar to what occurs in other European countries, such as Spain, Portugal (6.1%), Romania and Moldova (10.9%), and Russia (0.63–4.3%), where the risk of infection among humans has been studied (1, 3, 4, 7, 8, 26, 27).

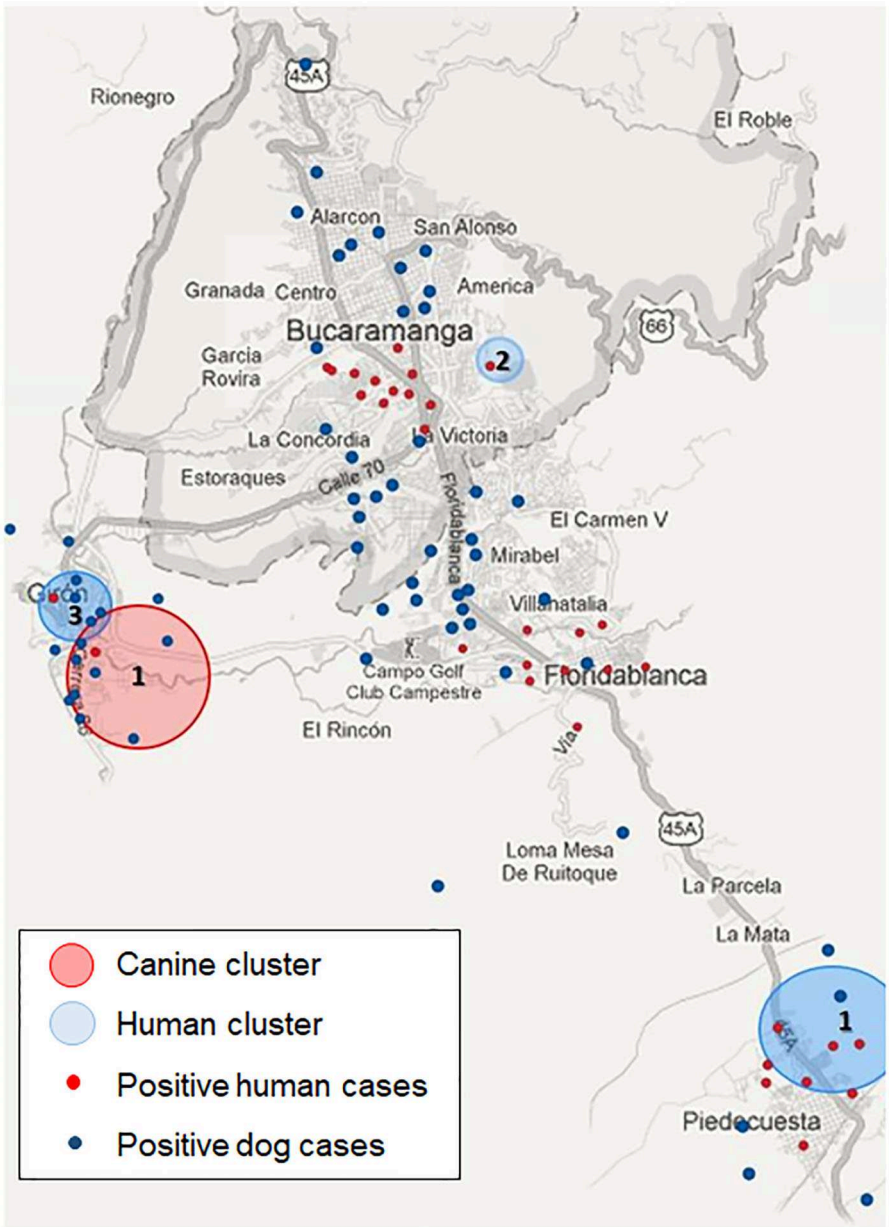

FIGURE 3 | Geospatial exploration of dogs and humans positive for *D. immitis* in the metropolitan area of Bucaramanga imported into Qgis program (version 3.8.0).

Regarding the variables evaluated, we observed that age can be a risk factor. In our study, the population with the highest seropositivity was that from the age group of 51–65 years-old. This result is similar to other studies that report that the risk of infection increases with age (3, 4, 7, 8, 26, 27). Furthermore, not only did this study allow us to address the problem from a biological point of view, but also from a socioeconomic point of view in the case of humans. The greatest seroprevalence was observed in stratum 1, where sanitary hygiene conditions are not adequate (20%), followed by stratum 2 (8.3%), stratum 3 (5.7%), and stratum 4 (4.1%). Seropositive individuals were not detected in the last two strata where the sanitary hygiene level is optimal. Socioeconomic status has been associated with mortality and the use of health services, which indicates that a lower income reduces the application of prophylactic and preventive measures to vectors and canines that live with humans (29). Environmental sanitation elements, such as water; sewage, garbage, and waste disposal; sanitary landfills; and garbage treatment, influence the prevalence of parasitosis. These data allow us to associate the lack of sanitary hygiene with the development of dirofilariosis, which may become a socially-determinant public health factor, as in the case of other vector-borne

diseases in Colombia, such as malaria, leishmaniosis and Chagas disease (9, 30, 31).

In conclusion, this study describes, for the first time, seropositivity to *D. immitis* and *Wolbachia* Surface Protein in one of the most populated areas of Colombia with a high presence of dogs infected with *D. immitis*. The corresponding authorities should take measures to monitor and control this emerging zoonotic disease to reduce prevalence in canines, while including human pulmonary dirofilariosis in the differential diagnosis of pulmonary nodules. It is necessary to perform further studies in Colombia regarding vectors, reservoirs and humans to clarify the risk of this infection.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The sampling process complied with the Helsinki Code of Ethics and Animal Welfare and was approved under resolution no. 040-2019 by the ethics committee of Universidad Cooperativa de Colombia. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ME-M, RM, and VA-Q designed the study, wrote the manuscript, participated in the discussion of the results, and corrected the manuscript. JA-N, IH, and MF-A performed the fieldwork and collected the data. All authors read and approved the final manuscript.

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REFERENCES

1. Simón F, Siles-Lucas M, Morchón R, González-Miguel J, Mellado I, Carretón E, et al. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. *Clin Microbiol Rev*. (2012) 25:507–44. doi: 10.1128/CMR.00012-12
2. Morchón R, Carretón E, González-Miguel J, Mellado-Hernández I. Heartworm disease (*Dirofilaria immitis*) and their vectors in Europe–new distribution trends. *Front Physiol*. (2012) 3:196. doi: 10.3389/fphys.2012.00196
3. Cabrera ED, Carretón E, Morchón R, Falcón-Cordón Y, Falcón-Cordón S, Simón F, et al. The Canary Islands as a model of risk of pulmonary dirofilariasis in a hyperendemic area. *Parasitol Res.* (2018) 117:933–6. doi: 10.1007/s00436-018-5774-1
4. Fontes-Sousa AP, Silvestre-Ferreira AC, Carretón E, Esteves-Guimarães J, Maia-Rocha C, Oliveira P, et al. Exposure of humans to the zoonotic nematode *Dirofilaria immitis* in northern Portugal. *Epidemiol Infect*. (2019) 147:e282. doi: 10.1017/S0950268819001687
5. Simón F, Kramer LH, Román A, Blasini W, Morchón R, Marcos-Atxutegi C, et al. Immunopathology of *Dirofilaria immitis* infection. *Vet Res Commun*. (2007) 31:161–71. doi: 10.1007/s11259-006-3387-0
6. Genchi C, Kramer LH, Sassera D, Bandi C. *Wolbachia* and its implications for the immunopathology of filariasis. *Endocr Metab Immune Disord Drug Targets*. (2012) 12:53–6. doi: 10.2174/187153012799279108
7. Ciuca L, Simón F, Rinaldi L, Kramer L, Genchi M, Cringoli G, et al. Seroepidemiological survey of human exposure to *Dirofilaria* spp. in Romania and Moldova. *Acta Trop*. (2018) 187:169–74. doi: 10.1016/j.actatropica.2018.07.012
8. Kartashev V, Afonin A, González-Miguel J, Sepúlveda R, Simón L, Morchón R, et al. Regional warming and emerging vector-borne zoonotic dirofilariasis in the Russian Federation, Ukraine, and other post-Soviet states from 1981 to 2011 and projection by 2030. *Biomed Res Int*. (2014) 2014:858936. doi: 10.1155/2014/858936
9. Maggi RG, Krämer F. A review on the occurrence of companion vector–borne diseases in pet animals in Latin America. *Parasit Vectors*. (2019) 12:145. doi: 10.1186/s13071-019-3407-x
10. Labarthe N, Guerrero J. Epidemiology of heartworm: what is happening in South America and Mexico? *Vet Parasitol*. (2005) 133:149–56. doi: 10.1016/j.vetpar.2005.04.006
11. Dantas-Torres F, Otranto D. Dirofilariasis in the Americas: a more virulent *Dirofilaria immitis*? *Parasit Vectors*. (2013) 6:288. doi: 10.1186/1756-3305-6-288
12. de Argôlo EGG, Reis T, Fontes DAT, Gonçalves EC, Giese EG, Melo FTV, et al. Canine filariasis in the Amazon: species diversity and epidemiology of these emergent and neglected zoonoses. *PLoS ONE*. (2018) 13:e0200419. doi: 10.1371/journal.pone.0200419
13. McCown ME, Monterroso VH, Cardona W. Surveillance for *Ehrlichia canis*, *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, and *Dirofilaria immitis* in dogs from three cities in Colombia. *J Spec Oper Med*. (2014) 14:86–90.
14. Beaver PC, Orihel TC, Leonard G. Pulmonary dirofilariasis: restudy of worms reported gravid. *Am J Trop Med Hyg*. (1990) 43:9–167. doi: 10.4269/ajtmh.1990.43.167
15. Vieira C, Montoya M, Agudelo S, Simón F. Human antibody response to a 56-kDa purified excretory/secretory product of *Dirofilaria immitis*. *Trop Med Int Heal*. (2000) 5:855–9. doi: 10.1046/j.1365-3156.2000.00663.x
16. Vieira C, Vélez ID, Montoya MN, Agudelo S, Alvarez MI, Genchi C, et al. *Dirofilaria immitis* in Tikuna Indians and their dogs in the Colombian Amazon. *Ann Trop Med Parasitol*. (1998) 92:123–5. doi: 10.1080/00034983.1998.11813270
17. Datos Climáticos Mundiales. *Clima Bucaramanga: temperatura, climograma y tabla climática para Bucaramanga*. (2020). Available online at: https://es.climate-data.org/america-del-sur/colombia/santander/bucaramanga-5923 (accessed May 12, 2020).
18. Albarracín Navas J, Cala FA. *Primer censo canino y felino del área urbana del municipio de Bucaramanga*. Colombia: Rev Colom Cienc Pecua (2011). Available online at: https://repository.ucc.edu.co/bitstream/20.500.12494/10799/1/2019_censo_canino_felino.pdf.
19. Departamento Administrativo Nacional de Estadística. *Clasificación socio-económica Colombia*. (2020). Available online at: https://www.dane.gov.co/files/geoestadistica/Preguntas_frecuentes_estratificacion.pdf (accessed May 3, 2020).

20. Acevedo RA, Theis JH, Kraus JF, Longhurst WM. Combination of filtration and histochemical stain for detection and differentiation of *Dirofilaria immitis* and *Dipetalonema reconditum* in the dog. *Am J Vet Res.* (1991) 42:537–40.
21. Simón F, Muro A, Cordero M, Martín JA. Seroepidemiologic survey of human dirofilariosis in western Spain. *Trop Med Parasitol.* (1991) 42:106–8.
22. Simón F, Prieto G, Morchón R, Bazzocchi C, Bandi C, Genchi C. Immunoglobulin G antibodies against the endosymbionts of filarial nematodes (*Wolbachia*) in patients with pulmonary dirofilariasis. *Clin Diagn Lab Immunol.* (2003) 10:180–1. doi: 10.1128/CDLI.10.1.180-181.2003
23. Perera L, Pérez-Arellano JL, Cordero M, Simón F, Muro A. Utility of antibodies against a 22 kD molecule of *Dirofilaria immitis* in the diagnosis of human pulmonary dirofilariasis. *Trop Med Int Health.* (1998) 3:151–5. doi: 10.1046/j.1365-3156.1998.00209.x
24. Santamaría B, Cordero M, Muro A, Simón F. Evaluation of *Dirofilaria immitis* excretory/secretory products for seroepidemiological studies on human dirofilariosis. *Parasite.* (1995) 2:269–73. doi: 10.1051/parasite/1995023269
25. Ruiz R, Gustavo A, Parra J, Duque JE. Oviposition dynamics of *Aedes* (*Stegomyia*) *aegypti* (Diptera: Culicidae), gonadotrophic state and coexistence with other culicidae in the metropolitan area of Bucaramanga. *Rev Univ Ind Santander Salud*. (2018) 50:308–9. doi: 10.18273/revsal.v50n4-2018004
26. Montoya-Alonso JA, Mellado I, Carretón E, Cabrera-Pedrero ED, Morchón R, Simón F. Canine dirofilariasis caused by *Dirofilaria immitis* is a risk factor for the human population on the island of Gran Canaria, Canary Islands, Spain. *Parasitol Res.* (2010) 107:1265–9. doi: 10.1007/s00436-010-1987-7
27. Montoya-Alonso JA, Carretón E, Corbera JA, Juste MC, Mellado I, Morchón R, et al. Current prevalence of *Dirofilaria immitis* in dogs, cats and humans from the island of Gran Canaria, Spain. *Vet Parasitol.* (2011) 176:291–4. doi: 10.1016/j.vetpar.2011.01.011
28. American Heartworm Society (2020). Available online at: https://www.heartwormsociety.org/ (accessed May 14, 2020).
29. Agudelo C, Villareal E, Cáceres E, López C, Eljach J, Ramírez N, et al. Human and dogs *Toxocara canis* infection in a poor neighborhood in Bogota. *Mem Inst Oswaldo Cruz.* (1990) 85:75–8. doi: 10.1590/S0074-02761990000100012
30. Caraballo L, Coronado S. Parasite allergens. *Mol Immunol.* (2018) 100:113–9. doi: 10.1016/j.molimm.2018.03.014
31. Feachem RGA, Chen I, Akbari O, Bertozzi-Villa A, Bhatt S, Binka F, et al. Malaria eradication within a generation: ambitious, achievable, and necessary. *Lancet.* (2019) 394:1056–112. doi: 10.1016/S0140-6736(19)31139-0
32. Esteban-Mendoza MV, Arcila-Quiceno V, Albarracín-Navas JH, Hernández I, Flecha-Alarcón MC, Morchón R. Current situation of the presence of *Dirofilaria immitis* in dogs and humans in Bucaramanga, Colombia. *Res Square.* (2020). doi: 10.21203/rs.2.19256/v3. [Epub ahead of print].

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Cystic Echinococcosis of Camels: 12S rRNA Gene Variation Revealed Changing Pattern of Genetic Diversity Within *Echinococcus granulosus* sensu lato in the Middle East and North/Sub-Saharan Africa

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Cystic echinococcosis (CE) is one of the most widespread zoonotic diseases, with considerable public health and economic importance. Camels play a significant role in transmission cycle of *Echinococcus granulosus* especially, in the Middle East and North Africa (MENA). The present study aimed to identify the genetic variation and haplotype distribution of camel isolates of *E. granulosus* sensu lato using all existing *E. granulosus* mitochondrial DNA data from camels in different parts of the world. Sequence data from 1,144 camel isolates of *E. granulosus* s.l. available in the NCBI GenBank including 57 camel hydatid cysts collected in central Iran were used to analyze the nature of genetic variation within the camel isolates of *E. granulosus* s.l. in MENA region. Fifty-seven camel isolates were also PCR-sequenced on mitochondrial 12S rRNA gene. Haplotype network analysis revealed seven different haplotypes clustered into four major groups. *E. intermedius* G6 was identified as the most commonly represented genotype in camels followed by G1. Mitochondrial 12S rRNA gene sequence analysis on 57 camel isolates identified three different genotypes, including *E. intermedius*/G6 (35/57, 61.4%), *E. granulosus* sensu stricto/G1-G3 (21/57, 36.8%) as well as one isolate identified as *E. ortleppi*/G5 (1/57, 1.8%). The number of base substitutions per site over 420 positions of partial 12S rRNA gene sequences were shown as 0.000 and 0.004 for *E. intermedius* (G6) corresponding to the Middle East and sub-Saharan isolates, respectively. Camel isolates of *E. granulosus* in the MENA region present moderate genetic diversity (Hd = 0.5540–0.6050). The Middle East isolates demonstrated a more diverse population than the North/sub-Saharan isolates, where six out of seven 12S rRNA haplotypes were identified in the former region. *E. intermedius* (G6 genotype) was shown to be the most common species in the world camel population. In conclusion, camels showed to be an

important intermediate host species in the MENA region with different patterns of genetic variation between the Middle East and Africa.

Keywords: hydatid disease, *Echinococcus intermedius*, *Echinococcus canadensis*, 12S rDNA, genetic variation, *Camelus dromedarius*, strain, MENA

INTRODUCTION

Tapeworms of the genus *Echinococcus*, causing a spectrum of infections known as echinococcosis, are members of the family Taeniidae. Cystic echinococcosis (CE) caused by the larval stages of *Echinococcus granulosus* sensu lato represents serious zoonotic infections in human and animals with a cosmopolitan distribution (1, 2). The life cycle involves dogs and other canids as definitive and domestic and wild ungulates as the intermediate hosts (3).

Extensive intraspecific variations have been documented within this species with significant epidemiological and clinical implications. *E. granulosus* genotype variation, may affect parasite life cycle and transmission patterns, host range and pathogenicity to humans (4, 5). Based on the biological and molecular genetic analyses using nuclear and mitochondrial DNA sequences 10 distinct genotypes with different host preferences have been identified for *E. granulosus* sensu lato and new nomenclature has been adopted for several genotypes (6) i.e., *E. granulosus* sensu stricto (G1–G3) with a wide range of intermediate hosts particularly sheep, goat and buffaloes, *E. equinus* (G4) of horses, *E. ortleppi* (G5) of cattle, *E. intermedius* (G6–G7) of camels and pigs, *E. canadensis* (G8 and G10) of Fennoscandian and subarctic cervids and *E. felidis* of African wild felid population (2).

According to Alvarez Rojas et al. about 88 and 11% of human CE infections are due to *E. granulosus* sensu stricto and *E. intermedius,* respectively (7). Therefore, camels are one of the most important intermediate hosts of the parasite especially in endemic regions of the Middle East and North Africa (MENA) where they play an important role in the transmission of *E. granulosus*. CE prevalence in camels has been estimated as 8–36% in different endemic countries (2, 8). In camels the presence of three *Echinococcus* species have been documented in different regions of the world, i.e., *E. granulosus* sensu stricto, *E. intermedius*, and *E. ortleppi* (2, 9). Several studies identified 100% of the parasites isolated from camels as *E. intermedius* (G6 genotype), however *E. granulosus* sensu stricto (G1–G3) has been shown to perpetuate in the camel-dog cycle as well (1, 10). Different studies showed 17.0–88.4% of camels harbored *E. granulosus* G1 genotype (11, 12). The nature and significance of *Echinococcus* variation in camels is poorly understood (6). Genotype G6 is more widespread in camels as a common intermediate host in the Middle East, Asia, and Africa, while G7 distribution has been reported among pigs and wild boars in Europe, the highly endemic Mediterranean areas and Central America (6). Both genotypes have been found to co-exist in Turkey, Argentina and Peru.

Mitochondrial DNA has been widely used for molecular epidemiological studies on helminth parasites mainly because of its conserved structure, mode of inheritance, and relatively high evolutionary rate. Currently one of the accepted markers for investigation of genetics and characterization of helminth parasites is mitochondrial 12S ribosomal RNA (12S rRNA) gene (13). The present study was conducted to provide a global outlook on the nature of genetic variation and haplotype distribution of *E. granulosus* sensu lato infecting camels using mitochondrial 12S rRNA gene sequence analysis.

MATERIALS AND METHODS

In this study all existing 12S rRNA gene sequences of camel isolates of *E. granulosus* sensu lato were retrieved form NCBI GenBank. To collect a dataset of 12S rDNA region, sequences data searched in and downloaded from NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/) using keywords "Echinococcus," "12S rRNA," and "camel" with mitochondrial records filtration for sequences longer than 400 bp. The search strategy was performed as follows: ((((("Echinococcus"[Organism] OR Echinococcus[All Fields]) AND 12S[All Fields]) AND mitochondrion[filter]) AND ("Camelus dromedarius"[Organism] OR camel[All Fields])) AND ("400"[SLEN]: "14000"[SLEN]). All sequence features were manually checked to confirm the parasite host as camel.

In addition, liver and lung samples were collected from 57 camels slaughtered in the municipal abattoirs of Kerman, Qom, and Tehran provinces. The protoscoleces and/or germinal layer were aspirated from each cyst and were preserved in −20°C after three-time washing in sterile saline solution. Total genomic DNA (gDNA) was extracted from individual cyst samples using High Pure PCR template preparation kit (Roche Diagnostic, Germany) and was stored at −20°C for future molecular analysis.

A partial 450-bp fragment of mitochondrial 12S rRNA gene was amplified using specific primers as previously described by Rostami et al. 12SRF (5′-AGGGGATAGGAC ACAGTGCCAGC-3′) as the forward and 12SRR (5′-CGG TGTGTACATGAGCTAAAC-3′) as reverse primers (14). No template controls were included in each experiment. The PCR products were electrophoresed on 1% (w/v) agarose gel containing ethidium bromide. Moreover, a partial mitochondrial Cytochrome c oxidase subunit 1 (cox1) gene was amplified from individual isolates using the primer sets JB3/JB4.5 for an accurate distinction between genotypes within *E. granulosus* sensu stricto (15). All amplicons were sequenced by an ABI-3730XL capillary machine (Macrogen Inc., South Korea).

Reference sequences in this study were collected form reference nucleotide dataset (RefSeq) for representative *Echinococcus* species. Sequence data were trimmed, aligned, and

TABLE 1 | The summary of global data on the frequency of *E. granulosus* sensu lato genotypes in camels (Camelus dromedarius) according to five major camel-rearing regions in the world.

Region	Genotypes (No., %)	Country	No. each genotype/No. examined	Genotype (%)	Gene marker(s)	References
North Africa	G1, G3 (55/289, 19) G6, G7 (233/289, 80.6) G5 (1/289, 0.4)	Algeria	6/6	G6 (100)	bg 1/3, cox1, nad1	(20)
			8/10	G6 (80)	cox1, nad1, act2 hbx2	(21)
			1/10	G1 (10)		
			1/10	G2 (10)		
		Egypt	47/47	G6 (100)	12S rRNA	(22)
			20/20	G6 (100)	nad1	(23)
			40/40	G6 (100)	12S rRNA	(24)
			26/28	G6 (92.9)	cox1, nad1, actin II	(5)
			1/28	G1 (3.57)		
			1/28	G5 (3.57)		
		Libya	5/5	G1 (100)	cox1	(25)
			83/83	G6 (100)	cox1, nad1	(26)
		Morocco	34/34	G1 (100)	cox1, nad1	(27)
		Tunisia	13/13	G1 (100)	cox1	(28)
			3/3	G6 (100)	ITS1, cox1	(29)
Sub-Saharan Africa	G1–G3 (55/530, 10.4) G6–G7 (474/530, 89.4) Unidentified (1/530,0.2)	Ethiopia	9/12	G1–G3 (75)	cox1	(30)
			3/12	G6–G10 (25)		
		Kenya	26/108	G1 (24.1)	12S rRNA, cox1, nad1	(31)
			82/108	G6–G7 (75.9)		
			17/100	G1–G3 (17.0)	nad1	(11)
			83/100	G6–G7 (83.0)		
			3/15	G1–G3 (20)	nad1	(32)
			11/15	G6–G7 (73.3)		
			1/15	Unidentified (6.7)		
		Mauritania	3/3	G6 (100)	bg 1/3, cox1, nad1	(33)
			1/1	G6–G7 (100)	12S rRNA	(34)
			17/17	G6 (100)	cox1, nad1, act2, hbx2	(21)
		Somalia	2/2	G6 (100)	cox1	(35)
		Sudan	35/35	G6–G7 (100)	12S rRNA, cox1, nad1	(31)
			207/207	G6–G7 (100)	12S rRNA, cox1, nad1	(36)
			30/30	G6 (100)	cox1, nad1	(37)
Central and East Asia	G1–G3 (1/2, 50) G6–G7 (1/2, 50)	China	1/1	G1 (100)	cox1	(35)
		Kazakhstan	1/1	G6 (100)	cob, nad3, nad1,cox1, cox2 and rrnS	(38)
Middle East	G1–G3 (144/318, 45.3) G6–G7 (172/318, 54.1) G5 (2/318, 0.6)	Iran	2/2	G6 (100)	cox1, nad1	(39)
			8/32	G1 (25.0)	ITS1	(40)
			24/32	G6 (75.0)		
			5/19	G1 (26.3)	cox1, nad1	(41)
			8/19	G3 (42.1)		
			6/19	G6 (31.6)		
			9/26	G1 (34.6)	ITS1	(42)
			17/26	G6 (65.4)		
			4/9	G1 (44.4)	nad1, cox1	(43)
			2/9	G3 (22.2)		
			3/9	G6 (33.3)		
			38/43	G1 (88.4)	cox1, nad1, atp6, 12S rRNA	(44)
			5/43	G6 (11.6)		
			4/14	G1 (28.6)	cox1, nad1	(9)
			4/14	G3 (28.6)		
			5/14	G6 (35.7)		
			1/14	G5 (7.1)		

(Continued)

TABLE 1 | Continued

Region	Genotypes (No., %)	Country	No. each genotype/No. examined	Genotype (%)	Gene marker(s)	References
			25/100 4/100 71/100	G1 (25) G3 (4) G6 (71)	cox1	(13)
			15/57 6/57 35/57 1/57	G1 (26.3) G3 (10.5) G6 (61.4) G5 (1.8)	12S rRNA, cox1	Present study
		Oman	11/15 4/ 15	G1 (73.3) G6 (26.7)	12S rRNA	(45)
		Turkey	1/1	G1 (100)	cox1, ITS1	(46)
South Asia	G1–G3 (5/5, 100)	Pakistan	5/5	G1 (100)	cox1	(47)
Total				G1-G3 (260/1144, 22.7) G6–G7 (880/1144, 76.9) G5 (3/1144, 0.3) Unidentified (1/1144, 0.1)		

edited manually in BioEdit software v.7.2 after equalizing each sample sequence length by the elimination of the PCR primers sequences and global sequence alignments using ClustalW algorithm (16). Phylogenetic analysis was performed with the best-fit model of nucleotide substitution on 12S rDNA sequences using the program Mega 7 software (17). Molecular phylogeny of *E. granulosus* isolates was determined by maximum likelihood method, and the corresponding phylogenetic tree was constructed. The heuristic tree search and tree topologies algorithm reliability test were estimated with bootstrap testing with 1,000 replicates.

Average evolutionary divergence of 12s rRNA sequences of all MENA camel *E. granulosus* sensu lato genotypes/haplotypes were also studied with MEGA7 software by using the Kimura 2-parameter model and the rate variation among sites was modeled with a gamma distribution (shape parameter = 1).

Population genetics analysis of haplotypes was performed on a consensus string of 420 positions in the aligned sequences for nucleotide diversity (π) and haplotype diversity (Hd) using DnaSP software (18). Associations between each haplotype of *Echinococcus* species form camel and their corresponding genotypes were inferred by constructing the TCS network using PopART software (19). The individual nucleotide sequence data reported in this study were submitted to the GenBank database.

RESULTS

Of 1,144 camel isolates of *E. granulosus* s.l. sequences retrieved from NCBI GenBank, 68 records for 12S rRNA gene were identified (**Table 1**). All 57 isolates were successfully PCR-sequenced on 12S rRNA gene and the sequences were submitted to the NCBI GenBank database under the accession numbers MH395757–MH395800 for 12sRNA and MH397251–MH397259 for cox1. Sequence analyses of 12S rRNA gene showed four genotypes including 35 G6 (61.4%), 15 G1 (26.3%), 6 G3 (10.5%), and one G5 (1.8%) representing three *Echinococcus* species, i.e., *E. intermedius*, *E. granulosus* sensu stricto, and *E. ortleppi*. Six camel isolates within G1-G3 complex were identified as G3 genotype using partial cox1 sequence analysis.

Phylogenetic analysis of 12S rRNA sequence data from 57 camel isolates compared with reference genotypes of *E. granulosus* s.l. is shown in **Figure 1**. Four genotypes, G1, G3, G6, and G5 exhibit distinct clusters along with corresponding reference sequences representing all of the sequences determined in the present study. The haplotype diversity for 57 camel isolates from Iran (categorized into six haplotypes) was calculated as Hd = 0.6050 ($\pm$0.063). However, the haplotype diversity for 68 camel records from MENA region (categorized into seven haplotypes) was estimated at Hd = 0.5540 ($\pm$0.064).

Global data on the frequency of *E. granulosus* s.l. genotypes in camels according to five major camel-rearing regions in the world, i.e., the Middle East, North Africa, sub-Saharan Africa, East/Central Asia, and South Asia are summarized in **Table 1**. As it is shown, three different genotype patterns could be obtained in camels infected by *E. granulosus* sensu lato. *E. intermedius* (G6, camel strain) retained its dominance in camels from sub-Saharan Africa, so that about 90% of *Echinococcus* species isolated from camels were *E. intermedius*. In the Middle East, *E. granulosus* sensu stricto replaced *E. intermedius*, interestingly

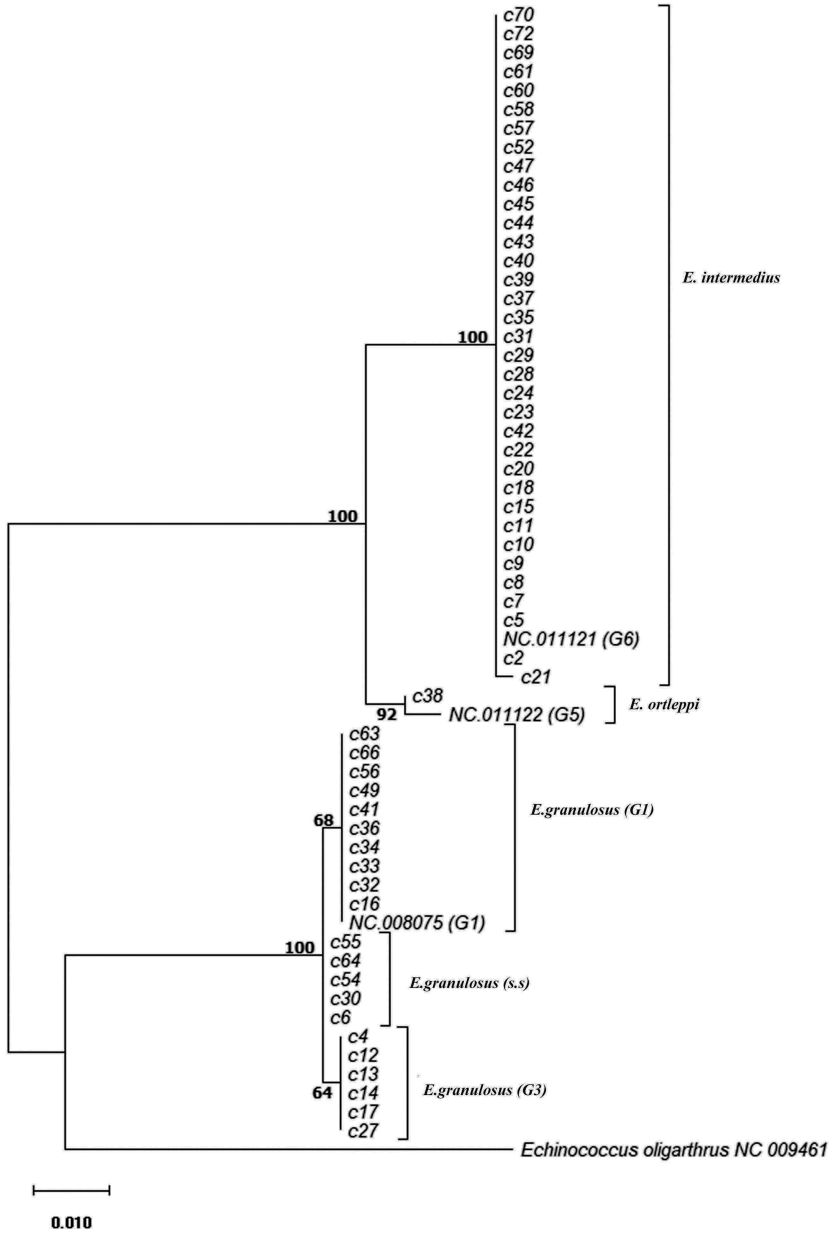

FIGURE 1 | Phylogenetic relationships of camel isolates of *Echinococcus granulosus* sensu lato based on 12S mitochondrial rRNA sequences based on Hasegawa-Kishino-Yano model. The heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The percentage of trees in which the associated samples clustered together is shown next to the branches. The tree is drawn to scale with branch lengths based on the number of substitutions per site. The analysis involved 60 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 420 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

half of *Echinococcus* species isolated from camels in this region have been identified as *E. granulosus* sensu stricto. The situation in North Africa is something in between, ∼80% of the parasites are G6 camel strain (**Figure 2**, **Table 1**).

The parsimony-based haplotype network analysis on all MENA records, indicated that *E. granulosus* sensu lato isolates were clustered into four major groups (**Figure 3**). The G6 genotype (NC011121) corresponds to the most commonly represented *E. granulosus* haplotype in camels originated from Central Asia, the Middle East and Africa, followed by G1 (NC008075) representing most of the isolates from Iran.

The number of base substitutions per site over all 420 positions of 12S rRNA partial sequence pairs were shown as 0.2–0.7% and 0.4% within the Middle Eastern and sub-Saharan

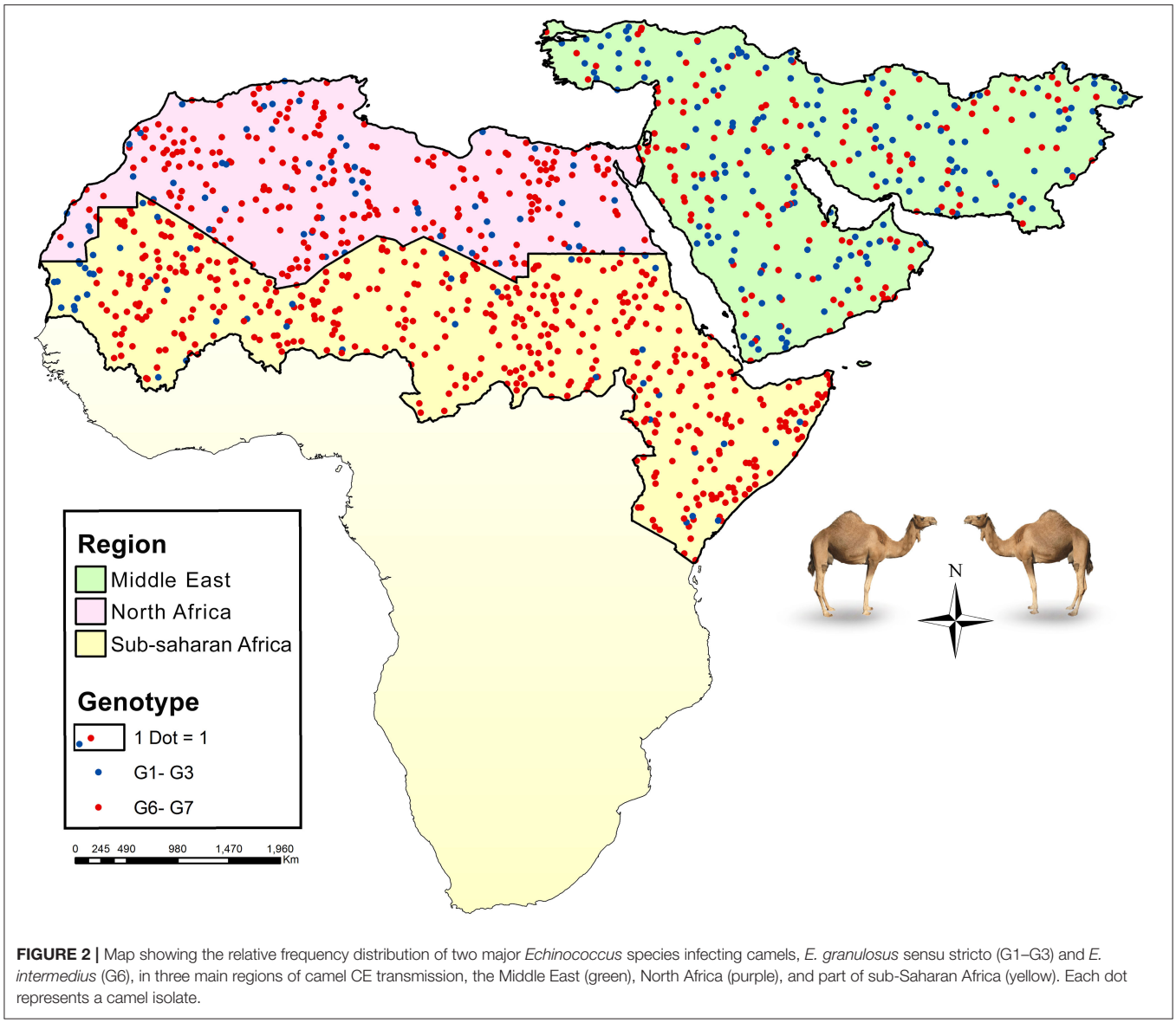

FIGURE 2 | Map showing the relative frequency distribution of two major *Echinococcus* species infecting camels, *E. granulosus* sensu stricto (G1–G3) and *E. intermedius* (G6), in three main regions of camel CE transmission, the Middle East (green), North Africa (purple), and part of sub-Saharan Africa (yellow). Each dot represents a camel isolate.

E. intermedius (G6) isolates, respectively. The average overall distance of all MENA *E. granulosus* s.l. isolates was 0.6% (**Figure 4**).

DISCUSSION

Control of cystic echinococcosis is complicated due to the fact that CE transmission occurs through different definitive/intermediate hosts systems (48). In this regard cystic echinococcosis of dromedaries has been received less attention among different livestock species. Camels play an essential role in the epidemiology and transmission of CE in the Middle East and North Africa. Little is known about the nature and significance of genetic variation of camel isolates of *E. granulosus* s.l. in the endemic areas. Most of the studies investigated a limited number of camel isolates which are not representatives of *E. granulosus* population in camels. In this study we present a global outlook of the significance of different species/genotypes of camel isolates of *E. granulosus* sensu lato in the molecular epidemiology of CE in the main camel breeding regions of the world using all available genetic markers including both nuclear and mitochondrial regions.

It has been already shown that 12S rRNA gene is a suitable marker for differentiating genotypes of E. granulosus, nonetheless few 12S rRNA nucleotide data are available in GenBank, particularly from camel CE. In the present study we investigated 12S rRNA gene diversity of 57 camel isolates of *E. granulosus* s.l. from Iran. The findings indicate that G6 genotype is the most common genotype (61.4%) in this Iranian

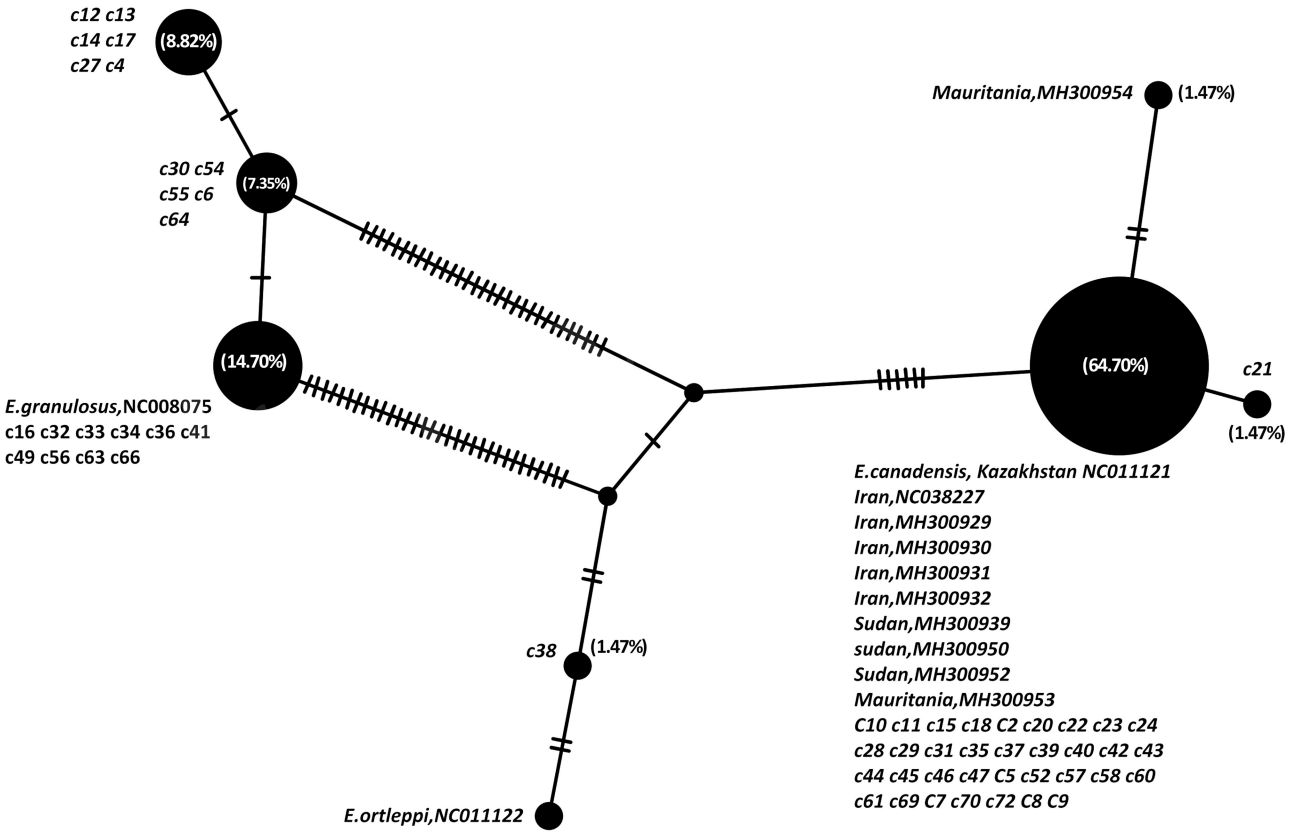

FIGURE 3 | Haplotype network analysis of *Echinococcus granulosus* sensu lato of partial 420 bp of 12S rRNA gene on all available camel records from the Middle East and Africa, based on statistical parsimony. The size of the circles indicates the frequency of the haplotypes. The nucleotide accession numbers and each individual isolate code in the present study are indicated next to each circle. Numbers in parenthesis are the percent frequency of the isolates in each haplotype.

Haplotype		1	2	3	4	5	6	7	Region
1	Hap1 (G6)								AF- ME
2	Hap2 (G6)	0.0048							AF
3	Hap3 (G6)	0.0024	0.0072						ME
4	Hap4 (G1-G3)	0.0987	0.1043	0.1016					ME
5	Hap5 (G1-G3)	0.0985	0.1042	0.1014	0.0048				ME
6	Hap6 (G1-G3)	0.0958	0.1014	0.0987	0.0024	0.0024			ME
7	Hap7 (G5)	0.0219	0.0269	0.0245	0.0901	0.0871	0.0873		ME

AF: Africa, **ME**: Middle East

FIGURE 4 | Pairwise comparison of nucleotide sequence differences (%) in mitochondrial 12S rRNA gene among seven available camel haplotypes within *Echinococcus granulosus* sensu lato.

dromedary population. The results also revealed the existence of three other genotypes including G1 (26.3%), G3 (10.5%), and G5 (1.8%).

The study showed that *E. granulosus* s.l. perpetuates in a camel-dog cycle mostly comprising of G6 genotype (*E. intermedius*) and several other less prevalent strains. Both species, *E. granulosus* s.s. and *E. intermedius* are believed to be overlapped in their cycle so that sheep and camels have contributed to the transmission of both species/genotypes. However, there are controversies on the contribution of the genotypes in each individual intermediate host species. Several studies showed the dominant species infecting camels is *E. granulosus* s.s. G1 genotype (10, 25, 28, 30, 43–45, 49), while several other studies reported higher frequency of *E. intermedius* in camels than *E. granulosus* s.s. (4, 9, 11, 20, 22, 25, 26, 29, 31, 33, 34, 36, 50, 51). In addition, sylvatic transmission of CE among camel populations should also be considered. Wild carnivores particularly wolves and golden jackals have long been considered as epidemiologically important definitive hosts in MENA region (52, 53). Regarding the nature of camel husbandry, the animals are usually roaming freely in vast geographical areas outside human dwellings during their long life span, therefore it is quite

probable for camels to get involved in the sylvatic life cycle of *E. granulosus* sensu lato.

Assuming that G6 parasites had primarily perpetuated in wild camels across the old world, this is probably an indication of different forces of infection with G1 sheep strain in different camel-rearing areas from the Iranian plateau to the Maghreb and sub-Saharan Africa. With the Middle East has already been occupied by *E. granulosus* s.s. G1 genotype (sheep strain) as the dominant genotype in the region, exerting remarkable forces of infection on the camels regularly infected by G6 genotype.

The camel domestication took place some 8000 years later than that of sheep and goat (54).

It is believed that camels were first domesticated in the Fertile Crescent and Arabian Peninsula in 2000–1000 BCE. Increasing trade of basic commodities including incense, myrrh and frankincense mainly transported by the camels across the region, facilitated the dispersion of *E. granulosus* s.s. We could not find any significant different nucleotide diversity among isolates from Middle East and sub-Saharan Africa. This may be due the fact that the emergence of domestic camel-dog cycle of *E. granulosus* s.l. following camel domestication is a relatively recent event, therefore the development of significant haplotype diversity within the camel isolates is not expected in a short period of time. Similar molecular epidemiological picture has been demonstrated in sheep-dog cycle in which the universal sheep strain (G1 genotype) is the dominant variant, however other genotypes including G3 and G6 are perpetuating in the G1 endemic areas.

It should be noted that the sample size in several camel studies is small and the findings are not conclusive (**Table 1**). Mitochondrial gene sequences are highly informative markers for molecular taxonomy and phylogenetic investigation of helminth parasites. Cytochrome c oxidase subunit 1 and 12S ribosomal RNA genes have been extensively used for genetic classification of tapeworms. However, few studies on *E. granulosus* 12S rRNA sequences have been performed on African camel isolates and obviously more 12S rRNA data is required from this important endemic region. It has been shown that 12S rRNA gene presents higher sequence variability than cox1, therefore it is assumed that the camel isolates of *E. granulosus* s.l. in Africa present even more homogeneous population than those of the Middle East (13).

In addition, the majority of the studies have used small partial fragments of mitochondrial genes. Using longer sequences in phylogeographical studies provides more reliable information on the molecular epidemiology of CE in camels (6). This study presents the second evidence of G5 presence in the Middle East camels. This suggests that *E. ortleppi*, the common cattle strain, could also be transmissible through a camel-dog cycle in the region. Previous studies in MENA indicated that camels usually harbor highly fertile/highly viable hydatid cysts reaching >90% fertility and >80% viability (14, 28, 55, 56). However, interestingly in our study few protoscoleces were found in the camel hydatid cyst harboring G5 genotype (*E. ortleppi*) and DNA was extracted from the cyst germinal layer. This is an indication of low susceptibility of camels to *E. ortleppi* and can explain the low prevalence of G5 in camels, however, to obtain a more comprehensive picture of the global epidemiology of *E. ortleppi*, further studies are required on the molecular epidemiology of camel CE in the MENA region.

CONCLUSION

Global data on the frequency of *E. granulosus* s.l. genotypes in camel populations of major camel-rearing regions of the world revealed a changing pattern of genotype distribution between the Middle East and African isolates. *E. intermedius* (G6 genotype) was identified as the most common species in camels, however the contribution of this species in camel populations in various areas of MENA is significantly different across the region from the Iranian plateau to sub-Saharan Africa. Mitochondrial 12S rDNA study of camel isolates of *E. granulosus* s.l. revealed significant species/genotype diversity. Camel isolates of *E. granulosus* in the MENA region present moderate genetic diversity with the Middle East isolates demonstrating a more diverse population than the North/sub-Saharan isolates, where three species, four genotypes and six different 12S rRNA haplotypes were identified in the region.

Camels are an important intermediate host species in Iran, harboring different species and genotypes of *E. granulosus* s.l. throughout their long lifetime. More in-depth large-scale studies using multiple large fragments of mitochondrial/nuclear gene sequences are required to elucidate the significance and actual contribution of camels in CE epidemiology.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This project was reviewed and approved by Research Review Committee of the National Institutes for Medical Research Development (NIMAD), No. 971237. The animals were slaughtered as part of the normal daily practice in the abattoirs.

AUTHOR CONTRIBUTIONS

MF, MM, and SR: conceptualization and study design. MD, SH, SN, and SR: data curation. MD, MM, SN, and SR: data analysis and laboratory experiments. MF: funding acquisition. MM, SH, SR, and MF: data validation. MD, MM, SH, SN, SR, and MF: writing—original draft preparation. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Cardona GA, Carmena D. A review of the global prevalence, molecular epidemiology and economics of cystic echinococcosis in production animals. *Vet Parasitol.* (2013) 192:10–32. doi: 10.1016/j.vetpar.2012.09.027
2. Deplazes P, Rinaldi L, Alvarez Rojas CA, Torgerson PR, Harandi MF, Romig T, et al. Global distribution of alveolar and cystic echinococcosis. *Adv Parasitol.* (2017) 95:315–493. doi: 10.1016/bs.apar.2016.11.001
3. Harandi MF, Budke CM, Rostami S, Fasihi Harandi M, Budke CM, Rostami S, et al. The monetary burden of cystic echinococcosis in Iran. *PLoS Negl Trop Dis.* (2012) 6:e1915. doi: 10.1371/journal.pntd.0001915
4. Thompson RCA. The taxonomy, phylogeny and transmission of *Echinococcus*. *Exp Parasitol.* (2008) 119:439–46. doi: 10.1016/j.exppara.2008.04.016
5. Amer S, Helal IB, Kamau E, Feng Y, Xiao L. Molecular characterization of *Echinococcus granulosus* sensu lato from farm animals in Egypt. *PLoS ONE.* (2015) 10:e0118509. doi: 10.1371/journal.pone.0118509
6. Laurimäe T, Kinkar L, Romig T, Omer RA, Casulli A, Umhang G, et al. The benefits of analysing complete mitochondrial genomes: deep insights into the phylogeny and population structure of *Echinococcus granulosus* sensu lato genotypes G6 and G7. *Infect Genet Evol.* (2018) 64:85–94. doi: 10.1016/j.meegid.2018.06.016
7. Alvarez Rojas CA, Romig T, Lightowlers MW. *Echinococcus granulosus* sensu lato genotypes infecting humans - review of current knowledge. *Int J Parasitol.* (2014) 44:9–18. doi: 10.1016/j.ijpara.2013.08.008
8. Ibrahim MM. Study of cystic echinococcosis in slaughtered animals in Al Baha region, Saudi Arabia: interaction between some biotic and abiotic factors. *Acta Trop.* (2010) 113:26–33. doi: 10.1016/j.actatropica.2009.08.029
9. Ebrahimipour M, Sadjjadi SM, Yousofi Darani H, Najjari M. Molecular studies on cystic echinococcosis of camel (*Camelus dromedarius*) and report of *Echinococcus ortleppi* in Iran. *Iran J Parasitol.* (2017) 12:323–31.
10. Sharbatkhori M, Mirhendi H, Harandi MF, Rezaeian M, Mohebali M, Eshraghian M, et al. *Echinococcus granulosus* genotypes in livestock of Iran indicating high frequency of G1 genotype in camels. *Exp Parasitol.* (2010) 124:373–9. doi: 10.1016/j.exppara.2009.11.020
11. Hüttner M, Siefert L, Mackenstedt U, Romig T. A survey of *Echinococcus* species in wild carnivores and livestock in East Africa. *Int J Parasitol.* (2009) 39:1269–76. doi: 10.1016/j.ijpara.2009.02.015
12. Rostami S, Shariat Torbaghan S, Dabiri S, Babaei Z, Ali Mohammadi M, Sharbatkhori M, et al. Genetic characterization of *Echinococcus granulosus* from a large number of formalin-fixed, paraffin-embedded tissue samples of human isolates in Iran. *Am J Trop Med Hyg.* (2015) 92:588–94. doi: 10.4269/ajtmh.14-0585
13. Rostami S, Salavati R, Beech RN, Sharbatkhori M, Babaei Z, Saedi S, et al. Cytochrome c oxidase subunit 1 and 12S ribosomal RNA characterization of Coenurus cerebralis from sheep in Iran. *Vet Parasitol.* (2013) 197:141–51. doi: 10.1016/j.vetpar.2013.07.001
14. Rostami S, Talebi S, Babaei Z, Sharbatkhori M, Ziaali N, Rostami H, et al. High resolution melting technique for molecular epidemiological studies of cystic echinococcosis: differentiating G1, G3, and G6 genotypes of *Echinococcus granulosus* sensu lato. *Parasitol Res.* (2013) 112:3441–7. doi: 10.1007/s00436-013-3523-z
15. Bowles J, Blair D, Mcmanus DPDPDPDPP, Bessho Y, Ohama T, Osawa S, et al. A molecular phylogeny of the genus *Echinococcus*. *Parasitology.* (1995) 110:317–28. doi: 10.1017/S0031182000080902
16. Hall TAT. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In *Nucleic Acids Symposium Series*, London (1999). p. 95–98.
17. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol.* (2016) 33:1870–4. doi: 10.1093/molbev/msw054
18. Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics.* (2009) 25:1451–2. doi: 10.1093/bioinformatics/btp187
19. Leigh JW, Bryant D. POPART: full-feature software for haplotype network construction. *Methods Ecol Evol.* (2015) 6:1110–6. doi: 10.1111/2041-210X.12410
20. Bardonnet K, Benchikh-Elfegoun MC, Bart JM, Harraga S, Hannache N, Haddad S, et al. Cystic echinococcosis in Algeria: Cattle act as reservoirs of a sheep strain and may contribute to human contamination. *Vet Parasitol.* (2003) 116:35–44. doi: 10.1016/S0304-4017(03)00255-3
21. Maillard S, Benchikh-Elfegoun MC, Knapp J, Bart JM, Koskei P, Gottstein B, et al. Taxonomic position and geographical distribution of the common sheep G1 and camel G6 strains of *Echinococcus granulosus* in three African countries. *Parasitol Res.* (2007) 100:495–503. doi: 10.1007/s00436-006-0286-9
22. Aaty HEA, Abdel-Hameed DM, Alam-Eldin YH, El-Shennawy SF, Aminou HA, Makled SS, et al. Molecular genotyping of *Echinococcus granulosus* in animal and human isolates from Egypt. *Acta Trop.* (2012) 121:125–8. doi: 10.1016/j.actatropica.2011.10.014
23. Khalifa N, F. Khater H, A. Fahmy H, E.I.Radwan M, S.A. Afify J. Genotyping and phylogenetic analysis of cystic *Echinococcosis* isolated from camels and humans in Egypt. *Am J Epidemiol Infect Dis.* (2014) 2:74–82. doi: 10.12691/ajeid-2-3-2
24. Alam-Eldin YH, Abdel Aaty HE, Ahmed MA. Molecular characterization of cystic echinococcosis: first record of G7 in Egypt and G1 in Yemen. *Acta Parasitol.* (2015) 60:662–5. doi: 10.1515/ap-2015-0094
25. Tashani OA, Zhang LH, Boufana B, Jegi A, McManus DP. Epidemiology and strain characteristics of *Echinococcus granulosus* in the Benghazi area of eastern Libya. *Ann Trop Med Parasitol.* (2002) 96:369–81. doi: 10.1179/000349802125000952
26. Abushhewa MH, Abushhiwa MHS, Nolan MJ, Jex AR, Campbell BE, Jabbar A, et al. Genetic classification of *Echinococcus granulosus* cysts from humans, cattle and camels in Libya using mutation scanning-based analysis of mitochondrial loci. *Mol Cell Probes.* (2010) 24:346–51. doi: 10.1016/j.mcp.2010.07.005
27. Azlaf R, Dakkak A. Epidemiological study of the cystic echinococcosis in Morocco. *Vet Parasitol.* (2006) 137:83–93. doi: 10.1016/j.vetpar.2006.01.003
28. Lahmar S, Debbek H, Zhang LH, McManus DP, Souissi A, Chelly S, et al. Transmission dynamics of the *Echinococcus granulosus* sheep-dog strain (G1 genotype) in camels in Tunisia. *Vet Parasitol.* (2004) 121:151–6. doi: 10.1016/j.vetpar.2004.02.016
29. M'rad S, Filisetti D, Oudni M, Mekki M, Belguith M, Nouri A, Sayadi T, et al. Molecular evidence of ovine (G1) and camel (G6) strains of *Echinococcus granulosus* in Tunisia and putative role of cattle in human contamination. *Vet Parasitol.* (2005) 129:267–72. doi: 10.1016/j.vetpar.2005.02.006
30. Hailemariam Z, Nakao M, Menkir S, Lavikainen A, Yanagida T, Okamoto M, et al. Molecular identification of unilocular hydatid cysts from domestic ungulates in Ethiopia: Implications for human infections. *Parasitol Int.* (2012) 61:375–7. doi: 10.1016/j.parint.2012.01.007
31. Dinkel A, Njoroge EM, Zimmermann A, Wälz M, Zeyhle E, Elmahdi IE, et al. A PCR system for detection of species and genotypes of the *Echinococcus granulosus*-complex, with reference to the epidemiological situation in eastern Africa. *Int J Parasitol.* (2004) 34:645–53. doi: 10.1016/j.ijpara.2003.12.013

32. Mbaya H, Magambo J, Njenga S, Zeyhle E, Mbae C, Mulinge E, et al. Echinococcus spp. in central Kenya: a different story. *Parasitol Res.* (2014) 113:3789–94. doi: 10.1007/s00436-014-4045-z
33. Bardonnet K, Piarroux R, Dia L, Schneegans F, Beurdeley A, Godot V, et al. Combined eco-epidemiological and molecular biology approaches to assess *Echinococcus granulosus* transmission to humans in Mauritania: occurrence of the 'camel' strain and human cystic echinococcosis. *Trans R Soc Trop Med Hyg.* (2002) 96:383–6. doi: 10.1016/S0035-9203(02)90369-X
34. Farjallah S, Busi M, Mahjoub MO, Slimane B Ben, Said K, D'Amelio S. Molecular characterization of *Echinococcus granulosus* in Tunisia and Mauritania by mitochondrial rrnS gene sequencing. *Parassitologia.* (2007) 49:239–46.
35. Bowles J, Blair D, McManus DP. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Mol Biochem Parasitol.* (1992) 54:165–73. doi: 10.1016/0166-6851(92)90109-W
36. Omer RA, Dinkel A, Romig T, Mackenstedt U, Elnahas AA, Aradaib IE, et al. A molecular survey of cystic echinococcosis in Sudan. *Vet Parasitol.* (2010) 169:340–6. doi: 10.1016/j.vetpar.2010.01.004
37. Ibrahim K, Thomas R, Peter K, Omer RA. A molecular survey on cystic echinococcosis in Sinnar area, blue Nile state (Sudan). *Chin Med J.* (2011) 124:2829–33. doi: 10.3760/cma.j.issn.0366-6999.2011.18.006
38. Nakao M, McManus DP, Schantz PM, Craig PS, Ito A. A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. *Parasitology.* (2007) 134:713–22. doi: 10.1017/S0031182006001934
39. Zhang L, Eslami A, Hosseini SH, McManus DP. Indication of the presence of two distinct strains of *Echinococcus granulosus* in Iran by mitochondrial DNA markers. *Am J Trop Med Hyg.* (1998) 59:171–4. doi: 10.4269/ajtmh.1998.59.171
40. Harandi MF, Hobbs RP, Adams PJ, Mobedi I, Morgan-Ryan UM, Thompson RCA. Molecular and morphological characterization of *Echinococcus granulosus* of human and animal origin in Iran. *Parasitology.* (2002) 125:367–73. doi: 10.1017/S0031182002002172
41. Sharbatkhori M, Fasihi Harandi M, Mirhendi H, Hajialilo E, Kia EB. Sequence analysis of cox1 and nad1 genes in *Echinococcus granulosus* G3 genotype in camels (Camelus dromedarius) from central Iran. *Parasitol Res.* (2011) 108:521–7. doi: 10.1007/s00436-010-2092-7
42. Shahnazi M, Hejazi H, Salehi M, Andalib AR. Molecular characterization of human and animal *Echinococcus granulosus* isolates in Isfahan, Iran. *Acta Trop.* (2011) 117:47–50. doi: 10.1016/j.actatropica.2010.09.002
43. Hajialilo E, Harandi MF, Sharbatkhori M, Mirhendi H, Rostami S. Genetic characterization of *Echinococcus granulosus* in camels, cattle and sheep from the south-east of Iran indicates the presence of the G3 genotype. *J Helminthol.* (2012) 86:263–70. doi: 10.1017/S0022149X11000320
44. Rostami Nejad M, Taghipour N, Nochi Z, Nazemalhosseini Mojarad E, Mohebbi SR, Fasihi Harandi M, et al. Molecular identification of animal isolates of *Echinococcus granulosus* from Iran using four mitochondrial genes. *J Helminthol.* (2012) 86:485–92. doi: 10.1017/S0022149X1100071X
45. Al Kitani FA, Al Riyami S, Al Yahyai S, Al Awahi AH, Al Aawali M, Hussain MH. Abattoir based surveillance of cystic echinococcosis (CE) in the Sultanate of Oman during 2010-2013. *Vet Parasitol.* (2015) 211:208–15. doi: 10.1016/j.vetpar.2015.06.011
46. Utuk AE, Simsek S, Koroglu E, McManus DP. Molecular genetic characterization of different isolates of *Echinococcus granulosus* in east and southeast regions of Turkey. *Acta Trop.* (2008) 107:192–4. doi: 10.1016/j.actatropica.2008.05.026
47. Latif AA, Tanveer A, Maqbool A, Siddiqi N, Kyaw-Tanner M, Traub RJ. Morphological and molecular characterisation of *Echinococcus granulosus* in livestock and humans in Punjab, Pakistan. *Vet Parasitol.* (2010) 170:44–9. doi: 10.1016/j.vetpar.2010.02.003
48. Tamarozzi F, Deplazes P, Casulli A. Reinventing the wheel of *Echinococcus granulosus* sensu lato transmission to humans. *Trends Parasitol.* (2020)36:427–34. doi: 10.1016/j.pt.2020.02.004
49. Sharbatkhori M, Mirhendi H, Jex AR, Pangasa A, Campbell BE, Kia EB, et al. Genetic categorization of *Echinococcus granulosus* from humans and herbivorous hosts in Iran using an integrated mutation scanning-phylogenetic approach. *Electrophoresis.* (2009) 30:2648–55. doi: 10.1002/elps.200900145
50. Schwenkenbecher JM, Wirth T, Schnur LF, Jaffe CL, Schallig H, Al-Jawabreh A, et al. Microsatellite analysis reveals genetic structure of *Leishmania tropica*. *Int J Parasitol.* (2006) 36:237–46. doi: 10.1016/j.ijpara.2005.09.010
51. Knapp J, Bart JM, Glowatzki M-L, Ito A, Gerard S, Maillard S, et al. Assessment of use of microsatellite polymorphism analysis for improving spatial distribution tracking of *Echinococcus multilocularis*. *J Clin Microbiol.* (2007) 45:2943–50. doi: 10.1128/JCM.02107-06
52. Dalimi A, Motamedi G, Hosseini M, Mohammadian B, Malaki H, Ghamari Z, et al. *Echinococcosis*/hydatidosis in western Iran. *Vet Parasitol.* (2002) 105:161–71. doi: 10.1016/s0304-4017(02)00005-5
53. Gholami S, Jahandar H, Abastabar M, Pagheh A, Mobedi I, Sharbatkhori M. *Echinococcus granulosus* sensu stricto in Dogs and Jackals from Caspian Sea region, northern Iran. *Iran J Parasitol.* (2016) 11:186–94.
54. Orlando L. Back to the roots and routes of dromedary domestication. *Proc Natl Acad Sci USA.* (2016) 113:6588–90. doi: 10.1073/pnas.1606340113
55. Casulli A, Zeyhle E, Brunetti E, Pozio E, Meroni V, Genco F, et al. Molecular evidence of the camel strain (G6 genotype) of *Echinococcus granulosus* in humans from Turkana, Kenya. *Trans R Soc Trop Med Hyg.* (2010) 104:29–32. doi: 10.1016/j.trstmh.2009.08.001
56. Njoroge EM, Mbithi PMF, Gathuma JM, Wachira TM, Gathura PB, Magambo JK, et al. A study of cystic echinococcosis in slaughter animals in three selected areas of northern Turkana, Kenya. *Vet Parasitol.* (2002) 104:85–91. doi: 10.1016/S0304-4017(01)00614-8

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Toxocara canis Differentially Affects Hepatic MicroRNA Expression in Beagle Dogs at Different Stages of Infection

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Toxocara canis is a neglected zoonotic parasite, which threatens the health of dogs and humans worldwide. The molecular mechanisms that underlie the progression of *T. canis* infection remain mostly unknown. MicroRNAs (miRNAs) are small non-coding RNAs that have been identified in *T. canis*; however, the regulation and role of miRNAs in the host during infection remain incompletely understood. In this study, we determined hepatic miRNA expression at different stages of *T. canis* infection in beagle dogs. Individual dogs were infected by 300 embryonated *T. canis* eggs, and their livers were collected at 12 hpi (hours post-infection), 24 hpi, and 36 dpi (days post-infection). The expression profiles of liver miRNAs were determined using RNA-sequencing. Compared to the control groups, 9, 16, and 34 differentially expressed miRNAs (DEmiRNAs) were detected in the livers of infected dogs at the three infection stages, respectively. Among those DEmiRNAs, the novel-294 and cfa-miR-885 were predicted to regulate inflammation-related genes at the initial stage of infection (12 hpi). The cfa-miR-1839 was predicted to regulate the target gene TRIM71, which may influence the development of *T. canis* larvae at 24 hpi. Moreover, cfa-miR-370 and cfa-miR-133c were associated with immune response at the final stage of infection (36 dpi). Some immunity-related Gene Ontology terms were enriched particularly at 24 hpi. Likewise, Kyoto Encyclopedia of Genes and Genomes pathway analysis showed that many significantly enriched pathways were involved in inflammation and immune responses. The expression level of nine DEmiRNAs was validated using quantitative real-time PCR (qRT-PCR). These results show that miRNAs play critical roles in the pathogenesis of *T. canis* during the hepatic phase of parasite development. Our data provide fundamental information for further investigation of the roles of miRNAs in the innate/adaptive immune response of dogs infected by *T. canis*.

Keywords: *Toxocara canis*, miRNAs, beagle dogs, liver, RNA-seq

INTRODUCTION

Toxocariasis, mainly caused by *Toxocara canis* infection in dogs (1), is a neglected zoonosis worldwide. Dogs, as the definitive hosts of *T. canis*, can excrete eggs with feces, leading to environment contamination (2, 3). Humans can be infected by *T. canis* via ingestion of food contaminated with embryonated eggs or larvae (2, 4). The larvae hatch inside the intestinal tract of the host and then migrate to the other parts of the body, leading to serious health problems, such as visceral larva migrans, ocular larva migrans, and neurotoxocariasis (5, 6). Owing to non-specific symptoms in human infection and the diagnostic challenges (7), the public health impact caused by *T. canis* infection may be ignored. More efforts are needed to achieve better understanding of the pathogenesis of toxocariasis (1).

In recent years, multiple approaches, such as genomics, transcriptomics, and proteomics, have been applied to characterize the biological and molecular features of *T. canis* (8–11). However, there is a dearth of information regarding the interaction between *T. canis* and its host. MicroRNAs (miRNAs) are endogenous, small, non-coding RNAs that have received significant attention from the scientific community due to their involvement in many and diverse biological processes, such as apoptosis, proliferation, metabolism, and immune response (12). miRNAs together with transcription factors have been considered key regulators that modulate the expression levels of almost all genes that mediate various pathophysiological processes (13, 14). Also, parasites can alter the expression level of host miRNAs in order to regulate gene expression of the target tissues (15–17). miRNAs can repress mRNA expression through binding to the 3′ untranslated regions of target genes or by enhancing mRNA degradation (18). A previous study showed that *T. canis* miRNAs, Tc-let-7-5p, Tc-miR-34, and Tc-miR-100, play roles in host–parasite interactions (8). However, the regulation of miRNAs and their roles in the pathogenesis of *T. canis* during the hepatic phase of infection remains mostly unknown.

Therefore, the present study aimed to investigate the alteration of miRNA expression profiles in the livers of beagle dogs infected by *T. canis* at different stages of infection using small RNA transcriptome sequencing and bioinformatics analysis. The study findings revealed that some differentially expressed miRNAs (DEmiRNAs) play roles in the regulation of inflammatory and immune responses of puppies against *T. canis* infection.

MATERIALS AND METHODS

Experimental Infection of Dogs

The adult *T. canis* worms were collected from naturally infected dogs in Rongchang District, Chongqing Municipality, China, and female adult *T. canis* were identified based on gross morphology. The eggs of *T. canis* were obtained from the uteri of female *T. canis* worms. The unembryonated eggs were incubated with 0.5% formalin solution at 28°C (85–95% humidity) for 28 days, and then the eggs were harvested and filtered through a 200-mesh screen. The finally embryonated eggs were stored in 1% formalin solution at 4°C. Eighteen beagle puppies (6–7 weeks old) were provided by the National Canine Laboratory Animal Resource Center and housed following Good Laboratory Practice (GLP) in an animal facility, according to the GB standard (GB 14922.2-2011) of China. All puppies had tested negative for *T. canis* infection by indirect ELISA, using larval ES antigen, before the start of the experiment. Feces of individual puppies were collected daily and examined using a standard sugar floatation method. Puppies were equally allocated into three experimental and three control groups (three puppies per group). The puppy groups were housed separately and were provided with similar conditions including access to the same water source and food supply. Each puppy was orally infected with 300 embryonated eggs in 1 ml normal saline solution, while the control puppies were inoculated with the same amount of saline but without any eggs.

Detection of *T. canis* Infection

Blood samples of each puppy were collected from the jugular vein into sterile tubes containing EDTA-K2 and tubes without anticoagulant. The *T. canis* IgG antibody was detected using indirect ELISA as previously described (19). Light microscopy was also used to observe whether *T. canis* larvae are present in the livers of puppies. The genomic DNA of infectious eggs (used to infect puppies), larvae (isolated from the liver of infected puppies), and adult *T. canis* (recovered from the small intestine of infected puppies) were isolated using a DNA extraction kit (TianGen™, Beijing, China) according to the manufacturer's instruction. The isolated DNA samples were analyzed using PCR with primer sequences specific to the internal transcribed spacer (ITS) region (partial sequence of ITS-1 and ITS-2) (20). The positive PCR products were sequenced by Sangon Biotech (Shanghai, China), and the obtained sequences were searched against similar sequences available in the GenBank database using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Sample Collection, RNA Extraction, and Quantification

Infected puppies ($n = 9$) and naive (control) puppies ($n = 9$) were killed by potassium chloride (KCl) under a general anesthetic [50 mg/kg tiletamine-zolazepam (Zoletil®), Virbac, France]. The liver samples were collected from all puppies at 12 h post-infection (hpi), 24 hpi, and 36 days post-infection (dpi) according to methods described in our previous study (21). Three biological replicates were examined in each group at each time point post-infection. The collected liver samples were quickly stored in liquid nitrogen until used for RNA extraction. The larvae were recovered from the remaining fresh liver sample of each puppy using the modified Baermann funnel method as previously described (22). The total RNA was extracted from the liver samples of puppies using TRIZOL (Life Technologies, CA, USA). The genomic DNA was removed using DNase I (NEB, Ipswich, USA). RNA concentration was measured using the Qubit® RNA Assay Kit and Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). The integrity of RNA was assessed by the RNA Nano 6000 Assay Kit and the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The purity of RNA was examined using

TABLE 1 | Primers used in microRNA (miRNA)–specific quantitative real-time PCR (qRT-PCR) analysis.

miRNAs	Primer	Sequence (5′ to 3′)
U6	Forward primer	CGCTTCGGCAGCACATATAC
Cfa-miR-381	Forward primer	CTGGGTCTGGTATACAAGGGCAAGCTCTC
Cfa-miR-10b	Forward primer	CTGGGTCTGGTATACAAGGGCAAGCTCTC
Cfa-miR-194	Forward primer	CTGGGTCTGGTGTAACAGCAACTCCATGT
Cfa-miR-125a	Forward primer	CTGGGTCTGGTCCCTGAGACCCTTTAAC
Cfa-miR-371	Forward primer	CTGGGTCTGGACTCAAAAAATGGCGGCA
Cfa-miR-16	Forward primer	CTGGGTCTGGTAGCAGCACGTAAATATTGG
Cfa-miR-10a	Forward primer	CTGGGTCTGGTACCCTGTAGATCCGAA
Cfa-miR-146a	Forward primer	CTGGGTCTGGTGAGAACTGAATTCCATGGG

the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). High-quality RNA samples with RNA integrity numbers (RINs) >8.0 were used to construct the sequencing library.

Small RNA Library Preparation and Sequencing

A total of 3 μg RNA of each sample was used for the construction of the small RNA library by using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA). The constructed libraries were sequenced on an Illumina Hiseq 2500 platform. The reads that contain poly N, 5′ adapter contaminants, without 3′ adapter or the insert tag (including poly G, C, A, or T and low-quality reads) were filtered from raw data using custom perl and python scripts. The clean reads that ranged from 18 to 35 nt were mapped against the reference sequence by Bowtie (23). The mapped sRNAs were searched against miRBase20.0 data to identify known miRNAs. In addition, mirdeep2 (24) and miREvo (25) were used to identify potential novel miRNAs. The novel miRNA was predicted by the characteristics of the hairpin structure of the miRNA precursor. The expression levels of miRNA were estimated by TPM (transcript per million) (26). Differential expression analysis was performed using the DESeq R package (1.8.3). A P-value < 0.05 was used as the significance threshold value of differential expression.

Bioinformatics Analysis of DEmiRNA

The target genes of miRNAs were predicted by RNAhybrid, PITA tools, and miRanda (27). To predict the function of the target gene of DEmiRNAs, GOseq R package (28), and KOBAS software (29, 30) were used for Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, respectively. A P-value < 0.05 was considered as significantly enriched.

Verification of miRNA Expression by Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was performed to confirm the upregulation and downregulation of nine miRNAs randomly selected for verification of the RNA-seq results. qRT-PCR was performed using an miRcute enhanced miRNA qRT-PCR Kit (TianGen, Beijing, China) on a LightCycler480 (Roche, Basle, Switzerland). The cDNA of miRNA was synthesized using an miRcute enhanced miRNA cDNA first chain synthesis Kit (TianGen, Beijing, China). Then, 1 μl cDNA was used for qRT-PCR as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of 94°C for 20 s and 60°C for 34 s. All primers are listed in **Table 1**. The U6 small nuclear RNA (snRNA) was used as an internal control gene. Melting curve analysis (95°C for 10 s, 65°C for 1 min, and progressive increase from 65°C to 95°C) was performed to ensure specific amplification in each reaction. Each reaction included a blank control to rule out the presence of contamination. The relative expression quantity was analyzed by the $^{2-\Delta\Delta}$Ct method (31, 32).

RESULTS

Detection of *T. canis* Infection in Beagle Dogs

A blood sample of each puppy was collected and tested for IgG antibody against *T. canis*. The IgG antibodies of *T. canis* were detected in infected puppies at 36 dpi. At 12 hpi, *T. canis* larvae were recovered from the livers of three infected puppies, and at 24 hpi, *T. canis* larvae were recovered from the livers of all infected puppies. At 36 dpi, *T. canis* larvae were detected in the liver of one infected puppy, and adult *T. canis* were recovered in the small intestine of all infected puppies. Furthermore, no *T. canis* larvae and anti–*T. canis* IgG antibodies were found in the control puppies. The sequences obtained from the embryonated eggs, larvae, and adult worms were found to match the sequence of *T. canis* (GenBank Accession No. JF837169.1).

Characteristics of the Sequenced Data

In each miRNA library, 142,605,411 raw reads and 7.131 Gb raw data were obtained from infected puppy groups, whereas 136,764,149 raw reads and 6.898 Gb raw data were obtained from the control groups. More than 99% of reads had sequencing quality >Q20 (**Table 2**). The clean reads with appropriate 18–35 nt lengths were selected for further analysis. Moreover, 52.72–65.17 and 0.17–0.24% unique reads were confirmed as known and novel miRNAs, respectively. In addition, 22.16–29.70% non-annotated reads were found in this study (**Supplementary Table 1**), suggesting that these non-annotated reads could be involved in the pathogenesis and progression of *T. canis* infection.

Differentially Expressed Hepatic miRNAs (DEmiRNAs) at Different Infection stages

A total of 59 DEmiRNAs were identified at three infection stages, including 9, 16, and 34 DEmiRNAs at 12 hpi, 24 hpi, and 36 dpi, respectively. Details of DEmiRNAs are shown in **Supplementary Table 2**. Among these DEmiRNAs, 23 DEmiRNAs were upregulated, whereas 36 DEmiRNAs were downregulated (**Figure 1**). However, no common miRNA was found at the three infection stages (**Figure 2**).

Target Gene Prediction and Functional Analysis

Candidate target genes of DEmiRNAs of beagle dog livers were predicted by RNAhybrid, miRanda, and PITA tools. The

TABLE 2 | Summary of the quality control parameters of the reads.

Groups	Samples	Raw reads	Clean reads	Bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
12 hpi	A12hT1	17,052,302	16,734,756	0.853G	0.01	99.72	99.28	49.61
	A12hT2	15,050,522	14,632,243	0.753G	0.01	99.69	99.24	49.39
	A12hT3	15,753,780	15,606,433	0.788G	0.01	99.70	99.21	49.92
	A12hC1	18,499,157	18,320,368	0.925G	0.01	99.72	99.27	49.75
	A12hC2	15,502,426	15,346,075	0.775G	0.01	99.64	99.09	49.73
	A12hC3	13,471,115	13,320,106	0.674G	0.01	99.65	99.13	49.71
24 hpi	B24hT1	16,220,657	16,074,374	0.811G	0.01	99.57	98.92	49.48
	B24hT2	14,587,167	14,466,523	0.729G	0.01	99.69	99.20	49.60
	B24hT3	17,146,312	16,980,582	0.857G	0.01	99.62	99.04	49.76
	B24hC1	14,239,211	14,125,501	0.712G	0.01	99.64	99.10	49.01
	B24hC2	15,619,834	15,495,519	0.781G	0.01	99.58	98.97	49.15
	B24hC3	14,913,621	14,810,484	0.746G	0.01	99.70	99.23	49.20
36 dpi	D36dT1	18,000,592	17,826,356	0.900G	0.01	99.57	98.93	49.39
	D36dT2	15,372,801	15,223,534	0.769G	0.01	99.72	99.26	49.52
	D36dT3	13,421,278	13,326,515	0.671G	0.01	99.79	99.45	49.33
	D36dC1	13,738,157	13,648,316	0.687G	0.01	99.73	99.31	49.23
	D36dC2	15,012,200	14,900,388	0.751G	0.01	99.73	99.32	49.51
	D36dC3	16,947,963	16,797,392	0.847G	0.01	99.75	99.36	49.26

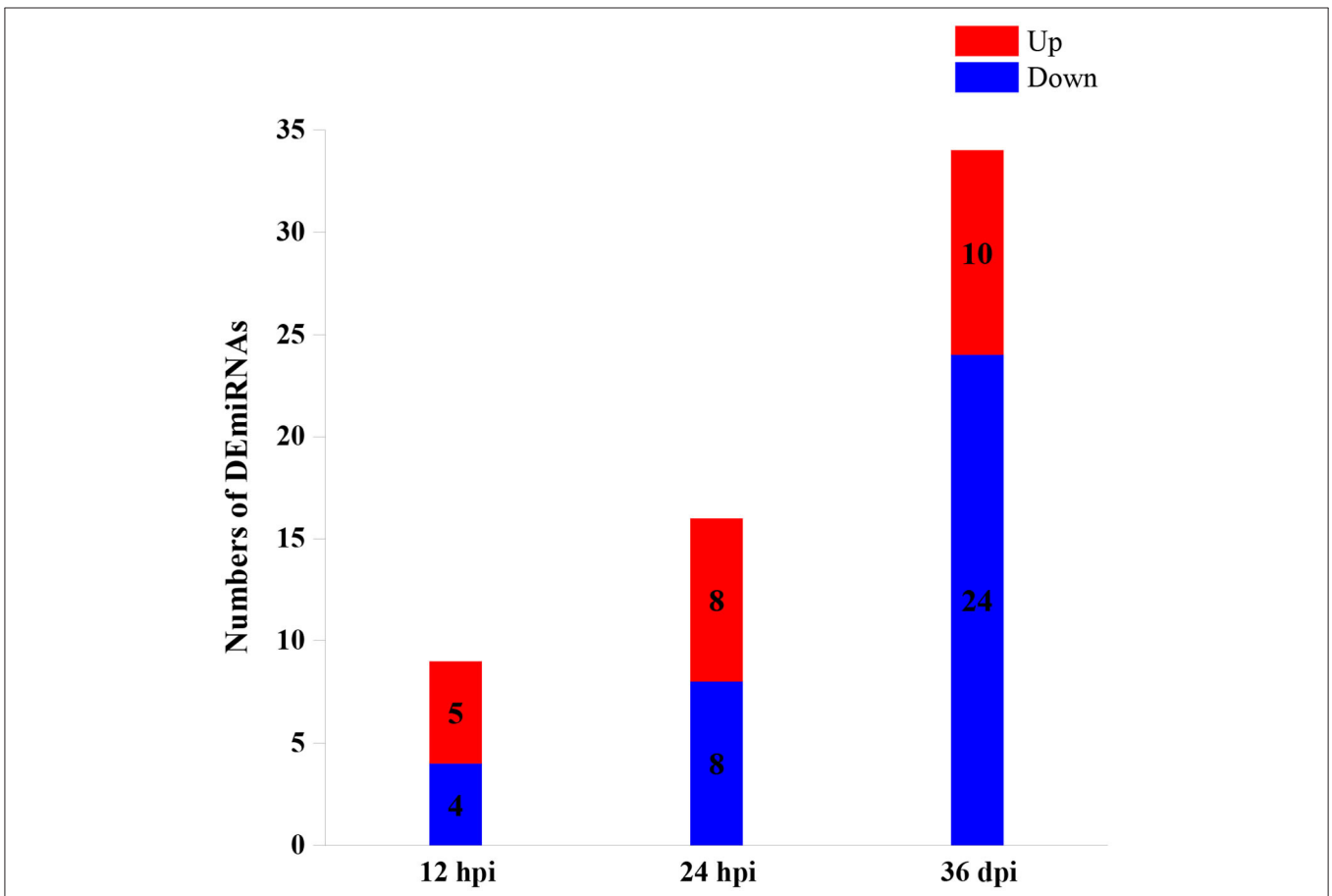

FIGURE 1 | The numbers of differentially expressed microRNAs (DEmiRNAs) at three infection stages [12 h post-infection (hpi), 24 hpi, and 36 days post-infection (dpi)]. The red and blue colors represent the upregulated and downregulated miRNAs, respectively.

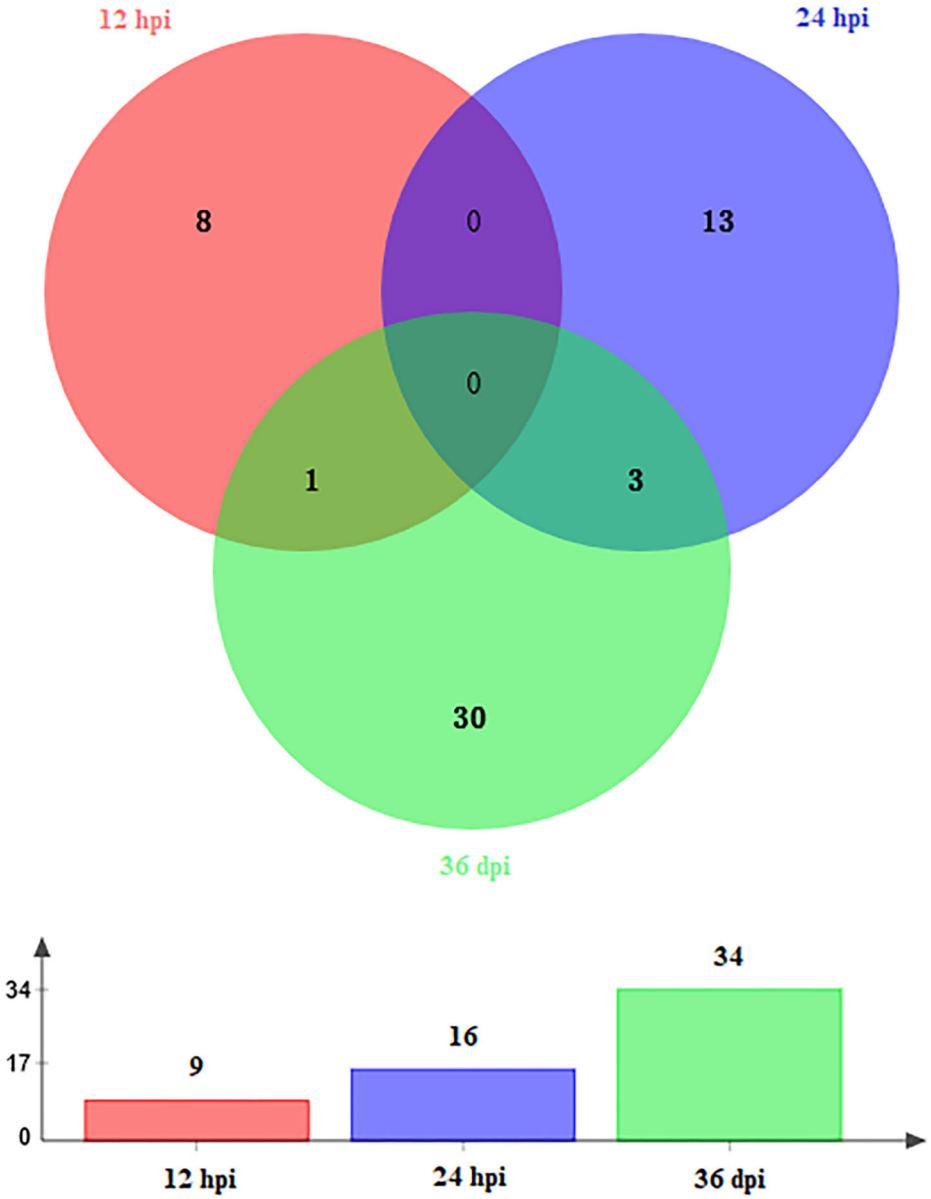

FIGURE 2 | Venn diagram showing the shared and unique DEmiRNAs at different time points after infection (12 hpi, 24 hpi, and 36 dpi).

prediction results showed that a total of 384, 518, and 1,225 hepatic genes were targeted by DEmiRNAs at 12 hpi, 24 hpi, and 36 dpi, respectively. The immunoglobulin superfamily (IgSF) is a molecular superfamily with an immunoglobulin-like domain, and most members of IgSF are found on the surface of lymphocytes and participate in various immune activities. In this study, the IgSF genes were predicted to be regulated by some miRNAs, such as novel-294, cfa-miR-25, cfa-miR-15b, cfa-miR-145, cfa-miR-150, cfa-miR-497, cfa-miR-1839 and cfa-miR-151, cfa-miR-133c, novel-337, cfa-miR-127, cfa-miR-205, and cfa-miR-194 (**Supplementary Table 3**). The GO enrichment analysis showed that 9 DEmiRNAs were significantly enriched in 797 GO terms at 12 hpi; 16 DEmiRNAs were significantly enriched in 768 GO terms at 24 hpi; and 34 DEmiRNAs were significantly enriched in 665 GO terms at 36 dpi ($P < 0.05$) (**Supplementary Table 4**). The top 30 most significant GO terms ($P < 0.05$) belonging to biological process and molecular function at each time point are shown in **Figure 3**. Among of these GO terms, the immune-related GO terms were found in infected livers (**Supplementary Table 5**). At 12 hpi, one DEmiRNA was related to three immune-related GO terms, including natural killer (NK) cell differentiation involved in immune response (GO:0002325), regulation of NK cell differentiation involved in immune response (GO:0032826),

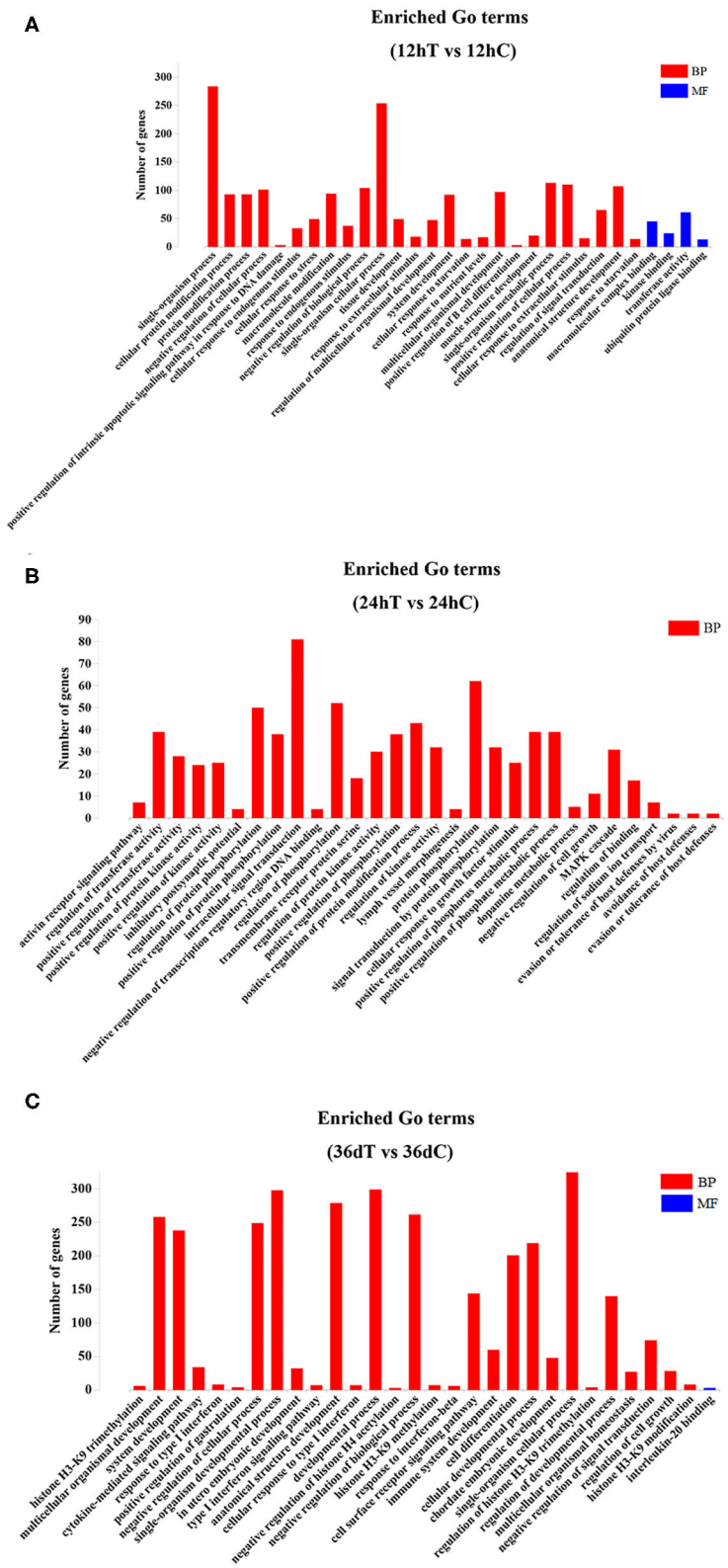

FIGURE 3 | The top 30 Gene Ontology (GO) terms of the DEmiRNAs. **(A)** The significantly enriched biological process (BP) and molecular function (MF) terms of the target genes of DEmiRNAs at 12 hpi. **(B)** The significantly enriched BP terms of target genes of DEmiRNAs at 24 hpi. **(C)** The significantly enriched BP and MF terms of target genes of DEmiRNAs at 36 dpi.

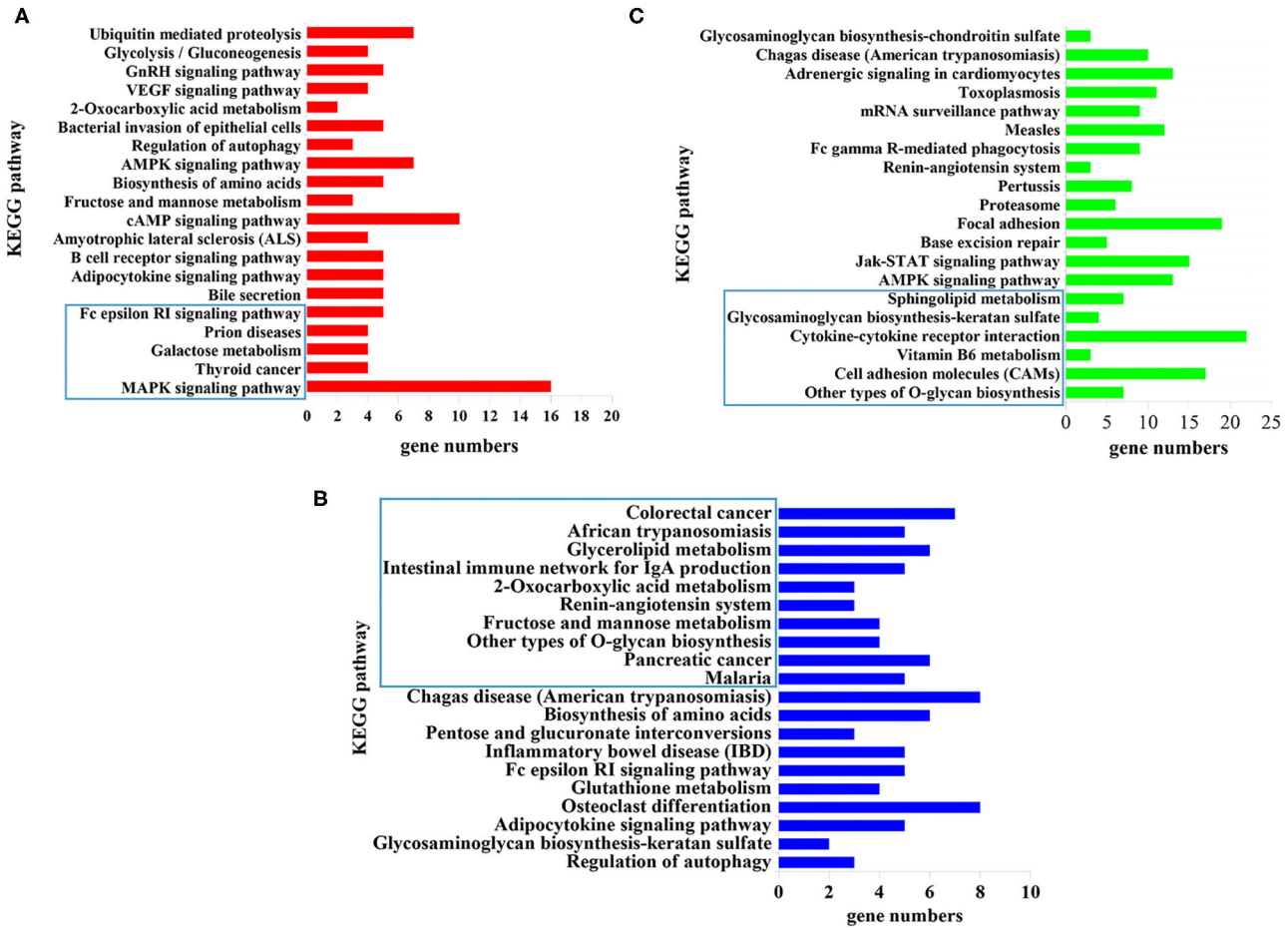

FIGURE 4 | The top 20 signaling pathways enriched by the target genes of differently expressed microRNAs (miRNAs) at **(A)** 12 hpi, **(B)** 24 hpi, and **(C)** 36 dpi. The X-axis labels denote the gene numbers, and the Y-axis labels represent the names of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The pathways enclosed within the blue rectangle represent the significantly enriched pathways.

and negative regulation of NK cell differentiation involved in immune response (GO:0032827). At 24 hpi, six DEmiRNAs were significantly related to 32 immune-related GO terms, including positive regulation of B-cell–mediated immunity (GO:0002714), positive regulation of immunoglobulin-mediated immune response (GO:0002891), positive regulation of leukocyte-mediated immunity (GO:0002705), positive regulation of lymphocyte-mediated immunity (GO:0002708), and regulation of innate immune response (GO:0045088). At 36 dpi, 12 DEmiRNAs were significantly related to four immune-related GO terms, including immune system development (GO:0002520), negative regulation of innate immune response (GO:0045824), positive regulation of immune system process (GO:0002684), and negative regulation of immune response (GO:0050777) (**Supplementary Table 5**).

KEGG Pathway Analysis

KEGG pathway analysis revealed that 5, 10, and 6 pathways were significantly enriched ($P < 0.05$) at 12 hpi, 24 hpi, and 36 dpi, respectively (**Supplementary Table 6**). The top 20 targeted pathways of DEmiRNAs are shown in **Figure 4**. At 12 hpi, some immune-related pathways were significantly enriched, including the mitogen-activated protein kinase (MAPK) signaling pathway (cfa04010) and Fc epsilon RI signaling pathway (cfa04664) (**Figure 4A**). The highly enriched pathways at 24 hpi (**Figure 4B**) included glycerolipid metabolism (cfa00561), the renin–angiotensin system pathway (cfa04614), and other types of O-glycan biosynthesis (cfa00514). Moreover, significantly inflammation-related pathways were found at 36 dpi, including cell adhesion molecules (CAMs) (cfa04514), vitamin B6 metabolism (cfa00750), cytokine–cytokine receptor interaction (cfa04060), and glycosaminoglycan biosynthesis–keratan sulfate (cfa00533) (**Figure 4C**).

qRT-PCR Validation of RNA-Sequencing Data

Nine miRNAs were randomly selected for qRT-PCR verification (**Figure 5**), including cfa-miR-381, cfa-miR-10b, cfa-miR-146,

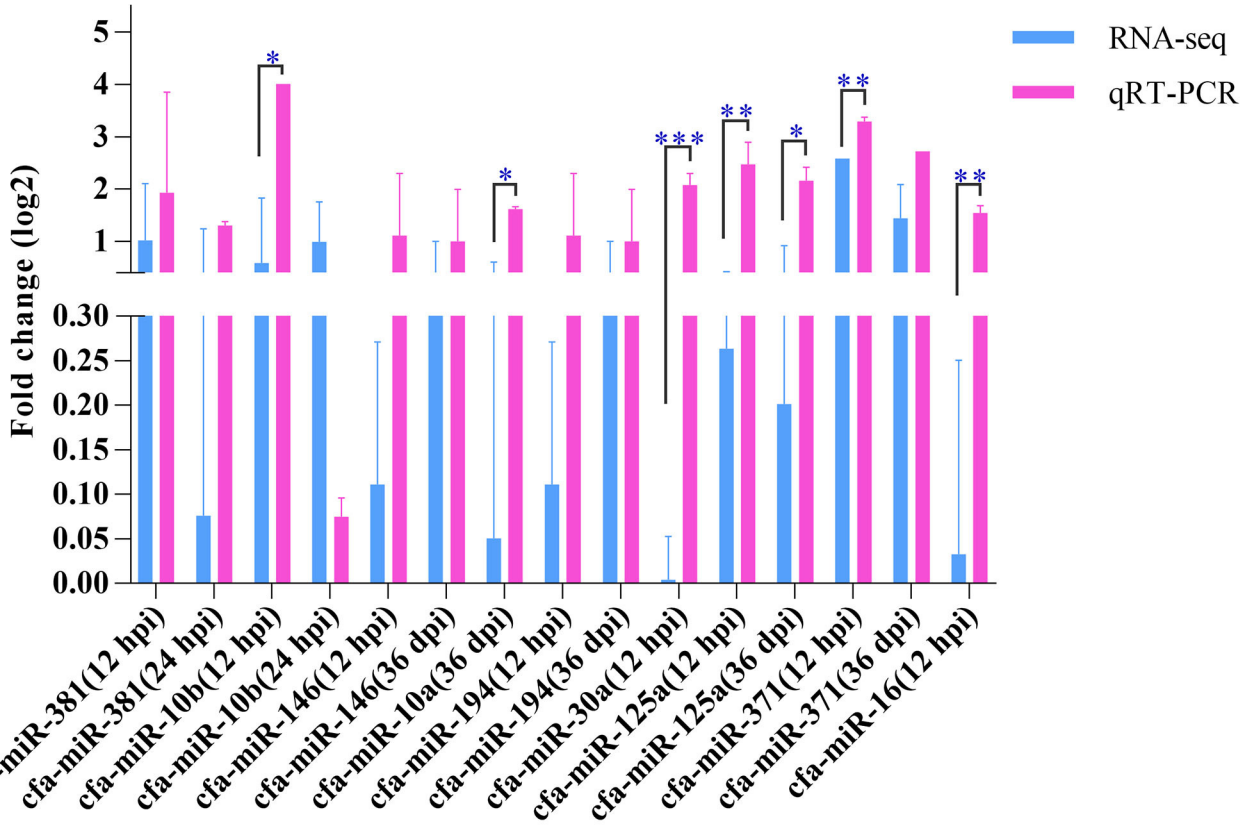

FIGURE 5 | Quantitative real-time PCR. The Y-axis shows the relative change of miRNA levels expressed as fold increase compared with the control U6 small nuclear RNA (snRNA). The X-axis shows the name of the nine miRNAs used in the cross-validation of expression analysis. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

cfa-miR-10a, cfa-miR-194, cfa-miR-30a, cfa-miR-125a, cfa-miR-371, and cfa-miR-16. Although the expression levels of the nine miRNAs obtained by qRT-PCR were slightly higher those obtained by RNA-sequencing, the expression trends obtained by both methods were consistent, which suggested an increased expression of the examined miRNAs.

DISCUSSION

In this study, hepatic miRNA expression patterns of beagle dogs during *T. canis* infection were investigated using RNA-sequencing analysis. A total of 59 DEmiRNAs were identified, including 9, 16, and 34 DEmiRNAs at 12 hpi, 24 hpi, and 36 dpi, respectively. Most of the downregulated DEmiRNAs were found in the infected group at 36 dpi (**Figure 1**). This finding suggested that miRNAs were negatively regulated to alter the expression of the target genes during the later stage of infection in infected puppies' livers. Moreover, among the dysregulated miRNAs, only one and three were common between 12 hpi and 36 dpi and between 24 hpi and 36 dpi, respectively (**Figure 2**). This finding indicates that the expression of hepatic miRNAs of beagle dogs evolves during the *T. canis* infection. Two important miRNAs, novel-294 and cfa-miR-885, were differentially expressed at the initial stage of infection (12 hpi) (**Supplementary Table 2**). Furthermore, the IgSF gene *FGFR1* was predicted to be regulated by miRNA novel-294 at 12 hpi (**Supplementary Table 3**). A previous study found that *FGFR1* expression improved beta-cell survival in cytokine-induced inflammation (33). Hence, we speculated that the upregulation of the novel-294 in puppies' livers promotes the immune response of puppies to *T. canis* infection *via* increasing the expression of *FGFR1*. miRNA cfa-miR-885 was predicted to regulate *IGSF3* gene of the IgSF (**Supplementary Table 3**). The IgSF and leukocyte integrins play an important role in the regulation of leukocyte recruitment to the inflammation sites (34). These findings suggest that cfa-miR-885 plays a protective role during *T. canis* infection.

At 24 hpi, 16 miRNAs were found differentially expressed in infected livers (e.g., cfa-miR-1839). The cfa-miR-1839 was downregulated at 24 hpi, and TRIM71, the predicted target gene of cfa-miR-1839 (**Supplementary Table 3**), regulates juvenile-to-adult transition events in nematodes and mammals (35). The majority of *T. canis* larvae reach the liver at 24 hpi (36); subsequently, the larvae migrate to lung, muscle, and brain tissue *via* the circulation (36). However, some larvae cannot continue to migrate and are trapped in the hepatic capillaries (36). Thus, we assumed that the downregulation of hepatic cfa-miR-1839

possibly regulates the juvenile-to-adult development of *T. canis* larvae in the puppies' livers. Whether cfa-miR-1839 regulates juvenile-to-adult transition of *T. canis* larvae remains to be further investigated.

cfa-miR-370 and cfa-miR-133c were significantly downregulated at 36 dpi (**Supplementary Table 2**). The target gene prediction showed that cfa-miR-370 targets the *CD3E* gene (**Supplementary Table 3**). *CD3E*, the T-cell antigen receptor epsilon subunit gene, is essential for TCR signaling and T-cell differentiation (37, 38). In our study, cfa-miR-370 was downregulated in the infected livers at 36 dpi, indicating that the downregulation of cfa-miR-370 may represent an immunoreaction to resist *T. canis* infection. In addition, two differently expressed miRNAs, cfa-miR-370 and cfa-miR-133c, were predicted to regulate the *ARRDC1* gene (**Supplementary Table 3**). Additionally, cfa-miR-133c was predicted to regulate the *IL18R1* gene. Previous research found that *IL-18R*/*MyD88* plays a crucial role in the development of a robust Th1 response during *Trypanosoma cruzi* infection (39). Therefore, the abnormal expression of cfa-miR-133c seems to be a potential candidate for further study of the role of cfa-miR-133c in promoting a protective Th1 immune response to *T. canis* infection.

GO enrichment analysis showed that 797, 769, and 665 GO terms were significantly enriched at 12 hpi, 24 hpi, and 36 dpi, respectively (**Supplementary Table 4**). Based on the analysis of the 797 significantly enriched GO terms at 12 hpi, nine DEmiRNAs were mainly involved in the signal-related biological process, including instance single-organism processes, single-organism cellular process, and single-organism metabolic process (**Figure 3A**). These processes are involved in acute liver injury (40). At 24 hpi, 16 DEmiRNAs were mainly associated with biological processes, such as protein phosphorylation, intracellular signal transduction, regulation of phosphorylation, and regulation of protein phosphorylation (**Figure 3B**). Some phosphorylated proteins were related to TNF-α signaling (41), which regulate immune response (42). At 36 dpi, 10 upregulated and 24 downregulated miRNAs were involved in response to stress, positive regulation of the immune system process, and regulation of nervous system development (**Figure 3C**). Moreover, a total of 39 immunization-related GO terms were significantly enriched at three time points (**Supplementary Table 5**). These results show that *T. canis* larvae can elicit a significant immune response after infecting the liver.

The top 20 enriched pathways are shown in **Figure 4**. The target genes of the DEmiRNAs were significantly enriched in the MAPK signaling pathway, galactose metabolism, and Fc epsilon RI signaling pathway at 12 hpi (**Figure 4A**). The MAPK signaling pathway participated in diverse cellular responses, such as inflammation, differentiation, proliferation, and apoptosis (43). Additionally, the MAPK signaling pathway is involved in the development, reproduction, and survival of *Schistosoma mansoni* (44). The Fc epsilon RI is the primary receptor in mast cells, which can influence the synthesis of proinflammatory cytokines and other molecules that are involved in inflammatory responses (45) (**Figure 4A**). These findings suggested that *T. canis* can cause inflammatory responses in puppy livers at the initial stage. At 24 hpi, the glycerolipid metabolism pathway, renin–angiotensin system pathway, and other types of O-glycan biosynthesis pathways were significantly enriched (**Figure 4B**). The glycerolipid metabolism pathway could perturb the host immune system and metabolism following anisakid infection (46). Further, the renin–angiotensin system pathway influences a range of processes from inflammation and immune responses to longevity (47). A previous study found that the O-glycoprotein biosynthesis pathway was associated with the immune response of dendritic cells (48). According to these findings, we assumed that *T. canis* larvae can trigger the immune responses in the infected host liver at 24 hpi. Some inflammation-related pathways were significantly enriched at 36 dpi, such as the cell adhesion molecules (CAMs) pathway, vitamin B6 metabolism pathway, cytokine–cytokine receptor interaction pathway, and glycosaminoglycan biosynthesis–keratan sulfate pathway (**Figure 4C**). The CAMs can direct mediate leukocyte migration, which is essential for generating effective inflammatory responses (49). Previous research found that vitamin B6 as a co-factor was involved in the inflammation response (50). It was reported that a high level of cytokine–cytokine motif chemokine ligand 1 could enhance and prolong the inflammatory response (51). The glycosaminoglycan pathway interacts with multiple ligands, which play an essential role in the inflammatory reaction (52). These results indicated that the puppies' livers may trigger inflammatory responses at the late stage of *T. canis* infection.

CONCLUSION

The present study, for the first time, revealed the hepatic miRNA expression patterns of beagle dogs at three *T. canis* infection stages. A total of 59 DEmiRNAs were identified in the infected livers. The functional enrichment analysis of predicted target genes showed that miRNA cfa-miR-1839 could be related to the juvenile-to-adult transition of *T. canis* larvae. KEGG pathway analysis found that some significantly enriched pathways were related to the inflammatory response at 12 hpi and 36 dpi. Several miRNAs, such as novel-294, cfa-miR-88, cfa-miR-370, and cfa-miR-133c, were associated with immune responses. These findings should enrich our understanding of the interactions between *T. canis* and its definitive host. Further studies to elucidate the detailed molecular mechanisms and the physiological functions of the DEmiRNAs in the pathogenesis of *T. canis* infection are warranted.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA630302.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Approval No. 2018-015). The dogs used in the study were handled in accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China.

AUTHOR CONTRIBUTIONS

Y-XL and X-QZ conceived and designed the experiments. YZ and W-BZ performed the experiments. YZ analyzed the data and wrote the paper. J-JH and HME participated in improving the English of the manuscript. HME, X-QZ, and Y-XL critically revised the manuscript. All authors have read and approved the final version of the manuscript. All authors contributed to the preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Chen J, Zhou DH, Nisbet AJ, Xu MJ, Huang SY, Li MW, et al. Advances in molecular identification, taxonomy, genetic variation and diagnosis of *Toxocara* spp. *Infect Genet Evol*. (2012) 12:1344–8. doi: 10.1016/j.meegid.2012.04.019
2. Overgaauw PA, van Knapen F. Veterinary and public health aspects of *Toxocara* spp. *Vet Parasitol*. (2013) 193:398–403. doi: 10.1016/j.vetpar.2012.12.035
3. Holland CV. Knowledge gaps in the epidemiology of *Toxocara*: the enigma remains. *Parasitology*. (2017) 144:81–94. doi: 10.1017/S0031182015001407
4. Macpherson CN. The epidemiology and public health importance of toxocariasis: a zoonosis of global importance. *Int J Parasitol*. (2013) 43:999–1008. doi: 10.1016/j.ijpara.2013.07.004
5. Ma G, Holland CV, Wang T, Hofmann A, Fan CK, Maizels RM, et al. Human toxocariasis. *Lancet Infect Dis*. (2018) 18:14–24. doi: 10.1016/S1473-3099(17)30331-6
6. Fan CK, Liao CW, Cheng YC. Factors affecting disease manifestation of toxocarosis in humans: genetics and environment. *Vet Parasitol*. (2013) 193:342–52. doi: 10.1016/j.vetpar.2012.12.030
7. Rubinsky-Elefant G, Hirata CE, Yamamoto JH, Ferreira MU. Human toxocariasis: diagnosis, worldwide seroprevalences and clinical expression of the systemic and ocular forms. *Ann Trop Med Parasitol*. (2010) 104:3–23. doi: 10.1179/136485910X12607012373957
8. Ma G, Luo Y, Zhu H, Luo Y, Korhonen PK, Young ND, et al. MicroRNAs of *Toxocara canis* and their predicted functional roles. *Parasit Vectors*. (2016) 9:229. doi: 10.1186/s13071-016-1508-3
9. Sperotto RL, Kremer FS, Aires Berne ME, Costa de Avila LF, da Silva Pinto L, Monteiro KM, et al. Proteomic analysis of *Toxocara canis* excretory and secretory (TES) proteins. *Mol Biochem Parasitol*. (2017) 211:39–47. doi: 10.1016/j.molbiopara.2016.09.002
10. Zheng WB, Zou Y, Zhu XQ, Liu GH. *Toxocara* "omics" and the promises it holds for medicine and veterinary medicine. *Adv Parasitol*. (2020) 109:89–108. doi: 10.1016/bs.apar.2020.01.002
11. Zhu XQ, Korhonen PK, Cai H, Young ND, Nejsum P, von Samson-Himmelstjerna G, et al. Genetic blueprint of the zoonotic pathogen *Toxocara canis*. *Nat Commun*. (2015) 6:6145. doi: 10.1038/ncomms7145
12. Cai Y, Yu X, Hu S, Yu J. A brief review on the mechanisms of miRNA regulation. *Genom Proteom Bioinform*. (2009) 7:147–54. doi: 10.1016/S1672-0229(08)60044-3
13. Chen K, Rajewsky N. The evolution of gene regulation by transcription factors and microRNAs. *Nat Rev Genet*. (2007) 8:93–103. doi: 10.1038/nrg1990
14. Hobert O. Gene regulation by transcription factors and microRNAs. *Science*. (2008) 319:1785–6. doi: 10.1126/science.1151651
15. Deng M, Lancto CA, Abrahamsen MS. *Cryptosporidium parvum* regulation of human epithelial cell gene expression. *Int J Parasitol*. (2004) 34:73–82. doi: 10.1016/j.ijpara.2003.10.001
16. Sturm A, Amino R, van de Sand C, Regen T, Retzlaff S, Rennenberg A, et al. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. *Science*. (2006) 313:1287–90. doi: 10.1126/science.1129720
17. McDonald V, Korbel DS, Barakat FM, Choudhry N, Petry F. Innate immune responses against *Cryptosporidium parvum* infection. *Parasite Immunol*. (2013) 35:55–64. doi: 10.1111/pim.12020
18. Ambros V. The functions of animal microRNAs. *Nature*. (2004) 431:350–5. doi: 10.1038/nature02871
19. Regis SC, Mendonça LR, Silva Ndos S, Dattoli VC, Alcântara-Neves NM, Barrouin-Melo SM. Seroprevalence and risk factors for canine toxocariasis by detection of specific IgG as a marker of infection in dogs from Salvador, Brazil. *Acta Trop*. (2011) 120:46–51. doi: 10.1016/j.actatropica.2011.05.011
20. Mikaeili F, Mathis A, Deplazes P, Mirhendi H, Barazesh A, Ebrahimi S, et al. Differentiation of *Toxocara canis* and *Toxocara cati* based on PCR-RFLP analyses of rDNA-ITS and mitochondrial cox1 and nad1 regions. *Acta Parasitol*. (2017) 62:549–56. doi: 10.1515/ap-2017-0066
21. Zheng WB, Zou Y, Elsheikha HM, Liu GH, Hu MH, Wang SL, et al. Serum metabolomic alterations in Beagle dogs experimentally infected with *Toxocara canis*. *Parasit Vectors*. (2019) 12:447. doi: 10.1186/s13071-019-3703-5
22. Takamiya S, Mita T. Large-scale purification of active liquid-cultured *Caenorhabditis elegans* using a modified Baermann apparatus. *Parasitol Int*. (2016) 65:580–3. doi: 10.1016/j.parint.2016.03.013
23. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol*. (2009) 10:R25. doi: 10.1186/gb-2009-10-3-r25
24. Friedländer MR, Mackowiak SD, Li N, Chen W, Rajewsky N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res*. (2012) 40:37–52. doi: 10.1093/nar/gkr688
25. Wen M, Shen Y, Shi S, Tang T. miREvo: an integrative microRNA evolutionary analysis platform for next-generation sequencing experiments. *BMC Bioinformatics*. (2012) 13:140. doi: 10.1186/1471-2105-13-140
26. Zhou L, Chen J, Li Z, Li X, Hu X, Huang Y, et al. Integrated profiling of microRNAs and mRNAs: microRNAs located on Xq27.3 associate with clear cell renal cell carcinoma. *PLoS ONE*. (2010) 5:e15224. doi: 10.1371/journal.pone.0015224
27. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in Drosophila. *Genome Biol*. (2003) 5:R1. doi: 10.1186/gb-2003-5-1-r1

28. Chen CM, Lu YL, Sio CP, Wu GC, Tzou WS, Pai TW. Gene Ontology based housekeeping gene selection for RNA-seq normalization. *Methods*. (2014) 67:354–63. doi: 10.1016/j.ymeth.2014.01.019
29. Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, et al. KEGG for linking genomes to life and the environment. *Nucleic Acids Res*. (2008) 36:D480–4. doi: 10.1093/nar/gkm882
30. Mao X, Cai T, Olyarchuk JG, Wei L. Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics*. (2005) 21:3787–93. doi: 10.1093/bioinformatics/bti430
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods*. (2001) 25:402–8. doi: 10.1006/meth.2001.1262
32. Adnan M, Morton G, Hadi S. Analysis of rpoS and bolA gene expression under various stress-induced environments in planktonic and biofilm phase using $2^{-\Delta\Delta C_T}$ method. *Mol Cell Biochem*. (2011) 357:275–82. doi: 10.1007/s11010-011-0898-y
33. Regeenes R, Silva PN, Chang HH, Arany EJ, Shukalyuk AI, Audet J, et al. Fibroblast growth factor receptor 5 (FGFR5) is a co-receptor for FGFR1 that is up-regulated in beta-cells by cytokine-induced inflammation. *J Biol Chem*. (2018) 293:17218–28. doi: 10.1074/jbc.RA118.003036
34. Wetzel A, Chavakis T, Preissner KT, Sticherling M, Haustein UF, Anderegg U, et al. Human Thy-1 (CD90) on activated endothelial cells is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J Immunol*. (2004) 172:3850–9. doi: 10.4049/jimmunol.172.6.3850
35. Aeschimann F, Neagu A, Rausch M, Großhans H. let-7 coordinates the transition to adulthood through a single primary and four secondary targets. *Life Sci Alliance*. (2019) 2:e201900335. doi: 10.26508/lsa.201900335
36. Schnieder T, Laabs EM, Welz C. Larval development of *Toxocara canis* in dogs. *Vet Parasitol*. (2011) 175:193–206. doi: 10.1016/j.vetpar.2010.10.027
37. Le Deist F, Hivroz C, Partiseti M, Rieux-Laucat F, Debatin KM, Choquet D, et al. T cell activation deficiencies. *Clin Immunol Immunopathol*. (1995) 76:163–4. doi: 10.1016/S0090-1229(95)90072-1
38. Soudais C, De Villartay JP, Le Deist F, Fischer A, Lisowska-Grospierre B. Genetic analysis of the human CD3-epsilon gene in a T cell receptor/CD3 immunodeficiency. *Immunodeficiency*. (1993) 4:117–9. doi: 10.1038/ng0193-77
39. Oliveira AC, Gomes-Neto JF, Barbosa CD, Granato A, Reis BS, Santos BM, et al. Crucial role for T cell-intrinsic IL-18R-MyD88 signaling in cognate immune response to intracellular parasite infection. *Elife*. (2017) 6:e30883. doi: 10.7554/eLife.30883
40. Wang G, Jin S, Ling X, Li Y, Hu Y, Zhang Y, et al. Proteomic profiling of LPS-induced macrophage-derived exosomes indicates their involvement in acute liver injury. *Proteomics*. (2019) 19:e1800274. doi: 10.1002/pmic.201800274
41. Oliveira KC, Carvalho ML, Bonatto JM, Schechtman D, Verjovski-Almeida S. Human TNF-α induces differential protein phosphorylation in Schistosoma mansoni adult male worms. *Parasitol Res*. (2016) 115:817–28. doi: 10.1007/s00436-015-4812-5
42. Pereira-Fonseca DCM, Oliveira-Rovai FM, Rodas LAC, Beloti CAC, Torrecilha RBP, Ito PKRK, et al. Dog skin parasite load, TLR-2, IL-10 and TNF-α expression and infectiousness. *Parasite Immunol*. (2017) 39:e12493. doi: 10.1111/pim.12493
43. Kim L, Butcher BA, Denkers EY. *Toxoplasma gondii* interferes with lipopolysaccharide-induced mitogen-activated protein kinase activation by mechanisms distinct from endotoxin tolerance. *J Immunol*. (2004) 172:3003–10. doi: 10.4049/jimmunol.172.5.3003
44. Gava SG, Tavares NC, Falcone FH, Oliveira G, Mourão MM. Profiling Transcriptional regulation and functional roles of schistosoma mansoni c-Jun N-Terminal Kinase. *Front Genet*. (2019) 10:1036. doi: 10.3389/fgene.2019.01036
45. Kraft S, Bieber T. Fc epsilon ri-mediated activation of transcription factors in antigen-presenting cells. *Int Arch Allergy Immunol*. (2001) 125:9–15. doi: 10.1159/000053791
46. Liu K, Yin D, Shu Y, Dai P, Yang Y, Wu H. Transcriptome and metabolome analyses of *Coilia nasus* in response to Anisakidae parasite infection. *Fish Shellfish Immunol*. (2019) 87:235–42. doi: 10.1016/j.fsi.2018.12.077
47. Sparks MA, Crowley SD, Gurley SB, Mirotsou M, Coffman TM. Classical Renin-Angiotensin system in kidney physiology. *Compr Physiol*. (2014) 4:1201–28. doi: 10.1002/cphy.c130040
48. Tomek MB, Maresch D, Windwarder M, Friedrich V, Janesch B, Fuchs K, et al. A general protein O-glycosylation gene cluster encodes the species-specific Glycan of the oral pathogen *Tannerella forsythia*: O-Glycan biosynthesis and immunological implications. *Front Microbiol*. (2018) 9:2008. doi: 10.3389/fmicb.2018.02008
49. Golias C, Tsoutsi E, Matziridis A, Makridis P, Batistatou A, Charalabopoulos K. Review. Leukocyte and endothelial cell adhesion molecules in inflammation focusing on inflammatory heart disease. *In Vivo*. (2007) 21:757–69.
50. Ueland PM, McCann A, Midttun Ø, Ulvik A. Inflammation, vitamin B6 and related pathways. *Mol Aspects Med*. (2017) 53:10–27. doi: 10.1016/j.mam.2016.08.001
51. Zhou Y, Qiao H, Yin N, Chen L, Xie Y, Wu J, et al. Immune and cytokine/chemokine responses of PBMCs in rotavirus-infected rhesus infants and their significance in viral pathogenesis. *J Med Virol*. (2019) 91:1448–69. doi: 10.1002/jmv.25460
52. Morla S. Glycosaminoglycans and Glycosaminoglycan mimetics in cancer and inflammation. *Int J Mol Sci*. (2019) 20:1963. doi: 10.3390/ijms20081963

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REVIEW
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Zoonotic Tick-Borne Pathogens in Temperate and Cold Regions of Europe—A Review on the Prevalence in Domestic Animals

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Ticks transmit a variety of pathogens affecting both human and animal health. In temperate and cold regions of Europe (Western, Central, Eastern, and Northern Europe), the most relevant zoonotic tick-borne pathogens are tick-borne encephalitis virus (TBEV), *Borrelia* spp. and *Anaplasma phagocytophilum*. More rarely, *Rickettsia* spp., *Neoehrlichia mikurensis,* and zoonotic *Babesia* spp. are identified as a cause of human disease. Domestic animals may also be clinically affected by these pathogens, and, furthermore, can be regarded as sentinel hosts for their occurrence in a certain area, or even play a role as reservoirs or amplifying hosts. For example, viraemic ruminants may transmit TBEV to humans via raw milk products. This review summarizes the role of domestic animals, including ruminants, horses, dogs, and cats, in the ecology of TBEV, *Borrelia* spp., *A. phagocytophilum*, *Rickettsia* spp., *N. mikurensis,* and zoonotic *Babesia* species. It gives an overview on the (sero-)prevalence of these infectious agents in domestic animals in temperate/cold regions of Europe, based on 148 individual prevalence studies. Meta-analyses of seroprevalence in asymptomatic animals estimated an overall seroprevalence of 2.7% for TBEV, 12.9% for *Borrelia burgdorferi* sensu lato (s.l.), 16.2% for *A. phagocytophilum* and 7.4% for *Babesia divergens*, with a high level of heterogeneity. Subgroup analyses with regard to animal species, diagnostic test, geographical region and decade of sampling were mostly non-significant, with the exception of significantly lower *B. burgdorferi* s.l. seroprevalences in dogs than in horses and cattle. More surveillance studies employing highly sensitive and specific test methods and including hitherto non-investigated regions are needed to determine if and how global changes in terms of climate, land use, agricultural practices and human behavior impact the frequency of zoonotic tick-borne pathogens in domestic animals.

Keywords: *Borrelia*, *Rickettsia*, *Anaplasma*, *Babesia*, *Neoehrlichia mikurensis*, tick-borne encephalitis, tick-borne diseases, vector-borne diseases

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INTRODUCTION

Many tick-borne diseases (TBDs) are so-called meta-zoonoses, i.e., they may be transmitted to humans as well as animals via their invertebrate tick host (1). In temperate/cold regions of Europe, the hard tick *Ixodes ricinus* is the most important vector of TBDs in terms of both animal and human (public) health, followed by *Dermacentor reticulatus* and *Dermacentor marginatus* (2).

Meta-zoonotic pathogens transmitted by *I. ricinus* include tick-borne encephalitis virus (TBEV), *Borrelia* spp., *Anaplasma phagocytophilum*, *Rickettsia* spp., *Neoehrlichia mikurensis* and zoonotic *Babesia* spp., while ticks of the genus *Dermacentor* may transmit *Rickettsia* spp. to both animals and humans, among others (3). In addition, *D. reticulatus* is now also recognized as a vector for TBEV (4).

Ixodes ricinus has a broad host spectrum, including birds, various wild and domestic animals as well as humans, and occurs in a wide variety of habitats throughout Europe, as far north as 66° N (Norway), close to the Arctic Circle (5). The species' range has continuously expanded northward as well as into higher altitudes during the past decades, probably driven by climatic and environmental changes (6, 7). Similarly, the distribution of *D. reticulatus* is expanding in several European countries (8, 9). A northward spread has been documented in the Baltic countries (10) as well as in Germany (11), but the species has not been documented in Scandinavia to date. In contrast, the distribution of *D. marginatus* seems to be comparatively stable with a northern distribution limit at ∼51° N in central Germany (11). In addition to the range expansion of different tick species, changes in human behavior toward more outdoor activities increase the risk of tick bites (2).

In consequence, the incidence and public health burden of TBDs seem to be increasing in Europe. Lyme borreliosis (LB), caused by spirochaetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex and transmitted primarily by *I. ricinus*, is the most common TBD in humans in the Northern Hemisphere. In Europe, annual incidence rates vary between 0.001 and 464 cases/100,000 inhabitants (12). In the Netherlands, a more than 2-fold increase of medical consultations and hospital admissions due to LB was noted from 1994 to 2005 (13). A similar increase in diagnosed LB cases was observed in the United Kingdom from 1998 to 2016 (14). Likewise, the incidence of tick-borne encephalitis, a flavivirus infection transmitted also primarily by *I. ricinus*, has increased significantly since the year 1990 in several European countries (15). The geographic pattern of this disease is also changing, with new transmission foci emerging in previously unaffected regions and countries, e.g., in the Netherlands in 2016 (16) and in the United Kingdom in 2018 (17).

In addition to rising disease incidences, several new tick-borne pathogens have been described in recent decades (18). Although known already since 1995 (19), pathogenicity of *Borrelia miyamotoi*, a tick-transmitted relapsing-fever spirochaete, was first reported in 2011 (20). Similarly, *N. mikurensis* was isolated from ticks and mammals (21) years before being recognized as a human (22) and probably veterinary (23) pathogen. Furthermore, the list of emerging zoonotic tick-borne pathogens relevant in Europe includes several *Rickettsia* spp. (18) and *Babesia* spp. (24). *Borrelia miyamotoi*, *N. mikurensis*, *Rickettsia helvetica* and the relevant zoonotic *Babesia* spp. are all transmitted by *I. ricinus*, which constitutes the main vector of zoonotic TBDs in central and northern Europe. In contrast, *D. marginatus*, the vector of *Rickettsia slovaca*, has a rather limited geographic distribution, and *D. reticulatus*, the vector of *Rickettsia raoultii*, rarely bites humans (11). Therefore, these tick species are of minor importance regarding zoonotic infections.

Domestic animals may also be clinically affected by these pathogens, and, furthermore, can be regarded as sentinel hosts for their occurrence in a certain area, or even play a role as reservoir hosts. Additionally, viraemic ruminants may directly transmit TBEV to humans via raw milk products, causing large outbreaks (25). In this review, we summarize the role of domestic animals, including ruminants, horses, dogs, and cats, in the ecology of TBEV, *Borrelia* spp., *A. phagocytophilum*, *Rickettsia* spp., *N. mikurensis,* and zoonotic *Babesia* species. Unlike for humans, no systematic surveillance of TBDs in domestic animals exists, making it difficult to assess whether the patterns of increasing disease incidence observed in humans can also be found in other species. Therefore (sero-)prevalence data on the mentioned pathogens in domestic animals in temperate and cold regions of Europe are compiled to analyze temporal and regional trends, the influence of the utilized diagnostic test and to identify knowledge gaps requiring further attention.

METHODS

Literature Survey

Systematic literature search on (sero-)prevalence data in temperate and cold regions of Europe [Northern, Western, Central, and Eastern Europe (excluding Russia); see **Figure 1** for included countries] was conducted in the PubMed database in May and July 2020, using combinations of the term "prevalence" with each of "animals," "ruminants," "horses," "dogs," "cats" and each of "TBEV," "*Borrelia*," "*Anaplasma*," "*Rickettsia*," "*Neoehrlichia*," and "*Babesia*."

Further records were obtained by searching the bibliographies of relevant articles and via incidental findings using other databases, e.g., Google Scholar. Original publications in English and different national languages (e.g., German, French, if available) were included. Articles that did not refer to the considered geographical region, did not contain (sero-)prevalence data, e.g., clinical case reports, or that presented data from only one herd/flock, were excluded.

Meta-Analyses

For TBEV, *B. burgdorferi* s.l., *A. phagocytophilum* and *Babesia divergens* seroprevalence, data based on healthy/asymptomatic animals or randomly selected diagnostic samples were subjected to meta-analyses, to gain a comprehensive picture on TBD prevalence in the general domestic animal population. As the number of studies retrieved for the remaining pathogens was low, no meta-analyses were conducted. If studies reported data on healthy and symptomatic groups, only data referring to the healthy group were extracted, because seroprevalences in symptomatic animals may be higher than in the general population.

Random-effects meta-analysis of proportions was conducted with the package "meta" (v. 4.13-0) (26) in R v. 4.0.2 (27), using the inverse variance method with logit transformation and restricted maximum likelihood estimation of the between-study variance (τ^2). To assess heterogeneity between studies, *Q*-tests were performed and the I^2 statistic was assessed, with values ≥50% considered heterogeneous. To evaluate possible sources

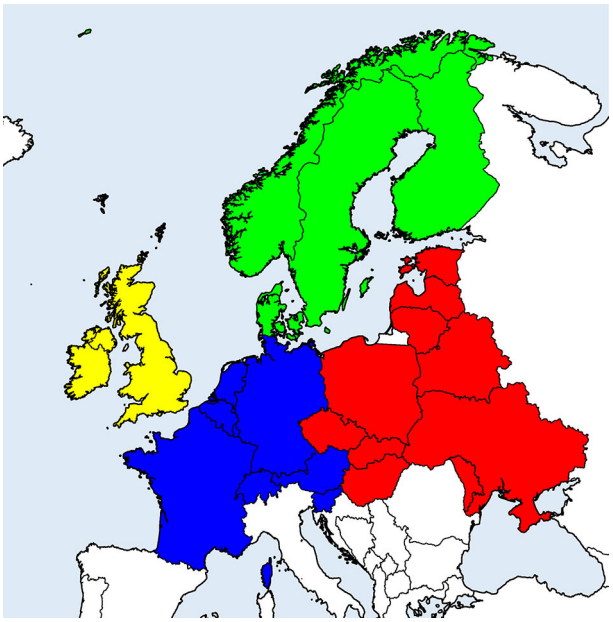

FIGURE 1 | European countries considered in the literature survey on (sero-)prevalence of tick-borne zoonotic diseases in domestic animals. Geographical regions are indicated by different colors: Scandinavia (green), British Isles (yellow), Western continental (blue), and Eastern continental Europe (red).

of heterogeneity, subgroup analyses were performed according to animal species, type of diagnostic test used, geographical region and decade of sampling. For analyses according to geographical region, the considered countries were classified into Eastern or Western continental Europe, Scandinavia or British Isles (**Figure 1**). In cases when studies did not report the period of sampling, it was assumed that the decade of sampling corresponded to the decade of data publication. In subgroup analyses, a common τ^2 was assumed across subgroups.

If studies reported more than one seroprevalence rate, referring to different species, geographic regions, or data acquisition periods, these were considered separately. Since observational prevalence studies are unlikely to suffer from publication bias, i.e., low prevalence rates have a similar probability of being published as higher prevalence rates (28), no assessment of publication bias was performed.

RESULTS

In total, 7,552 publications were assessed for eligibility and 7,404 were excluded because they were not relevant with regard to the considered pathogens or geographical range, consisted of clinical case reports referring to single animals or herds, dealt only with imported animals or did not contain sufficient data. The selection process during the literature survey is depicted in **Figure 2**, with a final dataset containing 148 articles. Of these, 65 reported data on *A. phagocytophilum,* 55 on *B. burgdorferi* s.l., 35 on TBEV, 18 on zoonotic *Babesia* species, 9 on *Rickettsia* spp., and 5 on *N. mikurensis*. Some publications contained data on more than one of these pathogens.

An overview of the roles of domestic animals regarding the considered tick-borne pathogens is given in **Figure 3**. In the following, these roles as well as the (sero-)prevalence rates are discussed in detail for each infectious agent.

Tick-Borne Encephalitis Virus

Tick-borne encephalitis is regarded as the most important arthropod-borne viral disease in Europe (29). It is caused by a flavivirus which is mainly transmitted by *I. ricinus*, but the vector potential of *D. reticulatus* has also been shown (4). Unlike other tick-associated pathogens, it is not consistently distributed throughout the range of its vectors, but occurs in a patchy pattern in delimited geographic areas, termed microfoci or "hotspots." In these foci, it circulates between rodents and ticks and occasionally spills over to domestic animals and humans (29). In recent decades, a geographical spread of the virus has been observed in Europe with new transmission foci having recently emerged in the Netherlands (16) and the United Kingdom (17).

TBEV may cause severe neurologic disease in humans, horses, dogs, and probably also in ruminants (**Figure 3**). Furthermore, most domestic animals are regarded as useful sentinels for human TBE risk (30), with the exception of cats for which no data exist, explaining the comparatively large number of retrieved studies.

In total, 36 studies were retrieved (eight containing data on cattle, seven on sheep, 10 on goats, seven on horses, and 13 on dogs). As cross-reactions with other flaviviruses (e.g., West Nile virus, Louping Ill virus) in serological tests are common, only studies which confirmed positive samples via seroneutralisation test (SNT), considered the gold standard of TBEV serology (31), were included in the meta-analysis of seroprevalence ($N = 20$, with 39 animal cohorts). Therefore, subgroup analysis according to diagnostic test was not performed. The estimated overall prevalence was 2.8%, with a significant level of heterogeneity ($I^2 = 95.8\%$; 95% CI: 95.0–96.5%; $P < 0.001$). No significant differences between animal species ($\chi^2 = 2.6$, df = 4, $P = 0.622$; **Figure 4**) nor between decades ($\chi^2 = 4.4$, df = 2, $P = 0.110$) or regions ($\chi^2 = 1.9$, df = 2, $P = 0.390$) were found.

Ruminants

Domestic ruminants develop viraemia upon TBEV infection, which usually lasts a few days, but remain mostly asymptomatic (32). However, they excrete TBEV in milk during the viraemic phase, potentially leading to human infection via raw milk products like unpasteurized milk or raw milk cheese. In goat milk, infective virus can be detected for up to 19 days post infection (33). Such alimentary transmission often causes clusters of cases [e.g., (34, 35)] and is regarded as the second most important route of human infection (25).

In addition, a few clinical cases of neurologic disease in small ruminants due to TBEV have been described (36, 37). Possibly, clinical cases in these species are often overlooked or misinterpreted, e.g., as *Listeria monocytogenes* infection (36), and may be more common than previously thought. Furthermore, ruminants are regarded as useful sentinel species for TBEV occurrence. They have a comparatively restricted range of

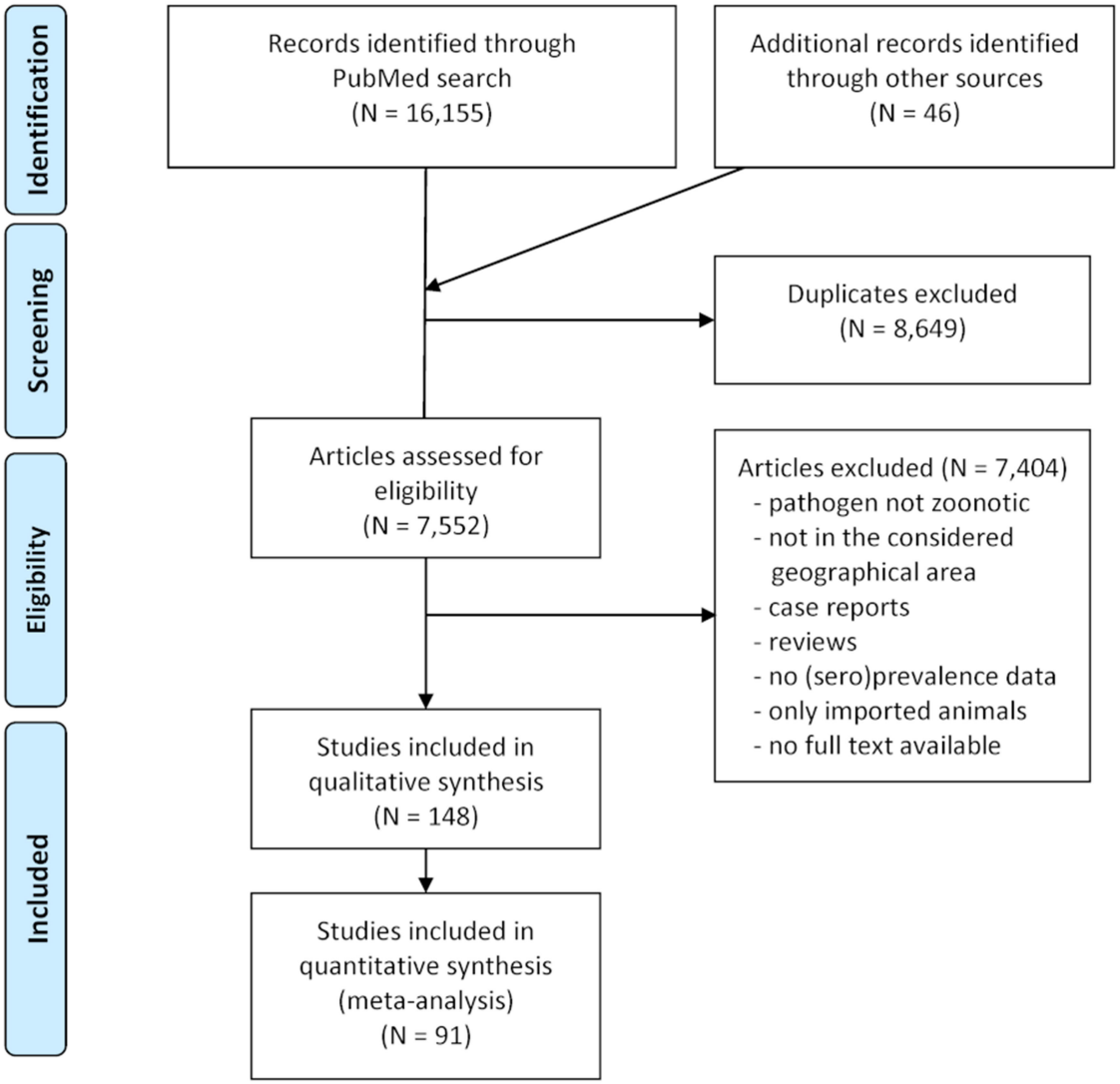

FIGURE 2 | PRISMA flow-chart detailing the selection process applied during the literature survey.

activity, i.e., they travel less than dogs and horses, and show persistence of antibodies for up to 28 months post infection (38). Therefore, several studies have been conducted on TBEV seroprevalence in domestic ruminants, particularly in goats and sheep (**Supplementary Table 1**).

Studies on cattle ($N = 8$, two from Norway, one each from Belgium, Finland, Hungary, Lithuania, the Netherlands, and Poland) reported seroprevalences ranging from 0.0% in the Netherlands (39) to 26.5% in Hungary (40) (**Supplementary Table 1**). One Norwegian study assessed TBEV excretion in milk and found 5.4% PCR-positive samples (41).

Seven studies reported seroprevalence data on sheep (two each from Sweden and Germany, one each from Hungary, Lithuania, and Slovakia), with values ranging from 0.0% in northern Germany (42) to 25.6% in farms with high lamb morbidity and mortality in Sweden (43). However, no confirmation of positive samples by SNT was performed in the latter study, so that cross-reactions with other flavivirus infections, e.g., louping ill, which has previously been detected in Norwegian sheep (44), cannot be ruled out. Seroprevalence in the 10 studies reporting data on goats (four from Germany, one each from Austria, Lithuania, the Netherlands, and Poland) ranged from 0.0% in the northern German federal state of Mecklenburg-Western Pomerania, which is not regarded as a TBEV risk area (45), to 14.6% in the Swiss canton of Ticino (46). Direct pathogen detection in sheep and goats, e.g., by PCR, was not reported in the considered studies.

Horses

As in ruminants, TBEV infections in horses are mostly asymptomatic (47). However, cases of encephalomyelitis with symptoms such as anorexia, ataxia, spasms, and epileptic seizures

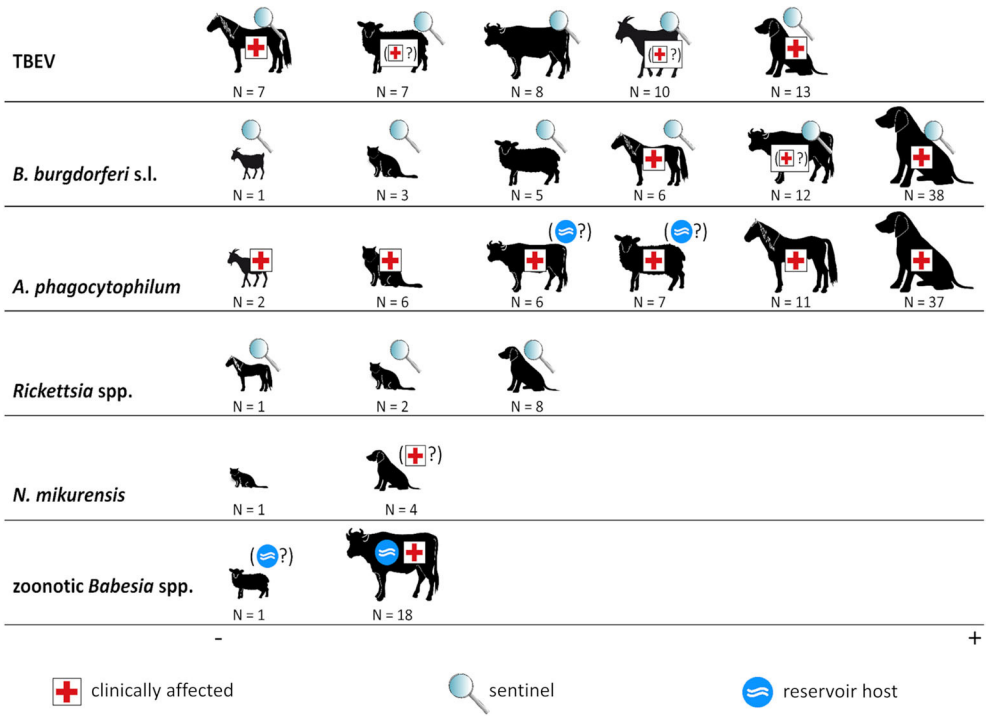

FIGURE 3 | Animal species and tick-borne pathogens. The size of animal icons corresponds to the number of studies. Symbols indicate the role of each animal species in the ecology of the respective pathogen. Symbols in brackets indicate that this role is uncertain. *Borrelia miyamotoi* is not shown as no (sero-)prevalence studies regarding this pathogen in domestic animals were retrieved.

have been described (48, 49). Mild neurologic deficits may persist after recovery (49).

The literature survey resulted in the identification of seven studies on TBEV seroprevalence in horses (three from Germany, two from Austria, one each from Hungary and Slovakia; **Supplementary Table 1**), with values ranging from 0.0% in Hungary (40) to 33.0% in a TBEV risk area in Bavaria, Germany (49). All of these studies confirmed positive samples by SNT (**Supplementary Table 1**).

Dogs and Cats

In dogs, a similar TBE disease course as in humans with severe, often fatal neurological manifestations due to encephalitis has been described (50). However, high seroprevalence rates in some areas indicate that only a small proportion of infected dogs develops disease, whereas most infections remain asymptomatic (47). Because dogs usually accompany their owners, they are regarded as valuable sentinels for human TBEV risk. However, as companions of man, they often have a travel history, which makes assessment of TBE risk in a certain area based on dog sera less reliable compared to other sentinel animals (30).

In the present survey, 13 studies presenting data on TBEV (sero-)prevalence in dogs were identified (three from Germany, two from the Czech Republic, one each from Austria, Belgium, Denmark, Finland, the Netherlands, Norway, and Poland, and one study reporting data on dogs from different European countries; **Supplementary Table 1**). Seroprevalence ranged from 0.0% in the Netherlands (39) to 53.6% in dogs with neurological signs in Germany (51). However, positive results were not confirmed by SNT in the latter study, so that the possibility of cross-reactions with other flaviviruses needs to be considered. Cross-reactions also appear probable in light of the high seroprevalence detected in healthy dogs (30.4%) in the same study, compared with an estimated overall seroprevalence of 1.4% based on asymptomatic dogs when positive samples were confirmed by SNT (**Figure 4**). Nevertheless, another study including dogs with neurological illness determined a TBEV infection rate of 12.6% by real-time PCR (52), indicating that TBEV prevalence among dogs with neurologic disease may be substantial.

No cases of TBEV infections in cats have been published to date. Preliminary data of a study including more than 200 cats from a TBE-endemic area in Germany showed no seropositive individuals (personal communication with Martin Pfeffer, Institute of Animal Hygiene and Veterinary Public Health, University of Leipzig, and Gerhard Dobler, Bundeswehr Institute of Microbiology, Munich).

Borrelia burgdorferi s.l.

The *B. burgdorferi* s.l. complex currently comprises 22 recognized species of spirochaetal bacteria (53), at least nine of which occur in European tick populations (54). Of those, *Borrelia afzelii* and *Borrelia garinii* are the most prevalent and constitute the most important agents of human LB throughout Europe (54, 55).

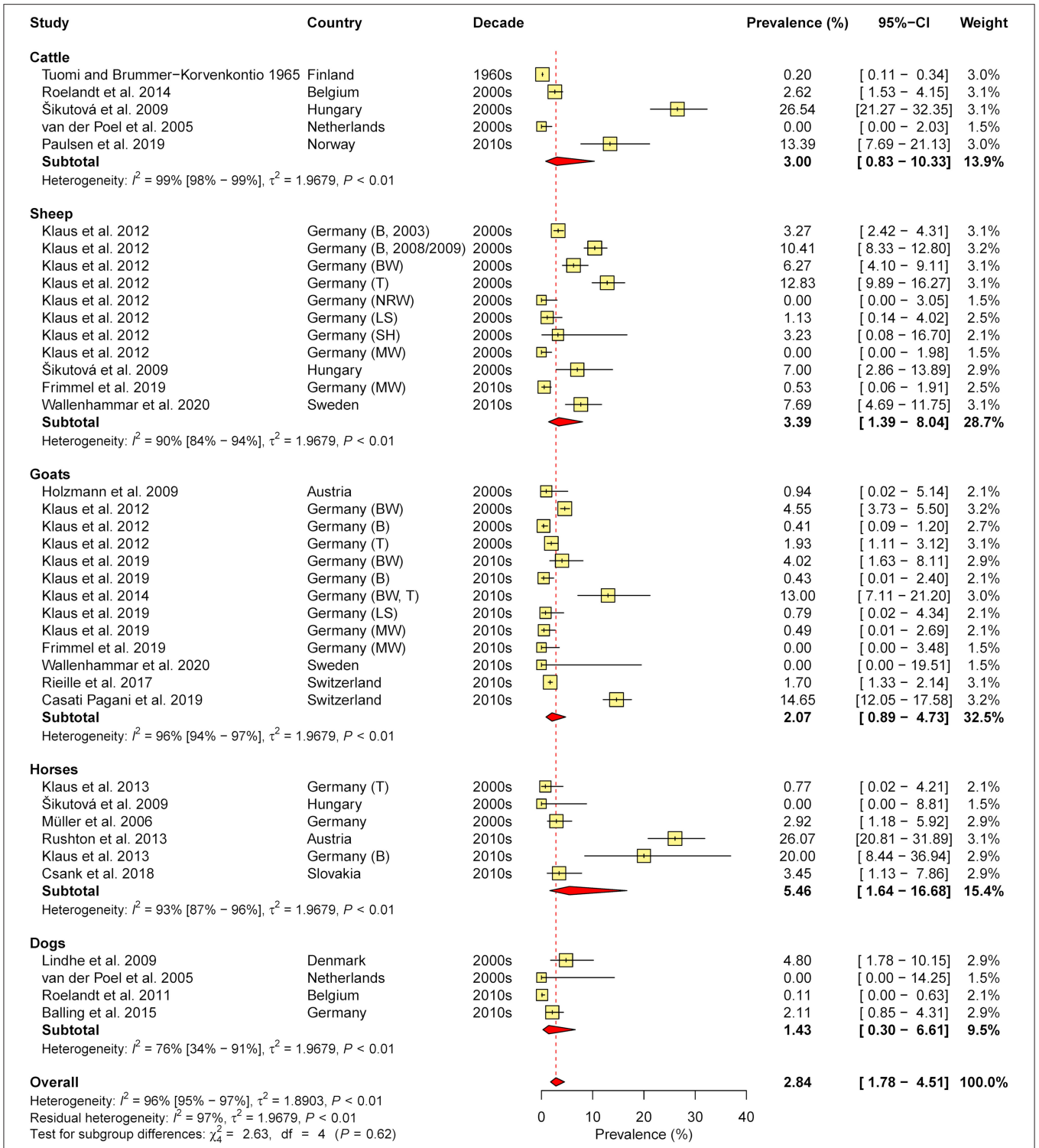

FIGURE 4 | Forest plot displaying the results of random-effects meta-analysis of tick-borne encephalitis virus seroprevalence in domestic animals, with subgroup analysis according to animal species. Individual study results are shown as yellow squares, corresponding in size to the weight of the study on the overall prevalence estimate. Error bars indicate 95% confidence intervals (CI). Pooled prevalences are shown as red diamonds and the red dotted vertical line indicates the estimated overall prevalence.

Borrelia burgdorferi s.l. predominantly circulates between *Ixodes* ticks (in Europe *I. ricinus*) and wild mammals (e.g., *B. afzelii*, *B. bavariensis*), birds (e.g., *B. garinii*, *B. valaisiana*), or reptiles (*B. lusitaniae*) as reservoir hosts (56).

In total, 53 studies reporting *B. burgdorferi* s.l. (sero-)prevalence rates in domestic animals were retrieved (**Supplementary Table 2**). Since a vaccine against borreliosis in dogs was introduced to the European market at the end of the 1990s, it is important to distinguish between vaccinated and naturally exposed dogs when evaluating seroprevalence (57). Antibodies against the variable major protein-like sequence expressed (VlsE) and one of its invariable regions, the C6 peptide, as well as the outer surface protein OspF indicate natural exposure, because these antigens are not present in the available vaccines (58). Therefore, dog seroprevalence studies conducted after 1995 were only considered in the meta-analysis if they were based on a C6 or OspF assay.

Meta-analysis of seroprevalence, including 48 animal cohorts (13 cattle, 3 sheep, 9 horse, 21 dog, and 2 cat cohorts) from 30 publications, estimated an overall seroprevalence of 12.4% (95% CI: 0.1–17.2%) with a significant level of heterogeneity (I^2 = 98.0%; 95% CI: 97.8–98.3%; $P < 0.001$). Subgroup analysis indicated a significant effect of animal species ($\chi^2 = 24.4$, df = 4, $P < 0.001$), with a lower seroprevalence in dogs (5.8%, 95% CI: 3.7–8.9%) than in cattle (23.6%, 95% CI: 14.8–35.4%) and horses (22.5%, 95% CI: 12.6–36.9%; **Figure 5**). In contrast, there were no significant differences according to decade ($\chi^2 = 4.7$, df = 3, $P = 0.193$), diagnostic test used ($\chi^2 = 3.3$, df = 2, $P = 0.193$), and geographical region ($\chi^2 = 2.9$, df = 2, $P = 0.230$) when analyzing all 48 cohorts.

Due to the significant effect of animal species, further analyses were conducted on the data subset for dogs, as this species had the largest sample size (21 cohorts from 16 studies). In the data subset on dogs, a significant effect of diagnostic test was found ($\chi^2 = 7.5$, df = 2, $P = 0.023$), with a lower seroprevalence determined by a C6-based rapid ELISA (3.1%, 95% CI: 1.5–6.0%) than by conventional ELISA (11.2%, 95% CI: 6.0–20.0%; **Supplementary Figure 1**). In contrast, no significant effect of geographical region ($\chi^2 = 2.4$, df = 2, $P = 0.302$) nor decade of sampling ($\chi^2 = 3.5$, df = 3, $P = 0.324$) was found in dogs.

Ruminants

Species-specific *Borrelia*-host associations are thought to be primarily driven by variation in resistance toward host defense mechanisms, particularly complement (59). Different species of the *B. burgdorferi* s.l. complex differ in their level of susceptibility toward inactivation of sera from certain animals *in vitro* [e.g., (60, 61)]. Notably, all tested members of the *B. burgdorferi* s.l. complex display high sensitivity toward serum complement from several ruminant species, including deer, bison, and cattle (59). Thus, these species seem to be irrelevant as *B. burgdorferi* s.l. reservoirs. In contrast, ticks feeding on these species may even lose their *Borrelia* infection, as suggested by prevalence patterns in engorged ticks recovered from deer, cattle and goats vs. prevalence in questing ticks (62, 63). Therefore, it has been suggested that an increase in grazing domestic ruminants may lower the risk of Lyme disease acquisition in a certain area (62). However, this does not apply to all ruminants, as several *B. burgdorferi* s.l. species are resistant toward serum of sheep and their wild relatives, the mouflon (59), and sheep may sustain natural *B. burgdorferi* s.l. cycles in the absence of other tick hosts (64).

Despite their apparent ability to eliminate *B. burgdorferi* s.l. spirochaetes, active *B. burgdorferi* sensu stricto (s.s.), and *B. afzelii* infections with associated symptoms (skin erythema, fever, acute lameness due to arthritis) have been described in cattle in rare cases (65, 66). Other studies draw a connection between serological evidence of *B. burgdorferi* s.l. infection and clinical signs such as lameness and swollen joints (67), but causality in these cases is extremely questionable. Experimental infections of cattle with *B. burgdorferi* s.s., *B. garinii*, and *B. afzelii* produced no clinical signs (68). Similarly, clinical manifestations of LB have been suspected in sheep (69), but experimental infections failed to produce any symptoms (70). Regarding goats, neither clinical cases nor infection experiments have been published to the authors' knowledge. Overall, clinical relevance of *B. burgdorferi* s.l. for ruminants is questionable. Nevertheless, they seroconvert upon contact with the pathogen (68) and may therefore be regarded as sentinels for pathogen presence.

In the present survey, 12 studies reporting seroprevalence rates in cattle were retrieved (six from Germany, one each from France, Poland, Slovakia, Sweden, and Switzerland and one reporting data from Poland as well as Slovakia; **Supplementary Table 2**). Reported seroprevalences ranged from 1.1% in northern Sweden (71) to 66.0% in Germany (72). Five studies reported data on sheep (two from Sweden, one each from France, Norway and Slovakia), with seroprevalences ranging from 0.0% in healthy sheep in central Sweden to 84.6% in lambs with arthritis on the island of Gotland, Sweden (71). The highest seroprevalence in asymptomatic sheep was determined in France (56.5%) (73). The only study containing data on goats reported a 17.2% seroprevalence rate in this species in Slovakia (74). Direct pathogen detection, e.g., by PCR, was not reported for ruminants in the considered studies.

Horses

In horses, a broad spectrum of clinical manifestations, including arthritis, lameness, anterior uveitis, encephalitis, and abortion, has been attributed to *B. burgdorferi* s.l. infection; however, in many cases a causal relationship has not been conclusively proven (75). Experimental inoculations of ponies led to systemic infection, persisting for at least 9 months, but did not induce any clinical signs nor histopathological alterations, except for skin lesions (76, 77). More recently, however, several case reports of equine neuroborreliosis with *B. burgdorferi* s.l. detection in the central nervous system have been published (78–81). In one of these studies, the species was identified as *B. burgdorferi* s.s. and high spirochaetal loads were demonstrated in tissues with inflammation (79). However, all of these cases occurred in North America, thus, it remains unclear if European *B. burgdorferi* s.s. isolates and other LB agents are capable of causing clinical manifestations in horses. Evidence from *in vitro* studies suggests that all tested *B. burgdorferi* s.l. species are susceptible to inactivation by equine complement,

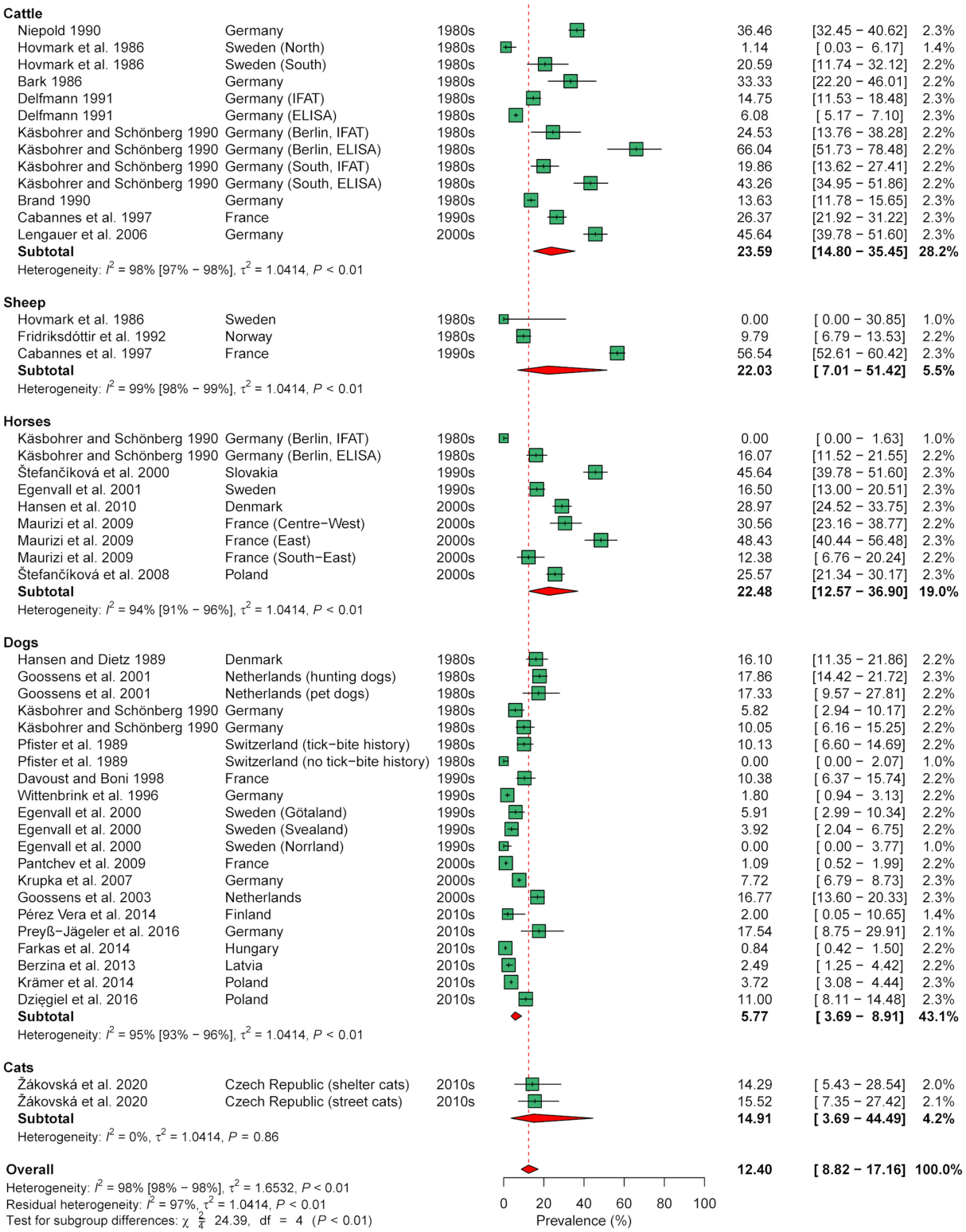

FIGURE 5 | Forest plot displaying the results of random-effects meta-analysis of *B. burgdorferi* s.l. seroprevalence in domestic animals, with subgroup analysis according to animal species. Individual study results are shown as green squares, corresponding in size to the weight of the study on the overall prevalence estimate. Error bars indicate 95% confidence intervals (CI). Pooled prevalences are shown as red diamonds and the red dotted vertical line indicates the estimated overall prevalence.

except for *B. burgdorferi* s.s. which displays an intermediate sensitivity (59).

In contrast to the paucity of conclusive clinical equine borreliosis cases in Europe, six studies evaluating seroprevalence rates in this species were retrieved (one each from Poland, Denmark, France, Germany, and Sweden and one reporting data from both Poland and Slovakia). Reported seroprevalence rates ranged from 12.4% in south-eastern to 48.4% in eastern France (82). Studies employing direct detection methods, e.g., PCR, were not retrieved.

Dogs and Cats

According to a consensus statement by the American College of Veterinary Internal Medicine (ACVIM), most *B. burgdorferi* s.l. seropositive dogs and cats display no clinical signs, neither after natural nor experimental infections (58). A small subset of dogs, however, may develop arthritis due to *B. burgdorferi* s.s. infection, as demonstrated by experimental infections (83–85). Furthermore, nephritis is putatively associated with *B. burgdorferi* s.s. infections, however, well-documented case reports are rare and no experimental studies exist in this regard (58). To the authors' knowledge, evidence for clinical manifestations due to other species of the *B. burgdorferi* s.l. complex in dogs is lacking to date, although *B. afzelii* has been isolated from a dog with clinical signs attributable to LB in Europe (86). Furthermore, DNA of *B. valaisiana* and *B. garinii* has been amplified from symptomatic dogs [(87, 88); see below]. In experimental studies, dogs have been shown to transmit borreliae to ticks, indicating a potential reservoir function (89). However, in the light of abundant wild reservoir hosts, such as rodents and birds, the impact of pet dogs on the natural epidemiological cycle is probably neglectable (90).

Cats have been infected experimentally and show seroconversion, but no clinical signs of infection (91, 92). Some case reports have attributed clinical signs in cats, such as cardiac arrhythmia and lameness, to *B. burgdorferi* s.l. infection based on seropositivity, PCR detection of the pathogen and/or resolution upon antibiotic treatment (93, 94); however, as in many other cases a causative relationship remains speculative.

Nevertheless, seroprevalence in domestic dogs and cats may provide an estimate of human LB risk. A strong association was found between canine seroprevalence and mean LB incidence on county level in the United States of America (95).

In the present survey, 38 studies reporting *B. burgdorferi* s.l. (sero-)prevalences in dogs were compiled (seven each from Germany and Poland, four from Sweden, three each from the Netherlands, Slovakia, and Switzerland, two each from the Czech Republic and France and one each from Austria, Denmark, Finland, Hungary, Latvia, Lithuania, and Norway). Seroprevalences ranging from 0.0% in northern Norway (96) to 57.5% in Bernese Mountain dogs in Switzerland (97) were reported. This dog breed seems to have a predisposition for *B. burgdorferi* s.l. infection, as demonstrated by several studies (97–99), although the reasons for this predisposition are unknown (58). As reported above, a significant effect of the utilized diagnostic test on the seroprevalence in dogs was found (section Tick-Borne Encephalitis Virus, **Supplementary Figure 1**), with lower rates determined by the most recently developed test, a C6-based rapid ELISA, than by conventional ELISA or IFAT (57).

Some studies also reported PCR detection rates in dogs, ranging from 0.0% in asymptomatic dogs (100) to 60.0% in animals with clinical signs attributable to borreliosis (87). DNA of *B. burgdorferi* s.s., *B. afzelii*, *B. valaisiana*, and *B. garinii* was amplified in these cases (87, 88).

Three studies reported data on cats (one each from the Czech Republic and the United Kingdom, one based on cats from different European countries). Two of these assessed seroprevalence, which ranged from 2.2% in symptomatic cats from different European countries, determined by a C6-based rapid ELISA (101), to 19.2% of cats presented at veterinary clinics in the Czech Republic, determined by conventional ELISA (102). The third study determined a 1.6% *B. burgdorferi* s.l. infection rate in systemically ill cats in the United Kingdom by PCR (103).

Borrelia miyamotoi

Borrelia miyamotoi was first isolated from *Ixodes persulcatus* in Japan in 1995 (19), but pathogenicity for humans was not recognized until 2011 (20). Since then, this pathogen has been known to cause a febrile illness (104) and, more recently, it has been associated with meningoencephalitis in immunocompromised patients (105, 106). Similar to *B. burgdorferi* s.l., rodents and birds seem to be reservoir hosts for this spirochaete (104). Little is known about the role of domestic animals regarding *B. miyamotoi* ecology. In contrast to *B. burgdorferi* s.l., prevalence of *B. miyamotoi* in ticks is not negatively affected by the presence of cattle (62, 107). *Borrelia miyamotoi* DNA has been detected in two healthy cats in the USA (108). However, infection of other domestic animals has not been documented so far to the authors' knowledge and no (sero-)prevalence studies have been conducted.

Anaplasma phagocytophilum

Anaplasma phagocytophilum is regarded as an important zoonotic pathogen, causing disease in humans, domestic ruminants, horses, dogs, and rarely in cats, while wild mammals act as reservoir hosts (109). This obligate intracellular rickettsial pathogen replicates in neutrophilic granulocytes and leads to thrombocytopenia, leukopenia, anemia, and immunosuppression associated with variable clinical signs (110). The epidemiology of *A. phagocytophilum* is complex due to the circulation of various strains and ecotypes, and shows considerable differences between Europe and North America (109). Human, equine, and canine granulocytic anaplasmosis have been described in Asia, Europe, and North America, while domestic ruminants only seem to be affected in Europe (109). Based on *groEL* genetic sequences, eight haplotype clusters of *A. phagocytophilum* were recently identified, and most isolates from humans and domestic animals belong to cluster 1, while other clusters, e.g., containing samples from roe deer, do not seem to be zoonotic (111).

Overall, 65 studies reporting (sero-)prevalence rates of *A. phagocytophilum* in domestic animals in the study region were retrieved (**Supplementary Table 3**). The meta-analysis based on seroprevalence rates of asymptomatic animals, including data

on 35 animal cohorts (2x cattle, 3x sheep, 9x horses, 18x dogs, 3x cats) from 28 publications, yielded an estimated overall seroprevalence of 16.2% (95% CI: 11.7–22.0%), with a significant level of heterogeneity (I^2 = 98.6%; 95% CI: 98.4–98.8%; P < 0.001). Although rather high seroprevalence rates were detected in sheep as compared to the other animal species, subgroup analyses indicated no significant difference (test for subgroup differences: χ^2 = 6.4, df = 4, P = 0.171; **Figure 6**). Likewise, significant differences according to geographical region (χ^2 = 0.38, df = 2, P = 0.828), the decades of sampling (χ^2 = 0.78, df = 2, P = 0.676; **Figure 6**) or the type of diagnostic test were not detected (χ^2 = 4.98, df = 2, P = 0.083).

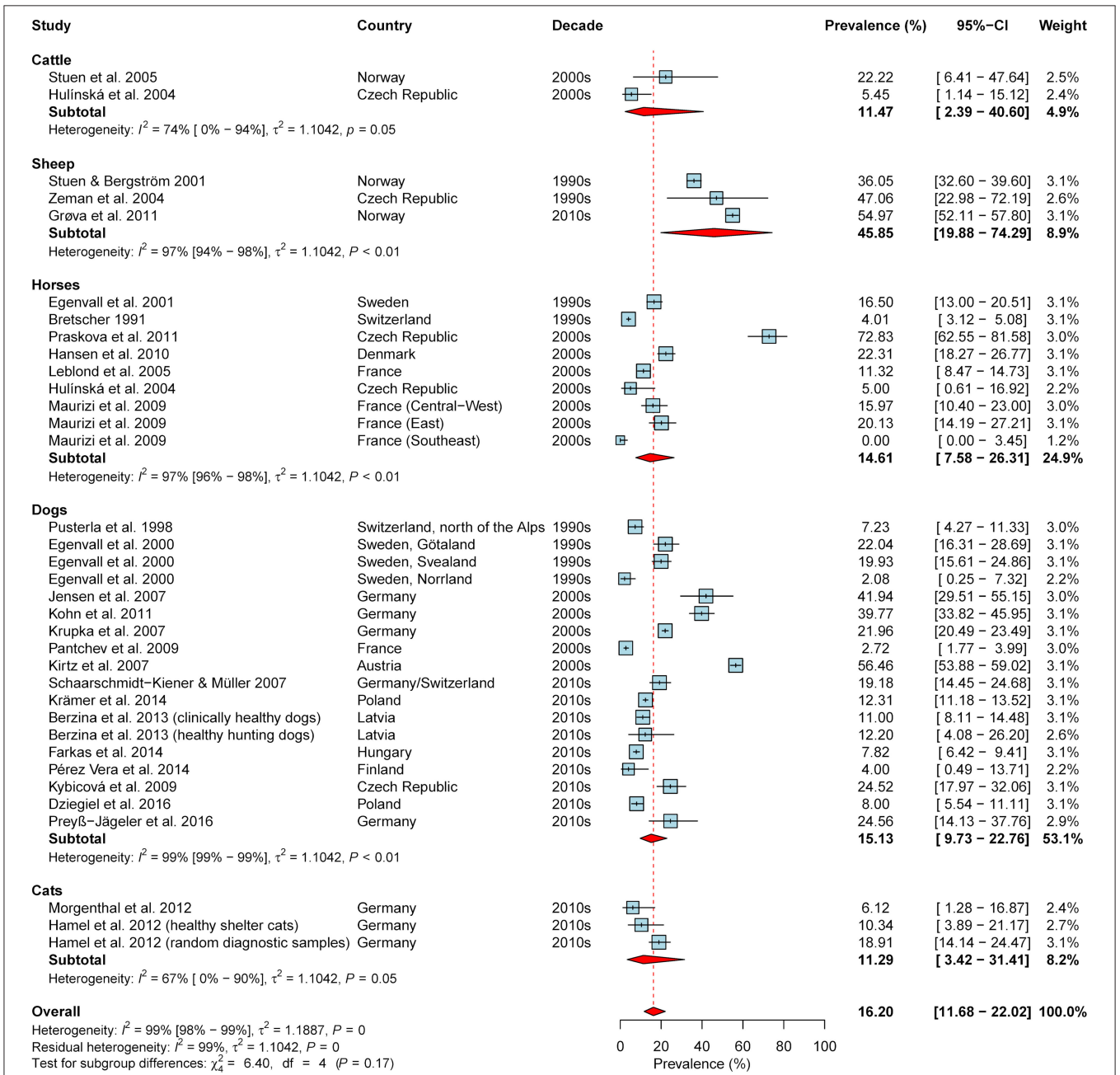

FIGURE 6 | Forest plot displaying the results of random-effects meta-analysis of *A. phagocytophilum* seroprevalence in domestic animals, with subgroup analysis according to animal species. Individual study results are shown as blue squares, corresponding in size to the weight of the study on the overall prevalence estimate. Error bars indicate 95% confidence intervals (CI). Pooled prevalences are shown as red diamonds and the red dotted vertical line indicates the estimated overall prevalence.

Ruminants

In domestic ruminants, the disease caused by *A. phagocytophilum* is known as tick-borne fever and presents with fever, anorexia, abortion and a drop in milk production (109). In sheep but not in cattle, immunosuppression is also common, frequently resulting in secondary infections (112), which may be fatal in some cases (113). After recovery, sheep develop persistent infections with recurrent phases of high bacteraemia for at least 1 year, suggesting they may also act as a pathogen reservoir (114, 115). Furthermore, field data suggest that cattle can also become persistently infected or are frequently re-infected, indicating a possible reservoir function, which needs to be explored further (116). Recent genetic analyses have shown that *A. phagocytophilum* isolates from sheep and cattle in Europe cluster with isolates from humans, dogs, and horses (117).

In total, six studies reported (sero-)prevalence rates for cattle (two studies from Sweden and one study each from Belgium, the Czech Republic, Norway, and Switzerland), seven for sheep (two each from the Czech Republic and Norway, one each from Denmark, Germany and Sweden), and two for goats (Switzerland and United Kingdom, **Supplementary Table 3**).

In cattle, seroprevalence rates varied from 5.5% in the Czech Republic (118) to 100% in a clinically-affected herd in Norway (119). The highest published seroprevalence rate in asymptomatic cattle was 63.0% in a Swiss study (120). Three studies reported prevalence rates based on PCR, which ranged from 5.5% in the Czech Republic (118) to 85.7% in symptomatic animals in Sweden (121).

In sheep, prevalence of *A. phagocytophilum* antibodies ranged from 36.0% in Norway (122) to 100% in a flock in the Czech Republic (123). PCR-determined prevalence rates in sheep varied from 2.9% in the Czech Republic/Slovakia (124) to 41.9% in sheep flocks with high lamb morbidity and mortality in Sweden (43). Regarding goats, a PCR-determined prevalence of 5.6% was reported from Switzerland (125). In addition, four of five feral goats caught in Northern Ireland, UK, were PCR-positive (126).

However, (sero-)prevalence rates in ruminants may be difficult to compare between studies, since marked seasonal variation has been found. For example, in a Swiss study, seroprevalence of two cattle herds varied between 16% before and 63% at the end of the grazing season (120). As most ruminants are housed during winter, determined (sero-)prevalence rates greatly depend upon the season of sampling.

In addition, serologic cross-reactivity with other *Anaplasma* spp., e.g., *Anaplasma marginale* in cattle (127) and probably also *Anaplasma ovis* in sheep (128), needs to be considered. In Europe, *A. marginale* occurs mainly in the Mediterranean region, but also in Switzerland, Austria, and Hungary (129). *Anaplasma ovis* has been detected in France (130) as well as Slovakia (124) and Hungary (129).

Horses

In horses, *A. phagocytophilum* may cause an acute febrile disease with depression, anorexia, ataxia, icterus, and lower limb oedema, which is usually self-limiting (131). Similar to sheep, horses may develop persistent subclinical infections with recurrent bacteraemia after spontaneous recovery from acute disease (132). Notably, equine *A. phagocytophilum* strains seem to be similar or identical to those causing disease in humans and dogs (133).

Most equine granulocytic anaplasmosis (EGA) cases have been reported from European countries (131). In the present investigation, 11 studies reporting (sero-)prevalence rates of *A. phagocytophilum* in horses were identified, two each from the Czech Republic, France, and Sweden and one each from Denmark, France, the Netherlands, Switzerland, and Sweden. Another two studies reported data from Poland, Slovakia and the Ukraine and from Germany, Poland and the Ukraine, respectively. Reported seroprevalence rates ranged from 4.0% in Switzerland (134) to 72.8% in the Czech Republic (135). Remarkably, the latter study was based on healthy horses. Prevalences determined by PCR ranged from 0.0% in the Ukraine (136) to 62.9% in Sweden, whereby the latter study was based on horses presenting symptoms attributable to EGA.

Dogs and Cats

Canine granulocytic anaplasmosis (CGA) is regarded as one of the most important vector-borne diseases in Europe. While many cases are probably subclinical, acute febrile illness may also occur (137). Frequent clinical signs are lethargy, anorexia, and pale mucous membranes, sometimes accompanied by enlarged lymph nodes, bleeding (petechias, epistaxis), and immune-mediated arthritis (137). Similar symptoms may be seen in cats infected with *A. phagocytophilum*, although experimental studies indicate that symptoms are usually mild (138). Experimental infections have been shown to persist for at least $5^1/_2$ months in dogs (139) and 3 months in cats (91). The *A. phagocytophilum* strains causing disease in dogs seem to be zoonotic (117).

In total, 37 studies on *A. phagocytophilum* (sero-)prevalence in dogs were retrieved from the literature, including 10 from Germany, six from Poland, five from Sweden, two each from Austria, the Czech Republic, Hungary Slovakia, and Switzerland, and one study each from Finland, France, Latvia, Lithuania, and the United Kingdom. One study included dogs from Germany and Switzerland. Reported seroprevalence rates, determined either by IFAT or (rapid) ELISA, ranged from 2.1% in northern Norway (96) to 56.5% in Austria (140) (**Supplementary Table 3**). However, the latter study included dogs with symptoms possibly related to CGA. Since infection may not lead to clinical disease, and CGA is characterized by rather unspecific symptoms, several studies did not find a significant difference in seroprevalence between apparently healthy animals and those presenting some form of illness [e.g., (100, 141)]. Nevertheless, cohorts of symptomatic dogs were excluded from the meta-analysis on seroprevalence. Serologic tests available for assessing *A. phagocytophilum* exposure may cross-react with *Anaplasma platys* antibodies, therefore, seroprevalence may be overestimated. However, as *A. platys* transmission in Europe is restricted to Mediterranean countries (137), this pathogen only plays a role as an imported bacterium in the countries considered in this review, with the exception of southern France. Nevertheless, with increased travel activity and import of dogs by animal welfare organizations, this should be kept in mind when interpreting seroprevalence rates.

Prevalence rates determined by PCR ranged from 0.0% in Switzerland and Poland (142, 143) to 66.7% in dogs with symptoms attributable to CGA in Sweden (121). The highest prevalence in apparently healthy dogs was 12.2% in a group of Latvian hunting dogs (144).

Regarding cats, six studies were found (four from Germany, one from the United Kingdom and one from Ireland). These studies reported IFAT-determined seroprevalence rates from 6.1% in healthy cats (145) to 18.9% in random diagnostic samples (146). PCR detection rates varied from 0.0% in healthy cats (145) to 4.3% in necropsy samples from shelter cats (147).

Neoehrlichia mikurensis

Neoehrlichia mikurensis, a member of the family Anaplasmataceae, was first discovered in the early 2000s, recognized as a human pathogen in 2010 and recently cultivated in tick cell lines as well as human endothelial cells (148). It occurs in *I. ricinus* populations throughout Europe and human cases, mainly involving immunosuppressed patients, have been reported from several countries (149). Regarding domestic animals, knowledge on the relevance of *N. mikurensis* as a pathogenic agent and respective prevalence data are scarce.

In the present literature survey, five studies investigating *N. mikurensis* occurrence in domestic animals (four in dogs and one in cats) by PCR, but no seroprevalence studies, were obtained (**Table 1**). Infections in dogs seem to be rare, as only 0.3% of 1,023 dogs in Germany (151) and 0.1% of 889 dogs in Switzerland were infected (142). The positive dog in Switzerland was splenectomised (142). Another positive dog died of haemolytic anemia in the Czech Republic (150) and in a case report from Germany, canine *N. mikurensis* infection was associated with neutropenia and thrombocytopenia (23). However, the clinical relevance of *N. mikurensis* in dogs is still unclear (23, 142). A single study also tested spleen samples from 141 cats in Germany, but *N. mikurensis* was not detected (147). Other domestic animal species have not been investigated so far to the authors' knowledge.

Rickettsia spp.

Several tick-transmitted human-pathogenic *Rickettsia* spp. occur in Europe. While the causative agent of Mediterranean spotted fever, *Rickettsia conorii*, has been known since the beginning of the twentieth century, several further *Rickettsia* spp. and their associated syndromes were described in the 1990s and 2000s (153). In central and northern Europe, *R. helvetica*, transmitted by *I. ricinus*, is probably the most frequent species. It causes a mild febrile illness in humans and is only sometimes associated with skin rash (154). In addition, *I. ricinus* may transmit *Rickettsia monacensis*, which leads to a clinical picture similar to Mediterranean spotted fever (153). *Rickettsia slovaca* and *R. raoultii,* causative agents of scalp eschar and neck lymphadenopathy (SENLAT), are transmitted by *Dermacentor* species (153).

With the exception of dogs, domestic animals do not seem to be susceptible to disease caused by human-pathogenic *Rickettsia* species. In dogs, infection with *Rickettsia rickettsii*, which causes Rocky Mountain spotted fever in North America, leads to clinical signs similar to those in humans (155). In addition, *R. conorii* has been associated with canine febrile illness (156). Dogs are also capable of transmitting *R. conorii* to ticks and may thus exert a reservoir function (157). However, canine disease due to the *Rickettsia* spp. relevant in central and northern Europe or a respective reservoir function have not been reported to the authors' knowledge.

Overall, domestic animals can mainly be regarded as sentinels for human exposure to *Rickettsia* spp. in central and northern Europe. However, only nine studies were identified (three from Germany, two from Switzerland, one each from the Czech Republic, Ireland, Poland, and Sweden), reporting data on horses, dogs, and cats (**Table 2**). Regarding domestic ruminants, no studies on *Rickettsia* (sero-)prevalence in the considered geographical region were obtained. The only study on horses reported a 36.5% *R. helvetica* seroprevalence in Sweden (158).

In dogs, a high level of exposure to spotted-fever group rickettsiae was reported, with seroprevalence rates ranging from 17.0 to 93.9% (158, 160). When *R. helvetica*-specific antigens were used, seroprevalences of 17.0% (158) and 66.0% (160) were determined. Despite this high level of exposure, *Rickettsia* DNA (mainly *R. raoultii*) was found in only 0.8% of tested dogs in Germany (151), whereas two PCR-based studies from Switzerland (142, 162) and one study from Poland (161) reported

TABLE 1 | (Sero-)prevalence studies on *Neoehrlichia mikurensis* in domestic animals in temperate and cold regions of Europe.

Country	Region	Year(s) of sampling	Method(s)	Positive/total	Prevalence	Comment(s)	References
DOGS							
Czech Republic	NA	2009–2012	PCR	1/19	5.3%	Dogs with fatal immunhaemolytic anemia	(150)
Germany	Brandenburg	2013–2014	HRM PCR	3/1,023	0.3%		(151)
Hungary	Somogy	NA	PCR	0/90	0.0%	*Candidatus* Neoehrlichia lotoris-like detected in 6 dogs	(152)
Switzerland	Zurich	2005–2006	Real-time PCR	1/889	0.1%	The positive dog was splenectomised	(142)
CATS							
Germany	Berlin	2006–2008	HRM PCR	0/141	0.0%	Spleen samples from shelter cats	(147)

HRM PCR, high-resolution melt PCR.

TABLE 2 | (Sero-)prevalence studies on tick-transmitted *Rickettsia* spp. in domestic animals in temperate and cold regions of Europe.

Country	Region	Year(s) of sampling	Method(s)	Positive/total	Prevalence	Comment(s)	References
HORSES							
Sweden	NA	2010–2011	IFAT	23/63	36.5%	*R. helvetica* used as antigen	(158)
DOGS							
Czech Republic	NA	2009–2012	PCR	0/19	0.0%	Dogs with fatal immunhaemolytic anemia	(150)
Germany	Nationwide	2012–2014	ELISA[a]	469/602	77.9%	Dogs that never left Germany	(159)
	Nationwide	2012–2014	Micro-IFAT	568/605	93.9%	Same samples as in (159); clearly differentiable samples: 66.0% *R. helvetica*, 2.8% *R. raoultii*, 1.6% *R. slovaca*	(160)
	Brandenburg	2013–2014	PCR	8/1,021	0.8%	Identified species: 7x *R. raoultii*, 1x *R. felis*	(151)
Poland	North-Western Poland	NA	PCR	0/100 (group 1), 0/92 (group 2), 0/50 (group 3)	0.0% (group 1), 0.0% (group 2), 0.0% (group 3)	Group 1: healthy shelter dogs, group 2: suspected borreliosis, group 3: diagnosed babesiosis	(161)
Sweden	NA	2010–2011	IFAT	17/100	17.0%	*R. helvetica* used as antigen	(158)
Switzerland	Zurich	2005–2006	Real-time PCR[b]	0/889	0.0%		(142)
	Zurich	NA	Real-time PCR[b]	0/884	0.0%		(162)
CATS							
Ireland	Dublin	2008	PCR	0/121	0.0%		(163)
Sweden	NA	2010–2011	IFAT	19/90	22.1%	*R. helvetica* used as antigen	(158)

[a] *Commercially available, detects all spotted-fever group rickettsiae.*
[b] *Specific for R. helvetica.*
ELISA, enzyme-linked immunosorbent assay; IFAT, immunofluorescence antibody test.

0.0% prevalence. Similarly, *R. helvetica* antibodies were detected in 22.1% of tested cats in Sweden (158), but no *Rickettsia* DNA was amplified from 121 tested cats in Ireland (163). Therefore, it seems unlikely that dogs and cats contribute to the epidemiology of tick-transmitted rickettsioses as reservoir hosts in northern and central Europe.

Zoonotic *Babesia* spp.

Piroplasms of the genus *Babesia* are tick-transmitted protozoan parasites, which usually display a high degree of host specificity. Nevertheless, a few species are zoonotic, predominantly affecting immunocompromised patients (24). In Europe, most human infections are caused by the cattle parasite *B. divergens* (154). On the American continent, human babesiosis due to *Babesia microti*, which is rodent-associated, is more common. *Babesia microti* also occurs in Europe. However, clinically symptomatic human *B. microti* infections reported in Europe were mostly acquired in the Americas, so it is unclear whether European *B. microti* strains are human-pathogenic (154). Furthermore, *Babesia venatorum*, a parasite of deer, has been recognized as a human pathogen in immunocompromised patients in Europe (154).

Regarding domestic animals, only cattle are affected by and act as reservoirs for a *Babesia* spp. with zoonotic relevance, namely *B. divergens*, whereas the species parasitizing horses, sheep, goats, and dogs are not zoonotic. No cat-specific *Babesia* species are distributed in Europe. *Babesia microti* DNA has been detected in cats in southern Europe (e.g., in Italy) but the relevance of this finding remains unclear (164).

In cattle, *B. divergens* infection may lead to severe haemolytic anemia, which can be fatal (165, 166). Symptoms consist of fever, pale or jaundiced mucous membranes, anorexia, weakness, elevated heart and respiratory rates and hemoglobinuria, hence the colloquial name of the disease, "redwater" (165). Recovering animals acquire immunity, which is maintained by repeated pathogen exposure (165). Calves under the age of ∼9 months display higher resistance toward clinical disease and are subsequently immunologically protected (167, 168), thus, clinical disease in endemic situations usually only occurs in immunologically naïve animals, which were either recently introduced to the area or had no access to pasture during the first year of life (165).

In the present survey, 18 studies reporting *Babesia* (sero-)prevalence data in cattle were retrieved (four each from France, Germany, and the United Kingdom, two from Belgium, and one each from Hungary, Norway, Sweden, and Switzerland; **Table 3**). Reported seroprevalence rates, determined mostly by IFAT, ranged from 0.0% in asymptomatic animals in Northern Germany (177) to 100% in animals presenting with acute babesiosis in France (172). The highest seroprevalence in randomly chosen individuals, but from a region where babesiosis was known to occur, was 90.6% at the end of the grazing season (167). The latter study also showed that seroprevalence varied throughout the year, similar to the pattern described above for *A. phagocytophilum* (120), making different studies difficult to compare. Keeping this draw-back in mind, an overall seroprevalence of 7.4% (95% CI: 2.6–19.2%) was estimated based on 11 healthy or randomly chosen

TABLE 3 | (Sero-)prevalence studies on zoonotic *Babesia* spp. in domestic animals in temperate and cold regions of Europe.

Country	Region	Year(s) of sampling	Method(s)	Positive/total	Prevalence	Comment(s)	Referencea
Cattle (*B. divergens*)							
Belgium	Central Belgium	1988	IFAT	136/1,721	7.9%		(169)*
	Southern Belgium	2010	IFAT	7/65 (spring), 13/65 (summer), 8/65 (autumn)	10.7% (spring), 20.0% (summer), 12.3% (autumn)	Farms with a known history of babesiosis or anaplasmosis	(170)
France	Nationwide	1988	Blood smears / inoculation in gerbils	374/424	88.2%	Animals treated for clinical babesiosis	(171)
	Sarthe	1991	ELISA	115/200	57.5%	Farms with clinical babesiosis during the last 5 years	(171)
	Ille-et-Vilaine	2001–2002	IFAT, *in vitro* culture	19/19 (IFAT, group 1), 31/77 (IFAT, group 2), 19/19 (culture, group 1), 31/77 (culture, group 2)	100% (IFAT, group 1), 40.3% (IFAT, group 2), 100% (culture, group 1), 40.3% (culture, group 2)	Group 1: acute babesiosis, group 2: asymptomatic	(172)*
	Mid-eastern France	2001–2002	IFAT, PCR	18/254 (IFAT), 12/254 (PCR)	7.1% (IFAT), 4.7% (PCR)		(173)*
	Western France	2007	IFAT	102/711	14.3%		(174)*
Germany	Bavaria	1982	IFAT, ELISA	211/1,616	13.1%		(175)*
	Northern Germany	1984–1985	IFAT	108/251	43.0%	Farms with history of babesiosis, includes vaccinated animals	(176)
	Northern Germany	1988–1990	IFAT	0/212 (group 1), 0/354 (group 2), 8/200 (group 3)	0.0% (group 1), 0.0% (group 2), 4.0% (group 3)	Group 1: *Borrelia*-positive animals, group 2: farms with suspected babesiosis, group 3: farms with history of babesiosis	(177)*
	Bavaria	2002	IFAT	1/287	0.4%		(178)*
Hungary	Northeastern Hungary	2005	IFAT	2/654	0.3%		(179)*
Norway	Southern Norway	2004–2005	IFAT	84/306	27.4%		(180)*
Sweden	Southern Sweden	NA	Real-time PCR	38/71	53.5%	Includes 39 cattle with symptoms of babesiosis	(181)
Switzerland	Jura Canton	1981	IFAT	98/289 (April), 190/327 (July), 309/341 (December)	33.9% (April), 58.1% (July), 90.6% (December)		(167)
United Kingdom	Scotland	1976	IFAT	290/368	78.8%	Two farms which experienced babesiosis outbreak	(182)
	Scotland	NA	IFAT	2,522/22,044	11.4%		(183)*
	Northern Ireland	1978	IFAT	5,731/18,000	31.8%		(184)*
	Scotland	2014	PCR	0/107	0.0%	Farms with a known history of babesiosis or anaplasmosis	(185)
SHEEP							
United Kingdom	Scotland	2014	PCR	11/93	11.8%	Identified as *B. venatorum*	(185)

**Included in meta-analysis of seroprevalence.*

ELISA, enzyme-linked immunosorbent assay, IFAT, immunfluorescence antibody test.

cattle cohorts (**Supplementary Figure 2**). A significant level of heterogeneity was detected (I^2 = 99.6%; 95% CI: 99.6–99.7%; $P < 0.001$). No significant temporal change in seroprevalence was detected from the 1970s to the 2000s (test for subgroup differences, $\chi^2 = 0.96$, df = 2, $P = 0.619$), although a decline in bovine babesiosis prevalence and/or clinical incidence has been postulated for several European countries (179, 180, 186). This may be due to the fact that several studies reporting this decline were based on clinical incidence (179, 186) and were thus not included in the calculation. Furthermore, no difference according to geographical region was found ($\chi^2 = 5.8$, df = 3, $P = 0.121$).

A few studies also reported detection of *B. divergens* by PCR, with infection rates ranging from 0.0% in Scotland (185) to 53.5% among clinically symptomatic cattle in Sweden (181). In randomly selected cattle in France, an infection rate of 4.7% was determined by PCR (173).

In addition to cattle, sheep might act as reservoirs for zoonotic *Babesia* spp., as *B. venatorum* infections have recently been detected in 11.8% of 93 studied sheep in the United Kingdom, whereas the 107 tested cattle were negative (185).

DISCUSSION

The present survey aimed at presenting an overview of the most important zoonotic TBDs in domestic animals in temperate and cold regions of Europe, where *I. ricinus* is the predominant tick vector. While a rather large number of studies on *B. burgdorferi* s.l., *A. phagocytophilum* and TBEV were retrieved, studies on *Rickettsia* spp. in domestic animals were few, probably because *Rickettsia* spp. have not (yet) been associated with clinical disease in these species. Therefore, the prevalence and clinical relevance of *Rickettsia* spp. in domestic animals represent a knowledge gap. Similarly, only few studies exist on the relatively recently discovered pathogen *N. mikurensis* in domestic animals, although first reports indicate that this pathogen may be of clinical relevance for dogs (150). Studies on relevant zoonotic *Babesia* spp. were only found with regard to *B. divergens* in cattle, while only one study investigated *B. venatorum*, which is usually deer-associated, in cattle and sheep. As *Babesia* spp. are characterized by a high level of host-specificity (187), it was not surprising that no prevalence studies were conducted on rodent-associated *B. microti* in domestic animals in the considered regions.

In general, a high level of heterogeneity was detected in the datasets. To limit this heterogeneity in meta-analysis of seroprevalence, only animal cohorts asymptomatic for the considered pathogen or random diagnostic samples were included, and further restrictions were applied regarding the diagnostic test used for TBEV in all domestic animals and *B. burgdorferi* s.l. in dogs. Nevertheless, heterogeneity remained high, even after conducting subgroup analyses according to species, diagnostic test (if applicable), geographic region and decade. A significant effect was only found regarding species differences in *B. burgdorferi* s.l. infections, with higher rates in domestic ruminants and horses than in dogs. On the one hand, this might be due to the close relationship between dogs and their owners, resulting in better protection from TBDs due to treatment with (repellent) acaricides, and shorter duration of tick attachment, as ticks are probably noticed sooner on dogs than on horses or cattle. On the other hand, the choice of diagnostic test may have contributed to the significantly lower *B. burgdorferi* s.l. seroprevalence in dogs. Although no significant effect of diagnostic test was found in the overall dataset, analyzing the data subset on dogs alone revealed a significant effect, with lower rates determined by the most recently developed test, a rapid ELISA with a high sensitivity and specificity for antibodies against the *Borrelia* C6 antigen (57). This test was almost exclusively used in dogs, except for two studies on horses. This indicates that cross-reactivity with other pathogens, e.g., *Leptospira* spp. (74), may have been an issue, especially in older and in non-canine studies.

Regarding *A. phagocytophilum*, conspicuously high seroprevalence rates in sheep were reported. However, as the meta-analysis included only three studies on sheep, no significant species differences were found when conducting subgroup analysis. However, this should be investigated further, as granulocytic anaplasmosis is a severe, possibly fatal disease in sheep (113). In addition, sheep might constitute a reservoir for human-pathogenic *A. phagocytophilum* strains (114, 115, 117). Thus, more studies on *A. phagocytophilum* (sero-)prevalence in sheep seem warranted.

In addition to species differences and different diagnostic tests used, the chosen cut-off level to determine seropositivity, the source of the antigen in serological tests or the timing of sampling—as seroprevalences may increase during seasons of tick exposure (120, 171)—may have further increased heterogeneity of study results. These aspects, hampering the comparability of studies, may have contributed to the fact that no temporal changes in TBD seroprevalence in domestic animals were found. Possibly, a larger number of studies may have been necessary to detect a temporal trend under these conditions. Furthermore, there were large temporal gaps in the available seroprevalence studies. For example, studies on TBEV considered suitable for meta-analysis were mainly conducted as of the year 2000, with only one comparable study from the 1960s and none from the decades in between. Regarding *Babesia* spp., there was a gap in available screening studies on asymptomatic animals concerning the 1990s as well as the 2010s. Regarding *B. burgdorferi* s.l., most studies were conducted in the 1980s, following the initial description of the pathogen (188).

In consequence, the trend of increasing TBD incidence in humans was not reflected by the available data from domestic animals. Apart from the mentioned drawbacks that may have masked such a trend, it is also possible that seroprevalences in domestic animals are rather stable and that the increased TBD incidence in humans is due to factors which are not relevant for animals. For example, increased exposure due to more outdoor activities or increased awareness of patients and physicians for TBDs may lead to an increased disease incidence or more diagnoses of TBD infections in humans (2).

With regard to the geographical spread of zoonotic TBDs, e.g., concerning TBE, (sero-)prevalence in animals is usually not studied in a certain area until there is an indication of pathogen presence due to human cases. Therefore, it is difficult to ascertain when the pathogen emerged in animal populations in this area. As an example, no studies on TBEV in domestic animals in the United Kingdom were retrieved, where TBEV has only recently been detected (17).

CONCLUSIONS

This survey revealed a high heterogeneity in (sero-)prevalences of zoonotic TBDs in domestic animals in temperate and cold regions of Europe. In addition, temporal gaps in available

studies were detected, e.g., for TBEV and *B. divergens*. The high level of heterogeneity as well as the temporal gaps make it difficult to assess long-term temporal trends for comparison with data on humans. Furthermore, only few studies were retrieved regarding *Rickettsia* spp. and the recently described pathogen *N. mikurensis*, and none regarding *B. miyamotoi*. Therefore, more studies investigating these neglected pathogens are warranted. Additionally, further surveillance studies employing highly sensitive and specific test methods and including hitherto non-investigated regions are needed to determine if and how a changing world impacts the frequency of neglected zoonotic tick-borne pathogens in domestic animals.

AUTHOR CONTRIBUTIONS

CS designed the study. AS, AG, and A-KT conducted the literature search. AS conducted statistical analyses and drafted the manuscript. All authors participated in data interpretation, reviewed the manuscript draft, 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.2020.604910/full#supplementary-material

REFERENCES

1. WHO/FAO. *Joint FAO/WHO Expert Committee on Zoonoses [Meeting Held in Geneva from 6 to 12 December 1966]: Third Report*.World Health Organization, Geneva (1967).
2. Vayssier-Taussat M, Cosson JF, Degeilh B, Eloit M, Fontanet A, Moutailler S, et al. How a multidisciplinary 'One health' approach can combat the tick-borne pathogen threat in Europe. *Future Microbiol.* (2015) 10:809–18. doi: 10.2217/fmb.15.15
3. Rubel F, Brugger K, Pfeffer M, Chitimia-Dobler L, Didyk YM, Leverenz S, et al. Geographical distribution of *Dermacentor marginatus* and *Dermacentor reticulatus* in Europe. *Ticks Tick Borne Dis.* (2016) 7:224–33. doi: 10.1016/j.ttbdis.2015.10.015
4. Ličková M, Fumačová Havlíková S, Sláviková M, Slovák M, Drexler JF, Klempa B. *Dermacentor reticulatus* is a vector of tick-borne encephalitis virus. *Ticks Tick Borne Dis.* (2020) 11:101414. doi: 10.1016/j.ttbdis.2020.101414
5. Hvidsten D, Frafjord K, Gray JS, Henningsson AJ, Jenkins A, Kristiansen BE, et al. The distribution limit of the common tick, *Ixodes ricinus*, and some associated pathogens in north-western Europe. *Ticks Tick Borne Dis.* (2020) 11:101388. doi: 10.1016/j.ttbdis.2020.101388
6. Jore S, Vanwambeke SO, Viljugrein H, Isaksen K, Kristoffersen AB, Woldehiwet Z, et al. Climate and environmental change drives *Ixodes ricinus* geographical expansion at the northern range margin. *Parasit Vectors.* (2014) 7:11. doi: 10.1186/1756-3305-7-11
7. Materna J, Daniel M, Metelka L, Harčarik J. The vertical distribution, density and the development of the tick *Ixodes ricinus* in mountain areas influenced by climate changes (The Krkonoše Mts., Czech Republic). *Int J Med Microbiol.* (2008) 298:25–37. doi: 10.1016/j.ijmm.2008.05.004
8. Mierzejewska EJ, Estrada-Peña A, Alsarraf M, Kowalec M, Bajer A. Mapping of *Dermacentor reticulatus* expansion in Poland in 2012–2014. *Ticks Tick Borne Dis.* (2016) 7:94–106. doi: 10.1016/j.ttbdis.2015.09.003
9. Dautel H, Dippel C, Oehme R, Hartelt K, Schettler E. Evidence for an increased geographical distribution of *Dermacentor reticulatus* in Germany and detection of *Rickettsia* sp. *RpA4*. *Int J Med Microbiol.* (2006) 296:149–56. doi: 10.1016/j.ijmm.2006.01.013
10. Paulauskas A, Radzijevskaja J, Mardosaite-Busaitiene D, Aleksandravičiene A, Galdikas M, Krikštolaitis R. New localities of *Dermacentor reticulatus* ticks in the baltic countries. *Ticks Tick Borne Dis.* (2015) 6:630–5. doi: 10.1016/j.ttbdis.2015.05.007
11. Drehmann M, Springer A, Lindau A, Fachet K, Mai S, Thoma D, et al. The spatial distribution of *Dermacentor* ticks (Ixodidae) in Germany - evidence of a continuing spread of *Dermacentor reticulatus*. *Front Vet Sci.* (2020) 7:578220. doi: 10.3389/fvets.2020.578220
12. Sykes RA, Makiello P. An estimate of lyme borreliosis incidence in Western Europe. *J Public Health.* (2017) 39:74–81. doi: 10.1093/pubmed/fdw017
13. Hofhuis A, van der Giessen JW, Borgsteede F, Wielinga PR, Notermans DW, van Pelt W. Lyme borreliosis in the Netherlands: strong increase in GP consultations and hospital admissions in past 10 years. *Eurosurveillance.* (2006) 11:2978. doi: 10.2807/esw.11.25.02978-en
14. Tulloch JSP, Christley RM, Radford AD, Warner JC, Beadsworth MBJ, Beeching NJ, et al. A descriptive epidemiological study of the incidence of newly diagnosed lyme disease cases in a UK primary care cohort, 1998-2016. *BMC Infect Dis.* (2020) 20:285. doi: 10.1186/s12879-020-05018-2
15. Süss J. Tick-borne encephalitis in Europe and beyond – the epidemiological situation as of 2007. *Eurosurveillance.* (2008) 13:18916. doi: 10.2807/ese.13.26.18916-en
16. Dekker M, Laverman GD, de Vries A, Reimerink J, Geeraedts F. Emergence of tick-borne encephalitis (TBE) in the Netherlands. *Ticks Tick Borne Dis.* (2019) 10:176–9. doi: 10.1016/j.ttbdis.2018.10.008
17. Holding M, Dowall SD, Medlock JM, Carter DP, Pullan ST, Lewis J, et al. Tick-borne encephalitis virus, United Kingdom. *Emerg Infect Dis.* (2020) 26:90–6. doi: 10.3201/eid2601.191085
18. Kernif T, Leulmi H, Raoult D, Parola P. Emerging tick-borne bacterial pathogens. In: Scheld WM, Hughes JM, Whitley RJ, editors. *Emerging Infections*. Washington, DC: American Society for Microbiology (2016). p. 295–310.
19. Fukunaga M, Takahashi Y, Tsuruta Y, Matsushita O, Ralph D, Mcclelland M, et al. Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme Disease in Japan. *Int J Syst Evol Microbiol.* (1995) 45:804–10. doi: 10.1099/00207713-45-4-804
20. Platonov AE, Karan LS, Kolyasnikova NM, Makhneva NA, Toporkova MG, Maleev VV, et al. Humans infected with relapsing fever spirochete *Borrelia miyamotoi*, Russia. *Emerg Infect Dis.* (2011) 17:1816–23. doi: 10.3201/eid1710.101474
21. Kawahara M, Rikihisa Y, Isogai E, Takahashi M, Misumi H, Suto C, et al. Ultrastructure and phylogenetic analysis of '*Candidatus* Neoehrlichia mikurensis' in the family Anaplasmataceae, isolated from wild rats and found in *Ixodes ovatus* ticks. *Int J Syst Evol Microbiol.* (2004) 54:1837–43. doi: 10.1099/ijs.0.63260-0
22. Welinder-Olsson C, Kjellin E, Vaht K, Jacobsson S, Wennerås C. First case of human "*Candidatus* Neoehrlichia mikurensis" infection in a febrile patient with chronic lymphocytic leukemia. *J Clin Microbiol.* (2010) 48:1956–9. doi: 10.1128/JCM.02423-09
23. Diniz PPVP, Schulz BS, Hartmann K, Breitschwerdt EB. "*Candidatus* Neoehrlichia mikurensis" infection in a dog from Germany. *J Clin Microbiol.* (2011) 49:2059–62. doi: 10.1128/JCM.02327-10

24. Gray J, Zintl A, Hildebrandt A, Hunfeld K-P, Weiss L. Zoonotic babesiosis: overview of the disease and novel aspects of pathogen identity. *Ticks Tick Borne Dis.* (2010) 1:3–10. doi: 10.1016/j.ttbdis.2009.11.003
25. Dobler G, Gniel D, Petermann R, Pfeffer M. Epidemiology and distribution of tick-borne encephalitis. *Wien Med Wochenschr.* (2012) 162:230–8. doi: 10.1007/s10354-012-0100-5
26. Schwarzer G. meta: an R package for meta-analysis. *R News.* (2007) 7:40–5.
27. R Core Team. *R: A Language and Environment for Statistical Computing.* 3.5.0 ed. Vienna: R Foundation for Statistical Computing (2018).
28. Maulik PK, Mascarenhas MN, Mathers CD, Dua T, Saxena S. Prevalence of intellectual disability: a meta-analysis of population-based studies. *Res Dev Disabil.* (2011) 32:419–36. doi: 10.1016/j.ridd.2010.12.018
29. Dobler G, Hufert F, Pfeffer M, Essbauer S. Tick-borne encephalitis: from microfocus to human disease. In: Mehlhorn H, editor. *Progress in Parasitology*. Berlin: Springer. (2011) p. 323–31. doi: 10.1007/978-3-642-21396-0_17
30. Imhoff M, Hagedorn P, Schulze Y, Hellenbrand W, Pfeffer M, Niedrig M. Review: sentinels of tick-borne encephalitis risk. *Ticks Tick Borne Dis.* (2015) 6:592–600. doi: 10.1016/j.ttbdis.2015.05.001
31. Roelandt S, Suin V, Gucht S, Stede Y, Roels S. Comparative tick-borne encephalitis (virus) surveillance in Belgium 2009-2015: experiences with diagnostic tests, sentinel species and surveillance designs. *J Zoonotic Dis Public Health.* (2017) 1:4.
32. Paulsen KM, Granquist EG, Okstad W, Vikse R, Stiasny K, Andreassen ÅK, et al. Experimental infection of lambs with tick-borne encephalitis virus and co-infection with *Anaplasma phagocytophilum*. *PLoS ONE.* (2019) 14:e0226836. doi: 10.1371/journal.pone.0226836
33. Balogh Z, Egyed L, Ferenczi E, Bán E, Szomor KN, Takács M, et al. Experimental infection of goats with tick-borne encephalitis virus and the possibilities to prevent virus transmission by raw goat milk. *Intervirology.* (2012) 55:194–200. doi: 10.1159/000324023
34. Balogh Z, Ferenczi E, Szeles K, Stefanoff P, Gut W, Szomor KN, et al. Tick-borne encephalitis outbreak in hungary due to consumption of raw goat milk. *J Virol Methods.* (2010) 163:481–5. doi: 10.1016/j.jviromet.2009.10.003
35. Brockmann S, Oehme R, Buckenmaier T, Beer M, Jeffery-Smith A, Spannenkrebs M, et al. A cluster of two human cases of tick-borne encephalitis (TBE) transmitted by unpasteurised goat milk and cheese in Germany, May 2016. *Eurosurveillance.* (2018) 23:17-00336. doi: 10.2807/1560-7917.ES.2018.23.15.17-00336
36. Böhm B, Schade B, Bauer B, Hoffmann B, Hoffmann D, Ziegler U, et al. Tick-borne encephalitis in a naturally infected sheep. *BMC Vet Res.* (2017) 13:267. doi: 10.1186/s12917-017-1192-3
37. Zindel W, Wyler R. Zeckenenzephalitis bei einer Ziege im untern Prättigau. *Schweiz Arch Tierheilkd.* (1983) 125:383–6.
38. Klaus C, Ziegler U, Kalthoff D, Hoffmann B, Beer M. Tick-borne encephalitis virus (TBEV) – findings on cross reactivity and longevity of TBEV antibodies in animal sera. *BMC Vet Res.* (2014) 10:78. doi: 10.1186/1746-6148-10-78
39. van der Poel WHM, van der Heide R, Bakker D, Looff MD, Jong JD, van Manen N, et al. Attempt to detect evidence for tick-borne encephalitis virus in ticks and mammalian wildlife in the Netherlands. *Vector Borne Zoonotic Dis.* (2005) 5:58–64. doi: 10.1089/vbz.2005.5.58
40. Šikutová S, Hornok S, Hubálek Z, DoleŽálková I, Juricová Z, Rudolf I. Serological survey of domestic animals for tick-borne encephalitis and Bhanja viruses in northeastern Hungary. *Vet Microbiol.* (2009) 135:267–71. doi: 10.1016/j.vetmic.2008.09.082
41. Paulsen KM, Stuen S, das Neves CG, Suhel F, Gurung D, Soleng A, et al. Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway. *Zoonoses Public Health.* (2019) 66:216–22. doi: 10.1111/zph.12554
42. Klaus C, Beer M, Saier R, Schau U, Moog U, Hoffmann B, et al. Goats and sheep as sentinels for tick-borne encephalitis (TBE) virus – epidemiological studies in areas endemic and non-endemic for TBE virus in Germany. *Ticks Tick Borne Dis.* (2012) 3:27–37. doi: 10.1016/j.ttbdis.2011.09.011
43. Grandi G, Aspán A, Pihl J, Gustafsson K, Engström F, Jinnerot T, et al. Detection of tick-borne pathogens in lambs undergoing prophylactic treatment against ticks on two Swedish farms. *Front Vet Sci.* (2018) 5:6. doi: 10.3389/fvets.2018.00072
44. Gao GF, Jiang WR, Hussain MH, Venugopal K, Gritsun TS, Reid HW, et al. Sequencing and antigenic studies of a Norwegian virus isolated from encephalomyelitic sheep confirm the existence of louping ill virus outside Great Britain and Ireland. *J Gen Virol.* (1993) 74:109–14. doi: 10.1099/0022-1317-74-1-109
45. Frimmel S, Löbermann M, Feldhusen F, Seelmann M, Stiasny K, Süss J, et al. Detection of tick-borne encephalitis virus antibodies in sera of sheep and goats in Mecklenburg-Western Pomerania (north-eastern Germany). *Ticks Tick Borne Dis.* (2019) 10:901–4. doi: 10.1016/j.ttbdis.2019.04.012
46. Casati Pagani S, Frigerio Malossa S, Klaus C, Hoffmann D, Beretta O, Bomio-Pacciorini N, et al. First detection of TBE virus in ticks and sero-reactivity in goats in a non-endemic region in the southern part of Switzerland (Canton of Ticino). *Ticks Tick Borne Dis.* (2019) 10:868–74. doi: 10.1016/j.ttbdis.2019.04.006
47. Salat J, Ruzek D. Tick-borne encephalitis in domestic animals. *Acta Virol.* (2020) 64:226–32. doi: 10.4149/av_2020_212
48. Waldvogel A, Matile H, Wegmann C, Wyler R, Kunz C. Tick-borne encephalitis in the horse. *Schweiz Arch Tierheilkd.* (1981) 123:227–33.
49. Klaus C, Hörügel U, Hoffmann B, Beer M. Tick-borne encephalitis virus (TBEV) infection in horses: clinical and laboratory findings and epidemiological investigations. *Vet Microbiol.* (2013) 163:368–72. doi: 10.1016/j.vetmic.2012.12.041
50. Pfeffer M, Dobler G. Tick-borne encephalitis virus in dogs - is this an issue? *Parasit Vectors.* (2011) 4:59. doi: 10.1186/1756-3305-4-59
51. Reiner B, Grasmück S, Steffen F, Djuric N, Schindler T, Müller W, et al. Prevalence of TBE antibodies in serum and CSF of dogs with inflammatory and non-inflammatory CNS disease. *Int J Med Microbiol.* (2002) 291(Suppl). 33:234. doi: 10.1016/S1438-4221(02)80069-8
52. Hekrlová A, Kubíček O, Lány P, Rosenbergová K, Schánilec P. Tick-borne encephalitis in dogs: application of "nested real-time RT-PCR" for intravital virus detection. *Berl Munch Tierarztl Wochenschr.* (2015) 128:397–401. doi: 10.23767/0005-9366-128-397
53. Margos G, Fedorova N, Becker NS, Kleinjan JE, Marosevic D, Krebs S, et al. *Borrelia maritima* sp. nov., a novel species of the *Borrelia burgdorferi* sensu lato complex, occupying a basal position to North American species. *Int J Syst Evol Microbiol.* (2020) 70:849–56. doi: 10.1099/ijsem.0.003833
54. Strnad M, Hönig V, Ružek D, Grubhoffer L, Rego ROM. Europe-wide meta-analysis of *Borrelia burgdorferi* sensu lato prevalence in questing *Ixodes ricinus* ticks. *Appl Environ Microbiol.* (2017) 83:e00609–17. doi: 10.1128/AEM.00609-17
55. Strle F, Stanek G, Strle K. Lyme borreliosis: the European perspective. In: Halperin JJ, editor. *Lyme Disease: An Evidence-based Approach*. 2 ed. Wallingford; Oxfordshire; Boston, MA: CAB International (2018). p. 105–23.
56. Tufts DM, Hart TM, Chen GF, Kolokotronis S-O, Diuk-Wasser MA, Lin Y-P. Outer surface protein polymorphisms linked to host-spirochete association in Lyme borreliae. *Mol Microbiol.* (2019) 111:868–82. doi: 10.1111/mmi.14209
57. Pantchev N, Pluta S, Huisinga E, Nather S, Scheufelen M, Vrhovec MG, et al. Tick-borne diseases (borreliosis, anaplasmosis, babesiosis) in German and Austrian dogs: status quo and review of distribution, transmission, clinical findings, diagnostics and prophylaxis. *Parasitol Res.* (2015) 114:19–54. doi: 10.1007/s00436-015-4513-0
58. Littman MP, Gerber B, Goldstein RE, Labato MA, Lappin MR, Moore GE. ACVIM consensus update on Lyme borreliosis in dogs and cats. *J Vet Intern Med.* (2018) 32:887–903. doi: 10.1111/jvim.15085
59. Lin Y-P, Diuk-Wasser MA, Stevenson B, Kraiczy P. Complement evasion contributes to Lyme borreliae–host associations. *Trends Parasitol.* (2020) 36:634–45. doi: 10.1016/j.pt.2020.04.011
60. Kurtenbach K, Sewell H-S, Ogden NH, Randolph SE, Nuttall PA. Serum complement sensitivity as a key factor in Lyme disease ecology. *Infect Immun.* (1998) 66:1248–51. doi: 10.1128/IAI.66.3.1248-1251.1998
61. Bhide MR, Travnicek M, Levkutova M, Curlik J, Revajova V, Levkut M. Sensitivity of *Borrelia* genospecies to serum complement from different animals and human: a host—pathogen relationship. *FEMS Immunol Med Microbiol.* (2005) 43:165–72. doi: 10.1016/j.femsim.2004.07.012
62. Richter D, Matuschka F-R. Elimination of Lyme disease spirochetes from ticks feeding on domestic ruminants. *Appl Environ Microbiol.* (2010) 76:7650–2. doi: 10.1128/AEM.01649-10

63. Lacombe E, Rand PW, Smith RP Jr. Disparity of *Borrelia burgdorferi* infection rates of adult *Ixodes dammini* on deer and vegetation. *J Infect Dis.* (1993) 167:1236–8. doi: 10.1093/infdis/167.5.1236
64. Ogden NH, Nuttall PA, Randolph SE. Natural Lyme disease cycles maintained via sheep by co-feeding ticks. *Parasitology*. (1997) 115:591–9. doi: 10.1017/S0031182097001868
65. Burgess EC, Gendron-Fitzpatrick A, Wright WO. Arthritis and systemic disease caused by *Borrelia burgdorferi* infection in a cow. *J Am Vet Med Assoc.* (1987) 191:1468–70.
66. Lischer CJ, Leutenegger CM, Braun U, Lutz H. Diagnosis of Lyme disease in two cows by the detection of *Borrelia burgdorferi* DNA. *Vet Rec.* (2000) 146:497. doi: 10.1136/vr.146.17.497
67. Štefančíková A, Štěpánová G, Derdáková M, Pet'ko B, Kysel'ová J, Cigánek J, et al. Serological evidence for *Borrelia burgdorferi* infection associated with clinical signs in dairy cattle in Slovakia. *Vet Res Commun.* (2002) 26:601–11. doi: 10.1023/A:1020912618950
68. Tuomi J, Rantamäki LK, Tanskanen R. Experimental infection of cattle with several *Borrelia burgdorferi* sensu lato strains; immunological heterogeneity of strains as revealed in serological tests. *Vet Microbiol.* (1998) 60:27–43. doi: 10.1016/S0378-1135(97)00200-9
69. Fridriksdóttir V, Overnes G, Stuen S. Suspected Lyme borreliosis in sheep. *Vet Rec.* (1992) 130:323–4. doi: 10.1136/vr.130.15.323
70. Stuen S, Fridriksdóttir V. Experimental inoculation of sheep with *Borrelia burgdorferi*. *Vet Rec.* (1991) 129:315. doi: 10.1136/vr.129.14.315
71. Hovmark A, Åsbrink E, Schwan O, Hederstedt B, Christensson D. Antibodies to *Borrelia* spirochetes in sera from Swedish cattle and sheep. *Acta Vet Scand.* (1986) 27:479–85.
72. Käsbohrer A, Schönberg A. Serologic studies of the occurrence of *Borrelia burgdorferi* in domestic animals in Berlin (West). *Berl Munch Tierarztl Wochenschr.* (1990) 103:374–8.
73. Cabannes A, Hernandez JC, Lucchese F, Appriou M, Tribouley-Duret J. Epidemiology of Lyme disease in cattle and sheep, in Gironde (France). *Med Mal Infect.* (1997) 27:878–83. doi: 10.1016/S0399-077X(97)80242-9
74. Štefančíková A, Derdáková M, Škardová I, Szestáková E, Cisláková L, Kováčová D, et al. Some epidemiological and epizootiological aspects of Lyme borreliosis in Slovakia with the emphasis on the problems of serological diagnostics. *Biologia.* (2008) 63:1135–42. doi: 10.2478/s11756-008-0177-x
75. Divers TJ. Equine Lyme disease. *J Equine Vet Sci.* (2013) 33:488–92. doi: 10.1016/j.jevs.2013.03.187
76. Chang Y-F, Novosol V, McDonough SP, Chang C-F, Jacobson RH, Divers T, et al. Experimental infection of ponies with *Borrelia burgdorferi* by exposure to ixodid ticks. *Vet Pathol.* (2000) 37:68–76. doi: 10.1354/vp.37-1-68
77. Chang Y-F, Ku Y-W, Chang C-F, Chang C-D, McDonough SP, Divers T, et al. Antibiotic treatment of experimentally *Borrelia burgdorferi*-infected ponies. *Vet Microbiol.* (2005) 107:285–94. doi: 10.1016/j.vetmic.2005.02.006
78. James FM, Engiles JB, Beech J. Meningitis, cranial neuritis, and radiculoneuritis associated with *Borrelia burgdorferi* infection in a horse. *J Am Vet Med Assoc.* (2010) 237:1180–5. doi: 10.2460/javma.237.10.1180
79. Imai DM, Barr BC, Daft B, Bertone JJ, Feng S, Hodzic E, et al. Lyme neuroborreliosis in 2 horses. *Vet Pathol.* (2011) 48:1151–7. doi: 10.1177/0300985811398246
80. Wagner B, Glaser A, Bartol J, Mahar O, Johnson A, Divers T. A new sensitive Lyme multiplex assay to confirm neuroborreliosis in horses: a case report. In: *Proceedings of the 57th Annual Convention of the American Association of Equine Practitioners.* San Antonio, TX: American Association of Equine Practitioners (AAEP) (2011). p. 70–5.
81. Pecoraro HL, Felippe MJB, Miller AD, Divers TJ, Simpson KW, Guyer KM, et al. Neuroborreliosis in a horse with common variable immunodeficiency. *J Vet Diagn Investig.* (2019) 31:241–5. doi: 10.1177/1040638718824146
82. Maurizi L, Marié J-L, Aoun O, Courtin C, Gorsane S, Chal D, et al. Seroprevalence survey of equine Lyme borreliosis in France and in Sub-Saharan Africa. *Vector Borne Zoonotic Dis.* (2009) 10:535–7. doi: 10.1089/vbz.2009.0083
83. Appel MJG, Allan S, Jacobson RH, Lauderdale TL, Chang YF, Shin SJ, et al. Experimental Lyme disease in dogs produces arthritis and persistent infection. *J Infect Dis.* (1993) 167:651–4. doi: 10.1093/infdis/167.3.651
84. Chang Y-F, Novosel V, Chang C-F, Summers BA, Ma D-P, Chiang Y-W, et al. Experimental induction of chronic borreliosis in adult dogs exposed to *Borrelia burgdorferi*–infected ticks and treated with dexamethasone. *Am J Vet Res.* (2001) 62:1104–12. doi: 10.2460/ajvr.2001.62.1104
85. Summers BA, Straubinger AF, Jacobson RH, Chang YF, Appel MJG, Straubinger RK. Histopathological studies of experimental Lyme disease in the dog. *J Compar Pathol.* (2005) 133:1–13. doi: 10.1016/j.jcpa.2004.11.006
86. Speck S, Reiner B, Wittenbrink M. Isolation of *Borrelia afzelii* from dog. *Vet Rec.* (2001) 149:19–20. doi: 10.1136/vr.149.1.19
87. Hovius KE, Stark LA, Bleumink-Pluym NM, van de Pol I, Verbeek-de Kruif N, Rijpkema SG, et al. Presence and distribution of *Borrelia burgdorferi* sensu lato species in internal organs and skin of naturally infected symptomatic and asymptomatic dogs, as detected by polymerase chain reaction. *Vet Q.* (1999) 21:54–8. doi: 10.1080/01652176.1999.9694992
88. Skotarczak B, Wodecka B. Identification of *Borrelia burgdorferi* genospecies inducing Lyme disease in dogs from western Poland. *Acta Vet Hung.* (2005) 53:13–21. doi: 10.1556/avet.53.2005.1.2
89. Mather TN, Fish D, Coughlin RT. Competence of dogs as reservoirs for Lyme disease spirochetes (*Borrelia burgdorferi*). *J Am Vet Med Assoc.* (1994) 205:186–8.
90. Hovius KE. The protection of European dogs against infection with Lyme disease spirochaetes. In: Braks MAH, van Wieren SE, Takken W, Sprong H, editors. E*cology and Prevention of Lyme borreliosis Ecology and Control of Vector-borne Diseases Vol 4*. Wageningen, NL: Wageningen Academic Publishers (2016). p. 409–26.
91. Lappin MR, Chandrashekar R, Stillman B, Liu J, Mather TN. Evidence of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* infection in cats after exposure to wild-caught adult *Ixodes scapularis*. *J Vet Diagn Investig.* (2015) 27:522–5. doi: 10.1177/1040638715593598
92. Burgess EC. Experimentally induced infection of cats with *Borrelia burgdorferi*. *Am J Vet Res.* (1992) 53:1507–11.
93. Tørnqvist-Johnsen C, Dickson S-A, Rolph K, Palermo V, Hodgkiss-Geere H, Gilmore P, et al. First report of Lyme borreliosis leading to cardiac bradydysrhythmia in two cats. *JFMS Open Reports.* (2020) 6:1–6. doi: 10.1177/2055116919898292
94. Hoyt K, Chandrashekar R, Beall M, Leutenegger C, Lappin MR. Evidence for clinical anaplasmosis and borreliosis in cats in Maine. *Top Companion Anim Med.* (2018) 33:40–4. doi: 10.1053/j.tcam.2018.05.002
95. Liu Y, Nordone SK, Yabsley MJ, Lund RB, McMahan CS, Gettings JR. Quantifying the relationship between human Lyme disease and *Borrelia burgdorferi* exposure in domestic dogs. *Geospat Health.* (2019) 14:750. doi: 10.4081/gh.2019.750
96. Egenvall A, Bonnett BN, Gunnarsson A, Hedhammar A, Shoukri M, Bornstein S, et al. Sero-prevalence of granulocytic *Ehrlichia* spp. and *Borrelia burgdorferi* sensu lato in Swedish dogs 1991-94. *Scand J Infect Dis.* (2000) 32:19–25. doi: 10.1080/00365540050164164
97. Gerber B, Eichenberger S, Wittenbrink MM, Reusch CE. Increased prevalence of *Borrelia burgdorferi* infections in Bernese mountain dogs: a possible breed predisposition. *BMC Vet Res.* (2007) 3:15. doi: 10.1186/1746-6148-3-15
98. Preyß-Jägeler C, Müller E, Straubinger RK, Hartmann K. Prävalenz von Antikörpern gegen *Borrelia burgdorferi*, *Anaplasma phagocytophilum* und bestimmte *Leptospira-interrogans*-Serovare bei Berner Sennenhunden. *Tierarztl Prax Ausg K Kleintiere Heimtiere.* (2016) 44:77–85. doi: 10.15654/TPK-140962
99. Barth C, Straubinger RK, Sauter-Louis C, Hartmann K. Prevalence of antibodies against *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* and their clinical relevance in dogs in Munich, Germany. *Berl Munch Tierarztl Wochenschr.* (2012) 125:337–44. doi: 10.2376/0005-9366-125-337
100. Kybicová K, Schánilec P, Hulínská D, Uherková L, Kurzová Z, Spejchalová S. Detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato in dogs in the Czech Republic. *Vector Borne Zoonotic Dis.* (2009) 9:655–61. doi: 10.1089/vbz.2008.0127
101. Pantchev N, Vrhovec MG, Pluta S, Straubinger RK. Seropositivity of *Borrelia burgdorferi* in a cohort of symptomatic cats from Europe based on a C6-peptide assay with discussion of implications in disease aetiology. *Berl Munch Tierarztl Wochenschr.* (2016) 129:333–9. doi: 10.2376/0005-9366-15088

102. Žákovská A, Schánilec P, Treml F, Dušková M, Agudelo RCF. Seroprevalence of antibodies against *Borrelia burgdorferi* s. l and *Leptospira interrogans* s. l. in cats in district of Brno and its environs, the Czech Republic. *Ann Agric Environ Med*. (2020) 27:356–60. doi: 10.26444/aaem/122804
103. Shaw SE, Binns SH, Birtles RJ, Day MJ, Smithson RC, Kenny MJ. Molecular evidence of tick-transmitted infections in dogs and cats in the United Kingdom. *Vet Rec.* (2005) 157:645. doi: 10.1136/vr.157.21.645
104. Cutler S, Vayssier-Taussat M, Estrada-Peña A, Potkonjak A, Mihalca AD, Zeller H. A new *Borrelia* on the block: *Borrelia miyamotoi* - a human health risk? *Eurosurveillance.* (2019) 24:1800170. doi: 10.2807/1560-7917.ES.2019.24.18.1800170
105. Gugliotta JL, Goethert HK, Berardi VP, Telford SR 3rd. Meningoencephalitis from *Borrelia miyamotoi* in an immunocompromised patient. *N Engl J Med.* (2013) 368:240-5. doi: 10.1056/NEJMoa1209039
106. Boden K, Lobenstein S, Hermann B, Margos G, Fingerle V. *Borrelia miyamotoi*-associated neuroborreliosis in immunocompromised person. *Emerg Infect Dis.* (2016) 22:1617–20. doi: 10.3201/eid2209.152034
107. Richter D, Matuschka F-R. Modulatory effect of cattle on risk for Lyme disease. *Emerg Infect Dis.* (2006) 12:1919–23. doi: 10.3201/eid1212.051552
108. Shannon AB, Rucinsky R, Gaff HD, Brinkerhoff RJ. *Borrelia miyamotoi*, other vector-borne agents in cat blood and ticks in Eastern Maryland. *EcoHealth.* (2017) 14:816–20. doi: 10.1007/s10393-017-1268-3
109. Dugat T, Lagree AC, Maillard R, Boulouis HJ, Haddad N. Opening the black box of *Anaplasma phagocytophilum* diversity: current situation and future perspectives. *Front Cell Infect Microbiol.* (2015) 5:61. doi: 10.3389/fcimb.2015.00061
110. Dumler JS, Choi K-S, Garcia-Garcia JC, Barat NS, Scorpio DG, Garyu JW, et al. Human granulocytic anaplasmosis and *Anaplasma phagocytophilum*. *Emerg Infect Dis.* (2005) 11:1828–34. doi: 10.3201/eid1112.050898
111. Jaarsma RI, Sprong H, Takumi K, Kazimirova M, Silaghi C, Mysterud A, et al. *Anaplasma phagocytophilum* evolves in geographical and biotic niches of vertebrates and ticks. *Parasit Vectors.* (2019) 12:328. doi: 10.1186/s13071-019-3583-8
112. Brown WC. Adaptive immunity to *Anaplasma* pathogens and immune dysregulation: implications for bacterial persistence. *Comp Immunol Microbiol Infect Dis.* (2012) 35:241–52. doi: 10.1016/j.cimid.2011.12.002
113. Stuen S, Nevland S, Moum T. Fatal cases of tick-borne fever (TBF) in sheep caused by several 16S rRNA gene variants of *Anaplasma phagocytophilum*. *Ann N Y Acad Sci.* (2003) 990:433–4. doi: 10.1111/j.1749-6632.2003.tb07407.x
114. Stuen S, Braten M, Bergström K, Bardsen K. Cyclic variation in lambs infected with *Anaplasma phagocytophilum*. *Vet Rec.* (2008) 163:338–9. doi: 10.1136/vr.163.11.338
115. Thomas RJ, Birtles RJ, Radford AD, Woldehiwet Z. Recurrent bacteraemia in sheep infected persistently with *Anaplasma phagocytophilum*. *J Compar Pathol.* (2012) 147:360–7. doi: 10.1016/j.jcpa.2012.02.005
116. Lagrée A-C, Rouxel C, Kevin M, Dugat T, Girault G, Durand B, et al. Co-circulation of different *A. phagocytophilum* variants within cattle herds and possible reservoir role for cattle. *Parasit Vectors.* (2018) 11:163. doi: 10.1186/s13071-018-2661-7
117. Scharf W, Schauer S, Freyburger F, Petrovec M, Schaarschmidt-Kiener D, Liebisch G, et al. Distinct host species correlate with *Anaplasma phagocytophilum* ankA gene clusters. *J Clin Microbiol.* (2011) 49:790–6. doi: 10.1128/JCM.02051-10
118. Hulínská D, Langrová K, Pejčoch M, Pavlásek I. Detection of *Anaplasma phagocytophilum* in animals by real-time polymerase chain reaction. *APMIS.* (2004) 112:239–47. doi: 10.1111/j.1600-0463.2004.apm11204-0503.x
119. Stuen S, Oppegaard AS, Bergström K, Moum T. *Anaplasma phagocytophilum* infection in north Norway. The first laboratory confirmed case. *Acta Vet Scand.* (2005) 46:167. doi: 10.1186/1751-0147-46-167
120. Pusterla N, Pusterla JB, Braun U, Lutz H. Serological, hematologic and PCR studies of cattle in an area of Switzerland in which tick-borne fever (caused by *Ehrlichia phagocytophila*) is endemic. *Clin Diagn Lab Immunol.* (1998) 5:325–7. doi: 10.1128/CDLI.5.3.325-327.1998
121. Engvall EO, Pettersson B, Persson M, Artursson K, Johansson KE. A 16S rRNA-based PCR assay for detection and identification of granulocytic *Ehrlichia* species in dogs, horses, and cattle. *J Clin Microbiol.* (1996) 34:2170–4. doi: 10.1128/JCM.34.9.2170-2174.1996
122. Stuen S, Bergström K. Serological investigation of granulocytic *Ehrlichia* infection in sheep in Norway. *Acta Vet Scand.* (2001) 42:331–8. doi: 10.1186/1751-0147-42-331
123. Zeman P, Januska J, Orolinova M, Stuen S, Struhar V, Jebavy L. High seroprevalence of granulocytic ehrlichiosis distinguishes sheep that were the source of an alimentary epidemic of tick-borne encephalitis. *Wien Klin Wochenschr.* (2004) 116:614–6. doi: 10.1007/s00508-004-0191-0
124. Derdáková M, Štefančíková A, Špitalská E, Taragelová V, Koštálová T, Hrklová G, et al. Emergence and genetic variability of *Anaplasma* species in small ruminants and ticks from central Europe. *Vet Microbiol.* (2011) 153:293–8. doi: 10.1016/j.vetmic.2011.05.044
125. Silaghi C, Scheuerle MC, Friche Passos LM, Thiel C, Pfister K. PCR detection of *Anaplasma phagocytophilum* in goat flocks in an area endemic for tick-borne fever in Switzerland. *Parasite.* (2011) 18:57–62. doi: 10.1051/parasite/2011181057
126. Harrison A, Brown KJ, Ian Montgomery W. *Anaplasma phagocytophilum* in feral goats in Northern Ireland. *Vet Rec.* (2012) 170:602–3. doi: 10.1136/vr.e3938
127. Dreher UM, de la Fuente J, Hofmann-Lehmann R, Meli ML, Pusterla N, Kocan KM, et al. Serologic cross-reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum*. *Clin Diagn Lab Immunol.* (2005) 12:1177–83. doi: 10.1128/CDLI.12.10.1177-1183.2005
128. de la Fuente J, Lew A, Lutz H, Meli ML, Hofmann-Lehmann R, Shkap V, et al. Genetic diversity of *Anaplasma* species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. *Anim Health Res Rev.* (2005) 6:75–89. doi: 10.1079/AHR2005104
129. Hornok S, Micsutka A, Fernández de Mera IG, Meli ML, Gönczi E, Tánczos B, et al. Fatal bovine anaplasmosis in a herd with new genotypes of *Anaplasma marginale*, *Anaplasma ovis* and concurrent haemoplasmosis. *Res Vet Sci.* (2012) 92:30–5. doi: 10.1016/j.rvsc.2010.10.011
130. Dahmani M, Davoust B, Rousseau F, Raoult D, Fenollar F, Mediannikov O. Natural Anaplasmataceae infection in *Rhipicephalus bursa* ticks collected from sheep in the French Basque country. *Ticks Tick Borne Dis.* (2017) 8:18–24. doi: 10.1016/j.ttbdis.2016.09.009
131. Saleem S, Ijaz M, Farooqi SH, Ghaffar A, Ali A, Iqbal K, et al. Equine granulocytic anaplasmosis 28 years later. *Microbial Pathog.* (2018) 119:1–8. doi: 10.1016/j.micpath.2018.04.001
132. Franzén P, Aspan A, Egenvall A, Gunnarsson A, Karlstam E, Pringle J. Molecular evidence for persistence of *Anaplasma phagocytophilum* in the absence of clinical abnormalities in horses after recovery from acute experimental infection. *J Vet Intern Med.* (2009) 23:636–42. doi: 10.1111/j.1939-1676.2009.0317.x
133. Zeman P, Jahn P. An entropy-optimized multilocus approach for characterizing the strains of *Anaplasma phagocytophilum* infecting horses in the Czech Republic. *J Med Microbiol.* (2009) 58:423–9. doi: 10.1099/jmm.0.007831-0
134. Bretscher R. Serologische Untersuchungen zur Häufigkeit und Verbreitung der equinen Ehrlichiose in der Schweiz. *Schweiz Arch Tierheilkd.* (1991) 133:185.
135. Praskova I, Bezdekova B, Zeman P, Jahn P. Seroprevalence of *Anaplasma phagocytophilum* in horses in the Czech Republic. *Ticks Tick Borne Dis.* (2011) 2:111–5. doi: 10.1016/j.ttbdis.2011.01.002
136. Slivinska K, Víchová B, Werszko J, Szewczyk T, Wróblewski Z, Petko B, et al. Molecular surveillance of *Theileria equi* and *Anaplasma phagocytophilum* infections in horses from Ukraine, Poland and Slovakia. *Vet Parasitol.* (2016) 215:35–7. doi: 10.1016/j.vetpar.2015.10.025
137. Sainz Á, Roura X, Miró G, Estrada-Peña A, Kohn B, Harrus S, et al. Guideline for veterinary practitioners on canine ehrlichiosis and anaplasmosis in Europe. *Parasit Vectors.* (2015) 8:75. doi: 10.1186/s13071-015-0649-0
138. Pennisi MG, Hofmann-Lehmann R, Radford AD, Tasker S, Belák S, Addie DD, et al. *Anaplasma, Ehrlichia* and *Rickettsia* species infections in cats: European guidelines from the ABCD on prevention and management. *J Feline Med Surg.* (2017) 19:542–8. doi: 10.1177/1098612X17706462
139. Egenvall A, Lilliehöök I, Bjöersdorff A, Engvall EO, Karlstam E, Artursson K, et al. Detection of granulocytic *Ehrlichia* species DNA by PCR in persistently infected dogs. *Vet Rec.* (2000) 146:186–90. doi: 10.1136/vr.146.7.186

140. Kirtz G, Czettel B, Thum D, Leidinger E. *Anaplasma phagocytophilum* in einer österreischichen Hundepopulation: eine Prävalenz-Studie (2001–2006). *Kleintierpraxis.* (2007) 7:562–8.
141. Egenvall A, Franzén P, Gunnarsson A, Engvall EO, Vågsholm I, Wikström U-B, et al. Cross-sectional study of the seroprevalence to *Borrelia burgdorferi* sensu lato and granulocytic *Ehrlichia* spp. and demographic, clinical and tick-exposure factors in Swedish horses. *Prev Vet Med.* (2001) 49:191–208. doi: 10.1016/S0167-5877(01)00187-8
142. Hofmann-Lehmann R, Wagmann N, Meli ML, Riond B, Novacco M, Joekel D, et al. Detection of *Candidatus* Neoehrlichia mikurensis and other Anaplasmataceae and Rickettsiaceae in Canidae in Switzerland and Mediterranean countries. *Schweiz Arch Tierheilkd.* (2016) 158:691–700. doi: 10.17236/sat00087
143. Bajer A, Mierzejewska EJ, Rodo A, Bednarska M, Kowalec M, Welc-Faleciak R. The risk of vector-borne infections in sled dogs associated with existing and new endemic areas in Poland: part 1: a population study on sled dogs during the racing season. *Vet Parasitol.* (2014) 202:276–86. doi: 10.1016/j.vetpar.2013.12.033
144. Berzina I, Capligina V, Bormane A, Pavulina A, Baumanis V, Ranka R, et al. Association between *Anaplasma phagocytophilum* seroprevalence in dogs and distribution of *Ixodes ricinus* and *Ixodes persulcatus* ticks in Latvia. *Ticks Tick Borne Dis.* (2013) 4:83–8. doi: 10.1016/j.ttbdis.2012.08.003
145. Morgenthal D, Hamel D, Arndt G, Silaghi C, Pfister K, Kempf VAJ, et al. Prävalenz von hämotrophen *Mycoplasma* spp., *Bartonella* spp. und *Anaplasma phagocytophilum* bei Katzen im Raum Berlin/Brandenburg. *Berl Munch Tierarztl Wochenschr.* (2012) 125:418–27. doi: 10.2376/0005-9366-125-11
146. Hamel D, Bondarenko A, Silaghi C, Nolte I, Pfister K. Seroprevalence and bacteremia [corrected] of *Anaplasma phagocytophilum* in cats from Bavaria and Lower Saxony (Germany). *Berl Munch Tierarztl Wochenschr.* (2012) 125:163–7. doi: 10.2371/0005-9311-125-113
147. Krücken J, Schreiber C, Maaz D, Kohn M, Demeler J, Beck S, et al. A novel high-resolution melt PCR assay discriminates *Anaplasma phagocytophilum* and *Candidatus* Neoehrlichia mikurensis. *J Clin Microbiol.* (2013) 51:1958–61. doi: 10.1128/JCM.00284-13
148. Wass L, Grankvist A, Bell-Sakyi L, Bergström M, Ulfhammer E, Lingblom C, et al. Cultivation of the causative agent of human neoehrlichiosis from clinical isolates identifies vascular endothelium as a target of infection. *Emerg Microbes Infect.* (2019) 8:413–25. doi: 10.1080/22221751.2019.1584017
149. Portillo A, Santibáñez P, Palomar AM, Santibáñez S, Oteo JA. *Candidatus* Neoehrlichia mikurensis in Europe. *New Microbes New Infect.* (2018) 22:30–6. doi: 10.1016/j.nmni.2017.12.011
150. Beck A, Huber D, Antolić M, Anzulović Ž, Reil I, Polkinghorne A, et al. Retrospective study of canine infectious haemolytic anaemia cases reveals the importance of molecular investigation in accurate postmortal diagnostic protocols. *Comp Immunol Microbiol Infect Dis.* (2019) 65:81–7. doi: 10.1016/j.cimid.2019.05.006
151. Liesner JM, Krücken J, Schaper R, Pachnicke S, Kohn B, Müller E, et al. Vector-borne pathogens in dogs and red foxes from the federal state of Brandenburg, Germany. *Vet Parasitol.* (2016) 224:44–51. doi: 10.1016/j.vetpar.2016.05.012
152. Hornok S, Horváth G, Takács N, Farkas R, Szoke K, Kontschán J. Molecular evidence of a badger-associated *Ehrlichia* sp., a *Candidatus* Neoehrlichia lotoris-like genotype and *Anaplasma marginale* in dogs. *Ticks Tick Borne Dis.* (2018) 9:1302–9. doi: 10.1016/j.ttbdis.2018.05.012
153. Oteo JA, Portillo A. Tick-borne rickettsioses in Europe. *Ticks Tick Borne Dis.* (2012) 3:271–8. doi: 10.1016/j.ttbdis.2012.10.035
154. Azagi T, Hoornstra D, Kremer K, Hovius JWR, Sprong H. Evaluation of disease causality of rare *Ixodes ricinus*-borne infections in Europe. *Pathogens.* (2020) 9:150. doi: 10.3390/pathogens9020150
155. Keenan KP, Buhles WC, Huxsoll DL, Williams RG, Hildebrandt PK. Studies on the pathogenesis of *Rickettsia rickettsii* in the dog: clinical and clinicopathologic changes of experimental infection. *Am J Vet Res.* (1977) 38:851–6.
156. Solano-Gallego L, Kidd L, Trotta M, Di Marco M, Caldin M, Furlanello T, et al. Febrile illness associated with *Rickettsia conorii* infection in dogs from Sicily. *Emerg Infect Dis.* (2006) 12:1985–8. doi: 10.3201/eid1212.060326
157. Levin ML, Killmaster LF, Zemtsova GE. Domestic dogs (*Canis familiaris*) as reservoir hosts for *Rickettsia conorii*. *Vector Borne Zoonotic Dis.* (2011) 12:28–33. doi: 10.1089/vbz.2011.0684
158. Elfving K, Malmsten J, Dalin A-M, Nilsson K. Serologic and molecular prevalence of *Rickettsia helvetica* and *Anaplasma phagocytophilum* in wild cervids and domestic mammals in the central parts of Sweden. *Vector Borne Zoonotic Dis.* (2015) 15:529–34. doi: 10.1089/vbz.2015.1768
159. Wächter M, Pfeffer M, Schulz N, Balling A, Chirek A, Bach JP, et al. Seroprevalence of spotted fever group *Rickettsiae* in dogs in Germany. *Vector Borne Zoonotic Dis.* (2015) 15:191–4. doi: 10.1089/vbz.2014.1715
160. Wächter M, Wölfel S, Pfeffer M, Dobler G, Kohn B, Moritz A, et al. Serological differentiation of antibodies against *Rickettsia helvetica, R. raoultii, R. slovaca, R. monacensis* and *R. felis* in dogs from Germany by a micro-immunofluorescent antibody test. *Parasit Vectors.* (2015) 8:126. doi: 10.1186/s13071-015-0745-1
161. Rymaszewska A, Małgorzata A. Molecular evidence of vector-borne pathogens coinfecting dogs from Poland. *Acta Vet Hung.* (2011) 59:215. doi: 10.1556/avet.2011.008
162. Boretti FS, Perreten A, Meli ML, Cattori V, Willi B, Wengi N, et al. Molecular investigations of *Rickettsia helvetica* infection in dogs, foxes, humans, and *Ixodes* ticks. *Appl Environ Microbiol.* (2009) 75:3230–7. doi: 10.1128/AEM.00220-09
163. Juvet F, Lappin MR, Brennan S, Mooney CT. Prevalence of selected infectious agents in cats in Ireland. *J Feline Med Surg.* (2010) 12:476–82. doi: 10.1016/j.jfms.2010.02.003
164. Spada E, Proverbio D, Galluzzo P, Perego R, Bagnagatti De Giorgi G, Roggero N, et al. Frequency of piroplasms *Babesia microti* and *Cytauxzoon felis* in stray cats from northern Italy. *Biomed Res Int.* (2014) 2014:943754. doi: 10.1155/2014/943754
165. Zintl A, Mulcahy G, Skerrett HE, Taylor SM, Gray JS. *Babesia divergens*, a bovine blood parasite of veterinary and zoonotic importance. *Clin Microbiol Rev.* (2003) 16:622–36. doi: 10.1128/CMR.16.4.622-636.2003
166. Springer A, Höltershinken M, Lienhart F, Ermel S, Rehage J, Hülskötter K, et al. Emergence and epidemiology of bovine babesiosis due to *Babesia divergens* on a northern German beef production farm. *Front Vet Sci.* (2020) 7:649. doi: 10.3389/fvets.2020.00649
167. Gern L, Kindler A, Brossard M. Annual evolution of cattle immunity against *Babesia divergens* in northern Switzerland. *Prev Vet Med.* (1988) 6:9–16. doi: 10.1016/0167-5877(88)90021-9
168. Zintl A, Gray JS, Skerrett HE, Mulcahy G. Possible mechanisms underlying age-related resistance to bovine babesiosis. *Parasite Immunol.* (2005) 27:115–20. doi: 10.1111/j.1365-3024.2005.00748.x
169. Losson B, Lefevre F. La babesiose bovine en Belgique. Une enquête sérologique en zone d'endémie. *Ann Med Vet.* (1989) 133:421–6.
170. Lempereur L, Lebrun M, Cuvelier P, Sépult G, Caron Y, Saegerman C, et al. Longitudinal field study on bovine *Babesia* spp. and *Anaplasma phagocytophilum* infections during a grazing season in Belgium. *Parasitol Res.* (2012) 110:1525–30. doi: 10.1007/s00436-011-2657-0
171. L'Hostis M, Chauvin A, Valentin A, Precigout E, Gorenflot A. Survey of *Babesia divergens* antibody kinetics in cattle in western France. *Vet Res.* (1997) 28:481–8.
172. Malandrin L, L'Hostis M, Chauvin A. Isolation of *Babesia divergens* from carrier cattle blood using in vitro culture. *Vet Res.* (2004) 35:131–9. doi: 10.1051/vetres:2003047
173. Devos J, Geysen D. Epidemiological study of the prevalence of *Babesia divergens* in a veterinary practice in the mid-east of France. *Vet Parasitol.* (2004) 125:237–49. doi: 10.1016/j.vetpar.2004.08.004
174. Agoulon A, Malandrin L, Lepigeon F, Vénisse M, Bonnet S, Becker CAM, et al. A vegetation index qualifying pasture edges is related to *Ixodes ricinus* density and to *Babesia divergens* seroprevalence in dairy cattle herds. *Vet Parasitol.* (2012) 185:101–9. doi: 10.1016/j.vetpar.2011.10.022
175. Ullmann B. *Untersuchungen zum Vorkommen und zur serologischen Differenzierung von Babesien bei Rindern im westlichen Allgäu* (Dissertation). Ludwig-Maximilians-Universität München, Munich, Germany (1983).

176. Ganse-Dumrath D, Hannover TH. *Epidemiology of Babesia divergens infection in cattle in northern Germany* (Dissertation). Univerisity of Veterinary Medicine Hannover, Hanover, Germany (1986).
177. Niepold J. *Untersuchung von Rinderseren auf das simultane Vorkommen von Infektionen mit Borrelien (Borrelia burgdorferi) und Babesien (Babesia divergens)*. (Dissertation). University of Veterinary Medicine Hannover, Hanover, Germany (1990).
178. Lengauer H, Just FT, Edelhofer R, Pfister K. Tick infestation and the prevalence of *Borrelia burgdorferi* and *Babesia divergens* in cattle in Bavaria. *Berl Munch Tierarztl Wochenschr*. (2006) 119:335–41.
179. Hornok S, Edelhofer R, Szotáczky I, Hajtós I. *Babesia divergens* becoming extinct in cattle of northeast Hungary: new data on the past and present situation. *Acta Vet Hung.* (2006) 54:493–501. doi: 10.1556/AVet.54.2006.4.7
180. Hasle G, Bjune GA, Christensson D, Røed KH, Whist AC, Leinaas HP. Detection of *Babesia divergens* in southern Norway by using an immunofluorescence antibody test in cow sera. *Acta Vet Scand.* (2010) 52:55. doi: 10.1186/1751-0147-52-55
181. Andersson MO, Víchová B, Tolf C, Krzyzanowska S, Waldenström J, Karlsson ME. Co-infection with *Babesia divergens* and *Anaplasma phagocytophilum* in cattle (*Bos taurus*), Sweden. *Ticks Tick Borne Dis.* (2017) 8:933–5. doi: 10.1016/j.ttbdis.2017.08.005
182. Adam KMG, Blewett DA, Collins TJ, Edgar JT. Outbreaks of babesiasis on two farms in Scotland. *Br Vet J.* (1978) 134:428–33. doi: 10.1016/S0007-1935(17)33384-5
183. Adam KM, Blewett DA. A serological survey for *Babesia* in cattle in Scotland II. The occurrence of antibody in the population and the distribution of infected herds. *Ann Trop Med Parasitol.* (1978) 72:417–28. doi: 10.1080/00034983.1978.11719342
184. Taylor SM, Kenny J, Strain A. The distribution of *Babesia divergens* infection within the cattle population of Northern Ireland. *Br Vet J.* (1982) 138:384–92. doi: 10.1016/S0007-1935(17)30983-1
185. Gray A, Capewell P, Loney C, Katzer F, Shiels BR, Weir W. Sheep as host species for zoonotic *Babesia venatorum*, United Kingdom. *Emerg Infect Dis.* (2019) 25:2257–60. doi: 10.3201/eid2512.190459
186. Zintl A, McGrath G, O'Grady L, Fanning J, Downing K, Roche D, et al. Changing incidence of bovine babesiosis in Ireland. *Ir Vet J.* (2014) 67:7. doi: 10.1186/2046-0481-67-19
187. Chauvin A, Moreau E, Bonnet S, Plantard O, Malandrin L. *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. *Vet Res.* (2009) 40:37. doi: 10.1051/vetres/2009020
188. Johnson RC, Schmid GP, Hyde FW, Steigerwalt AG, Brenner DJ. *Borrelia burgdorferi* sp. nov.: etiologic agent of Lyme disease. *Int J Syst Evol Microbiol.* (1984) 34:496–7. doi: 10.1099/00207713-34-4-496

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frontiers in Veterinary Science

BRIEF RESEARCH REPORT
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Leishmania infantum Seroprevalence in Cats From Touristic Areas of Italy and Greece

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Leishmaniosis by *Leishmania infantum* is a major zoonotic Vector-Borne Disease (VBD) in terms of geographic distribution, pathogenicity and zoonotic potential. While dogs are the main reservoir of *L. infantum*, the infection in cats is poorly understood although increasingly reported from enzootic and non-enzootic areas. The Mediterranean basin is a key area for leishmaniosis and includes touristic spots that require continuous surveillance for VBDs in consideration of the growing tendency of tourists to travel with their pets. This study evaluated *L. infantum* seroprevalence in cats living in selected touristic localities of Italy and Greece. A total of 269 cat serum samples from three Sites i.e., 76, 40, and 153 from Adriatic Coast of Abruzzo, Italy (Site A), Giglio Island, Tuscany, Italy (Site B), and Mykonos Island, Greece (Site C), respectively, were included in the survey. Sera samples were subjected to an indirect immunofluorescence antibody assay for the detection of anti-*L. infantum* specific IgG. Associations between possible risk factors and seropositivity to *L. infantum* were statistically evaluated. Antibodies against *L. infantum* were detected in eight out of 269 (3.0%) cats tested i.e., 4/76 (5.3%), 1/40 (2.5%), and 3/153 (2.0%), from sites A, B, and C, respectively. A statistical association between anti-*L. infantum* antibodies and cohabitation with dogs was shown. This study indicates that feline populations living in the examined Italian and Greek touristic areas are exposed to *L. infantum* and that they may contribute to the circulation of *L. infantum*, enhancing the risk of infection for dogs and humans.

Keywords: ***Leishmania infantum*, cats, seroprevalence, Italy, Greece, zoonosis**

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INTRODUCTION

Leishmaniosis caused by the protozoan parasite *Leishmania infantum* is a Vector-Borne Disease (VBD) of major veterinary and public health concern in the Mediterranean Basin (1, 2), where phlebotomine sandflies of the genus Phlebotomus transmit *L. infantum* to a vertebrate host during blood meal (3). Accordingly, geographic spread, epizootiology, and epidemiology of *L. infantum* are strictly associated with the distribution of these vectors (4, 5).

Current climate changes enhance the reproduction and spread of sandflies and, consequently, promote the rate of *L. infantum* transmission to a wide range of vertebrates, including humans (6, 7). Recent records have shown a rise of infection rates in companion animals (8–10) and wildlife (11–13). Also, autochthonous cases of infection by *L. infantum* in people are reported throughout Europe, including Italy and Greece (14). Southern Europe in particular is regarded as a high-burden area for human visceral leishmaniosis due to *L. infantum* (1).

Dogs are considered the most important domestic reservoir of *L. infantum* (15) while the role of cats as potential reservoir of this parasite requires further corroboration (16). Although cats are in general considered resistant to *L. infantum* (17), they may be infected and potentially act as a source of infection for phlebotomine sandflies (18, 19). In particular, infected cats may successfully pass *L. infantum* to *Phlebotomus perniciosus* in which the parasite further continues its development (20, 21). Additionally, different studies have documented that *L. infantum* circulates among feline populations of Mediterranean basin e.g., Greece, Portugal, Turkey, Cyprus, Spain, and Italy, (22–28). Case reports in cats imported from enzootic areas or traveling with their owners in such localities have shown that feline leishmaniosis may be introduced in non-enzootic countries (29, 30). This latter feature is important in regions with intense movements and travels of animals either with their owners (e.g. tourism), or in the frame of adoption programs of stray animals (animal rights associations activity).

Cats are exposed to *L. infantum* in Italy (28) and Greece (31), which are indeed key epizootiological hubs. Touristic travels are particularly intense in these countries and this is of importance considering the substantial correlation between pet movements and spread of *L. infantum* from enzootic to free areas (10, 31, 32). In such areas a continuous surveillance of the epizootiology and distribution of feline leishmaniosis is pivotal, toward the protection of animal and human health. Thus, the aim of the present study was to evaluate the exposure to *L. infantum* in cats from touristic localities of Italy and Greece, and to investigate possible risk factors associated with the seropositivity in feline populations.

MATERIALS AND METHODS

Animals and Study Areas

Overall, 269 cats (116 in Italy and 153 in Greece) living in three touristic areas were included in the study: 76 cats from Adriatic Coast of Abruzzo, Italy (Site A), 40 cats from Giglio Island, Tuscany, Italy (Site B), and 153 cats from Mykonos Island, Greece (Site C). Detailed information about sex, age, and lifestyle was recorded for each cat. Consent for sample collection and screening for leishmaniosis was obtained from the animal owners or the local municipality authorities and animal rights association authorities, as applicable.

Abbreviations: VBD, Vector-borne Diseases; IFAT, Immunofluorescence Antibody Test; CI, Confidence Interval.

TABLE 1 | Number and percentage of cats seropositive and seronegative at IFAT (cut-off dilution 1:80) for *Leishmania infantum* antibodies for each possible risk factor considered in the present study.

	Seropositive n/tot (%)	Seronegative n/tot (%)	*P*-value
Male	3/140 (2.1)	137/140 (97.9)	0.4862
Female	5/129 (3.9)	124/129 (96.1)	
Outdoor	7/224 (3.1)	217/224 (96.9)	>0.9999
Indoor	1/45 (2.2)	44/45 (97.8)	
<36 m	3/128 (2.3)	125/128 (97.7)	0.7251
≥36 m	5/141 (3.5)	136/141 (96.4)	
Cohabitation with dogs	3/27 (11.1)	24/27 (88.9)	0.0360
No cohabitation with dogs	5/242 (2)	237/242 (97.9)	

Associations with P-values of <0.05 were considered statistically significant.

Sampling and Serology

Blood samples were collected individually in tubes without anticoagulant and centrifuged after clot formation for serum separation and collection. Sera were subjected to an indirect immunofluorescence antibody test (IFAT) for the detection of specific IgG against *L. infantum*. Commercially available slides coated with promastigotes (MegaFLUO Leish—Megacor Diagnostik GmbH) and anti-cat IgG conjugate (FLUO FITC anti-cat IgG conjugate) were used, following the instructions provided by the manufacturer. The cut-off dilution of 1:80 was applied, as indicated in the LeishVet guidelines[1].

Statistical Analysis

A statistical analysis was carried out using GraphPad Prism 8 (GraphPad Software, LLC). The Fisher's exact test was used to evaluate the eventual significant associations ($p \leq 0.05$) between exposure to *L. infantum* and possible risk factors (age, sex, lifestyle, cohabitation with other animals).

Cats were divided in two age classes i.e., aging <36 months old (n. 128), and ≥36 months (n. 141). One hundred and forty cats were male and 129 were female. Overall, 45 cats were housed indoor and were not allowed to go outside, while 224 had constant outdoor access or lived permanently outdoor.

RESULTS

Overall, antibodies against *L. infantum* were detected in 8/269 cats (3.0%; 95% CI ±2.0) i.e., 4/76 (5.3%; 95% CI ±5.0) from site A, 1/40 (2.5%; 95% CI ±4.5) from site B, and 3/153 (2.0%; 95% CI ±2.2) from site C.

The Fisher's exact test revealed a statistically significant ($p < 0.05$) association between cat seropositivity and cohabitation with dogs ($p = 0.0360$). According to the statistical analysis no other relevant associations were found. The number of seropositive and seronegative cats for each possible risk factor

[1]http://www.leishvet.org/fact-sheet-feline-leishmaniosis/feline-leishmaniosis-clinical-diagnosis/

is detailed in **Table 1**. The median age of both seropositive and seronegative cats was 36 months.

DISCUSSION

The data generated in the present study add new information on the exposure to *L. infantum* of cat populations in the examined areas of Italy and Greece.

The overall seroprevalence (3.0%) recorded is in line with values registered in central Spain (1.3–3.2%), Portugal (2.8%) and, very recently, throughout Italy (3.3%) (28, 33, 34). A recent systematic review with meta-analysis of the literature of the last two decades has indicated higher seroprevalence rates in cats from these countries i.e., 11.0% in Greece and 24.0% in Italy (2). Indeed, studies are difficult to compare when different molecular/serological techniques are used, while differences in the prevalence rates could also be attributed to the selection of the sample population (e.g., age, lifestyle, and number of cats), to the selected antibody titer cut-off and to the sampling areas. In fact, IFAT is the most widely used method and, although different values have been applied in different studies (34–39), a cut-off of 1:80 is recommended in the LeishVet group guidelines (see text footnote 1).

The seroprevalence herein detected in Site A (5.6%) is higher than the rates registered recently in the same region (27). This may be attributed to the fact that 3/4 (75.0%) of the positive cats from Site A cohabited with dogs, as this was identified as a statistically significant risk factor by the statistical analysis in the present study. Accordingly, a recent study has demonstrated that a close contact with infected dogs can lead to high seroprevalence rates in cats (40). Nevertheless, this association should be interpreted with caution, because (i) it is generally accepted that the risk of infection does not increase in animals or humans cohabiting with infected dogs, but rather if a high seroprevalence occurs in local dog population and (ii) the parasitological status of dogs cohabiting with positive cats of this study was unknown. Another explanation could be that only cats living in coastal areas of Site A have been examined, while in the previous survey cats were sampled in both mountainous and coastal areas (27). Indeed, altitude and cold temperatures have a negative influence on the biology of *L. infantum* vectors (41, 42). Moreover, some efficient vectors of *L. infantum* e.g., *Phlebotomus neglectus*, find optimal habitat conditions at <1,000 m from the seashore (42, 43). The higher prevalence rates obtained in cats from Abruzzo 15 years ago by IFAT (16.3%) (44), can be explained by the low cut-off value (1:40) which has most likely overestimated the results.

The present data are the first generated on the exposure to *L. infantum* of feline populations living in Site B, a popular touristic island belonging to Tuscany regional territory. The prevalence rate recorded is in line with that detected in a previous study carried out in continental Tuscany in early 2000's (0.9%), (45). Importantly, the single cat that seroreacted was locally born and never moved to other areas. This confirms the presence of the pathogen on the island, which is not surprising, given that Tuscany is a known enzootic area of canine leishmaniosis (46). It should be also considered that Giglio island is a dog-friendly island for tourists[2] and dogs traveling with their owners from enzootic areas of continental Italy can constantly contribute to the circulation of *L. infantum* among the local fauna. This result thus represents a potential alarm bell ringing for pet populations for the near future.

In a recent study on feline VBDs performed in continental and insular Greece, no *L. infantum* seropositive cats were detected in Mykonos Island (31), a fact that could be attributed to the small size of sampled cats. Therefore, the present results provide new information for this geographic region. In fact, in previous studies in Greece, specific antibodies to *Leishmania* spp. in cats have been detected in Crete Island (14.7%) and in continental Greece i.e., Thessaloniki (3.8%), Athens (8.3%), Thessaly and Macedonia regions (10%) (22, 31, 47). As suggested for Italy, the circulation of *L. infantum* in Site C could have been favored by sociological factors. Indeed, in Greek Islands (including Site C) numerous colonies of stray cats managed by charities and animal welfare organizations arrange adoptions and re-homing of cats[3] (31). Nonetheless, these commendable initiatives may further concur to the spread of feline leishmaniosis, because cats are not tested before their re-location. The lack of studies on seropositivity in dog populations living in Mykonos Island does not allow a comparison of the prevalence between species. However, *L. infantum* is widely distributed in canine populations of Greece, and seroprevalence rates in regions of continental Greece range from 2% up to 50.2% (48, 49). An overall prevalence of 6.5% emerged in a recent serological and molecular study carried out on the Islands of Ios, Santorini, Tinos, and Skiathos (50). The prevalence of *L. infantum* in Greece and its presence in surrounding islands suggest that this protozoan most likely circulates also among canine populations of Mykonos.

Animals living outdoors are considered at high risk of infection with arthropod-transmitted pathogens, including *L. infantum* (51–53). Nevertheless, a significant correlation between outdoor lifestyle and the presence of *L. infantum* antibodies in the here examined cats was not observed. This is consistent with the results of a recent study, where 4/5 seropositive cats had, unexpectedly, an indoor lifestyle (27). Therefore, the present data suggest that indoor housing is not a sufficient preventative measure against leishmaniosis.

On the whole, this study confirms that cats living in the examined touristic areas of the Mediterranean basin are exposed to *L. infantum*. Infection rates in cats are generally lower than in dogs and the role of cats in the epidemiology of zoonotic leishmaniosis is still controversial and should be further investigated (16, 54). Recent findings have ultimately confirmed that dogs are better reservoir and spreaders of *L. infantum* than cats because under the same exposure conditions they display

[2]https://www.traghetti-giglio.it/en/tp-magazine/dog-friendly-holiday-giglio-island/

[3]www.mykonosanimals.org

higher parasitic load, a determining factor for infectivity to sandflies (40, 55). Nonetheless, the role of cats as persistent source of infection for vectors (54) should not be overlooked, as they may amplify infection chances for dogs and, subsequently, for people.

In conclusion, the awareness toward feline infections should be kept at higher levels. Further studies evaluating the occurrence of *L. infantum* both in cats and dogs living in touristic destinations like Mykonos and Giglio Islands are warranted. Also, it would be interesting to conduct molecular studies to evaluate if species other than *L. infantum* occur in animal populations living in Mediterranean touristic spots. The importance of a constant surveillance is underlined by the fact that leishmaniosis has been regarded as an emerging threat in travelers (56) and cases of human leishmaniosis in tourists that visited Italy and Greece have been documented (57, 58). As routine examinations for *Leishmania* infection and application of chemoprophylactic measures in dogs and cats traveling with their owners are not regulated by laws, the establishment of standard preventative protocols for traveling animals is of importance for limiting the spread of zoonotic leishmaniosis and to minimize Public Health risks.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because they are represented only by the number of seropositive/seronegative cats in each study areas. Requests to access the datasets should be directed to smorelli@unite.it.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because Blood samples were collected from cats upon a written consent obtained from the animal owners or the local municipality authorities and animal rights association authorities, in the framework of animal health screening programmes. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

DT has supervised the whole study and drafted the manuscript. SM, MC, and AD participated in all project activities and in drafting the manuscript, after critically revising it. DD, AB, ADC, IR, RS, and BP have participated in the field, laboratory work, and in drafting the manuscript in various ways. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Burza S, Croft S, Boelaert M. Leishmaniasis. *Lancet.* (2018) 392:951–70. doi: 10.1016/S0140-6736(18)31204-2
2. Asfaram S, Fakhar M, Teshnizi SH. Is the cat an important reservoir host for visceral leishmaniasis? A systematic review with meta-analysis. *J Venom Anim Toxins Incl Trop Dis.* (2019) 25:e20190012. doi: 10.1590/1678-9199-JVATITD-2019-0012
3. Torres-Guerrero E, Quintanilla-Cedillo M, Ruiz-Esmenjaud J, Arenas R. Leishmaniasis: a review. *F1000Res.* (2017) 6:750. doi: 10.12688/f1000research.11120.1
4. Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P, et al. A historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies. *PLoS Negl Trop Dis.* (2016) 10:e0004349. doi: 10.1371/journal.pntd.0004349
5. Steverding D. The history of leishmaniasis. *Parasit Vectors.* (2017) 10:82. doi: 10.1186/s13071-017-2028-5
6. Semenza J, Menne B. Climate change and infectious diseases in Europe. *Lancet Infect Dis.* (2009) 9:365–75. doi: 10.1016/S1473-3099(09)70104-5
7. Trájer A, Sebestyén V. The changing distribution of *Leishmania infantum* Nicolle, 1908 and its Mediterranean sandfly vectors in the last 140 kys. *Sci Rep.* (2019) 9:11820. doi: 10.1038/s41598-019-48350-7
8. Le Rutte EA, van Straten R, Overgaauw PAM. Awareness and control of canine leishmaniosis: a survey among Spanish and French veterinarians. *Vet Parasitol.* (2018) 253:87–93. doi: 10.1016/j.vetpar.2018.01.013
9. Escobar TA, Dowich G, Dos Santos TP, Zuravski L, Duarte CA, Lübeck I, et al. Assessment of *Leishmania infantum* infection in equine populations in a canine visceral leishmaniosis transmission area. *BMC Vet Res.* (2019) 15:381. doi: 10.1186/s12917-019-2108-1
10. Spada E, Perego R, Vitale F, Bruno F, Castelli G, Tarantola G, et al. Feline *Leishmania* spp. infection in a non-endemic area of northern Italy. *Animals.* (2020) 10:817. doi: 10.3390/ani10050817
11. Millán J, Ferroglio E, Solano-Gallego L. Role of wildlife in the epidemiology of *Leishmania infantum* infection in Europe. *Parasitol Res.* (2014) 113:2005–14. doi: 10.1007/s00436-014-3929-2
12. Tsakmakidis I, Pavlou C, Tamvakis A, Papadopoulos T, Christodoulou V, Angelopoulou K, et al. *Leishmania* infection in lagomorphs and minks in Greece. *Vet Parasitol Reg Stud Rep.* (2019) 16:100279. doi: 10.1016/j.vprsr.2019.100279
13. Cantos-Barreda A, Navarro R, Pardo-Marín L, Martínez-Subiela S, Ortega E, Cerón JJ, et al. Clinical leishmaniosis in a captive Eurasian otter (*Lutra lutra*) in Spain: a case report. *BMC Vet Res.* (2020) 16:312. doi: 10.1186/s12917-020-02509-x
14. Pace D. Leishmaniasis. *J Infect.* (2014) 20:1–9. doi: 10.1016/j.jinf.2014.07.016
15. Quinnell RJ, Courtenay O. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. *Parasitology.* (2009) 136:1915–34. doi: 10.1017/S0031182009991156
16. Dalvi APR, Carvalho TDG, Werneck GL. Is There an association between exposure to cats and occurrence of visceral leishmaniasis in humans and dogs? *Vector Borne Zoonotic Dis.* (2018) 18:335–42. doi: 10.1089/vbz.2017.2162
17. Day M. Cats are not small dogs: is there an immunological explanation for why cats are less affected by arthropod-borne disease than dogs? *Parasit Vectors.* (2016) 9:507. doi: 10.1186/s13071-016-1798-5
18. da Silva S, Rabelo P, Gontijo N, Ribeiro R, Melo M, Ribeiro V, et al. First report of infection of *Lutzomyia longipalpis* by *Leishmania* (*Leishmania*)

infantum from a naturally infected cat of Brazil. *Vet Parasitol.* (2010) 174:150–4. doi: 10.1016/j.vetpar.2010.08.005
19. Afonso M, Duarte R, Miranda J, Caranha L, Rangel E. Studies on the feeding habits of *Lutzomyia* (*Lutzomyia*) *longipalpis* (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae) populations from endemic areas of american visceral leishmaniasis in Northeastern Brazil. *J Trop Med.* (2012) 2012:858657. doi: 10.1155/2012/858657
20. Maroli M, Pennisi MG, Di Muccio T, Khoury C, Gradoni L, Gramiccia M. Infection of sandflies by a cat naturally infected with *Leishmania infantum*. *Vet Parasitol.* (2007) 145:357–60. doi: 10.1016/j.vetpar.2006.11.009
21. González E, Jiménez M, Hernández S, Martín-Martín I, Molina R. Phlebotomine sand fly survey in the focus of leishmaniasis in Madrid, Spain (2012–2014): seasonal dynamics, *Leishmania infantum* infection rates and blood meal preferences. *Parasit Vectors.* (2017) 10:368. doi: 10.1186/s13071-017-2309-z
22. Diakou A, Papadopoulos E, Lazarides K. Specific anti-*Leishmania* spp. antibodies in stray cats in Greece. *J Feline Med Surg.* (2009) 11:728–30. doi: 10.1016/j.jfms.2008.01.009
23. Maia C, Gomes J, Cristòvão J, Nunes M, Martins A, Rebêlo E, et al. Feline *Leishmania* infection in a canine leishmaniasis endemic region, Portugal. *Vet Parasitol.* (2010) 174:336–40. doi: 10.1016/j.vetpar.2010.08.030
24. Can H, Döşkaya M, Özdemir H, Sahar E, Karakavuk M, Pektaş B, et al. Seroprevalence of *Leishmania infection* and molecular detection of *Leishmania tropica* and *Leishmania infantum* in stray cats of Izmir, Turkey. *Exp Parasitol.* (2016) 167:109–14. doi: 10.1016/j.exppara.2016.05.011
25. Attipa C, Papasouliotis K, Solano-Gallego L, Baneth G, Nachum-Biala Y, Sarvani E, et al. Prevalence study and risk factor analysis of selected bacterial, protozoal and viral, including vector-borne, pathogens in cats from Cyprus. *Parasit Vector.* (2017) 10:130. doi: 10.1186/s13071-017-2063-2
26. Montoya A, García M, Gálvez R, Checa R, Marino V, Sarquis J, et al. Implications of zoonotic and vector-borne parasites to free-roaming cats in central Spain. *Vet Parasitol.* (2018) 251:125–30. doi: 10.1016/j.vetpar.2018.01.009
27. Morelli S, Crisi P, Di Cesare A, De Santis F, Barlaam A, Santoprete G, et al. Exposure of client-owned cats to zoonotic vector-borne pathogens: clinic-pathological alterations and infection risk analysis. *Comp Immunol Microbiol Infect Dis.* (2019) 66:101344. doi: 10.1016/j.cimid.2019.101344
28. Iatta R, Furlanello T, Colella V, Tarallo V, Latrofa M, Brianti E, et al. A nationwide survey of *Leishmania infantum* infection in cats and associated risk factors in Italy. *PLoS Negl Trop Dis.* (2019) 13: e0007594. doi: 10.1371/journal.pntd.0007594
29. Rüfenacht S, Sager H, Muller N, Schaerer V, Heier A, Welle M, et al. Two cases of feline leishmaniosis in Switzerland. *Vet Rec.* (2005) 156:542–5. doi: 10.1136/vr.156.17.542
30. Richter M, Schaarschmidt-Kiener D, Krudewig C. Ocular signs, diagnosis and long-term treatment with allopurinol in a cat with leishmaniasis. *Schweiz Arch Tierheilkd.* (2014) 156:289–94 doi: 10.1024/0036-7281/a000593
31. Diakou A, Di Cesare A, Accettura P, Barros L, Iorio R, Paoletti B, et al. Intestinal parasites and vector-borne pathogens in stray and free-roaming cats living in continental and insular Greece. *PLoS Negl Trop Dis.* (2017) 11: e0005335. doi: 10.1371/journal.pntd.0005335
32. Maia C, Cardoso L. Spread of *Leishmania infantum* in Europe with dog travelling. *Vet Parasitol.* (2015) 213:2–11. doi: 10.1016/j.vetpar.2015.05.003
33. Cardoso L, Lopes A, Sherry K, Schallig H, Solano-Gallego L. Low seroprevalence of *Leishmania infantum* infection in cats from northern Portugal based on DAT and ELISA. *Vet Parasitol.* (2010) 174:37–42. doi: 10.1016/j.vetpar.2010.08.022
34. Miró G, Rupérez C, Checa R, Álvez R, Hernández L, García M, et al. Current status of *L. infantum* infection in stray cats in the Madrid region (Spain): implications for the recent outbreak of human leishmaniosis? *Parasit Vectors.* (2014) 7:112. doi: 10.1186/1756-3305-7-112
35. Otranto D, Napoli E, Latrofa MS, Annoscia G, Tarallo VD, Greco G, et al. Feline and canine leishmaniosis and other vector-borne diseases in the Aeolian Islands: pathogen and vector circulation in a confined environment. *Vet Parasitol.* (2017) 236:144–51. doi: 10.1016/j.vetpar.2017.01.019
36. Morganti G, Veronesi F, Stefanetti V, Di Muccio T, Fiorentino E, Diaferia M, et al. Emerging feline vector-borne pathogens in Italy. *Parasit Vectors.* (2019) 12:193. doi: 10.1186/s13071-019-3409-8
37. Silaghi C, Knaus M, Rapti D, Kusi I, Shukullari E, Hamel D, et al. Survey of *Toxoplasma gondii* and *Neospora caninum*, haemotropic mycoplasmas and other arthropod-borne pathogens in cats from Albania. *Parasit Vectors.* (2014) 7:62. doi: 10.1186/1756-3305-7-62
38. Pennisi M, Lupo T, Malara D, Masucci M, Migliazzo A, Lombardo G. Serological and molecular prevalence of *Leishmania infantum* infection in cats from Southern Italy. *J Feline Med Surg.* (2012) 14:656–57. doi: 10.5402/2013/916376
39. Persichetti M, Solano-Gallego L, Serrano L, Altet L, Reale S, Masucci M, et al. Detection of vector-borne pathogens in cats and their ectoparasites in southern Italy. *Parasit Vectors.* (2016) 9:247. doi: 10.1186/s13071-016-1534-1
40. Baneth G, Nachum-Biala Y, Zuberi A, Zipori-Barki N, Orshan L, Kleinerman G, et al. *Leishmania* infection in cats and dogs housed together in an animal shelter reveals a higher parasite load in infected dogs despite a greater seroprevalence among cats. *Parasite Vectors.* (2020) 13:115. doi: 10.1186/s13071-020-3989-3
41. Koch LK, Kochmann J, Klimpel S, Cunze S. Modeling the climatic suitability of leishmaniasis vector species in Europe. *Sci Rep.* (2017) 7:13325.
42. Tsirigotakis N, Pavlou C, Christodoulou V, Dokianakis E, Kourouniotis C, Alten B, et al. Phlebotomine sand flies (Diptera: Psychodidae) in the Greek Aegean Islands: ecological approaches. *Parasit Vectors.* (2018) 11:97. doi: 10.1186/s13071-018-2680-4
43. Latrofa MS, Iatta R, Dantas-Torres F, Annoscia G, Gabrielli S, Pombi M, et al. Detection of *Leishmania infantum* DNA in phlebotomine sand flies from an area where canine leishmaniosis is endemic in southern Italy. *Vet Parasitol.* (2018) 253:39–42. doi: 10.1016/j.vetpar.2018.02.006
44. Vita S, Santori D, Aguzzi I, Petrotta E, Luciani A. Feline leishmaniasis and ehrlichiosis: serological investigation in Abruzzo region. *Vet Res Commun.* (2005) 2:319–21. doi: 10.1007/s11259-005-0071-8
45. Poli A, Abramo F, Barsotti P, Leva S, Gramiccia M, Ludovisi A, et al. Feline leishmaniosis due to *Leishmania infantum* in Italy. *Vet Parasitol.* (2002) 106:181–91. doi: 10.1016/s0304-4017(02)00081-x
46. Sauda F, Malandrucco L, Macrì G, Scarpulla M, De Liberato C, Terracciano G, et al. *Leishmania infantum*, *Dirofilaria* spp. and other endoparasite infections in kennel dogs in central Italy. *Parasite.* (2018) 25:2. doi: 10.1051/parasite/2018001
47. Chatzis M, Andreadou M, Leontides L, Kasabalis D, Mylonakis M, Koutinas A, et al. Cytological and molecular detection of *Leishmania infantum* in different tissues of clinically normal and sick cats. *Vet Parasitol.* (2014) 202:217–25. doi: 10.1016/j.vetpar.2014.02.044
48. Athanasiou LV, Kontos VI, Saridomichelakis MN, Rallis TS, Diakou A. A cross-sectional sero-epidemiological study of canine leishmaniasis in Greek mainland. *Acta Trop.* (2012) 122:291–5. doi: 10.1016/j.actatropica.2012.02.003
49. Ntais P, Sifaki-Pistola D, Christodoulou V, Messaritakis I, Pratlong F, Poupalos G, et al. Leishmaniases in Greece. *Am J Trop Med Hyg.* (2013) 89:906–15. doi: 10.4269/ajtmh.13-0070
50. Diakou A, Di Cesare A, Morelli S, Colombo M, Halos L, Simonato G, et al. Endoparasites and vector-borne pathogens in dogs from Greek islands: pathogen distribution and zoonotic implications. *PLoS Negl Trop Dis.* (2019) 13:e0007003 doi: 10.1371/journal.pntd.0007003
51. Beugnet F, Halos L. *Parasitoses and Vector Borne Diseases of Cats, 1st Edn.* Merial, Lyon: Press (2015).
52. Spada E, Canzi I, Baggiani L, Perego R, Vitale F, Migliazzo A, et al. Prevalence of *Leishmania infantum* and co-infections in stray cats in northern Italy. *Comp Immunol Microbiol Infect Dis.* (2016) 45:53–8. doi: 10.1016/j.cimid.2016.03.001
53. Persichetti M, Pennisi M, Vullo A, Masucci M, Migliazzo A, Solano-Gallego L. Clinical evaluation of outdoor cats exposed to ectoparasites and associated risk for vector-borne infections in southern Italy. *Parasit Vectors.* (2018) 11:136. doi: 10.1186/s13071-018-2725-8
54. Pennisi M, Cardoso L, Baneth G, Bourdeau P, Koutinas A, Miró G, et al. LeishVet update and recommendations on feline leishmaniosis. *Parasit Vectors.* (2015) 8:302. doi: 10.1186/s13071-015-0909-z
55. Borja LS, Sousa OMF, Solcà MDS, Bastos LA, Bordoni M, Magalhães JT, et al. Parasite load in the blood and skin of dogs naturally infected by *Leishmania infantum* is correlated with their capacity to infect sand fly vectors. *Vet Parasitol.* (2016) 229:110–7. doi: 10.1016/j.vetpar.2016.10.004

56. Mansueto P, Seidita A, Vitale G, Cascio A. Leishmaniasis in travelers: a literature review. *Travel Med Infect Dis.* (2014) 12:563–81. doi: 10.1016/j.tmaid.2014.09.007
57. Berens-Riha N, Fleischmann E, Pratlong F, Bretzel G, von Sonnenburg F, Löscher T. Cutaneous leishmaniasis (*Leishmania tropica*) in a German tourist after travel to Greece. *J Travel Med.* (2009) 16:220–2. doi: 10.1111/j.1708-8305.2008.00291.x
58. Ehehalt U, Schunk M, Jensenius M, van Genderen PJ, Gkrania-Klotsas E, Chappuis F, et al. Leishmaniasis acquired by travellers to endemic regions in Europe: a EuroTravNet multi-centre study. *Travel Med Infect Dis.* (2014) 12:167–72. doi: 10.1016/j.tmaid.2013.12.003

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Functional Characterization of Two Thioredoxin Proteins of *Toxoplasma gondii* Using the CRISPR-Cas9 System

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Toxoplasmosis caused by infection with *Toxoplasma gondii* is an important parasitic zoonosis with a worldwide distribution. In this study, we examined the functions of two thioredoxins (namely CTrp26 and CTrx1) of *T. gondii* tachyzoites by generation of HA tag strains or gene deficient parasites in Type I RH strain (ToxoDB#10). Immunofluorescence analysis (IFA) was used to investigate the subcellular localization of the thioredoxins (Trxs). Results of IFA showed that both CTrp26 and CTrx1 were located in the cytoplasm of *T. gondii*. Functional characterizations of CTrp26 and CTrx1-deficient parasites were performed by plaque assay, intracellular replication, egress, H_2O_2 resistance, detection of reactive oxygen species (ROS) level and total antioxidant capacity (T-AOC) assays *in vitro*, as well as mouse infection *in vivo.* Our results showed that deletion of CTrp26 or CTrx1 did not influence the ability of *T. gondii* RH strain to replicate, egress, form plaque, resist H_2O_2 exposure, maintain the ROS level, and T-AOC, and also did not serve as virulence factors in Kunming mice. Taken together, these results provide new properties of the two Trxs. Although they are not essential for RH strain, they may have roles in other strains of this parasite due to their different expression patterns, which warrants future research.

Keywords: *Toxoplasma gondii*, CRISPR-Cas9, thioredoxin, functional characterization, toxoplasmosis

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INTRODUCTION

Toxoplasma gondii is an obligate parasite that belongs to the apicomplexa with a worldwide distribution (1). This parasite can infect almost all warm-blooded animals and ∼1/3rd of the world population are seropositive (2). Horizontal transmission of *T. gondii* occurs via consumption of raw or undercooked meat containing tissue cysts or by ingestion of water, food, and soil contaminated with oocysts (1, 3). In immunologically healthy subjects, most people are asymptomatic (2). However, in immunocompromised individuals, especially in patients with AIDS and organ transplants, the symptoms that toxoplasmosis causes are severe, or even life-threatening (4). Toxoplasmic encephalitis is the primary manifestation in these patients, accompanied by headache, ataxia, loss of memory, fever, and other symptoms (1, 5). In addition, *T. gondii* can be transmitted via vertical transmission (6). It is noticeable that the placenta is not only a barrier to protect fetus

but also a target organ where *T. gondii* multiplication occurs. Once transplacental transmission occurs in the first and second trimesters, it leads to severe influence on the growth of fetus, probably resulting in microcephalus, hydrocephalus, cataract, strabismus, retinochoroiditis even abortion (1, 2, 7).

As an aerobiont, *T. gondii* can multiply rapidly within host cells at the infective stage of tachyzoites. To survive, *T. gondii* must undergo the redox stress which is induced by the microenvironment of host cells. This parasite not only eliminates oxidized material activity, but also resists the damage of reactive oxygen species (ROS) generated by host cells (8, 9). Thus, there should be a variety of mechanisms to balance the redox state in *T. gondii*, which is hypersensitive to redox imbalance.

Several peroxidases of *T. gondii*, including catalase, peroxiredoxin, superoxide dismutase (SOD), glutathione peroxidase, and thioredoxin (Trx) peroxidase, constitute the antioxidant network to protect this parasite from damage of ROS (9). *T. gondii* uses catalase to eliminate the by-products of peroxisome, such as H_2O_2. Peroxiredoxin can assist catalase to detoxify ROS, and it also protects *T. gondii* against H_2O_2 stress (10). There are two classical antioxidant systems in this parasite, namely glutaredoxin (Grx) system and thioredoxin (Trx) system, which serve as thiol-disulfide pairs to control the redox balance of *T. gondii* (9, 11). Glutaredoxin, glutathione (GSH), nictinamide adenine dinucleotide phosphate (NADPH), and glutathione reductase constitute one of the classical antioxidant systems, namely Grx system (9, 12). After reacting with target protein that contains disulfide bond, Grx becomes oxidized form, and then is reduced by GSH. NADPH and glutathione reductase operate together to reduce GSSG that means oxidized GSH, to become reduced form (13). Some Grx, such as human Grx2, can utilize electrons supplying from TrxR to keep redox balance (14). The basic biological functions of Grx are to maintain the ratio of GSH/GSSG at the normal level and the high concentration of thiol groups within cells, which overlaps the function of Trx to some extent (15). Thioredoxin system consists of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH, which is ubiquitous in eukaryotes and prokaryotes (16). Oxidized thioredoxin, Trx-S_2, forms after thiol-disulfide exchange reactions occurred *via* Trx-$(SH)_2$ and oxidized proteins, and Trx-S_2 is reduced by TrxR and NADPH that serves sufficient hydrogen electrons. Three components of this system participate mutually in several biological activities to protect organisms from oxidized damage and apoptosis (16, 17). The physiological function of Trx in virtually all organisms is to keep cytoplasmic proteins with reduced form, which means relatively stable (18).

The physiological functions of Trxs may be multiple in different organisms, evolving from catalyzing thiol-disulfide exchange reaction to specialized functions. It seems that Trx is "moonlighting protein," and the functions of Trxs in *T. gondii* are still unclear. Here, we used the CRISPR-Cas9 technique to study two Trx genes, namely CTrp26 (TGME49_290260) and CTrx1 (TGME49_266620), in the virulent *T. gondii* RH strain. The subcellular localization of the two Trxs, the abilities of Trx-deficient tachyzoites to form plaque, replicate, egress, resist H_2O_2 and maintain ROS level and total antioxidant capacity (T-AOC) *in vitro* and the virulence in mice *in vivo* were evaluated.

MATERIALS AND METHODS

Parasite and Cell Cultures

The tachyzoites of *T. gondii* RH strain (Type I, ToxoDB#10) were maintained *in vitro* in human foreskin fibroblast (HFF, ATCC®, SCRC-1041™) at 37°C,5% CO_2 atmosphere. HFF cells were cultured in Dulbecco's Modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum (FBS), 10 mM HEPES (pH 7.2), 100 Ug/ml streptomycin, and 100 U/ml penicillin (19). Infected cells were cracked by 27-gauga needle to release tachyzoites and then filtered using Millipore filter of 5 μm pore size.

Construction of Trx Mutant Strains

Trx knockout strains were generated using CRISPR-Cas9 approach as previously described (19). All primers used in this study are listed in **Supplementary Table 1**. Briefly, SgRNA of each Trx was inserted into the plasmid pSAG1-Cas9-SgUPRT to replace the UPRT targeting RNA using a Q5 Mutagenesis Kit (NEB). After sequencing, positive plasmids were extracted by using an Endo-Free Plasmid DNA Mini Kit (Omega). The 5′ and 3′ homologous arms of each Trx were amplified from the genomic DNA of *T. gondii* RH strain with each pair of specific primers listed in **Supplementary Table 1**. The plasmid pUPRT-DHFR-D was used as the template to generate DHFR fragment. Then three fragments mentioned above were engineered into the plasmid pUC19 with multi-fragment cloning method using a CloneExpress II one-step Cloning Kit (Vazyme). Positive plasmids were used to amplify the fragment of 5HR-DHFR-3HR to generate the homologous templates. About 40 μg positive plasmids of CRISPR-Cas9 and 15 μg purified fragment of 5HR-DHFR-3HR templates were co-transfected into tachyzoites of *T. gondii* RH strain by electroporation. Single strain clones were obtained by limiting dilution method and cultured in 96-well-plates with 3 μM pyrimethamine. Trx knockout strains were confirmed by PCR and RT-PCR as previously described (20).

C-Terminal Tagging

The CRISPR-Cas9 plasmids of each Trx that targets the 3′ region of each gene were constructed as previously described (21). The PCR product of each Trx that contains ∼1.5 kb of 3′ region of a Trx gene (except the STOP codon) and the fragment of 10×HA and DHFR was amplified from the p10×HA-LIC-DHFR plasmids as the template using a pair of specific primers. The purified fragment and C-terminal Trx-specific CRISPR-Cas9 plasmids were mixed and co-transfected into freshly egressed tachyzoites of *T. gondii* RH strain using electroporation. Single positive clones were identified by PCR and immunofluorescence analysis (IFA).

Western Blotting and Immunofluorescence Analyses

For Western blotting, freshly purified tachyzoites of *T. gondii* RH strain were lysed by RIPA lysis buffer on ice to obtain

proteins and were used for SDS-PAGE analysis, then transferred to polyvinylidene fluoride (PVDF) member. The primary antibodies used in Western blotting were rabbit anti-Aldolase (1:500) and rabbit anti-HA (1:1,000) (21).

For immunofluorescence analysis (IFA), HFF cells were infected with freshly egressed tachyzoites of *T. gondii* RH strain and were fixed with 4% paraformaldehyde, then penetrated with 0.2% Triton X-100 for 20 min. After blocked by 5% BSA diluted in PBS for 1 h, infected cells were incubated with rabbit anti-IMC1 (1:1,000) and mouse anti-HA (1:500) as primary antibodies at 4°C for overnight, and then incubated with Alexa Fluor 488 anti-rabbit IgG (1:1,000) and Alexa Fluor 594 goat anti-mouse IgG (1:1,000) as secondary antibodies at 37°C for 1 h. The samples were imaged using a Leica confocal microscope system (tcs sp52, Leica, Germany) (21).

Plaque Assay

About 200 tachyzoites of Trx knockout and wild-type (WT) RH strains were inoculated into 12-well-plates containing monolayer of HFF cells, and the 12-well-plates were inoculated for 1 week under 37°C, 5% CO_2 environment. The medium was removed and the infected cells were fixed with 4% paraformaldehyde for 30 min and stained with 0.5% crystal violet for 10 min at ambient temperature. The number and size of plaques were counted and analyzed (22).

Intracellular Replication

Tachyzoites of *T. gondii* RH strain were incubated into cell culture dishes containing monolayer of HFF cells for 1 h, with about 10^5 tachyzoites of each Trx knockout or WT strains per dish. After removing old medium, the dishes were washed three times to remove extracellular parasites. Then, infected cells were added with new culture medium and maintained at 37°C with 5% CO_2 atmosphere for 23 h. HFF cells were fixed with 4% paraformaldehyde for 30 min and stained with mouse anti-SAG1, followed by Alexa Fluor 594 goat-anti mouse IgG. Parasitophorous vacuoles (PVs) were selected randomly for analysis, and the numbers of parasites contained in PVs were recorded (22).

Egress Assay

Cell culture dishes containing monolayer of HFF cells were inoculated with freshly harvested tachyzoites of Trx knockout or WT strains, with about 10^5 tachyzoites per dish, and were maintained at 37°C with 5% CO_2 for 1 h. Then the dishes were washed three times with PBS to remove floating parasites and added with fresh culture medium to continue to culture for further 30–36 h. Then infected cells were treated with 3 μM calcium ionophore A23187 diluted in DMEM. The timing and images of parasites to egress from host cells were recorded by live cell microscopy (20).

H_2O_2 Resistance Analysis

Freshly egressed tachyzoites were cultured in regular DMEM or DMEM containing 1.6 mM H_2O_2 for 1 h at 37°C, 5% CO_2 atmosphere, then centrifuged at 1,000 g to remove the supernatants. Parasites were re-suspended in DMEM and inoculated into 12-well-plates containing monolayer of HFF cells to culture for 40 h. After being fixed with 4% paraformaldehyde, the samples were stained with mouse anti-SAG1, followed by Alexa Fluor 594 goat-anti mouse IgG. PVs were selected to count the number of parasites that PVs contained.

Detection of ROS Level in Trx Mutant Strains

Infected cells were cracked by 27-gauga needle to release tachyzoites. 1×10^6 purified tachyzoites were labeled with DCFH-DA (Beyotime, China) and suspended in DMEM. After incubated darkly at 37°C, 5% CO_2 atmosphere for 1 h, samples were centrifuged at 1,200 g to remove the supernatants and washed three times with PBS. Total fluorescence intensity of tachyzoites were determined with fluorescence microplate reader (Spectra Max M5, MD, USA) with 488 nm of excitation wavelength and 525 nm of emission wavelength.

Analysis of Total Antioxidant Capacity

Purified tachyzoites were resuspended in PBS and broken with ultrasonic cell disruptor (Uibra-Cell, Sonics, USA). The suspensions were centrifuged at 12,000 g under 4°C to extract proteins of *T. gondii*. T-AOC was measured by T-AOC assay kit (Beyotime, China) according to the manufacturer's instructions. The results were represented as total antioxidant capacity in per gram of protein.

Mouse Infection With Trx- Deficient Strains

Specific-pathogen-free (SPF) Kunming mice (female, 7 weeks old) were purchased from The Center of Laboratory Animals, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Mice (5 mice/cage) were habituated for 1 week to reduce stress response before experiment. Mice were infected with freshly egressed tachyzoites of Trx knockout or WT strains (10 mice/strain) by intraperitoneal injection, with 100 tachyzoites of *T. gondii* RH strain per mouse. All mice were recorded daily for the progression of illness and death time.

Statistical Analysis

The differences of experiments *in vitro* and *in vivo* were analyzed using Student's *t*-test and Gehan–Breslow–Wilcoxon test with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data obtained from three independent experiments were presented as means ± standard deviations (SD). The differences were considered statistically different when *P*-value was below 0.05.

RESULTS

Localization of CTrp26 and CTrx1

To determine the subcellular localization of CTrp26 and CTrx1 in *T. gondii*, 10×HA (~39 kDa) was inserted into the C-terminus of CTrp26 or CTrx1 gene endogenous locus. IFA results showed that CTrp26 and CTrx1 were both located in the cytoplasm of *T. gondii* (**Figure 1A**). The findings were consistent with the results of Western blotting analysis, with the bond of CTrp26 and CTrx1 occurred between 55 and 70 kDa (**Figure 1B**).

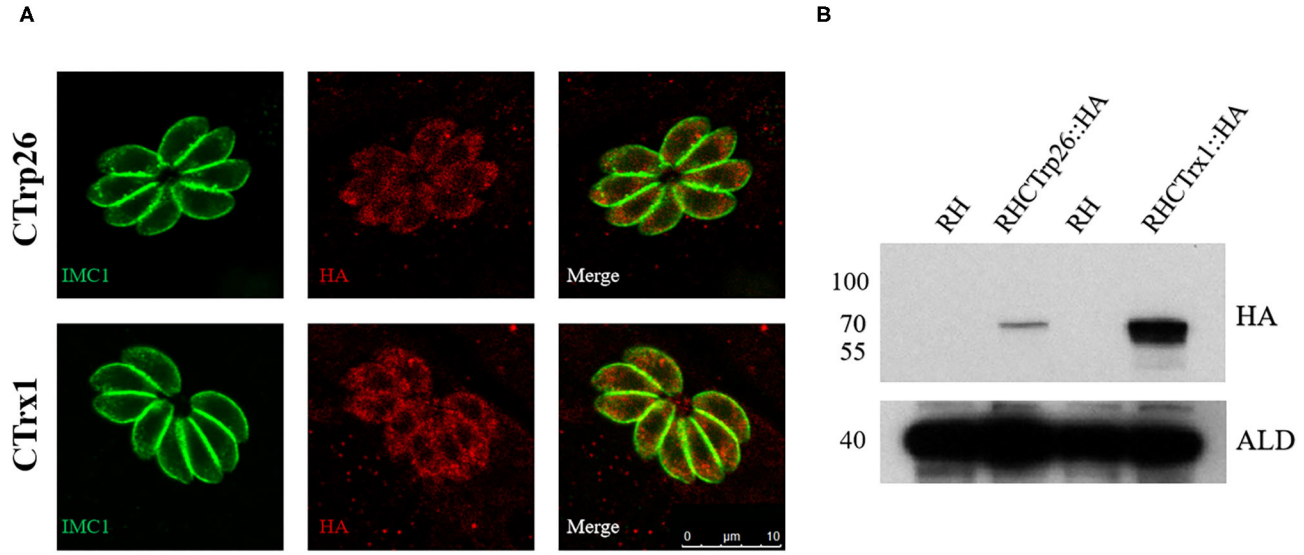

FIGURE 1 | Immunofluorescence analysis and Western blotting of CTrp26 and CTrx1. **(A)** Immunofluorescence analysis shows that both CTrp26 and CTrx1 are located in cytoplasm of *Toxoplasma gondii*. **(B)** Western blotting confirmed that the 10×HA were successfully inserted into the C terminal of CTrp26 or CTrx1. The 10×HA tagged CTrp26 and CTrx1 are about 62.3 and 61.7 kDa, respectively. Anti-adolase (ALD) was served as a loading control.

Construction of RHΔCTrp26 and RHΔCTrx1 Strains by CRISPR-Cas9

CRISPR-Cas9 technique was used to delete the CTrp26 and CTrx1 genes in type I RH strain, and CTrp26 or CTrx1 coding region was replaced by 5HR-DHFR-3HR fragment with homologous recombination technology (**Figure 2A**). Single clones obtained from drug selection and limiting dilution methods were confirmed by PCR, and ∼600 bp fragment was amplified in the WT strain but was not detected in KO strains because of the replacement by the 5HR-DHFR-3HR fragment (**Figure 2B**). RT-PCR method was also used to confirm the deletion of CTrp26 or CTrx1 gene at the mRNA level, with ∼200 bp fragment detected in the WT strains and no fragment found in KO strains (**Figure 2C**). Our results showed that CTrp26 and CTrx1 genes were disrupted by CRISPR-Cas9-mediated homologous recombination technology and that RHΔCTrp26 and RHΔCTrx1 strains were successfully constructed.

Disruption of CTrp26 or CTrx1 Gene Did Not Affect the Growth and Egress of *T. gondii*

Plaque assay was used to compare the ability of plaque formation between RHΔCTrp26 or RHΔCTrx1 knockout strains and WT strains (**Figure 3**). HFF cells grown in 12-well-plate were infected with about 200 tachyzoites of Trx mutants or WT strains to allow parasites to grow for 7 days, and the size and number of plaques were analyzed. Results showed that there were no significant differences in the size and number of plaques between cells infected with RHΔCTrp26 or RHΔCTrx1 knockout tachyzoites and WT strains.

Subsequently, we investigated the role of Trxs on egress process of *T. gondii*, 3 μM calcium ionophore A23187 were used to treat HFF cells infected with RHΔCTrp26 or RHΔCTrx1 knockout tachyzoites and WT strains, and the egress process was recorded by time-lapse microscopy over 5 min (**Figure 4A**). The results showed that most Trx mutants and WT strains egressed from host cells within 2 min, with no significant differences in egress process being observed among different strains.

To evaluate the effect of Trxs on the intracellular replication of *T. gondii*, Trx mutants, and their parental strains were incubated into cell dishes containing monolayer of HFF cells for 24 h, and were fixed with 4% paraformaldehyde to count tachyzoite numbers that PVs contained (**Figure 4B**). Results showed that the ability of intracellular growth of RHΔCTrp26 or RHΔCTrx1 knockout strains and their parental strains was similar.

Deletion of CTrp26 or CTrx1 Gene Did Not Affect the Sensitivity of *T. gondii* to H_2O_2 Exposure

Extracellular parasites treated with regular DMEM or DMEM containing H_2O_2 were inoculated into HFF monolayers to examine whether RHΔCTrp26 and RHΔCTrx1 knockout strains were more sensitive to oxidative stress compared with WT RH strain. The results showed that the proliferation rates of the three strains were similar under the same condition (control group or treatment group) (**Figure 5**); however, the ability of intracellular growth associated with treatment group was significantly lower than that in control group ($P < 0.05$), suggesting that oxidative stress inhibited the proliferation of tachyzoites, but RHΔCTrp26 and RHΔCTrx1 strains were not more sensitive to H_2O_2 exposure compared with WT RH strain.

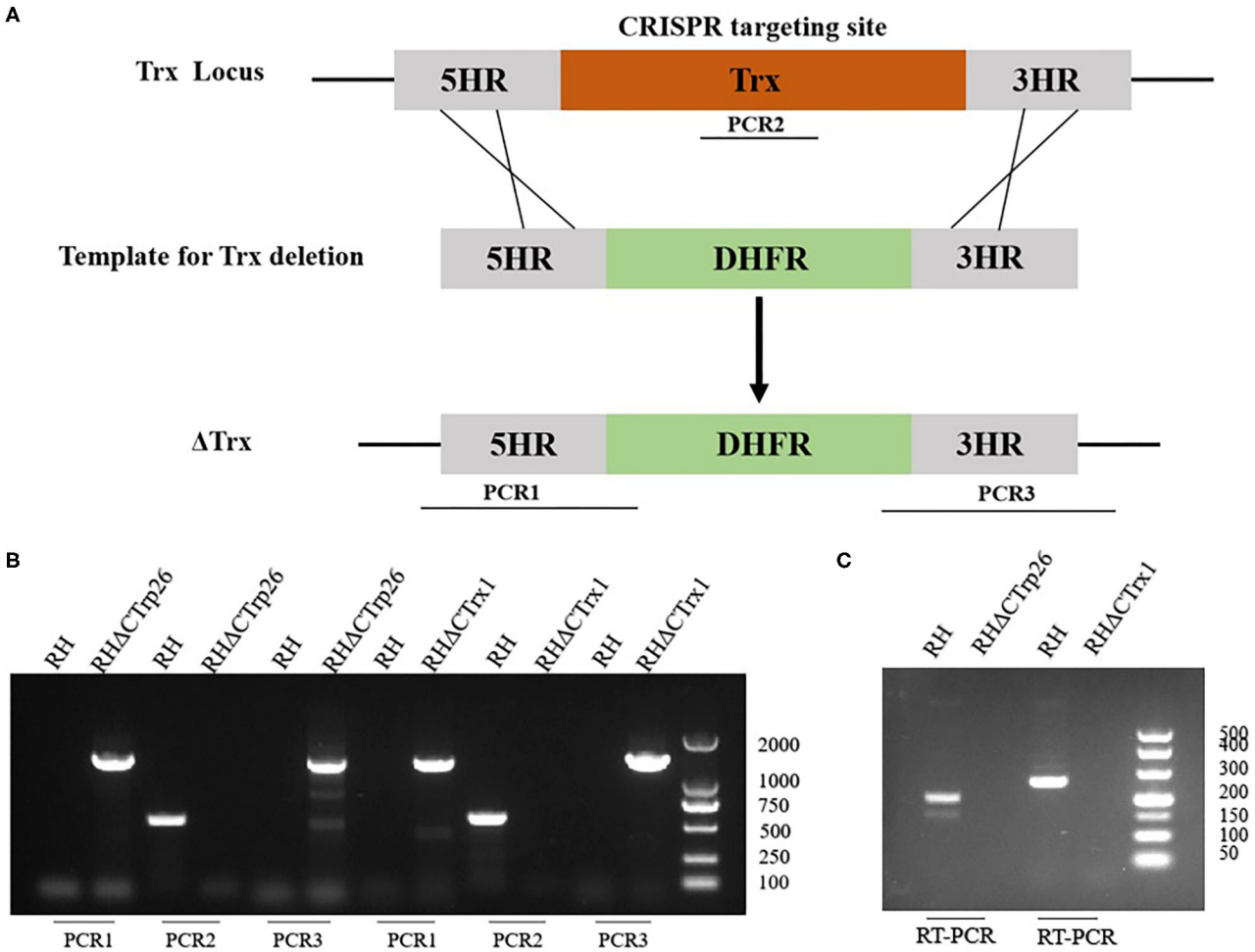

FIGURE 2 | Construction of CTrp26 or CTrx1 knockout strains. **(A)** Schematic representation of deleting CTrp26 or CTrx1 by CRISPR-Cas9-mediated homologous replacement. **(B)** Diagnostic PCRs show that CTrp26 or CTrx1 gene was successfully disrupted, which is confirmed by at mRNA level **(C)**.

Analysis of ROS Level and T-AOC in RHΔCTrp26 and RHΔCTrx1 Strains

To investigate ROS level in Trx mutants and WT strains, tachyzoites of the three strains were labeled with DCFH-DA (reduced form) and measured with fluorescence intensity of DCF (oxidized form) that reflects ROS level in *T. gondii*. The results showed that there were no significant differences in ROS level among RHΔCTrp26, RHΔCTrx1, and WT strains (**Figure 6A**), suggesting that disruption of CTrp26 or CTrx1 gene had no effect on ROS level in *T. gondii* RH strain.

Furthermore, we evaluated whether CTrp26 or CTrx1 deletion can affect the T-AOC of *T. gondii*. Extracted protein of tachyzoites was used to examine T-AOC using T-AOC assay kit (Beyotime, China). Our results revealed that T-AOC in per gram of protein among three strains were similar (**Figure 6B**), indicating that CTrp26 or CTrx1 plays no role in T-AOC in RH strain of *T. gondii*.

Deletion of CTrp26 or CTrx1 Gene Did Not Attenuate the Virulence of *T. gondii* in Mice

To evaluate whether the deletion of Trxs can attenuate the pathogenicity of the parasite to mice, Kunming mice were intraperitoneally injected with 100 tachyzoites of RHΔCTrp26, RHΔCTrx1, or WT strains, and the morbidity and mortality were recorded daily (**Figure 7**). The results showed that all mice died within 9–12 days, suggesting that Trxs are not virulence factors and do not alter lethal action of *T. gondii* RH strain in mice.

DISCUSSION

Some of the Apicomplexan parasites, such as *Plasmodium falciparum* and *T. gondii*, result in severe zoonoses like malaria and toxoplasmosis (8). These parasites are aerobian protozoans that are supersensitive to the microenvironment of the host cells (8). They adapt to environmental conditions they live, thus have

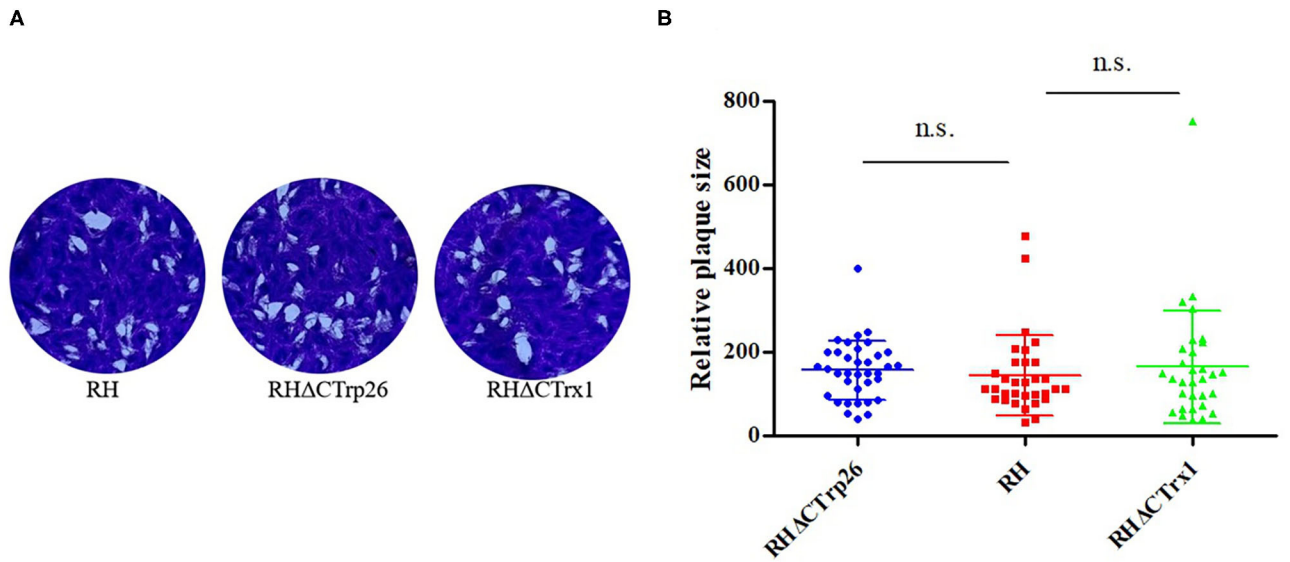

FIGURE 3 | The lytic cycle of RHΔCTrp26 or RHΔCTrx1 knockout strains and WT strain *in vitro*. **(A)** Plaque assay of Trx mutant strains and WT strain. **(B)** Relative size of the plaques generated by Trx mutant strains and WT strain showed no significant differences between WT strain and any of Trx mutant strains. n. s, not significant.

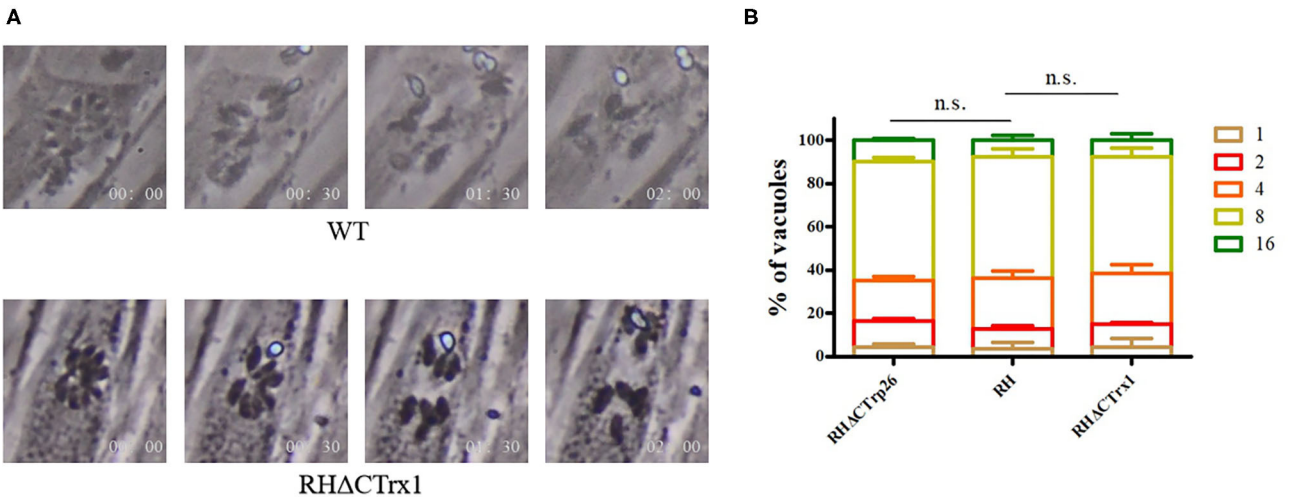

FIGURE 4 | Egress process and intracellular replication of CTrp26 or CTrx1 knockout strains and WT strain *in vitro*. **(A)** Representative image shows that one of Trx mutant strains (Δ*CTrx1*) and WT strain egress within 2 min after addition of 3 μM calcium ionophore A23187. **(B)** WT strain and Trx mutant strains have similar intracellular replication dynamic. n. s, not significant.

developed several antioxidant mechanisms to tackle the oxidative stress generated by the host cells.

There are three classic thioredoxins (PfTrx1–3) and two thioredoxin-like proteins (PfTlp1–2) that have been identified in *P. falciparum* (23). PfTrx1 is a cytoplasmic protein that can directly reduce oxidized glutathine and deloxify H_2O_2 to counteract oxidative stress (24). Although PfTrx2 was reported originally to locate in mitochondria, recent research revealed that PfTrx2 is the component of the translocon of exported proteins (PTEX) in PVs and involved in the protein trafficking from *P. falciparum* to host cells (25). PfTrx3 is located in the endoplasmic reticulum (ER) to fold target proteins *via* formation of disulfide band (24). However, limited studies have focused on the two thioredoxin-like proteins of *P. falciparum*, and the physiological functions of PfTlp1–2 remain unclear (23).

Catalase (CAT) is lacking in most protozoa, whereas it exists in *T. gondii* (26). A previous study indicated that CAT plays a role in the proliferation of *T. gondii* within PVs, because when CAT gene was deleted, parasites appeared more sensitive to H_2O_2 exposure and was less virulent to mice (27). Peroxiredoxin 2

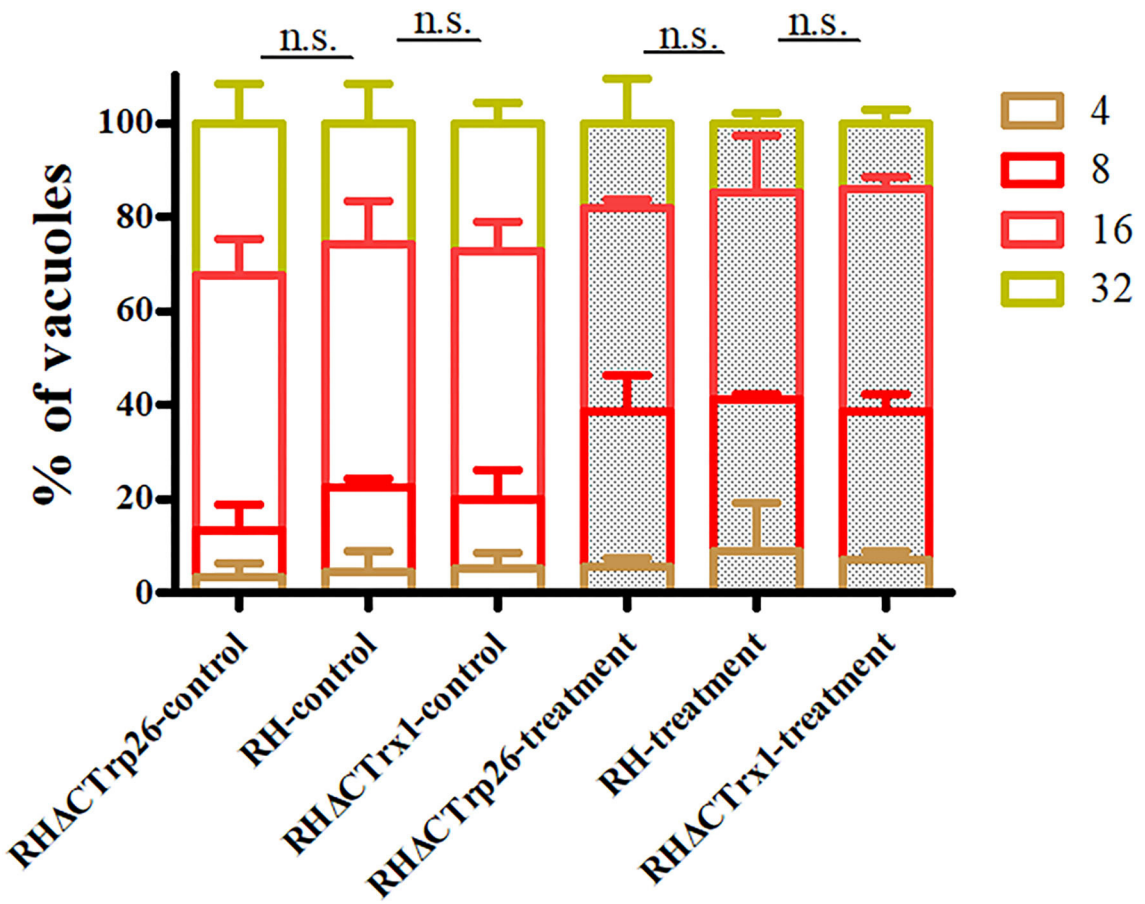

FIGURE 5 | Effect of H_2O_2 on *T. gondii* proliferation associated with RHΔCTrp26 or RHΔCTrx1 knockout strains and WT strain *in vitro.* Control and treatment represent parasites treated with regular DMEM and DMEM containing H_2O_2, respectively. n. s, not significant.

(Prx2) is also an antioxidant enzyme that locates in the cytoplasm of *T. gondii*, it not only helps CAT to detoxify ROS, but also can eliminate H_2O_2 by itself (28). Prx2-overexpression parasites showed strong resistance to oxidative stress (27). In addition, there are also classical antioxidant systems in *T. gondii*, especially the thioredoxin (Trx) system, which contains three members, namely thioredoxin (Trx), thioredoxin reductase (TrxR) and nictinamide adenine dinucleotide phosphate (NADPH). The three members of the Trx system operate mutually in order to balance the redox state in *T. gondii*. However, studies of the Trx system in *T. gondii* is limited, and the functions of Trx in *T. gondii* infection remain unclear.

Only a few studies have focused on the biological functions of Trx in *T. gondii* RH strain. To date, two Trxs have been identified in *T. gondii.* TgATrx1 is located in the outermost compartment of apicoplast, cytoplasm and ER (29). TgATrx2 is an apicoplast periplastid protein, whose phenotype enrichment score is −2.87 (8, 30). Both TgATrx1 and TgATrx2 are essential for the growth of *T. gondii*, and conditional depletion of either of two Trxs leads to loss of plaque formation, which indicated that generated parasites displayed severe grow defect in the absence of TgATrx1 or TgATrx2 protein (8), suggesting that TgATrx1 is in control of protein trafficking from ER to apicoplast, and TgATrx2 affects genome copy number of apicoplast (8).

In the present study, we generated a transgenic parasite by inserting a 10×HA tag into C-terminal of CTrp26 or CTrx1 to investigate the subcellular localization of CTrp26 and CTrx1 in *T. gondii*. IFA showed that both CTrp26 and CTrx1 were located in the cytoplasm of *T. gondii* tachyzoites. Furthermore, results of Western blotting confirmed that CTrp26 and CTrx1 were expressed successfully with the HA tags.

CRISPR-Cas9 technique was used to disrupt CTrp26 and CTrx1 in type I RH strain of *T. gondii,* and RHΔCTrp26 strain and RHΔCTrx1 strain were constructed. The number and size of plaques that generated by RHΔCTrp26, RHΔCTrx1 and WT RH strains were not significantly different, suggesting that CTrp26 and CTrx1 did not play role in several lytic cycles of *T. gondii* RH strain. Furthermore, intracellular replication and egress assay revealed that the replication and egress efficiencies of CTrp26 mutant and CTrx1 mutant were comparable to that of RH WT strain. Moreover, the survival of mice was recorded

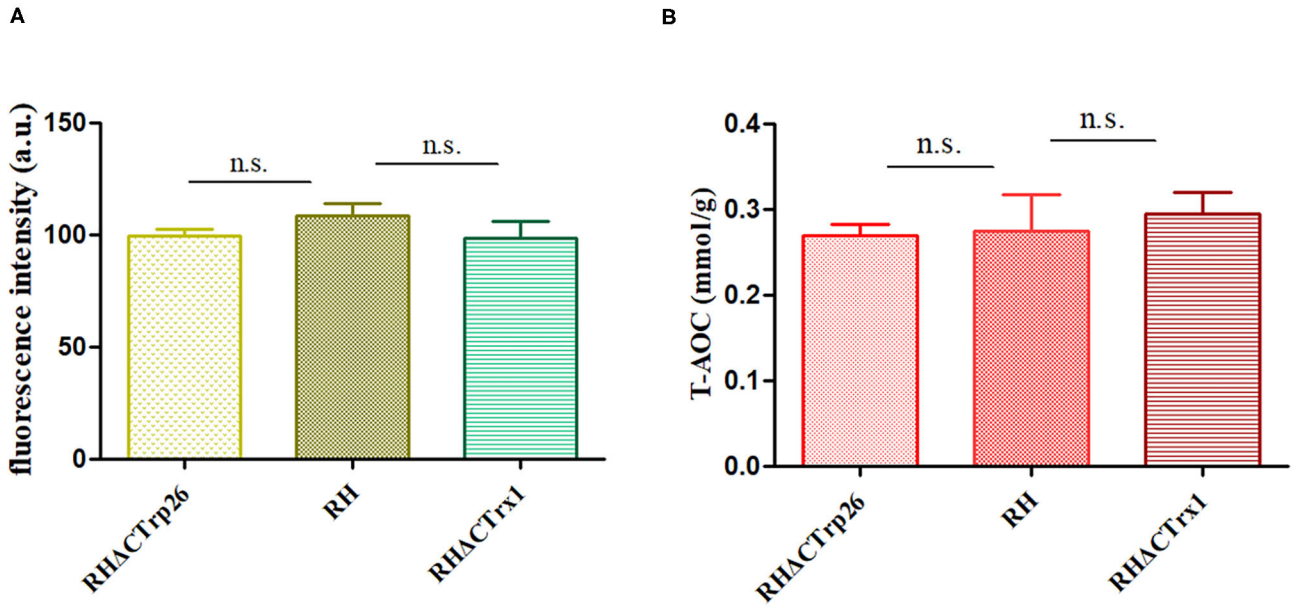

FIGURE 6 | Responses of oxidative stress on RHΔCTrp26 or RHΔCTrx1 knockout strains *in vitro*. **(A)** The reactive oxygen species level in RHΔCTrp26 or RHΔCTrx1 knockout strains and WT strain. **(B)** The total antioxidant capacity in RHΔCTrp26 or RHΔCTrx1 knockout strains and WT strain. n. s, not significant.

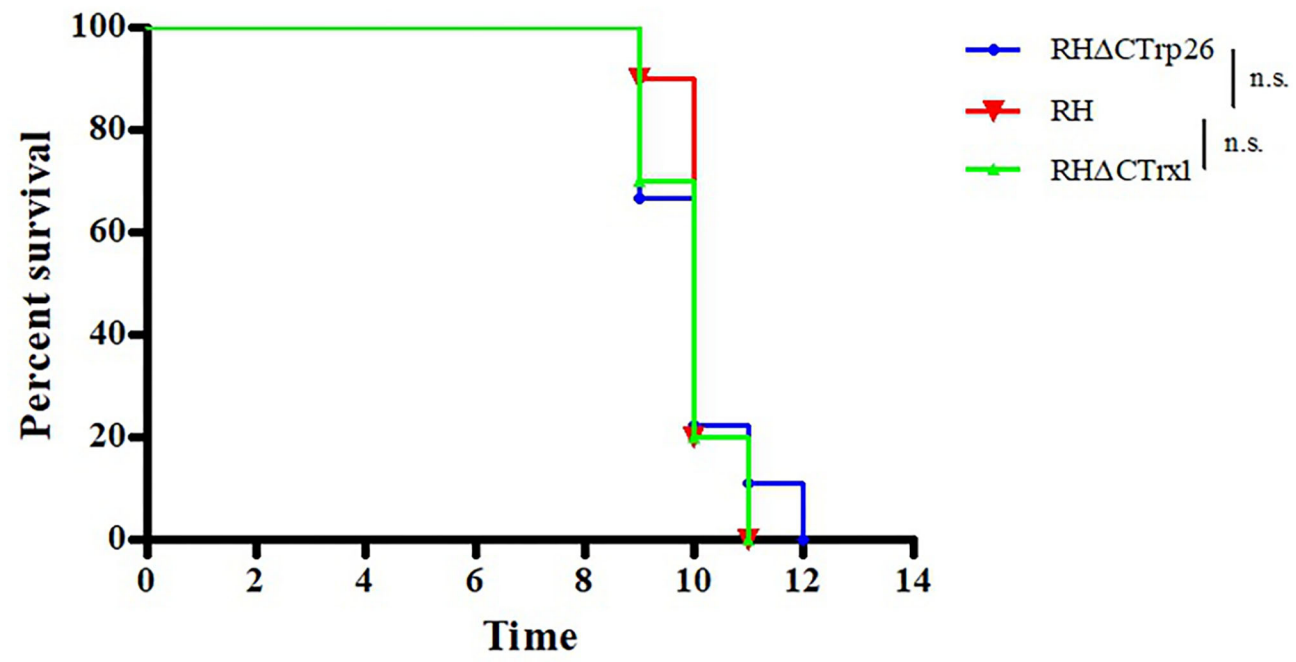

FIGURE 7 | Survival of Kunming mice infected with Trx mutant strains and WT strain of *T. gondii.* The mice (10/group) were intraperitoneally injected with 100 tachyzoites of each strain. The death time of mice were recorded until all mice died.

in order to investigate the functions of CTrp26 and CTrx1 *in vivo*. All mice died within 9-12 days, indicating that CTrp26 and CTrx1 were not important virulence factors and deletion of either of the two genes did not attenuate the virulence of *T. gondii* RH strain in mice. The phenotype enrichment scores of CTrp26 and CTrx1 are 0.42 and 1.28, respectively, which indicates that CTrp26 and CTrx1 are not essential genes in RH strain.

Considering that the function of Trx is keeping the redox balance, we also investigated the resistance of *T. gondii* to H_2O_2 exposure, ROS level and T-AOC of *T. gondii*. H_2O_2 exposure could inhibit the proliferation of tachyzoites of RHΔCTrp26, RHΔCTrx1 mutants and RH WT strain; however, the sensitivity of the three strains was similar, suggesting that CTrp26 and CTrx1 do not play role in resistance of H_2O_2 damage in *T. gondii* RH strain. Our results revealed that the disruption of CTrp26 or CTrx1 in *T. gondii* RH strain did not influence the ROS level in Trx mutant strains. The deletion of CTrp26 or CTrx1 did not reduce T-AOC of *T. gondii* RH strain.

CONCLUSION

Using CRISPR-Cas9 technique, the present study examined the basic biological functions of CTrp26 and CTrx1 in *T. gondii* RH strain *in vitro* and *in vivo*. Our results showed that CTrp26 and CTrx1 are both located in the cytoplasm of tachyzoites. Deletion of either of the two Trxs did not affect intracellular replication, egress process, plaque formation, H_2O_2 resistance, ROS level, and T-AOC of this parasite *in vitro*, and these two Trxs did not serve as virulence factors in *T. gondii* RH strain in Kunming mice. Further research will focus on the functions of CTrp26 and CTrx1 in other genotypes of *T. gondii* and the roles these two Trxs play when *T. gondii* undergo the damage of other ROS.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

AUTHOR CONTRIBUTIONS

J-LW and X-QZ designed the study. Z-WZ performed the experiments, analyzed the data, and wrote the manuscript. J-LW, Q-LL, and X-QZ revised the manuscript. T-TL, H-SZ, and L-XS participated in implementation of the study. All authors read and approved the final 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.2020.614759/full#supplementary-material

REFERENCES

1. Robert-Gangneux F, Dardé ML. Epidemiology of and diagnostic strategies for toxoplasmosis. *Clin Microbiol Rev.* (2012) 25:264–96. doi: 10.1128/CMR.05013-11
2. Montoya JG, Liesenfeld O. Toxoplasmosis. *Lancet.* (2004) 363:1965–76. doi: 10.1016/S0140-6736(04)16412-X
3. Wang JL, Zhang NZ, Li TT, He JJ, Elsheikha HM, Zhu XQ. Advances in the development of anti-*Toxoplasma gondii* vaccines: challenges, opportunities, and perspectives. *Trends Parasitol.* (2019) 35:239–53. doi: 10.1016/j.pt.2019.01.005
4. Feleke DG, Gebreweld A, Zewde G. Toxoplasmosis in pregnant women and HIV/AIDS patients in Ethiopia: a systematic review and meta-analysis. *J Parasitol Res.* (2019) 2019:4670397. doi: 10.1155/2019/4670397
5. Elsheikha HM, Marra CM, Zhu XQ. Epidemiology, pathophysiology, diagnosis, and management of cerebral toxoplasmosis. *Clin Microbiol Rev.* (2021) 34:e00115–19. doi: 10.1128/CMR.00115-19
6. Wallon M, Peyron F, Cornu C, Vinault S, Abrahamowicz M, Bonithon Kopp C, et al. Congenital toxoplasma infection: monthly prenatal screening decreases transmission rate and improves clinical outcome at age 3 years. *Clin Infect Dis.* (2013) 56:1223–31. doi: 10.1093/cid/cit032
7. Rico-Torres CP, Vargas-Villavicencio JA, Correa D. Is *Toxoplasma gondii* type related to clinical outcome in human congenital infection? Systematic and critical review. *Eur J Clin Microbiol Infect Dis.* (2016) 35:1079–88. doi: 10.1007/s10096-016-2656-2
8. Biddau M, Bouchut A, Major J, Saveria T, Tottey J, Oka O, et al. Two essential thioredoxins mediate apicoplast biogenesis, protein import, and gene expression in *Toxoplasma gondii*. *PLoS Pathog.* (2018) 14:e1006836. doi: 10.1371/journal.ppat.1006836
9. Bosch SS, Kronenberger T, Meissner KA, Zimbres FM, Stegehake D, Stegehake NM, et al. Oxidative stress control by apicomplexan parasites. *Biomed Res Int.* (2015) 2015:351289. doi: 10.1155/2015/351289
10. Hofmann B, Hecht HJ, Flohé L. Peroxiredoxins. *Biol Chem.* (2002) 383:347–64. doi: 10.1515/BC.2002.040
11. Jones DP, Go YM. Redox compartmentalization and cellular stress. *Diabetes Obes Metab.* (2010) 12(Suppl 2):116–25. doi: 10.1111/j.1463-1326.2010.01266.x
12. Luo M, Jiang YL, Ma XX, Tang YJ, He YX, Yu J, et al. Structural and biochemical characterization of yeast monothiol glutaredoxin Grx6. *J Mol Biol.* (2010) 398:614–22. doi: 10.1016/j.jmb.2010.03.029
13. Cooper AJ, Pinto JT, Callery PS. Reversible and irreversible protein glutathionylation: biological and clinical aspects. *Expert Opin Drug Metab Toxicol.* (2011) 7:1183. doi: 10.1517/17425255.2011.577738
14. Johansson C, Lillig CH, Holmgren A. Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons

from either glutathione or thioredoxin reductase. *J Biol Chem.* (2004) 279:7537–43. doi: 10.1074/jbc.M312719200
15. Shelton MD, Chock PB, Mieyal JJ. Glutaredoxin: role in reversible protein s-glutathionylation and regulation of redox signal transduction and protein translocation. *Antioxid Redox Signal.* (2005) 7:348–66. doi: 10.1089/ars.2005.7.348
16. Arnér ES, Holmgren A. Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem.* (2000) 267:6102–09. doi: 10.1046/j.1432-1327.2000.01701.x
17. Powis G, Montfort WR. Properties and biological activities of thioredoxins. *Annu Rev Pharmacol Toxicol.* (2000) 41:261–95. doi: 10.1146/annurev.pharmtox.41.1.261
18. Collet JF, Messens J. Structure, function, and mechanism of thioredoxin proteins. *Antioxid Redox Signal.* (2010) 13:1205–16. doi: 10.1089/ars.2010.3114
19. Wang JL, Huang SY, Li TT, Chen K, Ning HR, Zhu XQ. Evaluation of the basic functions of six calcium-dependent protein kinases in *Toxoplasma gondii* using CRISPR-Cas9 system. *Parasitol Res.* (2016) 115:697–702. doi: 10.1007/s00436-015-4791-6
20. Bai MJ, Wang JL, Elsheikha HM, Liang QL, Chen K, Nie LB, et al. Functional characterization of dense granule proteins in *Toxoplasma gondii* RH strain using CRISPR-Cas9 system. *Front Cell Infect Microbiol.* (2018) 8:300. doi: 10.3389/fcimb.2018.00300
21. Cao XZ, Wang JL, Elsheikha HM, Li TT, Sun LX, Liang QL, et al. Characterization of the role of amylo-alpha-1,6-glucosidase protein in the infectivity of *Toxoplasma gondii*. *Front Cell Infect Microbiol.* (2019) 9:418. doi: 10.3389/fcimb.2019.00418
22. Wang JL, Bai MJ, Elsheikha HM, Liang QL, Li TT, Cao XZ, et al. Novel roles of dense granule protein 12 (GRA12) in *Toxoplasma gondii* infection. *FASEB J.* (2020) 34:3165–78. doi: 10.1096/fj.201901416RR
23. Joetzik E, Becker K. Thioredoxin and glutathione systems in *Plasmodium falciparum*. *Int J Med Microbiol.* (2012) 302:187–94. doi: 10.1016/j.ijmm.2012.07.007
24. Kehr S, Sturm N, Rahlfs S, Przyborski JM, Becker K. Compartmentation of redox metabolism in malaria parasites. *PLos Pathog.* (2010) 6:e1001242. doi: 10.1371/journal.ppat.1001242
25. Sharma A, Sharma A, Dixit S, Sharma A. A structural insights into thioredoxin-2: a component of malaria parasite protein secretion machinery. *Sci Rep.* (2011) 1:179. doi: 10.1038/srep00179
26. Ding M, Clayton C, Soldati D. *Toxoplasma gondii* catalase: are there peroxisomes in toxoplasma? *J Cell Sci.* (2000) 113:2409–19.
27. Ding M, Kwok LY, Schlüter D, Clayton C, Soldati D. The antioxidant systems in *Toxoplasma gondii* and the role of cytosolic catalase in defence against oxidative injury. *Mol Microbiol.* (2004) 51:47–61. doi: 10.1046/j.1365-2958.2003.03823.x
28. Akerman SE, Müller S. Peroxiredoxin-linked detoxification of hydroperoxides in *Toxoplasma gondii*. *J Biol Chem.* (2005) 280:564–70. doi: 10.1074/jbc.M406367200
29. DeRocher AE, Coppens I, Karnataki A, Gilbert LA, Rome ME, Feagin JE, et al. A thioredoxin family protein of the apicoplast periphery identifies abundant candidate transport vesicles in *Toxoplasma gondii*. *Eukaryot Cell.* (2008) 7:1518–29. doi: 10.1128/EC.00081-08
30. Sheiner L, Demerly JL, Poulsen N, Beatty WL, Lucas O, Behnke MS, et al. A systematic screen to discover and analyze apicoplast proteins identifies a conserved and essential protein import factor. *PLoS Pathog.* (2011) 7:e1002392. doi: 10.1371/journal.ppat.1002392

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Asian Admixture in European *Echinococcus multilocularis* Populations: New Data From Poland Comparing EmsB Microsatellite Analyses and Mitochondrial Sequencing

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The cestode *Echinococcus multilocularis* is the causative agent of a severe zoonotic disease: alveolar echinococcosis (AE). The parasite is distributed over a vast area in northern Eurasia and North America, but the impact of AE on human health is highly uneven between different regions. One hypothetical reason for this difference in virulence may be the genetic structure of *E. multilocularis* which—based on mitochondrial sequences and EmsB microsatellite profiles—forms four distinct clades. These clades correspond approximately to their continents of origin: Asia, Europe, and North America, with a fourth clade apparently restricted to Mongolia and neighboring regions, even though this clade has not yet been described by EmsB genotyping. However, there are various records of genetic variants from the "wrong" region, e.g., "European" haplotypes in Western Canada, which may be the result of introduction or natural migration of host animals. One such example, prompting this study, is the recent record of an "Asian" mitochondrial haplotype in worms from foxes in Poland. At the time, this could not be confirmed by EmsB microsatellite analysis, a method that has proven to possess greater discriminatory power with the *E. multilocularis* nuclear genome than sequencing of mitochondrial markers. Therefore, worms collected from foxes in Poland were examined both by EmsB analysis and sequencing of the full mitochondrial *cox1* gene in order to allocate the samples to the European or Asian cluster. Based on EmsB analyses of 349 worms from 97 Polish red foxes, 92% of the worms clearly showed "European-type" EmsB profiles, but 27 worms (8%) from seven foxes showed profiles that clustered with samples of Asian origin. According to *cox1* sequences, a total of 18 worms from 8 foxes belonged to the Asian cluster of haplotypes. The two methods did not fully agree: only 13 worms from three foxes belonged to Asian clusters by both EmsB and *cox1*, whereas 18

worms from nine foxes belonged to different clusters, according to each marker. Cross-fertilization between worms of Asian origin and those from the European Polish population may explain these conflicting results. The presence of clearly Asian elements in the Polish *E. multilocularis* population could be the result of introduction of *E. multilocularis* with host animals (e.g., domestic dogs), or the migration of foxes. In the absence of genetic data from eastern European countries, especially those bordering Poland, it cannot be concluded whether this Asian admixture is typical for a larger area toward central/eastern Europe, or the Polish parasite population is the western extreme of a gradient where both European and Asian elements mingle. Further studies are needed on this subject, preferably using both mitochondrial sequencing and EmsB microsatellite analysis.

Keywords: *Echinococcus multilocularis*, Poland, EmsB microsatellite, mitochondrial sequencing, Asian origin, cross-fertilization

INTRODUCTION

The cestode *Echinococcus multilocularis* is the causative agent of a severe parasitic disease: alveolar echinococcosis (AE). This zoonotic disease is widely distributed throughout the northern hemisphere. China is considered the main focus of human AE cases, with an estimated 91% of all new cases per year worldwide; in contrast, the European proportion of the global load was estimated at <1%, representing 168 new AE cases annually (1). After the initial infection event, human disease is characterized by a long asymptomatic period (5–15 years), during which parasitic lesions develop in the liver, potentially extending or metastasizing to other organs. The mortality rate is >90% in untreated or inadequately treated cases within 10–15 years after diagnosis (2). Humans become infected through oral ingestion of *E. multilocularis* eggs dispersed into the environment. In the typical parasitic lifecycle, small mammals (often arvicoline rodents) ingest these eggs, leading to the development of hepatic lesions producing protoscoleces. The prey-predator relationship between these rodents and carnivores, in central and eastern Europe mainly red foxes, results in the colonization of the small intestines of carnivores by these protoscoleces, where they evolve to adult worms releasing eggs into the environment via the feces.

The impact of AE on human health is highly uneven between different regions. One hypothetical reason for this difference in virulence may be the genetic structure of *E. multilocularis*. A study based on sequencing of three complete mitochondrial genes *cox1*, *cob* and *nad2* (3,558 bp) of *E. multilocularis* samples resulted in the identification of four different geographical clades: Europe, Asia and North America, with a fourth clade apparently restricted to Mongolia and neighboring regions (3). The correlation between mitochondrial haplotype groups and geographical areas was recently demonstrated by different studies to be more complex, despite a still limited and inadequate geographical and numerical sampling coverage (4), with various records of genetic variants from the "wrong" region. European mitochondrial haplotypes were identified in Canada (5–8), but also in a captive primate from Russia (Moscow zoo), even though the latter probably did not correspond to autochthonous infection (9). Mongolian and North American mitochondrial haplotypes have been identified in Southern and Northern Siberia, respectively (9). Asian mitochondrial haplotypes were reported from the European part of Russia (9) and northwestern America (Alaska, Saint Lawrence island) (3).

Investigations of the genetic diversity of *E. multilocularis* were simultaneously to mitochondrial sequencing carried out using the EmsB microsatellite marker. This molecular tool has a very high discriminative power due to the quantitative exploitation of the amplification of about 40 copies located on chromosome 5 (10–12). EmsB studies confirmed the same geographical clades as those obtained by mitochondrial sequencing for Europe and Asia, although samples from various circumpolar locations above the Arctic Circle clustered with North American isolates, possibly due to long-distance mobility of Arctic foxes (11, 13, 14). EmsB profiles of the Mongolian clade have currently not been described, most probably due to the absence of EmsB genotyping of samples from this area. As for mitochondrial haplotypes, there are also some samples where the EmsB profiles do not correspond to their geographical origin. One *E. multilocularis* rodent sample from Canada and another from a human patient from Alaska shared the same EmsB profile with a Japanese isolate in the Asian clade (11). The exclusive presence in Svalbard of a single EmsB profile from the Arctic clade is coherent, despite it being geographically a part of Europe (14). Further European studies on the presence of *E. multilocularis* based on EmsB have revealed only profiles clustering together, therefore designated "European" profiles. The expansion of the parasite in Europe was investigated in the historical Alpine focus, with peripheral areas revealing mainland-island transmission ruled by founder events due to migration of red foxes (13). The presence of the parasite across France, but also in Denmark and Sweden, has confirmed this transmission scheme due to the identification of EmsB profiles previously reported from the historical focus in south-central Europe (15, 16). Microsatellite investigations of 301 worms from 87 foxes (one to five worms per fox) originating from all endemic provinces of Poland have resulted in the identification of 29 EmsB profiles and highlighted the influence of neighboring countries in the spatial expansion of the

parasite (17). In the same period of the cited study, mitochondrial sequencing (*cox1*, *cob*, *nad2*) was carried out on 83 worms isolated from red foxes (one worm per fox) (18), almost all had previously been characterized by EmsB. Seven of these worms, all from the northeastern part of Poland, belonged to a haplotype with very close genetic proximity to haplotypes typical for Asia. However, five of these seven worms were considered to belong to EmsB profiles (Pol01, Pol03, Pol17, and Pol19) typical for Europe, while for technical reasons, no profile was obtained for the other two worms (17).

In many studies using EmsB as a tool to investigate genetic diversity in *E. multilocularis*, attention was focused on the national or regional context, especially in the construction of dendrograms which only include samples from that study. However, due to the nature of the marker and the unweighted pair group method with arithmetic mean (UPGMA) used, the clustering structure of the dendrogram may be influenced by the number and the individual variation of the samples used (19). To obtain information for a larger geographical context, it is therefore necessary to combine larger and spatially distant EmsB data sets.

The identification of mitochondrial haplotypes of the Asian cluster in Poland and the availability of EmsB data from the same worms attributed to the European cluster has prompted this study, focusing on Poland, where the described inconsistencies between mitochondrial sequencing and EmsB results had been observed. Additional sequencing of a mitochondrial target (full *cox1*) was carried out in order to evaluate the current epidemiological situation in Poland regarding a potential Asian admixture in European *E. multilocularis* populations.

MATERIALS AND METHODS

Sample Collection

The 301 worms from 87 Polish foxes previously analyzed for EmsB microsatellite genetic diversity in the studies carried out by Umhang et al. (17) were added to 46 worms from ten foxes genotyped by Knapp et al. (13) to construct a dendrogram that also includes samples of Asian and Arctic origin [i.e., China, Japan, Canada, Alaska, and Svalbard from (11, 14)]. The foxes were each identified by a number and each worm by the number of the fox followed by the number of the individual worm (from one to five), as practiced previously (13, 17). Worms with an Asian mitochondrial haplotype identified previously (18) without available EmsB profiles were re-examined by EmsB genotyping. In addition, EmsB microsatellite data from all other *E. multilocularis* samples previously genotyped [EWET Project, (20)] in Europe (13, 15, 16, 21) and available from the EmsB database (20) were used to visualize potential Asian origins by performing a hierarchical clustering analysis represented in a dendrogram.

EmsB Microsatellite Analyses and Clustering Dendrogram

EmsB PCR amplification was performed as previously reported (17). Capillary electrophoresis of PCR products was performed on a 3500 genetic analyzer (Life Technologies, Foster City, CA, USA). The size and height of each peak of the electrophoretic profile constituting the EmsB profiles were determined with the use of GeneMapper 4.1. The characterization of EmsB profiles composed of several peaks or alleles from 209 to 241 bp was carried out as previously described (11, 20). The hierarchical clustering analysis was done using the Euclidean distance and the average link clustering method (UPGMA) (22). The uncertainty of clusters was evaluated by multiscale bootstrap resampling (B = 1,000) and given by approximately unbiased *p*-values (AU), according to Shimodaira (23, 24). Clustering analyses were performed using R statistical software (25) and the pvclust library (26). In each dendrogram, EmsB microsatellite data from previously genotyped samples from the Arctic and Asian groups (11, 13) were added. The genetic threshold of 0.08 was used to determine the genotyping status of each sample (11), while two *E. granulosus sensu stricto* (G1) samples were used as the outgroup.

Mitochondrial Sequencing and Haplotype Analysis

The worm samples with an Asian EmsB profile identified were submitted to full *cox1* sequencing when the haplotype was not previously obtained by Karamon et al. (18). Sequencing of the full mitochondrial *cox1* gene (1,608 bp) was performed from amplicons obtained by PCR, as previously reported (27, 28). Nucleotide sequences of the *cox1* gene obtained were used in addition to those previously reported (3, 9, 18) to construct a TCS haplotype network (29) generated with PopART (http://popart.otago.ac.nz). The previously reported *cox1* haplotypes from Russia (9) and from Europe (Austria, France, Belgium, Slovakia, Germany), Asia (Kazakhstan, China), North America, and Mongolia (China: Inner Mongolia) (3) with their original identification were integrated into this network. Polish *cox1* haplotypes were designated bythe letter referring to the specific *cox1* haplotype [e.g., POL_Efor *cox1* E haplotype from Poland as in (18)].

RESULTS

The retrospective analyses of 935 EmsB genotyped samples from all European countries except Poland (France, Sweden, Denmark, Estonia, Germany, Switzerland, Czech Republic, Austria, and Slovakia) did not reveal the presence of any Asian EmsB profiles and confirmed the exclusive presence of European profiles (data not shown). A dendrogram was constructed including the 349 worms from 97 Polish red foxes previously genotyped using EmsB (13, 17) and also including the two worms with the Asian cox_E haplotype for *cox1* reported by Karamon et al. (18) (worms 13.1 and 76.1 not available in Umhang et al. (17) (**Figure 1**). The analysis confirmed that 92% of the Polish worms belonged to the European group, but 27 worms isolated from seven foxes clustered in the three EmsB profiles APol1 to APol3 from the Asian group. These three profiles cluster together and are more distant from the other samples of Asian origin from China and Japan. Profile APol1 is exclusively composed of six worms from two foxes from North Poland (13) (**Table 1**). Profile APol2 is represented by only one worm (76.1) and is close to profile APol3,

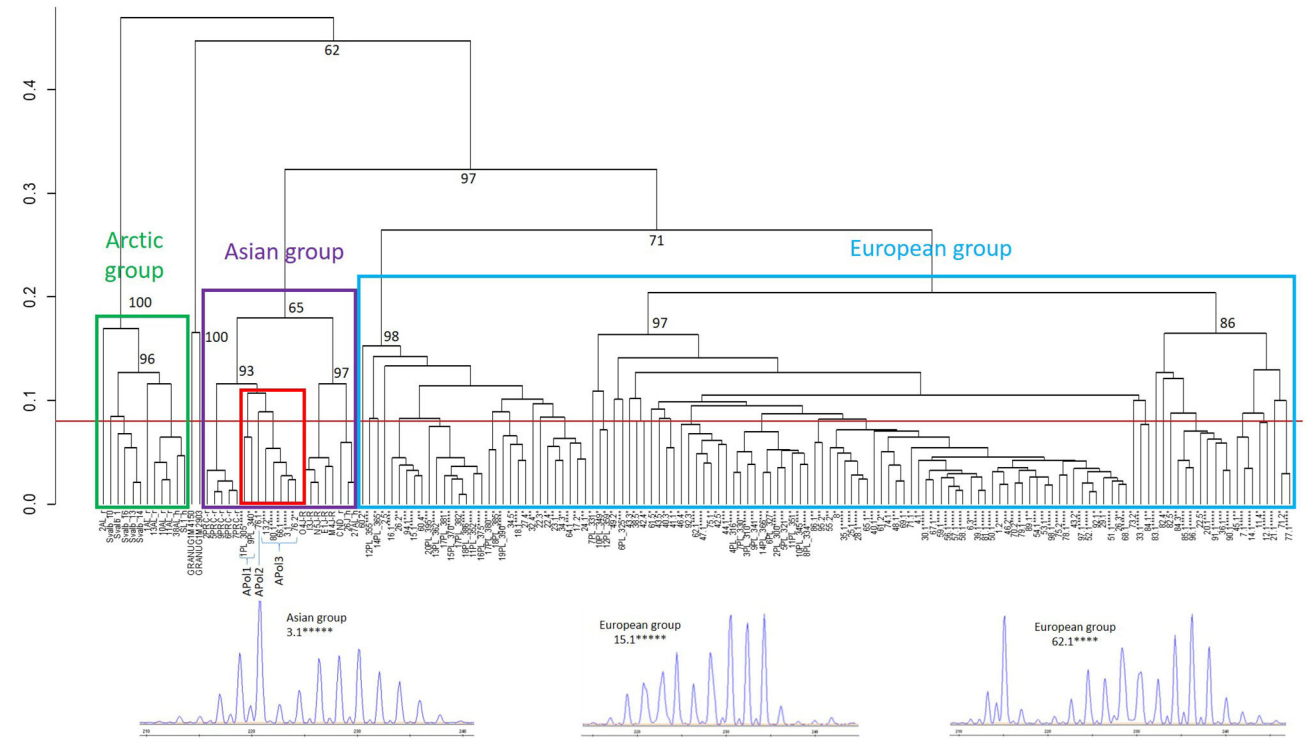

FIGURE 1 | Dendrogram constructed from EmsB data for Polish worm samples from Knapp et al. (13) and Umhang et al. (17). Worms from the same fox with the same profile were pooled to simplify the dendrogram, with the number of asterisks representing the number of worms concerned. Polish samples are indicated by the number of the fox and the representative worm, spaced by PL concerning samples from Knapp et al. (13). The outgroup control is made up of two samples of *Echinococcus granulosus sensu stricto* (G1). Samples from the EWET database were used for Asian (J: Japan, PRC: China, CND: Canada, AL: Alaska) and Arctic groups (AL: Alaska, Svalbard). The Polish samples with Asian profiles APol1 to APol3 are framed in red. The approximately unbiased *p*-values (black numbers on nodes, in percent) were calculated with multiscale bootstrap resampling (B = 1,000). A representative EmsB electrophoregram is provided for one Asian and two European profiles.

which is composed of 20 worms from 6 foxes genotyped in the national Polish study (17), with the exception of worm 13.1 which grouped with other worms from fox #13.

In addition to the ten nucleotide sequences of the full *cox1* gene already available (18), 19 others (including four partial sequences) were obtained from worms with an Asian EmsB profile (**Table 1**). The three *cox1* haplotypes A, B, and E previously reported by Karamon et al. (18) were identified in the 27 worms from the seven foxes, with the same haplotype for worms from the same fox. Haplotypes A and B of the European group differ in only one mutation and were identified in worms from foxes #1PL, #9PL, #13, and #80 (**Figure 2**). Haplotype E corresponds to a previously reported haplotype from Sichuan (China) and Altai (Russia) in the Asian group, and was identified in worms from foxes #3, #66, and #76. Karamon et al. (18) reported this haplotype previously from one worm each from five different foxes (40.1, 44.1, 77.1, 78.1, and 45.1), all of them showing European EmsB profiles, but also from one worm each from four different foxes (3.1, 13.1, 66.1, 76.1) showing Asian EmsB profiles (APol2 and Apol3), like the other worms from these foxes.

In total, worms from 12 foxes harbor an Asian mitochondrial haplotype and/or an Asian EmsB profile (**Table 1**, **Supplementary Table 1**). Thirteen worms isolated from three foxes (#3, #66, #76) belonged to Asian clusters by both EmsB and *cox1*. An Asian EmsB profile but a European mitochondrial haplotype was found in 13 worms from four foxes (#1PL, #9PL, #13, and #80), whereas an Asian mitochondrial haplotype and a European EmsB profile was obtained from the remaining five foxes. The geographic location of the foxes harboring worms from the Asian cluster (*cox1* haplotype and/or EmsB) concerned the provinces of Lubuskie (LB), Warmińsko-Mazurskie (WM), Kujawsko-Pomorskie (KP), Podlaskie (PD), and Mazowieckie (MZ).

DISCUSSION

Genetic variants of *E. multilocularis* from the "wrong" regions had previously been described from North America and Asia,

TABLE 1 | Results of full *cox1* sequencing and EmsB microsatellite analyses for Polish worm samples of Asian origin identified by at least one of the two molecular methods.

ID sample (fox_worm)	Province of Poland	Original study concerned	EmsB genotyping			Full *cox1* sequencing			Consensus origin
			Original profile	Study profile	Origin	Haplotype	GENBANK ID	Origin	EmsB/*cox1*
1PL_305	WM	Knapp et al. (13)	G01	APol1	Asian	POL_B (partial)	NA	European	Asian/European
1PL_306			G01	APol1	Asian	POL_B	MW255891	European	Asian/European
1PL_307			G01	APol1	Asian	NA	NA	NA	Asian/European
1PL_308			G01	APol1	Asian	POL_B	MW255892	European	Asian/European
1PL_309			G01	APol1	Asian	POL_B	MW255893	European	Asian/European
9PL340	WM	Knapp et al. (13)	G01	APol1	Asian	POL_A	MW255894	European	Asian/European
3.1	WM	Umhang et al. (17) and Karamon et al. (18)	P01	Apol3	Asian	POL_E	KY205685	Asian	Asian
3.2		Umhang et al. (17)	P01	Apol3	Asian	POL_E	MW255896	Asian	Asian
3.3			P01	Apol3	Asian	POL_E	MW255897	Asian	Asian
3.4			P01	Apol3	Asian	POL_E (partial)	NA	Asian	Asian
3.5			P01	Apol3	Asian	POL_E	MW255898	Asian	Asian
13.1	LB	Karamon et al. (18) and this study	NA	APol3	Asian	POL_A	KY205677	European	Asian/European
13.2		Umhang et al. (17)	P01	APol3	Asian	POL_A	MW255900	European	Asian/European
13.4			P01	APol3	Asian	POL_A	MW255901	European	Asian/European
66.1	PD	Umhang et al. (17) and Karamon et al. (18)	P01	APol3	Asian	POL_E	KY205685	Asian	Asian
66.2		Umhang et al. (17)	P01	APol3	Asian	POL_E (partial)	NA	Asian	Asian
66.3			P01	APol3	Asian	POL_E	MW255903	Asian	Asian
66.4			P01	APol3	Asian	POL_E	MW255904	Asian	Asian
66.5			P01	APol3	Asian	POL_E	MW255905	Asian	Asian
76.1	KP	Karamon et al. (18) and this study	NA	APol2	Asian	POL_E	KY205685	Asian	Asian
76.2		Umhang et al. (17)	P01	APol3	Asian	POL_E	MW255907	Asian	Asian
76.4			P01	APol3	Asian	POL_E (partial)	NA	Asian	Asian
80.1	MZ	Umhang et al. (17) and Karamon et al. (18)	P01	APol3	Asian	POL_A	KY205677	European	Asian/European
80.2		Umhang et al. (17)	P01	APol3	Asian	POL_A	MW255909	European	Asian/European
80.3			P01	APol3	Asian	POL_A	MW255910	European	Asian/European
80.4			P01	APol3	Asian	POL_A	MW255911	European	Asian/European
80.5			P01	APol3	Asian	NA	NA	NA	NA
40.1	WM	Umhang et al. (17) and Karamon et al. (18)	P19	EPol26	European	POL_E	KY205685	Asian	European/Asian
44.1	WM	Umhang et al. (17) and Karamon et al. (18)	P17	EPol22	European	POL_E	KY205685	Asian	European/Asian
77.1	MZ	Umhang et al. (17) and Karamon et al. (18)	P03	EPol34	European	POL_E	KY205685	Asian	European/Asian
78.1	MZ	Umhang et al. (17) and Karamon et al. (18)	P19	EPol26	European	POL_E	KY205685	Asian	European/Asian
45.1	WM	Umhang et al. (17) and Karamon et al. (18)	P24	EPol31	European	POL_E	KY205685	Asian	European/Asian

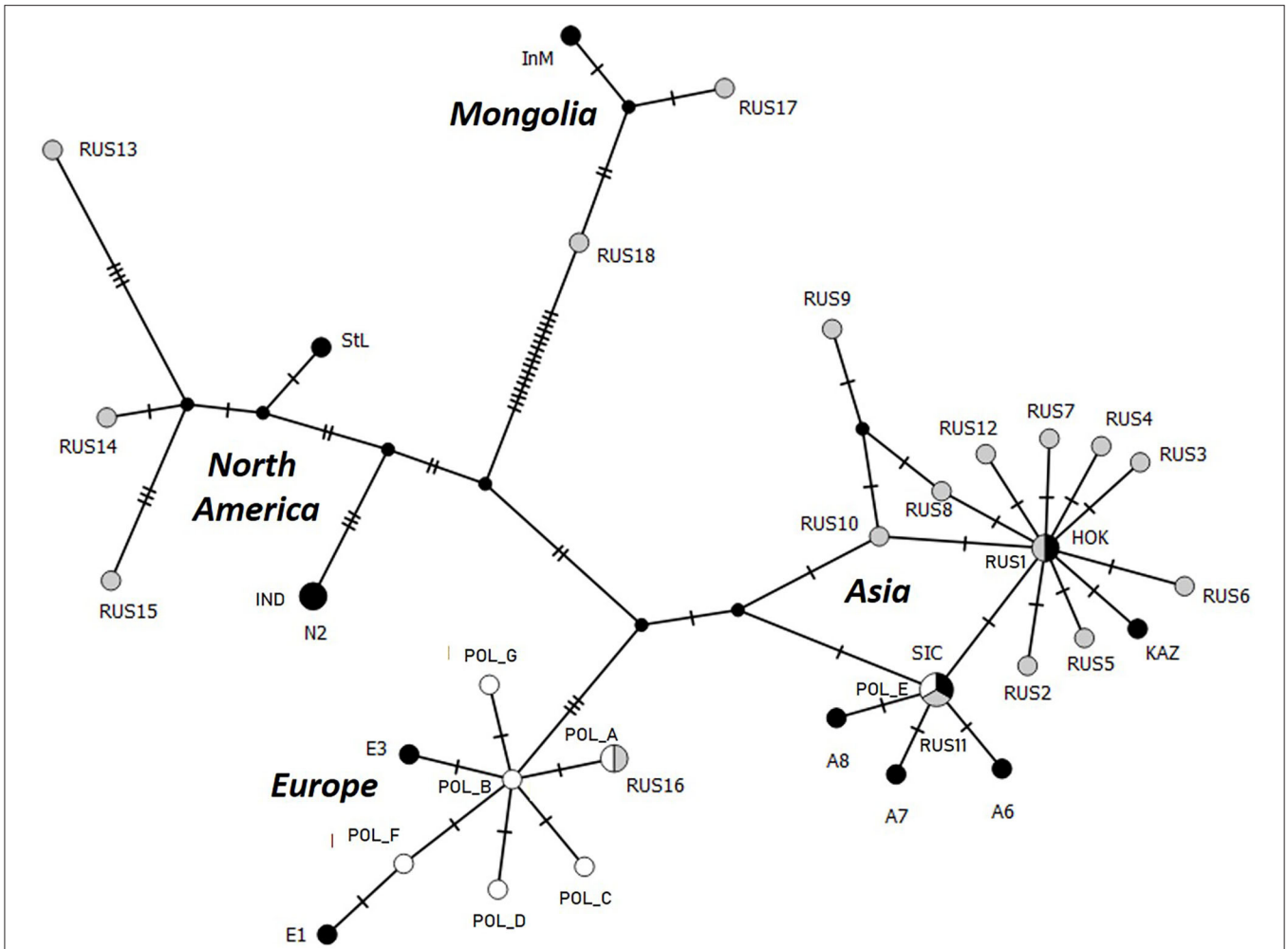

FIGURE 2 | Parsimonious network of *cox1* haplotypes of *E. multilocularis* samples from Poland (white) completed by others previously reported by Konyaev et al. (9) (gray) and Nakao et al. (3) (black), with their original identification of haplotypes. E, Europe; POL, Poland; RUS, Russia; InM, Chinese Inner Mongolia; StL, Alaska (St-Lawrence Island); SIC, China (Sichuan); HOK, Japan (Hokkaido); KAZ, Kazakhstan.

but this had never before concerned samples from non-Arctic Europe until the identification of a mitochondrial haplotype of Asian origin in Polish worms (18). Previous genetic analyses performed by mitochondrial sequencing had consistently identified European haplotypes in European samples (3, 21). In the same way, all the EmsB profiles previously reported from Europe were attributed to the European cluster (13, 15, 16, 20, 21). Here for the first time, both methods (representing the nuclear and the mitochondrial genomes) were used consistently with a larger set of samples. The results reveal the widespread and rather frequent presence in Poland of variants that belong to mitochondrial and/or EmsB clusters typical for Asia, and not known from anywhere else in Europe. Erroneously, profiles of certain samples had previously been allocated to the European cluster of EmsB profiles (13, 17), which could be shown to be an artifact due to the absence of samples from the Asian group in the dendrograms in that study. Attribution to one of the different clades (Europe, Asia, Arctic) using EmsB analyses requires systematic visual analysis of the electrophoretic profile and a hierarchical clustering analysis resulting in a dendrogram that includes samples from each of the different clades: Europe, Asia, and Arctic (including North America). Mitochondrial sequencing, unexpectedly, did not in all cases correspond to the EmsB status regarding the European or Asian clades. Only 13 worms from three foxes belonged to Asian clusters by both EmsB and *cox1*, whereas 18 worms from nine foxes belonged to different clusters according to each marker. However, the large majority (92%) of worms from Poland belonged to European clusters by both methods.

The two methods target mitochondrial or nuclear genomes corresponding to coding and non-coding regions, respectively. In contrast to the mitochondrial genome, the nuclear genome is subjected to recombination and is inherited by both male and female. Recent studies have confirmed that

both cross-fertilization and self-fertilization occur within species of *Echinococcus*, including *E. multilocularis* (11, 30–32). Cross-fertilization between worms from two different strains may occur in the intestines, leading to production of eggs with the mitochondrial genome of female origin, but with a nuclear genome integrating genetic material of male origin ("male introgression"). Worms with discrepancies between mitochondrial sequencing and EmsB microsatellite analyses are assumed to result from cross-fertilization between worms of typically "European" and typically "Asian" genomes.

The presence of clusters of genetic variants (both mitochondrial and nuclear) that correspond to continental origins indicates prolonged evolution in these geographical areas, without significant genetic exchange. Our study from Poland is the first large-scale investigation where a zone of apparent overlap or co-existence of such variants was identified. Our data do not allow for an unequivocal explanation of this observation, i.e., whether the presence of different variants was caused by recent introduction (e.g., via traveling domestic dogs, or via migration of wild foxes), or whether this situation represents an ancient polymorphism that has been present in the area for a longer period. The presence of only three EmsB profiles and one mitochondrial haplotype of the Asian cluster argues for (a) sporadic introduction event(s), although our observation of a "mosaic" distribution of mitochondrial and nuclear variants appears to indicate prolonged presence of these variants in Poland with sufficient time for recombination. However, it cannot be ruled out that the Polish worms are the westernmost representatives of Asian variants, and there may be a gradient of progressively decreasing "Asian" components in the genome of *E. multilocularis* populations from East to West. To decide on this, additional genetic analyses in Western Asia and Eastern Europe will be needed. To date, only few data are available from this region: European haplotypes and EmsB profiles were obtained from worms isolated in raccoon dogs from Estonia (21), but no genetic data are available for neighboring countries such as Latvia, Belarus and Ukraine. Concerning Russia, the only *E. multilocularis* sample reported that belongs to the European cluster (according to full *cox1* sequencing) was from a captive primate (*Galago senegalensis*) from the Moscow zoo, where there was a strong suspicion of an infection source from Baltic countries through imported mulch spread as ground cover in the enclosure (9). All the other *E. multilocularis* samples from the European or Asian parts of the Russian Federation belonged to Asian, North American or Mongolian genetic clusters.

It will be interesting to study the geographical limits of this Asian admixture in European *E. multilocularis* populations outside of Poland. As shown in this study, the simultaneous use of both mitochondrial sequencing and EmsB analysis is relevant in order to increase sensitivity and to detect introgression events. As we mentioned, future studies will be particularly relevant in the eastern part of Europe, but sample sizes using the described approach are not large, even in well-known endemic areas of central Europe. Given the fact that Asian genetic components in Poland were found not to be limited to the Northeast of the country but extend as far as Lubuskie (LB) province on the border with Germany, it would not be surprising to detect this kind of admixture even further to the West.

It has been speculated that the presence of certain genetic variants of *E. multilocularis* may have an impact on public health via differences in infectivity or pathogenicity to humans (33). This hypothesis has for instance served as a hypothetical explanation for the low number of human AE cases in North America, despite widespread presence of the parasite in animal hosts. However, no conclusions have been reached on this, and our detection of widespread recombinations between nuclear and mitochondrial markers calls for an examination of human samples using both approaches, as pathogenicity factors will most likely be situated in the nuclear genome rather than the mitochondria, which are at present far more frequently used for genetic characterization of isolates and allocation to clusters. This is highly relevant for the area under study, as, like elsewhere in Europe, an increasing prevalence in red foxes was observed followed by an increase of AE human cases associated with high morbidity and mortality, resulting in a public health situation that is of concern (34, 35).

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

GU, JKa, JKn, and TR: conceptualization. GU, JKa, and JKn: methodology. VB, CP, MW, GU, and JKa: investigation. JKa, TC, JKn, TR, MW, and GU: ressources. GU: writing original draft preparation and project administration. JKn, JKa, TR, MW, and FB: writing-review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Torgerson PR, Keller K, Magnotta M, Ragland N. The global burden of alveolar echinococcosis. *PLoS Negl Trop Dis*. (2010) 4:e722. doi: 10.1371/journal.pntd.0000722
2. Torgerson PR, Schweiger A, Deplazes P, Pohar M, Reichen J, Ammann RW, et al. Alveolar echinococcosis: from a deadly disease to a well-controlled infection. Relative survival and economic analysis in Switzerland over the last 35 years. *J Hepatol.* (2008) 49:72–7. doi: 10.1016/j.jhep.2008.03.023
3. Nakao M, Xiao N, Okamoto M, Yanagida T, Sako Y, Ito A. Geographic pattern of genetic variation in the fox tapeworm *Echinococcus multilocularis*. *Parasitol Int*. (2009) 58:384–9. doi: 10.1016/j.parint.2009.07.010
4. Deplazes P, Rinaldi L, Alvarez Rojas CA, Torgerson PR, Harandi MF, Romig T, et al. Global distribution of alveolar and cystic echinococcosis. *Adv Parasitol*. (2017) 95:315–493. doi: 10.1016/bs.apar.2016.11.001
5. Gesy K, Hill JE, Schwantje H, Liccioli S, Jenkins EJ. Establishment of a European-type strain of *Echinococcus multilocularis* in Canadian wildlife. *Parasitology*. (2013) 140:1133–7. doi: 10.1017/S0031182013000607
6. Christiansen EF, Himsworth CG, Hill JE, Haulena M. Infection of a Goeldi's monkey (*Callimico goeldii*) with a european strain of *Echinococcus multilocularis* in a canadian institution. *J Zoo Wildl Med*. (2015) 46:378–81. doi: 10.1638/2014-0123R1.1
7. Massolo A, Klein C, Kowalewska-Grochowska K, Belga S, MacDonald C, Vaughan S, et al. European *Echinococcus multilocularis* identified in patients in Canada. *N Engl J Med*. (2019) 381:384–5. doi: 10.1056/NEJMc1814975
8. Schurer JM, Tsybina P, Gesy KM, Kolapo TU, Skinner S, Hill JE, et al. Molecular evidence for local acquisition of human alveolar echinococcosis in Saskatchewan, Canada. *J Infect Dis*. (2020) jiaa473. doi: 10.1093/infdis/jiaa473
9. Konyaev SV, Yanagida T, Nakao M, Ingovatova GM, Shoykhet YN, Bondarev AY, et al. Genetic diversity of Echinococcus spp. in Russia. *Parasitology*. (2013) 140:1637–47. doi: 10.1017/S0031182013001340
10. Bart JM, Knapp J, Gottstein B, El-Garch F, Giraudoux P, Glowatzki ML, et al. EmsB, a tandem repeated multi-loci microsatellite, new tool to investigate the genetic diversity of *Echinococcus multilocularis*. *Infect Genet Evol*. (2006) 6:390–400. doi: 10.1016/j.meegid.2006.01.006
11. Knapp J, Bart JM, Glowatzki ML, Ito A, Gerard S, Maillard S, et al. Assessment of use of microsatellite polymorphism analysis for improving spatial distribution tracking of *Echinococcus multilocularis*. *J Clin Microbiol*. (2007) 45:2943–50. doi: 10.1128/JCM.02107-06
12. Valot B, Knapp J, Umhang G, Grenouillet F, Millon L. Genomic characterization of EmsB microsatellite loci in *Echinococcus multilocularis*. *Infect Genet Evol*. (2015) 32:338–41. doi: 10.1016/j.meegid.2015.03.040
13. Knapp J, Bart JM, Giraudoux P, Glowatzki ML, Breyer I, Raoul F, et al. Genetic diversity of the cestode *Echinococcus multilocularis* in red foxes at a continental scale in Europe. *PLoS Negl Trop Dis*. (2009) 3:e452. doi: 10.1371/journal.pntd.0000452
14. Knapp J, Staebler S, Bar JM, Stien A, Yoccoz NG, Drögemüller C, et al. *Echinococcus multilocularis* in Svalbard, Norway: microsatellite genotyping to investigate the origin of a highly focal contamination. *Infect Genet Evol*. (2012) 12:1270–4. doi: 10.1016/j.meegid.2012.03.008
15. Umhang G, Knapp J, Hormaz V, Raoul F, Boué F. Using the genetics of *Echinococcus multilocularis* to trace the history of expansion from an endemic area. *Infect Genet Evol*. (2014) 22:142–9. doi: 10.1016/j.meegid.2014.01.018
16. Knapp J, Umhang G, Wahlström H, Al-Sabi MNS, Ågren EO, Enemark HL. Genetic diversity of *Echinococcus multilocularis* in red foxes from two Scandinavian countries: Denmark and Sweden. *Food Waterborne Parasitol*. (2019) 14:e00045. doi: 10.1016/j.fawpar.2019.e00045
17. Umhang G, Karamon J, Hormaz V, Knapp J, Cencek T, Boué F. A step forward in the understanding of the presence and expansion of Echinococcus multilocularis in Eastern Europe using microsatellite EmsB genotyping in Poland. *Infect Genet Evol*. (2017) 54:176–82. doi: 10.1016/j.meegid.2017.07.004
18. Karamon J, Stojecki K, Samorek-Pierog M, Bilska-Zajac E, Rozycki M, Chmurzynska E, et al. Genetic diversity of *Echinococcus multilocularis* in red foxes in Poland: the first report of a haplotype of probable Asian origin. *Folia Parasitol*. (2017) 64:2017.007. doi: 10.14411/fp.2017.007
19. Knapp J, Gottstein B, Bretagne S, Bart JM, Umhang G, Richou C, et al. Genotyping *Echinococcus multilocularis* in human alveolar echinococcosis patients: an EmsB microsatellite analysis. *Pathogens*. (2020) 9:4. doi: 10.3390/pathogens9040282
20. Knapp J, Damy S, Brillaud J, Tissot JD, Navion J, Mélior R, et al. EWET: data collection and interface for the genetic analysis of *Echinococcus multilocularis* based on EmsB microsatellite. *PLoS ONE*. (2017) 12:e0183849. doi: 10.1371/journal.pone.0183849
21. Laurimaa L, Süld K, Moks E, Valdmann H, Umhang G, Knapp J, et al. First report of the zoonotic tapeworm *Echinococcus multilocularis* in raccoon dogs in Estonia, and comparisons with other countries in Europe. *Vet Parasitol*. (2015) 212:200–5. doi: 10.1016/j.vetpar.2015.06.004
22. Legendre P, Legendre L. *Numerical Ecology*. Amsterdam: Elsevier (1998).
23. Shimodaira H. An approximately unbiased test of phylogenetic tree selection. *Syst Biol*. (2002) 51:492–508.
24. Shimodaira H. Approximately unbiased tests of regions using multistep-multiscale bootstrap resampling. *Ann Stat*. (2004) 32:2616–41.
25. R Development Core Team. *R: A language and environment for statistical computing*. Vienna: The R Foundation for Statistical Computing (2005).
26. Suzuki R, Shimodaira H. Pvclust: an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* (2006) 22:1540–2.
27. Hüttner M, Nakao M, Wassermann T, Siefert L, Boomker JD, Dinkel A, et al. Genetic characterization and phylogenetic position of *Echinococcus felidis* (Cestoda: Taeniidae) from the African lion. *Int J Parasitol*. (2008) 38:861–8. doi: 10.1016/j.ijpara.2007.10.013
28. Addy F, Wassermann M, Banda F, Mbaya H, Aschenborn J, Aschenborn O, et al. Genetic polymorphism and population structure of *Echinococcus ortleppi*. *Parasitology*. (2017) 144:450–8. doi: 10.1017/S0031182016001840
29. Clement M, Posada D, Crandall, KA. TCS: a computer program to estimate gene genealogies. *Mol Ecol*. (2000) 9:1657–9. doi: 10.1046/j.1365-294x.2000.01020.x
30. Nakao M, Sako Y, Ito A. Isolation of polymorphic microsatellite loci from the tapeworm *Echinococcus multilocularis*. *Infect Genet Evol*. (2003) 3:159–63. doi: 10.1016/S1567-1348(03)00070-4
31. Knapp J, Guislain MH, Bart JM, Raoul F, Gottstein B, Giraudoux P, et al. Genetic diversity of *Echinococcus multilocularis* on a local scale. *Infect Genet Evol*. (2008) 8:367–73. doi: 10.1016/j.meegid.2008.02.010
32. Lymbery AJ. Phylogenetic pattern, evolutionary processes and species delimitation in the genus Echinococcus. *Adv Parasitol*. (2017) 95:111–45. doi: 10.1016/bs.apar.2016.07.002
33. Romig T, Deplazes P, Jenkins D, Giraudoux P, Massolo A, Craig PS, et al. Ecology and life cycle patterns of Echinococcus species. *Adv Parasitol*. (2017) 95:213–314. doi: 10.1016/bs.apar.2016.11.002
34. Marcinkute A, Šarkunas M, Moks E, Saarma U, Jokelainen P, Bagrade G, et al. Echinococcus infections in the Baltic region. *Vet Parasitol*. (2015) 213:121–31. doi: 10.1016/j.vetpar.2015.07.032
35. Bagrade G, Deksne G, Ozolina Z, Howlett SJ, Interisano M, Casulli A, et al. *Echinococcus multilocularis* in foxes and raccoon dogs: an increasing concern for Baltic countries. *Parasit Vectors*. (2016) 9:615. doi: 10.1186/s13071-016-1891-9

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Trichuris trichiura (Linnaeus, 1771) From Human and Non-human Primates: Morphology, Biometry, Host Specificity, Molecular Characterization, and Phylogeny

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Human trichuriasis is a Neglected Tropical Disease, which affects hundreds of millions of persons worldwide. Several studies have reported that non-human primates (NHP) represent important reservoirs for several known zoonotic infectious diseases. In this context, *Trichuris* infections have been found in a range of NHP species living in natural habitats, including colobus monkeys, macaques, baboons, and chimpanzees. To date, the systematics of the genus *Trichuris* parasitizing humans and NHP is unclear. During many years, *Trichuris trichiura* was considered as the whipworm present in humans and primates. Subsequently, molecular studies suggested that *Trichuris* spp. in humans and NHP represent several species that differ in host specificity. This work examines the current knowledge of *T. trichiura* and its relationship to whipworm parasites in other primate host species. A phylogenetic hypothesis, based on three mitochondrial genes (*cytochrome c oxidase* subunit 1, *cytochrome* b, and large subunit rRNA-encoding gene) and two fragments of ribosomal DNA (Internal Transcribed Spacer 1 and 2), allowed us to define a complex of populations of *T. trichiura* hosting in a large variety of NHP species, in addition to humans. These populations were divided into four phylogenetic groups with a different degree of host specificity. From these data, we carry out a new morphological and biometrical description of the populations of *Trichuris* based on data cited by other authors as well as those provided in this study. The presence of *T. trichiura* is analyzed in several NHP species in captivity from different garden zoos as possible reservoir of trichuriasis for humans. This study contributes to clarify questions that lead to identification of new taxa and will determine parasite transmission routes between these primates, allowing the implementation of appropriate control and prevention measures.

Keywords: ***Trichuris trichiura*, non-human primates, ribosomal DNA, mitochondrial DNA, zoonoses**

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INTRODUCTION

Worldwide, ∼1.5 billion people, nearly 24% of the world's population, are infected with soil-borne helminths. Soil-borne helminthiasis is widely distributed in tropical and subtropical areas, especially in sub-Saharan Africa, The Americas, China, and East Asia. More than 267 million preschoolers and more than 568 million school-age children live in areas with

severe transmission of these parasites and need treatment and preventive interventions. The main species of soil-borne helminths that infect humans are *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), and *Necator americanus* and *Ancylostoma duodenale* (hookworms) (1).

T. trichiura, is the etiological agent of the parasitic disease known as "trichuriasis," which is considered as a Neglected Tropical Disease. *T. trichiura* is the second most common helminth in humans, and Trichuriasis has a worldwide geographical distribution. The prevalence is higher in places with warm and humid weather, where there is a lack of basic sanitation services. Between 30 and 80% of cases are recorded in children, who suffer the greatest parasitic burden and those with the most significant symptoms (2). Transmission of this parasite occurs after ingestion of embryonated eggs. These eggs can enter new hosts through contaminated hands, food, soil, and water. Then these hatch in the intestine, where L1 larvae are released. Larvae penetrate the epithelial layer of the large intestine and grow to adult stage. After mating, the non-embryonated eggs are released from the females and again reach the environment through the host's feces.

Up to date, whipworms isolated from humans and other primates have traditionally been regarded as *T. trichiura* (3–5), while those recovered from pigs and wild boars are known as *Trichuris suis* (6, 7). It is well-known that differentiation between closely related species of *Trichuris* is very difficult due to the phenotypic plasticity of the organisms themselves; host-induced variation, paucity of morphological features, and overlapping morphological characteristics that occur among species (8–11). Thus, many studies on *Trichuris* have focused on the morphological and molecular differentiation of *T. trichiura* and *T. suis*, which are molecularly different but morphologically similar (7, 12–14).

On the other hand, the relationship between *Trichuris* from humans and non-human primates (NHP) in terms of genetic and evolutionary aspects is poorly understood. In recent years, some publications addressed the question of whether *Trichuris* species are shared between humans and NHP or whether there are different species. The genus *Trichuris* is likely a candidate to harbor cryptic species as it has a wide geographical distribution and infects several host species (15). As revealed by recent studies, there is more than one taxon capable of infecting humans and other primates, including individuals in captivity, suggesting that *T. trichiura* should be considered a complex species that includes different cryptic units (16, 17). In addition, based on morpho-biometric and molecular parameters, new species of *Trichuris* have been described in primates, such as *Trichuris rhinopiptheroxella* (18) that was found in the golden snub-nosed monkey (*Rhinopithecus roxellana*), *Trichuris colobae* from *Colobus guereza kikuyuensis* (19), and *Trichuris ursinus* from *Papio ursinus* (20). Therefore, these studies confirmed that *T. trichiura* is not the only whipworm found in primates.

Currently, the systematics of the genus *Trichuris* shows significant gaps. This is due to two main reasons: (i) the lack of comparative morpho-biometric data through the use of multiple parameters and statistical tests applied to the taxonomic study of these species and (ii) the paucity of published research on the genetics of the different *Trichuris* species in humans, NHP, and pigs. Nowadays, researchers have not yet managed to finally establish the degree of divergence between the different genetic lineages that appear to exist in *Trichuris* species parasitizing these hosts.

In this paper, we carried out an update of the morphological and biometric characteristics of *T. trichiura* isolated from human and NHP. Besides, the molecular phylogenetic relationships between these populations are analyzed based on molecular data (mitochondrial and nuclear markers). Furthermore, some phylogenetic hypotheses were inferred for *Trichuris* spp. to shed light on the degree of divergence between different genetic lineages. In addition, the presence of *T. trichiura* was analyzed in several NHP species in captivity from different garden zoos as possible reservoir of trichuriasis for humans.

MATERIALS AND METHODS

Ethics Statement

This study does not require approval by an ethics committee. *Macaca sylvanus* and *C. g. kikuyensis*, from which *Trichuris* specimens were collected from their caeca *postmortem*, died of natural death. The specimens were handled and housed in a zoo in strict accordance with good animal practices. The other specimens and eggs of *Trichuris* sp. were recovered from the feces after routine anthelmintic treatment.

Isolation of Material

In this study, we sampled *Trichuris*'s adults and eggs of a total of five NHP host species, including the Barbary macaque (*M. sylvanus*), vervet monkey (*Chlorocebus aethiops*), patas monkey (*Erythrocebus patas*), Guinea baboon (*Papio papio*), and black and white colobus (*C. g. kikuyuensis*) from the Zoo Castellar (Cádiz, Spain), Selwo Aventura (Málaga, Spain), Zoo Barcelona (Barcelona, Spain), Parque de la Naturaleza de Cabárceno (Cantabria, Spain), and Bioparc Fuengirola (Málaga, Spain), respectively (**Table 1**).

Only three adult whipworm specimens (two females and one male) were collected from Guinea baboon, sixty-five adults (32 females and 33 males) from a male Barbary macaque (17, 21) and five adults from a *C. g. kikuyuensis*. Adult's worms were washed separately in saline solution (0.9% w/v), then frozen at −20°C until posterior studies. Whipworm's eggs were isolated from feces of all NHP species. The sequences successfully obtained of different molecular markers are summarized in the **Table 1**.

The fecal eggs were concentrated using a Sheather's sugar solution (22) and then embryonated at 32°C for 3–4 weeks added with potassium dichromate 0.2% w/v solution to give humidity to the medium and prevent fungal and bacterial growth (23). Subsequently, the worms were measured and genomic DNA, from both worms and eggs, was extracted.

Morphological Study

Three adult whipworms from Guinea baboon were identified according to previous studies (7, 19, 20). We carried out morphological studies as described Oliveros et al. (24) and Skrjabin et al. (25). *Trichuris* specimens were measured according

TABLE 1 | DNA obtained from the samples.

Zoo gardens	Hosts species		N° adults/eggs analyzed	*Trichuris* species	Haplotypes obtained					Number of base pairs/G+C				
	Scientific name	Common name			*cox*1	*cob*	*rrn*L	ITS1	ITS2	*cox*1	*cob*	*rrn*L	ITS1	ITS2
Zoo Castellar	*Macaca sylvanus*	Barbary macaque	43 adults	*T. trichiura*	4 H (*)	5 H (*)	5 H	6 H	8 H (*)	370/38–39.2	520/30.2–31	386–387/29.7–31.3	586–597/63.1–64.3	514–587/63.7–65.2
Selwo Aventura	*Chlorocebus aethiops*	Vervet monkey	5 sample batches of eggs (40–65 eggs/batch)		1 H	1 H	-	2 H	5 H	370/38.6	522/30.1	–	594–601/61.4–62.3	556–580/62.1–64.1
Zoo Barcelona	*Erythrocebus patas*	Patas monkey	1 sample batch of eggs		1 H	1 H	-	1 H	1 H	332/38	520/29.6	–	593/64.1	436/65.6
Parque de la Naturaleza de Cabarceno	*Papio papio*	Guinea baboon	3 adults and 9 sample batches of eggs (40–200 eggs/batch)		1 H	4 H	1 H	2 H	5 H	370/38.6	520/29.6–30.8	387/30	588–607/61.4–64.3	602–611/62.8–63.2
Bioparc Fuengirola	*Colobus guereza kikuyuensis*	Black and white colobus	5 adults	*T. colobae*	-	-	5 H	-	-	-	-	395/30.1–30.9	-	-

H, haplotype; (*) analyzed by Rivero et al. (17).

to parameters reported by Spakulová and Lýsek (26), Suriano and Navone (27), and Robles et al. (28), and a comparative study was carried out with *T. trichiura*'s specimens previously analyzed biometrically and molecularly (7, 14, 17, 21).

Molecular Study

PCR and Sequencing of Specimens

Genomic DNA from adult worms and a pack of 45–200 isolated eggs were extracted using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Quality of extractions was assessed using 0.8% agarose gel electrophoresis infused with SYBR® Safe DNA gel stain.

All molecular markers sequenced in the present study [*cox*1, *cob* and *rrn*L mitochondrial DNA (mtDNA) and ITS1 and ITS2 ribosomal DNA (rDNA)] were amplified using the polymerase chain reaction (PCR) by a thermal cycler (Eppendorf AG; Hamburg, Germany). PCR mix, PCR conditions, and PCR primers are summarized in the **Supplementary Table 1**.

The PCR products were checked on SYBR® Safe stained with 2% w/v Tris–borate–EDTA (TBE) agarose gels. Bands were eluted and purified from the agarose gel using the QWizard SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, U.S.A.). Once purified and concentrated, the products were sequenced by Stab Vida (Lisbon, Portugal).

Phylogenetic Analysis

To assess the similarity among all marker sequences of *Trichuris* sp. obtained in the present study and other *Trichuris* species, the number of nucleotide differences per sequence was analyzed using Compute Pairwise Distances based on the number of differences method of MEGA v7.0 (29).

To obtain a nucleotide sequence alignment file, the MUSCLE alignment method was used in MEGA v7.0 (29). Additional sequences from the National Center for Biotechnology Information (NCBI) GenBank® database were incorporated into the alignments (**Supplementary Table 2**).

Assessment of nucleotide substitution saturation, an indicator of whether the genetic marker is useful, was performed using DAMBE v5.3.32 (30, 31). Saturation was based on the values of Iss (index of substitution saturation) and Iss.c (critical Iss value), where Iss < Iss.c indicated that the genetic marker was not saturated and vice versa. Haplotype diversity (h) and nucleotide diversity (π) were calculated using DnaSP v6.12.03 (32).

All phylogenetic trees were inferred based on nucleotide data and obtained by two methods: Maximum Likelihood (ML) and Bayesian Inference (BI). PHYML package was used to generate ML trees (33), and MrBayes v3.2.6 to generate BI (34). jModelTest was employed to resolve the best-fit substitution model for the parasite data (35). Models of evolution were selected for subsequent analysis according to the Akaike Information Criterion (36). To examine the dataset containing the concatenation of four markers used (ITS1, ITS2, *cox*1, and *cob*), analyses based on BI were partitioned by gene and models for individual genes within partitions were those selected by jModelTest. For ML inference, best-fit nucleotide substitution models included the general time-reversible (GTR) model with gamma-distributed rate variation (G) and a proportion of

invariable sites (I), GTR + G (ITS1 and ITS2), GTR + I + G (*cox1*), GTR + I + G (*cob*), and GTR + G (*rrn*L). Support for the topology was examined using bootstrapping (heuristic option) (37) over 1,000 replications to assess the relative reliability of clades. The commands used in MrBayes for BI were *nst* = 6 with gamma rates (ITS1, ITS2, and *rrn*L), *nst* = 6 with invgamma rates (*cox*1 and *cob*), and *nst* = mixed (concatenated phylogenetic trees). The standard deviation of split frequencies was used to determine whether the number of generations completed was sufficient; the chain was sampled every 500 generations, and each dataset was run for 10 million generations. Trees from the first million generations were discarded based on an assessment of convergence. Burn-in was determined empirically by examination of the log likelihood values of the chains. The Bayesian Posterior Probabilities (BPP) comprise the percentage converted.

RESULTS

Molecular Analysis

Annotation and Features of Ribosomal and Mitochondrial Genomes

The successfully sequenced specimens, the length of the different sequences, the content of G + C%, and haplotypes of ribosomal and mitochondrial markers analyzed at the present study are shown in **Table 1**. Different repetitive nucleotide sequences, termed ≪ microsatellites ≫, were found in the ITS2 sequences of *Trichuris* sp. from human and different NHP. Thus, Poly (GCA), Poly (CGA), and Poly (GCG) were observed in positions 280, 302, and 344, respectively. Furthermore, Poly (GCA) and Poly (GGC) were found in the ITS1 sequences in positions 77 and 203, respectively. Also, a common area to all species of *Trichuris* was observed in positions 280 (GATCTGGGTGT) and 286 (GCCGCCGGTT) in this ITS1 sequence.

Nucleotide sequence data reported in this study were deposited at the GenBank™, EMBL, and DDBJ databases, and the accession numbers are available in **Supplementary Table 2**.

Phylogenetic Analysis

All phylogenetic trees based on ribosomal and mitochondrial markers (partitioned and concatenated) confirmed the existence of two main clades: clade 1: "*Trichuris suis*" and clade 2: "*Trichuris trichiura*" (**Figures 1–3** and **Supplementary Figures 1–3**).

The alignment of 46 ITS1 and 79 ITS2 rDNA sequences of *Trichuris* species yielded a dataset of 876 and 863 characters, respectively. Based on ITS1 and ITS2 sequences, the concatenated phylogenetic tree revealed the existence of two highly supported phylogenetic groups within clade 2 "*T. trichiura*": One group corresponded to *T. trichiura* lineage (100% ML and 100% BPP) (**Supplementary Table 3**) clustering *Trichuris* sp. from different hosts and geographical regions in six subclades (100% ML and 100% BPP) that included *Trichuris* sp. from *Trachypithecus francoisi* (**Figure 1**). The other 5 subclades (except *Trichuris* sp. from *T. francoisi*) showed a high homology each other, ranging 94.65–99.65% (**Supplementary Table 4**).

The phylogeny inferred on mitochondrial datasets (partitioned and concatenated) revealed similar topologies (**Figure 2** and **Supplementary Figures 1–3**). Thus, four main clades were observed in "*T. trichiura* lineage" where *Trichuris* sp. from *P. papio*, *C. aethiops*, and *E. patas* clustered together in the subclade named 2c with *T. trichiura* from *Homo sapiens* from Uganda and *Trichuris* sp. from other hosts from Africa and Europe (**Figure 2** and **Supplementary Figures 1, 2**).

The multiple alignments of 48 *cox*1 nucleotide sequences (including outgroups) yielded a dataset of 296 characters. The phylogenetic tree based on *cox*1 showed *Trichuris* from *E. patas*, *C. aethiops*, and *P. papio* clustering in subclade 2c (European and African origin) and related with *Macaca fuscata* (subclade 2d). This marker did not resolve subclade 2a (Asian and USA origin) appearing in polytomy (**Supplementary Figure 1** and **Supplementary Table 3**).

The multiple alignments of 47 *cob* nucleotide sequences (including outgroups) yielded a dataset of 444 characters. The phylogenetic tree based on *cob* was in congruence with *rrn*L phylogenetic inferences of *T. trichiura* lineage. Nevertheless, the sister relationship between subclades 2a, 2b, and 2d was not supported (**Supplementary Figure 2** and **Supplementary Table 3**).

The *rrn*L dataset included 358 aligned positions and 76 taxa, including outgroups. ML and BI methods showed congruence between each other revealing two main clades ("*T. suis* lineage" and "*T. trichiura* lineage") and respect to the sister-group relationships between *Trichuris* spp. from NHP, humans and pigs (**Supplementary Figure 3** and **Supplementary Table 3**). Within clade 2 "*T. trichiura* lineage," phylogenetic trees confirmed the existence of four different subclades highly supported clustering subclade 2b: *T. trichiura* from *H. sapiens* from China and *Papio anubis* from the USA and subclade 2a including the minority haplotype of *T. trichiura* from *M. sylvanus* from Spain; subclade 2c: *T. trichiura* from *P. papio*, *Chlorocebus sabaeus* and *M. sylvanus* from Spain, *H. sapiens* from Uganda, *Papio hamadryas* from Europe, and two haplotypes of *Trichuris* sp. from *M. fuscata* from Europe; and subclade 2d: *T. trichiura* from *M. fuscata* from Europe (**Supplementary Figure 3**). Curiously, the minority haplotype of *M. sylvanus* (subclade 2a) and *M. fuscata* (subclade 2d) (European origin) appeared related with those from Asia and USA (subclade 2b) (**Supplementary Figure 3** and **Supplementary Table 3**). In addition, a sister relationship between *T. trichiura* and *Trichuris* sp. from *T. francoisi* was observed, both species within "*T. trichiura* lineage" (clade 2).

The concatenated dataset of mitochondrial gene (*cox*1 and *cob*) sequences (**Figure 2**) revealed the subclade 2c as the main one clustering the majority of *T. trichiura* parasitizing African humans and different NHP from Africa and Europe, showing a sister relationship between 2c and 2d and besides between 2a and 2b (Asian and USA origins) (**Figure 2**).

The concatenated dataset of ribosomal (ITS1 and ITS2) and mitochondrial (*cox*1 and *cob*) gene sequences included 2,479 aligned sites and only 13 taxa, since only we could concatenate sequences of the same individual. Phylogenetic analyses of this dataset yielded a tree with branches strongly supported. Thus, the *T. trichiura* population was separated in only three different

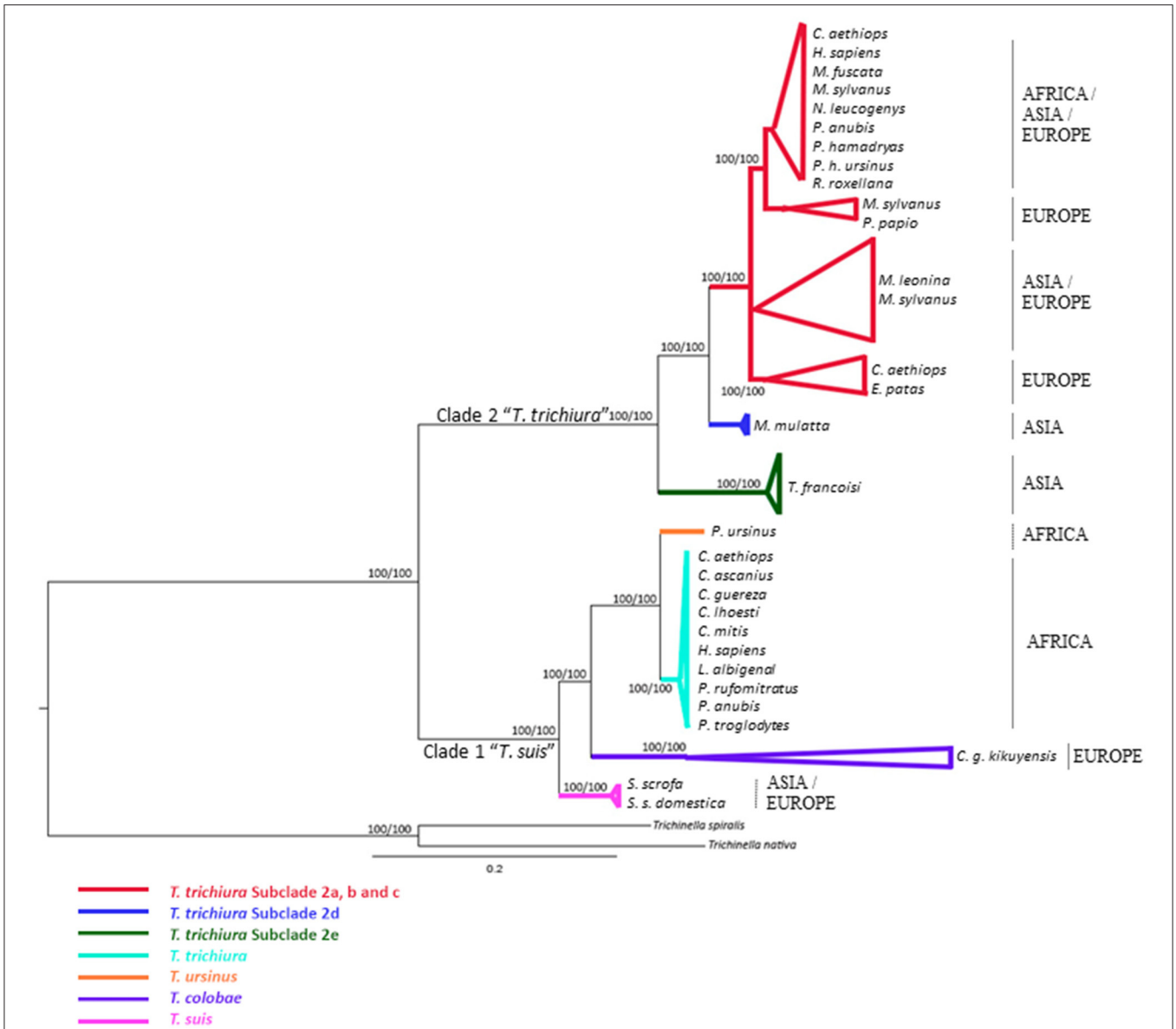

FIGURE 1 | Phylogenetic tree of *Trichuris* species based on combined analysis of ribosomal DNA (ITS1 and ITS2) inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

subclades, the subclade 2d not being represented due to the absence of sequences in all the markers. Subclade 2c was the most representative subclade including populations from a high variety of host species (**Figure 3** and **Supplementary Table 3**).

The phylogenetic inferences revealed that the populations of *Trichuris* from *P. papio*, *C. aethiops*, and *E. patas* analyzed in the present study clustered mainly in the subclade of *T. trichiura* (subclade 2c).

Comparative Sequence Analysis

In order to analyze the intraspecific and interspecific similarity in *T. trichiura* and between *T. trichiura* and *Trichuris* spp. parasitizing NHP as well as *T. suis*, we carried out a comparative study considering the different clades and subclades previously described for *Trichuris* spp. hosting humans, NHP, and swine (**Supplementary Tables 4–7**).

Thus, by examining ITS1 and ITS2 sequences, specimens obtained from *P. papio*, *C. aethiops*, and *E. patas* from Spain revealed a high similarity with populations of *T. trichiura* corresponding to subclades 2a, 2b, and 2c and subclade *Macaca mulatta* from different geographical origins (94.65 to 100%) (**Supplementary Table 4**). Further, the similarity was lower when clade 2 (*T. trichiura* lineage) and clade 1 (*T. suis*) were compared (90.62–92.23%).

Mitochondrial sequences (*cox*1 and *cob*) from *T. trichiura* from *P. papio*, *C. aethiops*, and *E. patas* from Spain showed the

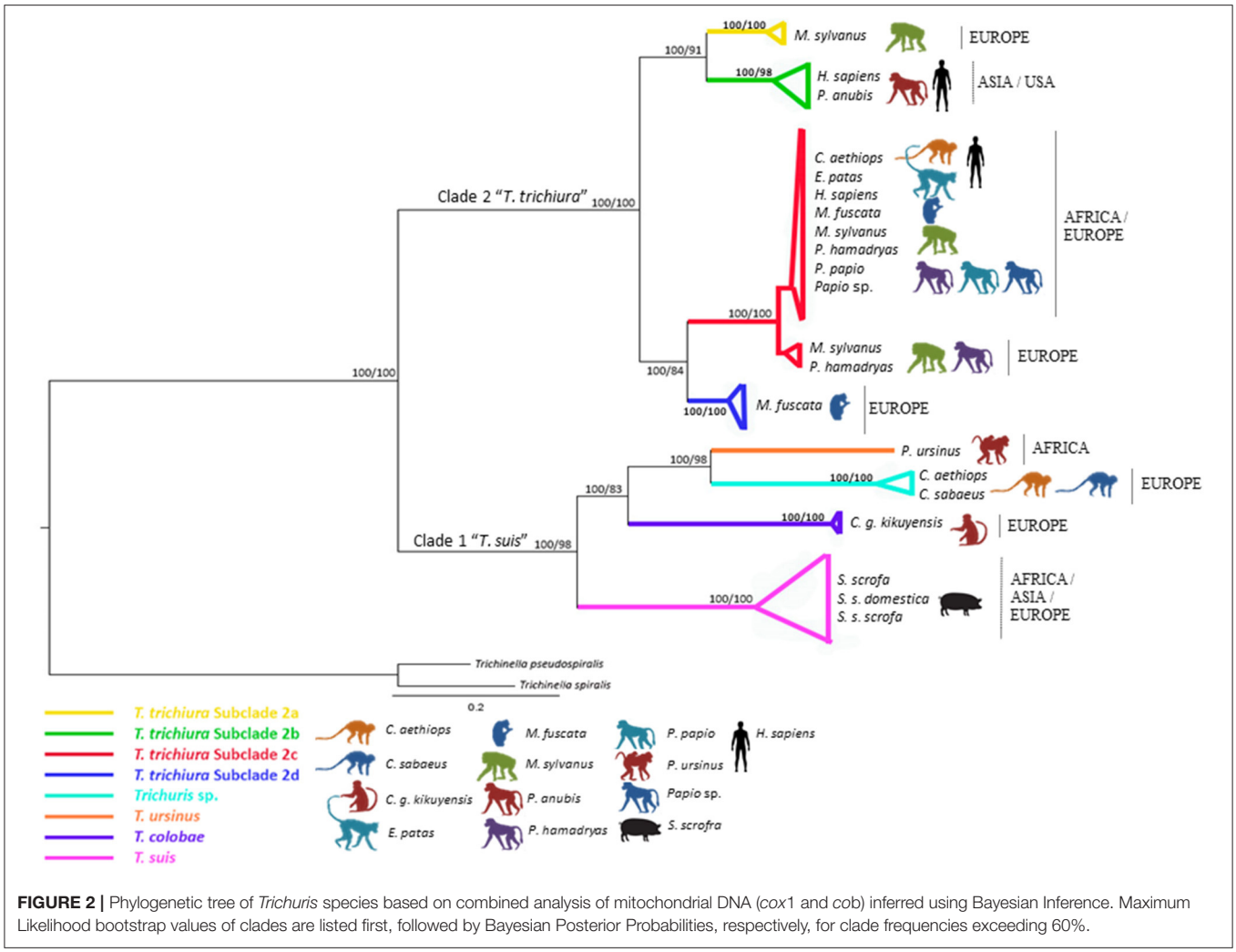

FIGURE 2 | Phylogenetic tree of *Trichuris* species based on combined analysis of mitochondrial DNA (*cox*1 and *cob*) inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

highest intraspecific similarity within subclade 2c (97.3 to 100% and 92.12 to 100%, respectively) (**Supplementary Tables 5, 6**). For *rrn*L sequences, similar results were observed for *T. trichiura* from *M. sylvanus* and *P. papio* (97.77–100%). However, based on three mitochondrial markers, *T. trichiura* from *M. sylvanus* showed two different lineages corresponding to subclades 2a and 2c (**Supplementary Table 7**). Based on three mitochondrial markers, the similarity between clade 2 and *T. suis* ranged from 68.47 to 82.68%, values lower than those shown within clade 2.

Analysis of genetic diversity for clade 2 based on ITS and mitochondrial sequences revealed a haplotype diversity of 1.0 and 0.95–0.93, respectively (**Table 2**). In addition, nucleotide diversity based on ITS and *rrn*L sequences was 0.05; nevertheless, mitochondrial genes (*cox*1 and *cob* genes) revealed a higher nucleotide diversity (0.09–0.11) with the maximum values for *cox*1 (0.11) (**Table 2**). Within clade 2 (subclades 2a, 2b, 2c, and 2d), haplotype diversity of mitochondrial genes ranged 0.71–1.0 for *cox*1, 0.67–1.0 for *cob*, and 0.88–1.0 for *rrn*L with the maximum values for subclade 2a. Nucleotide diversity of mitochondrial genes ranged 0.01–0.04 for *cox*1 and *cob* with the maximum values for subclade 2b, and the value for *rrn*L was 0.01 (**Table 2**).

The analysis of nucleotide substitution saturation based on nuclear markers showed Iss > Iss.c ($P < 0.005$), indicating that ITS regions were saturated. Nevertheless, mitochondrial genes (*cox*1, *cob*, and *rrn*L) were not saturated (Iss < Iss.c, $P < 0.005$). Genetic diversity measures for all markers are summarized in **Table 2**.

Update Morphological Description of *T. trichiura* (Linnaeus, 1771)

Taxonomic summary

- Class Enoplea (Order Trichocephalida) (12, 38).
- Host: *H. sapiens* (14), *Pan troglodytes* (7), *M. sylvanus* (17, 21), *P. papio* (present study).

General: This is a parasite with a filiform anterior part and a broad and handle-like posterior part. The thin anterior portion of the parasite displays 2 types of cuticle patterns. One side is distinctly striated with transverse grooves and the other side

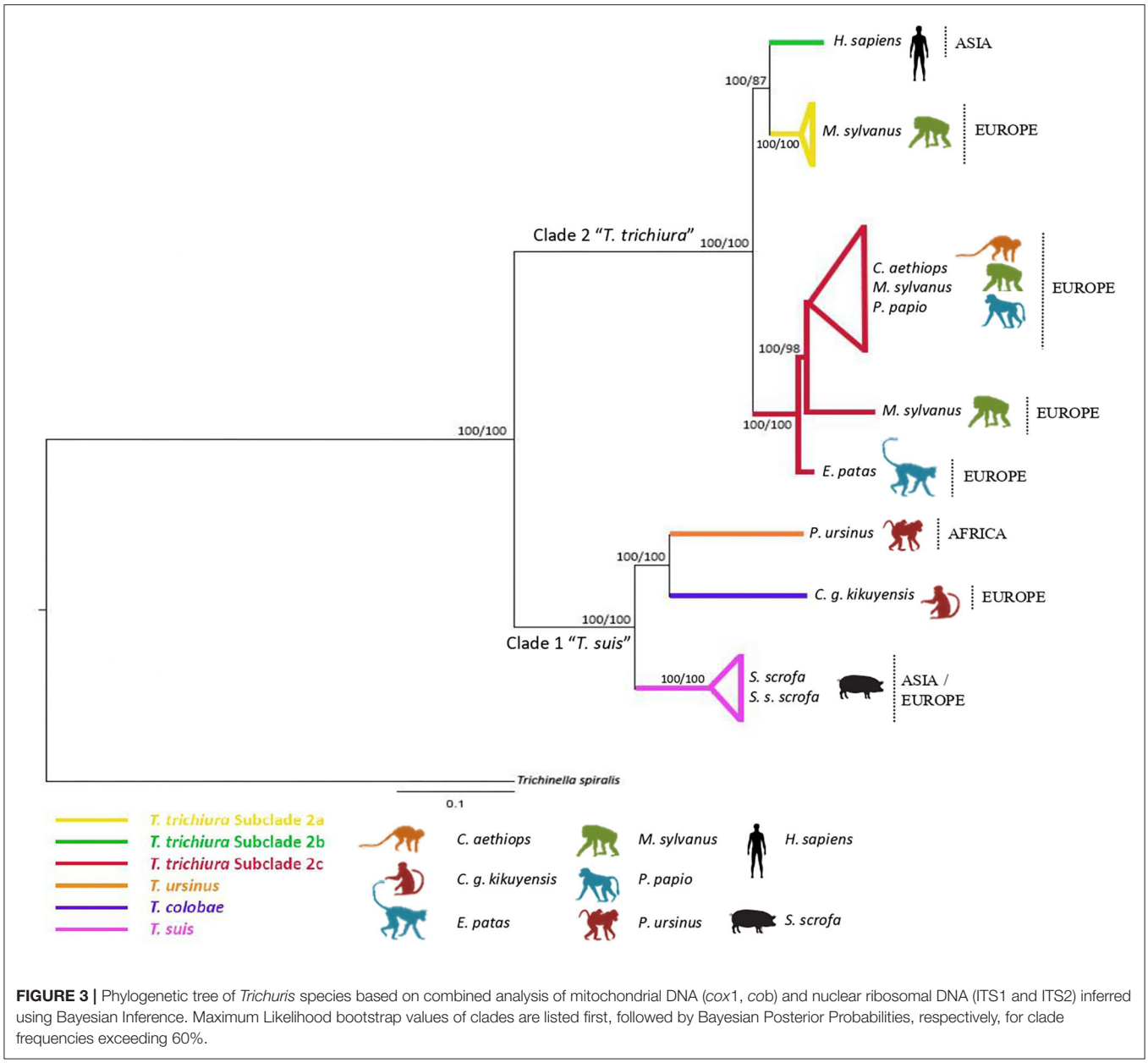

FIGURE 3 | Phylogenetic tree of *Trichuris* species based on combined analysis of mitochondrial DNA (*cox*1, *cob*) and nuclear ribosomal DNA (ITS1 and ITS2) inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

in a finely tuberculate band under higher magnification reveals small circular elevated bodies, which are evenly distributed (**Figure 4A**). The ventral side of the anterior part of the body is slightly tapered toward the cephalic end which presents a broad longitudinal elongated "bacillary band" showing typical cuticle inflations (**Figure 4B**). The mouth is surrounded by a group of cephalic papillae arranged in two circles (an inner circle and a lateral circle) with a conspicuous organ, the stylet (**Figure 4C**), protruding from the middle portion of the mouth cavity. Adults present a moniliform esophagus constituted with a short muscular zone and a long stichosome with 1 row of stichocytes (**Figure 4D**), and 1 pair of conspicuous cells at esophagus-intestinal junction level (**Figures 4D**, **5A,B**). The site where the esophagus transforms into the intestine corresponds to the place of transition of the thin anterior part into the thick posterior and shows typical glandular cells at this point (**Figures 4D**, **5A,B**).

Male: The body is 25–49.3 mm long. The ratio between anterior and posterior body length is 2.6–4:1. The thin anterior part is 1:1.4–1.7 of the entire length of the body. The length of the esophagus is 18–28.9 mm. The width of the body in the esophageal end is 0.15–0.49 mm, and the maximum width (in the posterior part of the body) is 0.39–0.74 mm (**Table 3**). Posterior end of the body ventrally incurved (**Figures 4E–G**).

The genital apparatus of the male is a long tube whose sections differ from each other in structure bearing different functions (**Figure 5A**). The first section of the genital apparatus is the testis, which is very long and strongly convoluted, beginning

TABLE 2 | Summary of genetic measures for *cox*1, *cob*, *rrn*L genes, and ITS region sequences.

	Subclade 2a			Subclade 2b			Subclade 2c			Subclade 2d			*Trichuris trichiura* complex (Clade 2)			
	***cox*1**	**cob**	***rrn*L**	***cox*1**	**cob**	***rrn*L**	***cox*1**	**cob**	***rrn*L**	***cox*1**	**cob**	***rrn*L**	***cox*1**	**cob**	***rrn*L**	**ITS**
Number of haplotypes/number of sequences	3/3	3/3	2/2	3/4	2/3	10/13	5/13	14/18	15/29	6/6	4/5	5/6	17/26	23/29	31/50	21/22
Haplotype diversity	1.0	1.0	1.0	0.83	0.67	0.95	0.71	0.95	0.88	1.0	0.90	0.93	0.93	0.98	0.95	1.0
Nucleotide diversity	0.02	0.02	0.01	0.04	0.04	0.01	0.01	0.02	0.01	0.02	0.01	0.01	0.11	0.09	0.05	0.05
Nucleotide saturation*	-	-	-	-	-	-	-	-	-	-	-	-	No	No	No	Yes

**Substitution saturation test for partial cox1, cob, rrnL genes, and ITS sequences of Trichuris trichiura (Clade 2). "Yes" indicates that most of this sites have already been changed before (Iss>Iss.c), indicating nucleotide saturation.*

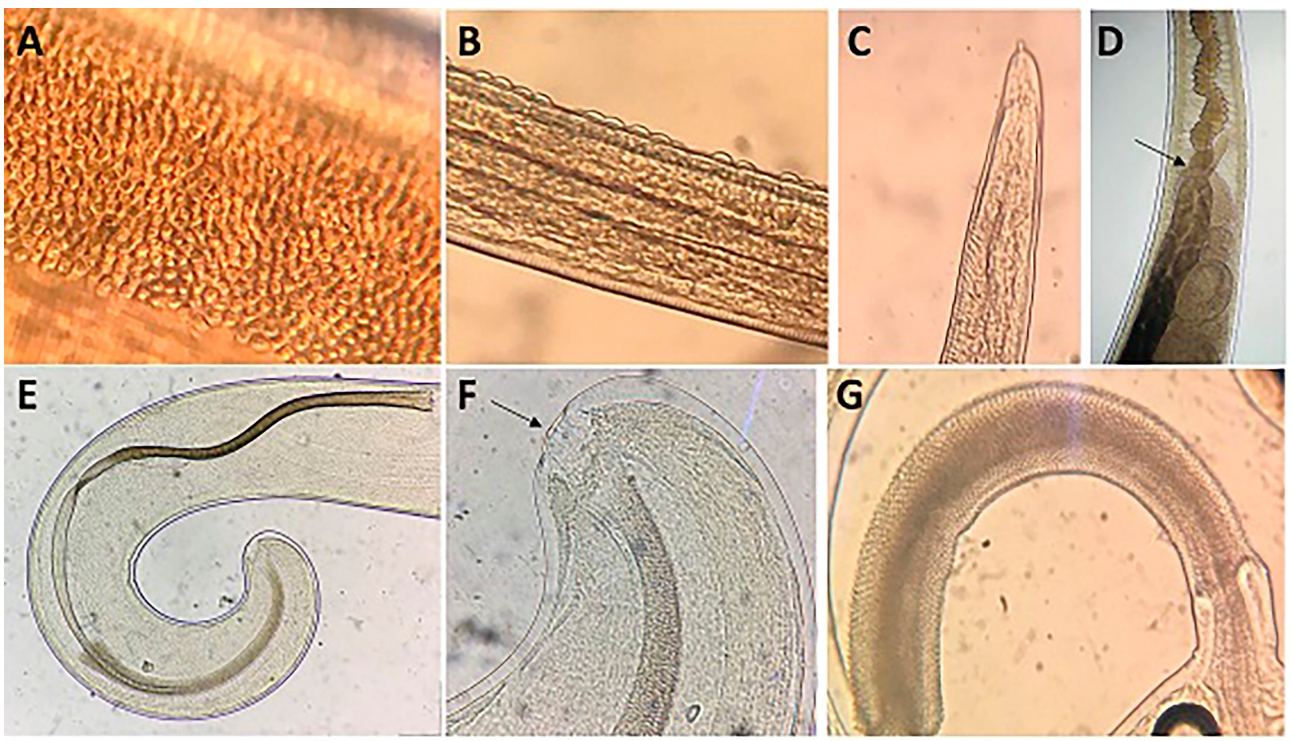

FIGURE 4 | Morphology of males of *T. trichiura*. **(A)** Tuberculate band. **(B)** Bacillary band showing typical cuticular inflations. **(C)** The mouth with the stylet. **(D)** Stichosome with 1 row of stichocytes and 1 pair of conspicuous cells at esophagus–intestinal junction level (arrowed). **(E)** Spicule. **(F)** Papillae pericloacal. **(G)** Spicule sheath.

in the posterior part of the male body, directed anteriorly, and lying along the long axis of the body terminating at a short distance from the transition of the esophagus into the intestine (**Figure 5A**). The testis ends near the union of the ejaculator conduct and intestine. The testis is followed by the vas deferens which at first runs somewhat anteriorly along the intestine, and then, at the level of the esophageal end and somewhat short of it, describes a convolution, turns backward, and terminates in a small constriction, connecting it with the following section of the genital apparatus, the seminal vesicle. The seminal vesicle runs parallel to the intestine but does not describe sharp convolutions and via a narrow tube with thick muscular walls joins the ejaculatory duct, which is 1.07–2.34 mm long (**Figure 5A**). The ejaculatory duct joins the intestine to form the cloaca, which opens at the posterior end of the male body. The cloaca with anus subterminal and one pair of paracloacal papilla not ornamented (**Figure 4F**) was observed when the spicule sheath was invaginated. No cluster of papillae was observed. The proximal cloacal tube is wide and continued with the distal cloacal tube (1.10–2.75 mm) that contains the spicule which projects into the anterior portion of the body in a spicule tube (**Figure 5A**). There is only one spicule, which is elongated with a pointed tip. This spicule presents two chitinized extreme zones and a light central part and is 1.61–3.81 mm long

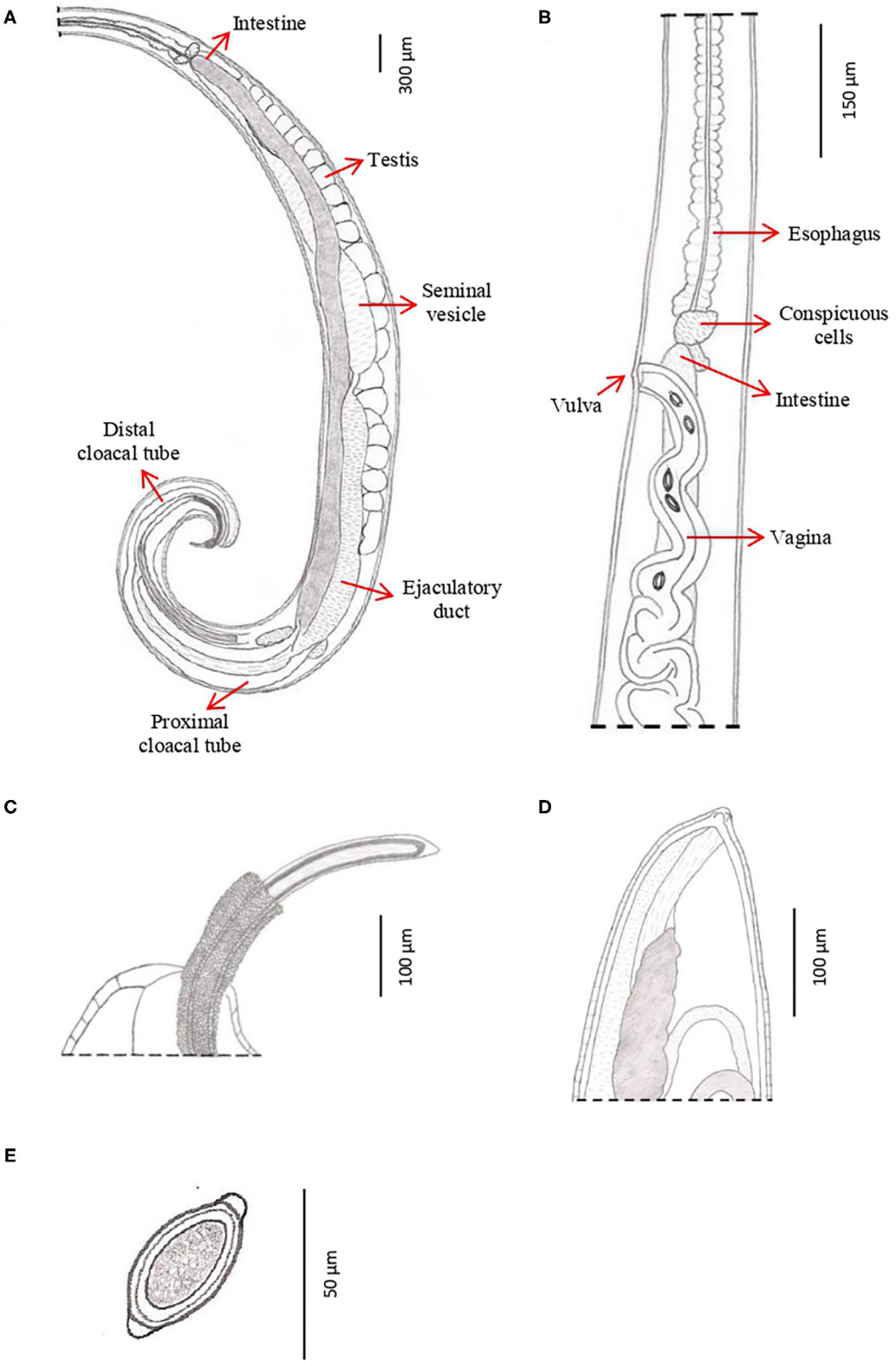

FIGURE 5 | Drawings of *T. trichiura* from *P. papio*. **(A)** Male, posterior end, spiny spicule sheath, spicule, spicule tube and proximal and distal cloacal tube, lateral view. **(B)** Female, esophagus–intestine junction, vulva and vagina, lateral view. **(C)** Male, detail of the posterior end, lateral view. **(D)** Female, posterior end, lateral view. **(E)** Eggs.

TABLE 3 | Biometrical data of males of *T. trichiura*.

	***T. trichiura* (Present study, updated)**	***T. trichiura* from *P. troglodytes* [Cutillas et al., (7)]**				***T. trichiura* from *Homo sapiens* [Nissen et al. (14)]**			***T. trichiura* from *M. sylvanus* [García-Sánchez et al. (21)]**				***T. trichiura* from *P. papio* (Present study)**		
	MIN-MAX	**MIN**	**MAX**	**X**	**δ**	**MIN**	**MAX**	**X**	**MIN**	**MAX**	**X**	**δ**	**MIN-MAX**	**X**	**δ**
M1	25.0–49.3	32.0	36.0	33.5	0.19	37.2	49.3	43.9	30.0	39.0	34.5	0.25	25.0	25.0	-
M2	18.0–28.9	18.0	23.0	20.5	0.21	20.9	28.9	25.6	19.0	28.0	21.9	0.27	18.0	18.0	-
LP	7.0–20.4	12.0	14.0	13.0	0.05	14.9	20.4	18.4	8.0	17.0	1.25	0.22	7.0	7.0	-
M3	0.09–0.31	0.09	0.31	0.15	0.02	0.10	0.20	0.15	0.12	0.18	0.14	0.02	0.12	0.12	-
M4	0.39–0.74	0.39	0.60	0.50	0.08	0.50	0.70	0.63	0.49	0.74	0.61	0.07	0.56	0.56	-
M5	0.15–0.49	0.15	0.24	0.2	0.04				0.25	0.49	0.37	0.06	0.35	0.35	-
M6	0.33–0.64	-	-	-	-				0.33	0.64	0.49	0.09	0.50	0.50	-
M7	1.10–1.62	-	-	-	-				1.10	1.57	1.33	0.14	1.62	1.62	-
M8	1.61–3.81	1.61	2.22	1.90	0.23	2.88	3.81	3.19	2.23	3.23	2.65	0.23	2.86	2.86	-
M9	0.22–1.23	0.22	0.22	0.22	-				0.53	1.23	0.93	0.20	0.63	0.63	-
M10	0.01–0.08	0.01	0.06	0.02	0.02				0.04	0.08	0.06	0.01	0.07	0.07	-
M11	0.04–0.09	0.05	0.09	0.07	0.02				0.04	0.08	0.06	0.01	0.05	0.05	-
M12	0.06–0.11	0.09	0.11	0.09	0.01				0.06	0.09	0.07	0.01	0.07	0.07	-
M13	1.79–5.19	1.79	1.95	1.88	0.07				2.90	5.19	4.06	0.58	3.31	3.31	-
M14	1.07–2.34	1.07	1.07	-	-				1.32	2.34	1.97	0.29	1.74	1.74	-
M15	1.10–2.75	1.10	1.10	-	-				1.55	2.75	2.20	0.36	1.35	1.35	-

M1, total body length of adult worm; M2, length of the esophageal region of the body; LP, length of the posterior region of the body; M3, width of the esophageal region of the body; M4, maximum width of the posterior region of the body (thickness); M5, body width in the place of the junction of the esophagus and the intestine; M6, Distance from the head end to beginning of bacillary stripes; M7, length of bacillary stripes; M8, length of spicule; M9, maximum length of spicule sheath; M10, width of the proximal end of the spicule; M11, width of the spicule sheath at the tail end of the body; M12, maximum width of the spicule sheath; M13, distance between the posterior part of the testis and tail end of body; M14, length of ejaculatory duct; M15, length of distal cloacal tube. Б*, arithmetic mean.* σ*, standard deviation.*

(**Figures 4E**, **5A**). The spicule is surrounded by a peculiar spicule sheath (0.22–1.23 mm long), which may protrude externally together with the spicule (**Figure 4G**). The maximum width of the spicule sheath is 0.06–0.11 mm and is covered throughout its length by densely located chitinous spines from the proximal to distal portion and is cylindrical without a distal bulb (**Figures 4G**, **5C** and **Table 3**).

Female: The body is 20–48.6 mm long. The ratio between anterior and posterior body length is 2.1–2.2:1. The thin anterior part is 1: 0.65–0.67 of the entire length of the body. The length of the esophagus is 13–33 mm. The width of the body around the esophageal end is 0.13–0.48 mm, and the maximum width (in the posterior part of the body) is 0.38–0.90 mm (**Table 4**). The uterus is unpaired. The vulva is located at esophagus–intestine junction level (**Figures 5B**, **6A,B**). This part of body thereafter changes into a transversely striated body cuticle. The vulva is non-protrusive and has no ornamentation (**Figures 6A,B**). The vagina has strong walls and, when everted, shows small papillae (**Figure 6B**). This vagina is long and presents one zone straight near the vulva while presenting circumvolutions nearly the uterus (**Figures 5B**, **6A**). The ovary is long and continues with the oviduct in the back of the body (**Figure 6C**). The anus lies, subterminal, at the tip of the tail (**Figures 5D**, **6D**). The eggs are barrel-shaped with clear, mucoid-appeared polar plugs, in addition to a vitelline membrane, and have a triple shell, the outermost layer of which is brown (**Figure 5E**).

DISCUSSION

In the present paper, we address an updated morphological and biometric description of *T. trichiura*, based on the results provided in the present study as well as on previous studies by different authors who characterized this species combining the analysis of morphological, biometric, and molecular characteristics. Thus, for the emendation of the description of *T. trichiura*, we have considered the populations of *P. troglodytes* (7), *H. sapiens* (14), *M. sylvanus* (17, 21), and *P. papio* (present study). For many years, different authors have based on the description of *T. trichiura* isolated from humans and NHP, on morphological and biometric characteristics exclusively (6, 25, 39–43), which was not molecularly confirmed to have corresponded to *T. trichiura*.

Thus, we found that females of *T. trichiura* are characterized by a non-protrusive vulva without ornamentation. However, some authors have described the vulva with a surface covered with spines like those of the male's spicule sheath (6, 25). Likewise, based on the morphological characteristics of males, *T. trichiura* is characterized by the presence of a lanceolate spicule that tapers at the end (41). However, Tenora et al. (42) observed a spicule with a cylindrical end in isolated samples of *H. sapiens*. Furthermore, males of *T. trichiura* isolated from *P. papio* and *M. sylvanus* presented a spicule with two chitinized extreme zones and a lighter central part (17). This feature was not observed in

TABLE 4 | Biometrical data of females of *T. trichiura*.

	T. trichiura (Present study, updated)	*T. trichiura* from *P. troglodytes* [Cutillas et al. (7)]				*T. trichiura* from *H. sapiens* [Nissen et al. (14)]			*T. trichiura* from *M. sylvanus* [García-Sánchez et al. (21)]				*T. trichiura* from *P. papio* (Present study)			
	MIN-MAX	**MIN**	**MAX**	**X**	**δ**	**MIN**	**MAX**	**X**	**MIN**	**MAX**	**X**	**δ**	**MIN**	**MAX**	**X**	**δ**
F1	20–48.6	20.0	42.0	33.4	0.78	29.1	48.6	38.4	30.0	38.0	34.1	0.25	24.0	28.0	26	0.28
F2	13–33	13.0	33.0	25.3	0.68	16.2	32.1	25.6	18.0	26.0	21.9	0.23	17.0	20.0	18.5	0.21
LP	6–15.60	6.0	10.0	8.1	0.14	10.5	15.60	13.7	09.0	14.0	12.1	0.16	7	8	7.5	0.07
F3	0.09–0.20	0.09	0.19	0.14	0.04	0.11	0.20	0.17	0.13	0.18	0.15	0.01	0.14	0.15	0.14	0.00
F4	0.38–0.90	0.38	0.64	0.45	0.08	0.52	0.90	0.73	0.64	0.81	0.72	0.05	0.60	0.63	0.62	0.02
F5	0.13–0.48	0.13	0.23	0.17	0.03				0.36	0.48	0.42	0.03	0.31	0.41	0.36	0.07
F6	0.38–0.76	0.48	0.64	0.56	0.11				0.42	0.76	0.50	0.09	0.38	0.42	0.40	0.03
F7	0.36–1.71	0.36	0.63	0.50	0.19				0.90	1.71	1.44	0.21	1.08	1.35	1.22	0.19
F8	0.68–1.99	0.68	1.29	0.96	0.20				0.73	1.99	1.12	0.35	0.87	0.91	0.89	0.03
F9	0.02–0.09	0.03	0.05	0.04	0.01				0.02	0.09	0.05	0.02	0.05	0.05	0.05	0.00
F10	0.33–0.11	0.11	0.24	0.15	0.05				0.15	0.33	0.25	0.05	0.18	0.19	0.18	0.01
F11	0.13–0.84	0.13	0.24	0.21	0.04				0.40	0.84	0.61	0.14	0.35	0.37	0.36	0.01
F12	0.19–0.62	0.32	0.53	0.44	0.11				0.19	0.48	0.30	0.09	0.56	0.62	0.59	0.04
F13	0.05–0.81	0.66	0.67	0.67	0.01				0.05	0.14	0.11	0.04	0.73	0.81	0.77	0.06

F1, total body length of adult worm; F2, length of the esophageal region of the body; LP, length of the posterior region of body; F3, width of the esophageal region of the body; F4, maximum width of the posterior region of the body (thickness); F5, body width in the place of the junction of the esophagus and the intestine; F6, distance from the head end to beginning of bacillary stripes; F7, length of bacillary stripes; F8, length of vagina; F9, diameter of vulva turned over the surface of the body; F10, distance of vulva from the place of junction of the esophagus and the intestine; F11, distance of the posterior loop of the uterus from the tail end of the body; F12, distance of the tail end of the body and posterior fold of the seminal receptacle; F13, length of the muscular zone of the esophagus. Б*, arithmetic mean.* σ*, standard deviation.*

males of *T. trichiura* described by several authors who exclusively carried out morphological and biometric studies (6, 7, 25, 39–42).

On the other hand, the spicule sheath is cylindrical without a distal bulb with triangular spines; however, other authors described these spines with different shapes and sizes, some of them with blunt points in *T. trichiura* from humans and NHP (25, 42). Besides, we found that the males of *T. trichiura* are characterized by the presence of a pair of paracloacal papillae which are observed when the sheath is invaginated (17); however, a proximal group of small papillae is not observed, as described by Ooi et al. (6) in a morpho-biometric study carried out on samples of monkeys, baboons, and humans. These papillae cluster was described in *T. colobae* by Cutillas et al. (19) who characterized a new species of *Trichuris* present in *C. g. kikuyuensis* based on morphological, biometric, and molecular data. This fact could suggest that the descriptions carried out by Ooi et al. (6) of *Trichuris* populations from humans and NHP based exclusively on morphological and biometric characters could correspond to different species closed to *T. trichiura*.

Biometrically, there is a concordance between the measures of *T. trichiura* obtained by different authors and those provided in the present paper, since all the values overlapped within the range of defined measures. However, regarding males, Dinnik et al. (40) reported that the maximum total body length and the maximum length of the anterior part [total body length of adult worm (M1) and length of esophageal region of body (M2)] were slightly larger (52 and 34 mm, respectively). Furthermore, the maximum width of the posterior region of body (thickness) (M4), length of spicule (M8), and length of ejaculatory duct (M14) has also been reported as slightly higher by Skrjabin et al. (25) (M4 = 0.76 mm, M8 = 3.9 mm, and M14 = 3.9 mm). Regarding females, the maximum total body length of adult worm (M1) showed slightly higher values in the studies of Dinnik et al. (40), Skrjabin et al. (25), and Vigot-Fréres (39) (50, 52.7, and 50 mm, respectively). In addition, the maximum length of the esophageal region of body (M2) and the maximum body width in the place of junction of esophagus and the intestine (F5) were slightly higher (35.6 and 0.50 mm, respectively) in the data provided by Skrjabin et al. (25).

In addition, there are differences in the population of *Trichuris* sp. from chimpanzees showing a shorter size of the males and females collected from chimpanzees with respect to *T. trichiura* from *H. sapiens*, *M. sylvanus*, and *P. papio*. García-Sánchez et al. (21) who reported the differentiation of *Trichuris* species using a morphometric approach cited these results previously. The occurrence of different biometrical measurements in the same species may be explained by the phenotypic plasticity of these organisms themselves (8–11).

Morphological and biometric data for *T. trichiura* provided in the present study allow the differentiation of this species with respect to other species of *Trichuris* parasitizing NHP such as *T. colobae* (19) and *T. ursinus* (20). The typical *T. trichiura* spicule has a clear central part, which is not present in that of *T. colobae* and *T. ursinus*. Furthermore, the typical papilla group is only present in *T. colobae* (19). Females of *T. trichiura* and *T. ursinus* appear to have a non-protrusive vulva (20); nevertheless, females of *T. colobae* present a vulva like a crater with papillae (19). The vagina is very long and straight in *T. ursinus* (20) but appear with circumvolutions in *T. trichiura*.

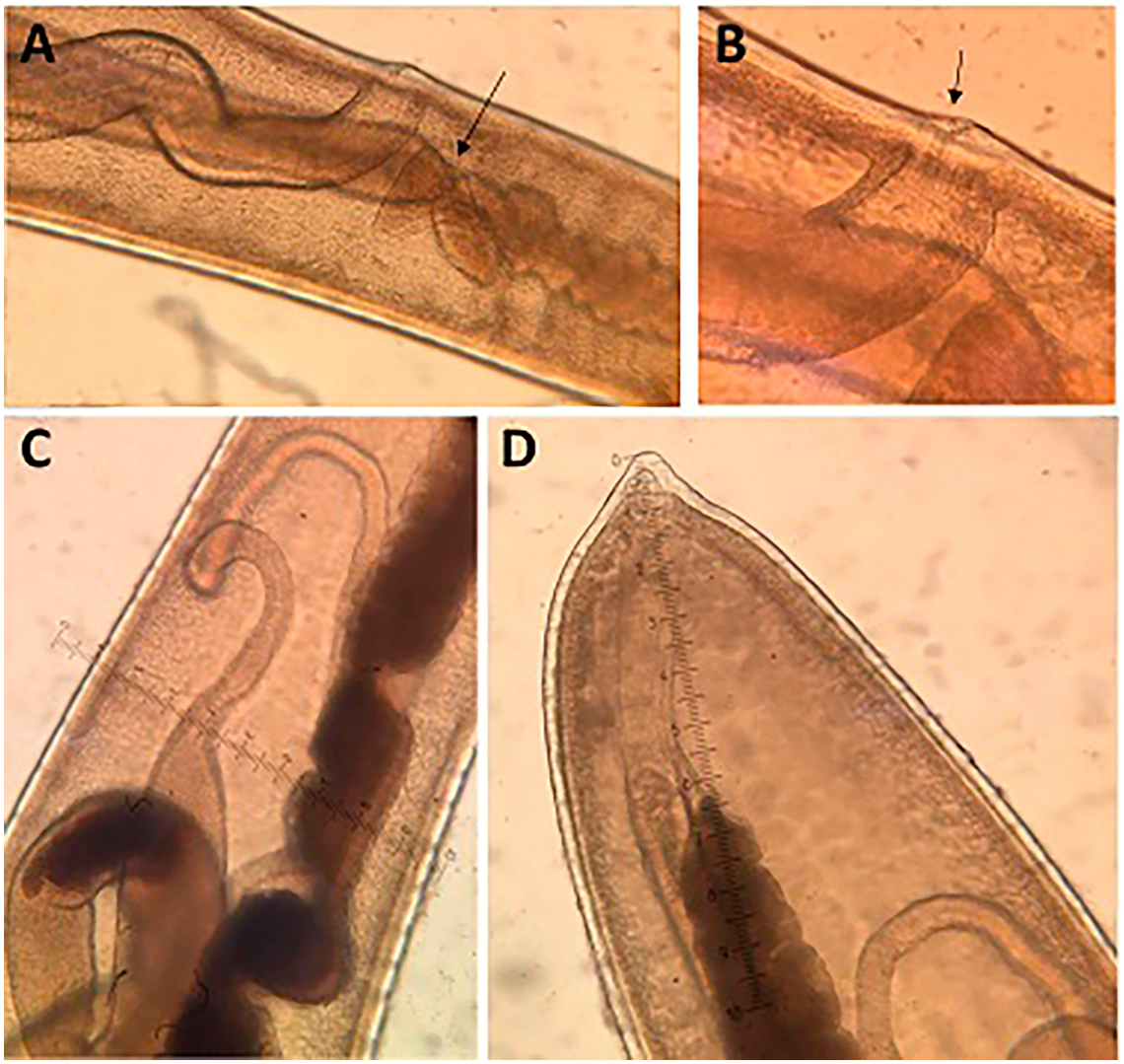

FIGURE 6 | Morphology of females of *T. trichiura*. **(A)** Esophagus–intestinal junction level (arrowed), vulva and vagina. **(B)** Vulva (arrowed). **(C)** Oviduct. **(D)** Posterior end.

With respect to the biometric characteristics, most of the measurements do not allow the specific differentiation between *T. trichiura* and other *Trichuris* spp. of NHP since these values overlapped for most of the measurements. However, males of *T. trichiura* have a smaller range of values in terms of distance from the end of the head to beginning of bacillary stripes and length of bacillary stripes (M6 and M7) compared to *T. colobae*, and values of length of minor bacillary stripes regarding *T. ursinus*. Regarding the specific differentiation of females based on biometric measurements, *T. trichiura* presents a lower range of values of length of bacillary stripes and distance of the tail end of the body and posterior fold of the seminal receptacle (F7 and F12) respect to *T. colobae*. In addition, the maximum and minimum values of distance of the vulva from the place of junction of the esophagus and the intestine were below than those observed in *T. ursinus* (19, 20).

From a molecular point of view, molecular markers which have been used by different authors to resolve species-level questions in *Trichuris* include the ITS1 and ITS2 nuclear regions (7, 44–53), 18S nuclear rRNA gene (38, 53, 54), mtDNA 16S rRNA gene (*rrn*L) (17, 50, 55), and protein-coding mitochondrial genes, including the 13 common genes obtained from the mitochondrial complete genome (12, 16, 56), *cox*1 mtDNA partial gene (17, 20, 38, 53, 55, 57), and *co*b mtDNA partial gene (17, 20, 55, 57, 58).

Different ITS rDNA types have been cited by different authors in some species of *Trichuris* (45–47). These sequence differences among ITS repeats in the rDNA array appeared to be a consequence of (intrachromosomal) mutational exchange during DNA replication (59). Thus, different reports suggest that different sequence types are most likely to be a result of base changes at certain positions in the sequence of a proportion of rDNA repeats because of mutational exchange during DNA replication, the extent of which appears to differ depending on the taxonomic group (60–63).

The results observed in ITS sequences revealed a percentage of similarity between 2a, 2b, 2c, and 2d subclades higher than those previously observed by other authors for species of the genus *Trichuris* (50, 57). Genetic analysis revealed that ITS regions were saturated and showed poor nucleotide diversity for clade 2 (*T. trichiura*). Thus, ITS sequences were not useful to infer the phylogenetic relationships between the different populations of *T. trichiura* (clade 2). On the other hand, some sequences from *T. trichiura* from Uganda and Cameroon (4) appeared within clade 1, suggesting that this population could be included on *T. ursinus* due to the high interpopulation similarity observed between both populations (**Supplementary Table 4** and **Figure 1**). Therefore, the different genetic lineages within *T. trichiura* were delimited exclusively based on analysis of the mitochondrial genes in agreement with Chan et al. (64), who evaluated the utility of mitochondrial and ribosomal genes for molecular systematics of parasitic nematodes. ITS regions accumulated substitutions substantially more slowly than mtDNA and showed nucleotide saturation.

Studies of interpopulation similarity between the different 2a, 2b, 2c, and 2d subclades, based on the mitochondrial markers (*cox*1 and *co*b), revealed a range of similarity lower than those observed by ITS sequences, and similar to those observed among other clearly defined species such as for example *T. suis* and *T. colobae*, *T. colobae*, and *T. ursinus* [cited by present authors, **Supplementary Tables 5, 6** and previously by Callejón et al. (20) and Rivero et al. (17)]. However, this similarity range showed higher values between *rrn*L sequences presenting a strong resolution of the different *T. trichiura* lineages (**Supplementary Table 7**). These results agree with those of Chan et al. (64), who evaluated the utility of mitochondrial ribosomal genes for molecular systematics of parasitic nematodes. These authors cited that 18S and 28S rRNA genes as well as 12S (*rrn*S) and 16S (*rrn*L) rRNA and *cox*1 genes showed a higher resolution for phylogenetic studies indicating that these five genes have potentially to be used as markers. Furthermore, they demonstrated that mitochondrial 12S and 16S genes present a resolution power at both lower and higher taxonomic levels for species and clade discrimination (64). For this reason and considering the similarity values observed in the different markers, we suggest that the populations of *T. trichiura* corresponding to the 2a, 2b, 2c, and 2d subclades correspond to different genetic lineages.

On the other hand, within clade 2 "*T. trichiura* lineage," results based on *rrn*L revealed an interpopulation similarity of 83.80–86.31% between *T. trichiura* populations and *Trichuris* sp. from *T. francoisi*. Hence, we suggest that this species has a close relationship with *T. trichiura*. Our results agree with Liu et al. (12) who considered that *Trichuris* populations from *T. francoisi* are a new species of *Trichuris*.

The phylogeny inferred from mitochondrial datasets revealed the same topology of those based on rDNA with respect to the two main clades (clade 1 and clade 2). We were able to identify several genetically distinct subgroups (subclades) of whipworms, which were present in the sampled primates. The subclades 2b and 2c showed a broad host range and were not restricted to NHP species. However, the subclades 2a and 2d showed a higher host specificity corresponding with the *T. trichiura* population from *M. sylvanus* and *H. sapiens* (subclade 2a) and *M. fuscata* (subclade 2d) exclusively. In agreement to this study, similar results were observed on *Trichuris* sp. from *M. fuscata* (44, 55). This population also showed two potentially distinct entities of *Trichuris* present in two different subclades: subclade 2d [analogous to subclade MF reported by Cavallero et al. (44, 55)] and subclade 2c. These authors suggested the possibility of two different sources of infection for Japanese macaques corresponding with two *Trichuris* taxa, one potentially able to also infect humans.

In our study, all phylogenetic trees (partitioned and concatenated) reported the existence of two main clades, which has been previously reported (3, 7, 12, 17, 20, 33, 44, 55, 58). A clear differentiation between *T. suis* (clade 1) and *T. trichiura* (clade 2) can be confirmed according to our results. Within clade 2 "*T. trichiura* lineage," *T. trichiura* can be also divided into 4 subclades, suggesting a complex of different genetic lineages. The analysis of the intraspecific similarity between the populations of *T. trichiura* from *P. papio*, *C. aethiops*, and *E. patas* from Spain revealed their highest value when compared with the populations of *T. trichiura* belonging to subclade 2c [previously described by Rivero et al. (17)] in all analyzed markers.

In addition to *T. trichiura*, a complex of *Trichuris* species showed infecting primates (*Trichuris* sp. from *T. francoisi* within clade 2 "*T. trichiura* lineage" and from *C. aethiops* from Italy and *C. sabaeus* from Czech Republic within clade 1 "*T. suis* lineage"). Different authors (17, 65) have previously reported the presence of several groups of *T. trichiura*. These results emphasize that the taxonomy and genetic variations of *Trichuris* are more complicated than previously acknowledged (65). Ravasi et al. (3) identified two distinct *Trichuris* genotypes that infect both humans and NHP. These authors identified "heterozygotes" confirming the identification of two distinct *Trichuris* genotypes in primates. On the other hand, Nissen et al. (14) identified "heterozygote" worms isolated from humans suggesting that *T. trichiura* might consist of several subspecies, some being found mainly in NHP. Ghai et al. (66) suggested that the *Trichuris* taxon should be considered a multi-host pathogen that is capable of infecting wild primates and humans. Finally, Betson et al. (5) reported that *Trichuris* infecting primates represents a complex of cryptic species with some species being able to infect both humans and NHP.

On the other hand, the existence of populations of *Trichuris* sp. associated with clade 1 "*T. suis* lineage" (based on all partitioned and concatenated markers) could indicate the possible origin of the change of host from NHP to pigs and, therefore, the origin of a new species, *T. suis*. This fact has been suggested by Hawash et al. (16) who found evidence of an African origin of *T. trichiura* that was later transmitted with human ancestors to Asia and then to South America. These authors suggested the possibility of a change of host to pigs in Asia where *T. suis* appears to have been transmitted globally by a combination of natural host dispersion and anthropogenic factors.

The present work examines the taxonomy, genetics, and phylogeny of *T. trichiura* parasitizing human and NHP and its

relationship to *Trichuris* spp. from other NHP host species. A similar analysis was carried out on whipworms from humans (5). These authors suggested the existence of zoonotic transmission, especially regarding *T. trichiura* infections in NHP and, possibly, also for *T. suis* from pigs and *Trichuris vulpis* from dogs. In consequence, *Trichuris* may represent different species with the potential differences in endemicity, which may have important implications for implementing effective control strategies (5).

Several studies have reported that NHP represent an important reservoir for several known zoonotic infectious diseases (67–69). In this context, *Trichuris* infections have been found in a range of NHP species living in natural habitats including colobus monkeys, macaques, baboons, and chimpanzees (70–78). Based on molecular studies described above, some *Trichuris* species seem to be specific to a particular NHP, while others likely have the potential to circulate between humans and NHP, as they are genetically identical. This is particularly important when humans and NHP live in close proximity, as it is becoming increasingly common with human encroachment on pristine habitats and NHP accessing gardens and farms in search of food, and it has significant implications for human health and wildlife conservation (5).

The taxonomic, genetic, and phylogenetic results obtained in the present study confirm that *T. trichiura* exists as a complex (four subclades) with different host affinities and cross-infection capabilities corresponding with four different genetic lineages. Specifically, two of the four subclades show little host specificity and can develop trichuriasis in a wider variety of NHP species (*C. sabaeus*, *C. aethiops*, *M. fuscata*, *M. sylvanus*, *E. patas*, *P. papio*, *P. hamadryas*, *Papio* sp., and *P. anubis*) shared with humans. For this reason, we suggest the existence of a possible reservoir in the previously mentioned NHP species for human trichuriasis, which constitutes a serious public health risk (17). However, previous studies showed that the majority of the population of *M. fuscata* was included in a specific subclade [2d, subclade MF cited by Cavallero et al. (44, 55)] including only specimens from macaques.

Therefore, considering the following arguments for the taxonomic and phylogenetic study of populations of *T. trichiura*: (i) ITS regions were saturated and accumulated substitutions substantially slowly than mtDNA; therefore, they are not good genetic markers to delimit different genetic lineages. This is in agreement with previous studies where intraspecific variation was not observed when using nuclear DNA (64) suggesting that is not a useful genetic marker for intraspecies discrimination. (ii) The mitochondrial genes *cox*1, *co*b, and *rrn*L were not saturated, indicating that these three genes could have potential to be used as markers. Nevertheless, although *cox*1 presents the advantage for the extensive availability of database sequences, allowing for thorough comparisons of unknown sequences, *cox*1 sequences showed a high intraspecific variability which hinders resolution between closely related species. (iii) Nucleotide diversity showed that the *rrn*L gene present a lower intraspecific variability than *cox*1 and *co*b genes. However, *rrn*L interspecific genetic distance values allowed the phylogenetic resolution of the different *T. trichiura* subclades. Whence, *rrn*L was used successfully for inter-lineage discrimination of closely related populations within the *T. trichiura* lineage.

We suggest the utility of *rrn*L rRNA gene as a useful genetic marker for *Trichuris* species discrimination. Different authors who evaluated the utility of different mitochondrial genes for phylogenetic analyses (64, 79–81) have cited similar results. Future studies should focus on developing sequence databases for rRNA genes, which can serve as alternative genetic.

In conclusion, the present work provides an extensive study of biometric, morphological and molecular data for the unification of criteria that allows an update description of *T. trichiura* as well as a complex taxonomic, genetic, and phylogenetic study of the cited species applying multiple genetic markers to whipworm populations collected from humans and NHP from sympatric areas and worldwide locations. This study provides useful results for future studies aimed at the identification of new subspecies and hybridization events between existing species and enables a much clearer and more detailed understanding of dispersal patterns. This will reveal parasite transmission routes between these primates and will allow the implementation of appropriate control and prevention measures.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JR, RC, and CC contribute conception, design of the study, and wrote the manuscript. All the authors contributed to the manuscript revision and read and approved the submitted version.

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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.2020.626120/full#supplementary-material

REFERENCES

1. World Health Organization. Soil-transmitted helminth infections (2020). Available online at: https://www.who.int/es/news-room/fact-sheets/detail/soil-transmitted-helminth-infections (accessed March 2, 2020).
2. Mcdowell MA, Rafati S, editors. *Neglected Tropical Diseases – Middle East and North Africa*. Wien: Springer-Verlag Wien (2014). p. 281.
3. Ravasi DF, O'Riain MJ, Davids F, Illing N. Phylogenetic evidence that two distinct *Trichuris* genotypes infect both humans and non-human primates. *PLoS ONE*. (2012) 7:e44187. doi: 10.1371/journal.pone.0044187
4. Ghai RR, Chapman CA, Omeja PA, Davies TJ, Goldberg TL. Nodule worm infection in humans and wild primates in Uganda: cryptic species in a newly identified region of human transmission. PLoS Negl Trop Dis. (2014) 8:e2641. doi: 10.1371/journal.pntd.0002641
5. Betson M, Søe M, Nejsum P. Human trichuriasis: whipworm genetics, phylogeny, transmission and future research directions. *Curr Trop Med Rep*. (2015) 2:209–17. doi: 10.1007/s40475-015-0062-y
6. Ooi HK, Tenora F, Itoh K, Kamiya M. Comparative study of *Trichuris trichiura* from nonhuman primates and from man, and their differences with *Trichuris suis*. *J Vet Med Sci*. (1993) 55:363–6. doi: 10.1292/jvms.55.363
7. Cutillas C, Callejón R, De Rojas M, Tewes B, Úbeda JM, Ariza C, et al. *Trichuris suis* and *Trichuris trichiura* are different nematode species. *Acta Trop*. (2009) 111:299–307. doi: 10.1016/j.actatropica.2009.05.011
8. Knight RA. Morphological differences in *Trichuris ovis* associated with different host species. *J Parasitol*. (1984) 70:842–3.
9. Spakulová M. Discriminant analysis as a method for the numerical evaluation of taxonomic characters in male trichurid nematodes. *Syst Parasitol*. (1994) 29:113–9. doi: 10.1007/BF00009807
10. Robles MdR, Navone GT. Redescription of *Trichuris laevitestis* (Nematoda: trichuridae) from *Akodon azarae* and *Scapteromys aquaticus* (Sigmodontinae: Cricetidae) in Buenos Aires province, Argentina. *J Parasitol*. (2006) 92:1053–7. doi: 10.1645/GE-827R.1
11. Robles MdR. New species of *Trichuris* (Nematoda: Trichuridae) from *Akodon montensis* Thomas, 1913, of the Paranaense Forest in Argentina. *J Parasitol*. (2011) 97:319–27. doi: 10.1645/GE-2434.1
12. Liu GH, Gasser RB, Su A, Nejsum P, Peng L, Lin RQ, et al. Clear genetic distinctiveness between human-and pig-derived *Trichuris* based on analysis of mitochondrial datasets. *PLoS Negl Trop Dis*. (2012) 6:e1539. doi: 10.1371/journal.pntd.0001539
13. Liu GH, Zhou W, Nisbet AJ, Xu MJ, Zhou DH, Zhao GH, et al. Characterization of *Trichuris trichiura* from humans and *T. suis* from pigs in China using internal transcribed spacers of nuclear ribosomal DNA. *J Helminthol*. (2014) 88:64–8. doi: 10.1017/S0022149X12000740
14. Nissen S, Aljubury A, Hansen TV, Olsen A, Christensen H, Thamsborg SM, et al. Genetic analysis of *Trichuris suis* and *Trichuris trichiura* recovered from humans and pigs in a sympatric setting in Uganda. *Vet Parasitol*. (2012) 188:68–77. doi: 10.1016/j.vetpar.2012.03.004
15. Nadler S, De Leon G. Integrating molecular and morphological approaches for characterizing parasite cryptic species: implications for parasitology. *Parasitology*. (2011) 138:1688–709. doi: 10.1017/S003118201000168X
16. Hawash MB, Andersen LO, Gasser RB, Stensvold C, Nejsum P. Mitochondrial genome analyses suggest multiple *Trichuris* species in humans, baboons, and pigs from different geographical regions. *PLoS Negl Trop Dis*. (2015) 9:e0004059. doi: 10.1371/journal.pntd.0004059
17. Rivero J, García-Sánchez A, Zurita A, Cutillas C, Callejón R. *Trichuris trichiura* isolated from *Macaca sylvanus*: morphological, biometrical, and molecular study. *BMC Vet Res*. (2020) 16:445. doi: 10.1186/s12917-020-02661-4
18. Wang H, Zhang H, Song L, Zhu L, Chen M, Ren G, et al. Morphological and molecular confirmation of the validity of *Trichuris rhinopiptheroxella* in the endangered golden snub-nosed monkey (*Rhinopithecus roxellana*). *J Helminthol*. (2019) 93:601–7. doi: 10.1017/S0022149X18000500
19. Cutillas C, De Rojas M, Zurita A, Oliveros R, Callejón R. *Trichuris colobae* n. sp. (Nematoda: Trichuridae), a new species of *Trichuris* from *Colobus guereza kikuyensis*. *Parasitol Res*. (2014) 113:2725–32. doi: 10.1007/s00436-014-3933-6
20. Callejón R, Halajian A, Cutillas C. Description of a new species, *Trichuris ursinus* n. sp. (Nematoda: Trichuridae) from *Papio ursinus* Keer, 1792 from South Africa. *Infect Genet Evol*. (2017) 51:182–93. doi: 10.1016/j.meegid.2017.04.002
21. García-Sánchez ÁM, Rivero J, Zurita A, Callejón R, Cutillas C. Differentiation of *Trichuris* species using a morphometric approach. *Int J Parasitol Parasites Wildl*. (2019) 9:218–23. doi: 10.1016/j.ijppaw.2019.05.012
22. Gupta SK, Singla LD. Veterinary diagnostics. In: Gupta RP, Garg SR, Nehra V, Lather D, editors. Chapter 6: *Diagnostic Trends in Parasitic Diseases of Animals*. Delhi: Statish Serial Publishing House (2013). p. 81–112.
23. Horii Y, Usui M. Experimental transmission of *Trichuris ova* from monkeys to man. *Trans R Soc Trop Med Hyg*. (1985) 79:423. doi: 10.1016/0035-9203(85)90403-1
24. Oliveros R, Cutillas C, Aris P, Guevara D. Morphologic, biometric and isoenzyme characterization of *Trichuris suis*. *Parasitol Res*. (1998) 84:513–5. doi: 10.1007/s004360050438
25. Skrjabin KI, Shikhobalova NP, Orlow IV. Essentials of Nematodology, vol. VI. In: Greenberg D, editors. Translated by Birron A. *Trichocephalidae* and *Capillariidae* of animals and the man and the diseases caused by them. Jerusalem: Keter Press Wiener Binder Ltd. (1957).
26. Spakulová M, Lýsek H. A biometric study of two populations of *Trichocephalus suis* Schrank, 1788 from swine and wild boars. *Helminthologia*. (1981) 18:91–8.
27. Suriano DM, Navone GT. Three new species of the genus *Trichuris* Roederer, 1761 (Nematoda: Trichuridae) from Cricetidae and Octodontidae rodents in Argentina. *Res Re Parasitol*. (1994) 54:39–46.
28. Robles MdR, Navone GT, Notarnicola J. A new species of *Trichuris* (Nematoda: Trichuridae) from *Phyllotini rodents* in Argentina. *J Parasitol*. (2006) 92:100–4. doi: 10.1645/GE-GE-552R.1
29. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol*. (2016) 33:1870–4. doi: 10.1093/molbev/msw054
30. Xia X, Xie Z, Salemi M, Chen L, Wang Y. An index of substitution saturation and its application. *Mol Phylogenetics Evol*. (2003) 26:1–7. doi: 10.1016/s1055-7903(02)00326-3
31. Xia X, Lemey P. Assessing substitution saturation with DAMBE. 2nd ed. In: Lemey P, Salemi M, Vandamme AM, editors. *The Phylogenetic Handbook: A Practical Approach to DNA and Protein Phylogeny*. Oxford: Cambridge University Press. (2009). p. 615–30.
32. Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, et al. DnaSP v6: DNA sequence polymorphism analysis of large datasets. *Mol Biol Evol*. (2017) 34:3299–302. doi: 10.1093/molbev/msx248
33. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol*. (2003) 52:696–704. doi: 10.1080/10635150390235520
34. Ronquist F, Huelsenbeck JP. MrBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. (2003) 19:1572–4. doi: 10.1093/bioinformatics/btg180
35. Posada D. jModelTest: phylogenetic model averaging. *Mol Biol Evol*. (2008) 25:1253–6. doi: 10.1093/molbev/msn083
36. Posada D, Buckley TR. Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. *Syst Biol*. (2004) 53:793–808. doi: 10.1080/10635150490522304
37. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*. (1985) 39:783–91. doi: 10.1111/j.1558-5646.1985.tb00420.x
38. Callejón R, Nadler S, De Rojas M, Zurita A, Petrášová J, Cutillas C. Molecular characterization and phylogeny of whipworm nematodes inferred from DNA sequences of *cox1* mtDNA and 18S rDNA. *Parasitol Res*. (2013) 112:3933–49. doi: 10.1007/s00436-013-3584-z
39. Frères V, Neveu-Lemaire M. Traité d'helminthologie médicale et vétérinaire. (1936) S1–16:641–2. doi: 10.4269/ajtmh.1936.s1-16.641
40. Dinnik NN. Meditsinskaya Parazitologiya i Parazitarnye Boleznl, 1. (1938). p. 1907.
41. Zaman V. Scanning electron microscopy of *Trichuris trichiura* (Nematoda). *Acta Trop*. (1984) 41:287–92.
42. Tenora F, Hovorka I, Hejlkova D. A supplement to the scanning electron microscopy of some *Trichocephalus* spp. (Nematoda). *Helminthologia*. (1988) 25:227–34.

43. Tenora F, Stanek M, Spakulová M. On the possibility of morphological differentiation of *Trichocephalus trichiurus* (L., 1771) and *T. suis* (Schrank, 1788) species. In: *Conference Aktuálne otázky humánnej parazitológie*. (1992).
44. Cavallero S, De Liberato C, Friedrich KG, Di Cave D, Masella V, D'Amelio S, et al. Genetic heterogeneity and phylogeny of *Trichuris* spp. from captive non-human primates based on ribosomal DNA sequence data. *Infect Genet Evol*. (2015) 34:450–6. doi: 10.1016/j.meegid.2015.06.009
45. Oliveros R, Cutillas C, de Rojas M, Arias P. Characterazition of four species of *Trichuris* (Nematoda: Enoplida) by their second internal transcribed spacer ribosomal DNA sequence. *Parasitol Res*. (2000) 86:1008–13. doi: 10.1007/pl00008519
46. Cutillas C, Oliveros R, De Rojas M, Guevara DC. Determination of *Trichuris muris* from murid hosts and *Trichuris* arvicolae (Nematoda) from arvicolid rodents by amplification and sequentiation of the ITS1-5.8S-ITS2 segment of the ribosomal DNA. *Parasitol Res*. (2002) 88:574–82. doi: 10.1007/s00436-002-0596-5
47. Cutillas C, Oliveros R, De Rojas M, Guevara DC. Determination of *Trichuris skrjabini* by sequencing of the ITS1-5.8S-ITS2 segment of the ribosomal DNA: comparative molecular study of different species of trichurids. *J Parasitol*. (2004) 90:648–52. doi: 10.1645/GE-3295RN
48. Cutillas C, De Rojas M, Ariza C, Úbeda JM, Guevara DC. Molecular identification of *Trichuris vulpis* and *Trichuris suis* isolated from different hosts. *Parasitol Res*. (2007) 100:383–9. doi: 10.1007/s00436-006-0275-z
49. Callejón R, Gutiérrez-Avilés L, Halajian A, Zurita A, de Rojas M, Cutillas C. Taxonomy and phylogeny of *Trichuris globulosa* Von Linstow, 1901 from camels. A review of *Trichuris* species parasitizing herbivorous. *Infect Genet Evol*. (2015) 34:61–74. doi: 10.1016/j.meegid.2015.06.011
50. Callejón R, Halajian A, De Rojas M, Marrugal A, Guevara DC, Cutillas C. 16S partial gene DNA and internal transcribed spacers ribosomal DNA as differential markers of *Trichuris discolor* populations. *Vet Parasitol*. (2012) 186:350–63. doi: 10.1016/j.vetpar.2011.11.033
51. Salaba O, Rylková K, Vadlejch J, Petrtýl M, Schánková Š, Brožová A, et al. The first determination of *Trichuris* sp. from roe deer by amplification and sequentiation of the ITS1-5.8S-ITS2 segment of ribosomal DNA. *Parasitol Res*. (2013) 112:955–60. doi: 10.1007/s00436-012-3215-0
52. Robles MdR, Cutillas C, Panei CJ, Callejón R. Morphological and molecular characterization of a new *Trichuris* species (Nematoda-Trichuridae), and phylogenetic relationships of *Trichuris* species of cricetid rodents from Argentina. *PLoS ONE*. (2014) 9:e112069. doi: 10.1371/journal.pone.0112069
53. DoleŽalová J, Oborník M, Hajdušková E, Jirku M, PetrŽelková KJ, Bolechová P, et al. How many species of whipworms do we share? Whipworms from man and other primates form two phylogenetic lineages. *Folia Parasitol (Praha)*. (2015) 62:1–12. doi: 10.14411/fp.2015.063
54. Guardone L, Deplazes P, Macchioni F, Magi M, Mathis A. Ribosomal and mitochondrial DNA analysis of Trichuridae nematodes of carnivores and small mammals. *Vet Parasitol*. (2013) 197:364–9. doi: 10.1016/j.vetpar.2013.06.022
55. Cavallero S, Nejsum P, Cutillas C, Callejón R, DoleŽalová J, Modrý D, et al. Insights into the molecular systematics of *Trichuris* infecting captive primates based on mitochondrial DNA analysis. *Vet Parasitol*. (2019) 272:23–30. doi: 10.1016/j.vetpar.2019.06.019
56. Liu GH, Gasser RB, Nejsum P, Wang Y, Chen Q, Song HQ, et al. Mitochondrial and nuclear ribosomal DNA evidence supports the existence of a new *Trichuris* species in the endangered françois' leaf-monkey. *PLoS ONE*. (2013) 8:e66249. doi: 10.1371/journal.pone.0066249
57. Callejón R, Robles MdR, Panei CJ, Cutillas C. Molecular diversification of *Trichuris* spp. from Sigmodontinae (Cricetidae) rodents from Argentina based on mitochondrial DNA sequences. *Parasitol Res*. (2016) 115:2933–45. doi: 10.1007/s00436-016-5045-y
58. Callejón R, Cutillas C, Nadler SA. Nuclear and mitochondrial genes for inferring *Trichuris* phylogeny. *Parasitol Res*. (2015) 114:4591–9. doi: 10.1007/s00436-015-4705-7
59. Schlötterer C, Tautz D. Chromosomal homogeneity of *Drosophila* ribosomal DNA arrays suggests intrachromosomal exchanges drive concerted evolution. *Curr Biol*. (1994) 4:777–83. doi: 10.1016/s0960-9822(00)00175-5
60. Wesson DM, Porter CH, Collins FH. Sequence and secondary structure comparisons of ITS rDNA in mosquitoes (Diptera: Culicidae). *Mol Phylogenet Evol*. (1992) 1:253–69. doi: 10.1016/1055-7903(92)90001-w
61. Campbell AJ, Gasser RB, Chilton NB. Differences in a ribosomal DNA sequence of *Strongylus* species allows identification of single eggs. *Int J Parasitol*. (1995) 25:359–65. doi: 10.1016/0020-7519(94)00116-6
62. Hoste H, Chilton NB, Gasser RB, Beveridge I. Differences in the second internal transcribed spacer (ribosomal DNA) between five species of *Trichostrongylus* (Nematoda: Trichostrongylidae). *Int J Parasitol*. (1995) 25:75–80. doi: 10.1016/0020-7519(94)00085-3
63. Gasser RB, Stewart LE, Speare R. Genetic markers in ribosomal DNA for hookworm identification. *Acta Trop*. (1996) 62:15–21. doi: 10.1016/s0001-706x(96)00015-0
64. Chan AHE, Chaisiri K, Morand S, Saralamba N, Thaenkham U. Evaluation and utility of mitochondrial ribosomal genes for molecular systematics of parasitic nematodes. *Parasit Vectors*. (2020) 13:364. doi: 10.1186/s13071-020-04242-8
65. Xie Y, Zhao B, Hoberg EP, Li M, Zhou X, Gu X, et al. Genetic characterisation and phylogenetic status of whipworms (*Trichuris* spp.) from captive non-human primates in China, determined by nuclear and mitochondrial sequencing. *Parasit Vectors*. (2018) 11:516. doi: 10.1186/s13071-018-3100-5
66. Ghai RR, Simons ND, Chapman CA, Omeja PA, Davies TJ, Ting N, et al. Hidden population structure and cross-species transmission of whipworms (*Trichuris* sp.) in humans and non-human primates in Uganda. *PLoS Negl Trop Dis*. (2014) 8:e3256. doi: 10.1371/journal.pntd.0003256
67. Geerts S, Gryseels B. Anthelmintic resistance in human helminths: a review. *Tropical Med Int Health*. (2001) 6:915–21. doi: 10.1046/j.1365-3156.2001.00774.x
68. World Health Organization. Helminth control in school-age children. A guide for managers of control programmes. Available online at: https://apps.who.int/iris/bitstream/handle/10665/44671/9789241548267_eng.pdf?sequence=1 (2011) (accessed September, 2011).
69. Global Burden of Disease Study 2013 Collaborators. *Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study*. *Lancet*. (2015) 386:743–800. doi: 10.1016/S0140-6736(15)60692-4
70. Arizono N, Yamada M, Tegoshi T, Onishi K. Molecular identification of *Oesophagostomum* and *Trichuris* eggs isolated from wild Japanese macaques. *Korean J Parasitol*. (2012) 50:253–7. doi: 10.3347/kjp.2012.50.3.253
71. Gillespie TR, Lonsdorf EV, Canfield EP, Meyer DJ, Nadler Y, Raphael J, et al. Demographic and ecological effects on patterns of parasitism in eastern chimpanzees (*Pan troglodytes* schweinfurthii) in Gombe National Park, Tanzania. *Am J Phys Anthropol*. (2010) 143:534–44. doi: 10.1002/ajpa.21348
72. Kooriyama T, Hasegawa H, Shimozuru M, Tsubota T, Nishida T, Iwaki T. Parasitology of five primates in Mahale Mountains National Park Tanzania. *Primates*. (2012) 53:365–75. doi: 10.1007/s10329-012-0311-9
73. Levecke B, Dorny P, Vercammen F, Visser LG, Van EM, Vercruysse J, et al. Transmission of *Entamoeba nuttalli* and *Trichuris trichiura* from non-human primates to humans. *Emerg Infect Dis*. (2015) 21:1871–2. doi: 10.3201/eid2110.141456
74. Li M, Zhao B, Li B, Wang Q, Niu L, Deng J, et al. Prevalence of gastrointestinal parasites in captive non-human primates of twenty-four zoological gardens in China. *J Med Primatol*. (2015) 44:168–73. doi: 10.1111/jmp.12170
75. Muriuki SMK, Murugu RK, Munene E, Karere GM, Chai DC. Some gastro-intestinal parasites of zoonotic (public health) importance commonly observed in old world non-human primates in Kenya. *Acta Trop*. (1998) 71:73–82. doi: 10.1016/s0001-706x(98)00040-0
76. Pourrut X, Diffo JLD, Somo RM, Bilong Bilong CF, Delaporte E, LeBreton M, et al. Prevalence of gastrointestinal parasites in primate bushmeat and pets in Cameroon. *Vet Parasitol*. (2011) 175:187–91. doi: 10.1016/j.vetpar.2010.09.023
77. Yao C, Walkush J, Shim D, Cruz K, Ketzis J. Molecular species identification of *Trichuris trichiura* in African green monkey on St. Kitts, West Indies. *Vet Parasitol Reg Stud Reports*. (2018) 11:22–6. doi: 10.1016/j.vprsr.2017.11.004

78. Zanzani SA, Gazzonis AL, Epis S, Manfredi MT. Study of the gastrointestinal parasitic fauna of captive non-human primates (Macaca fascicularis). *Parasitol Res*. (2016) 115:307–12. doi: 10.1007/s00436-015-4748-9
79. Medina M, Walsh PJ. Molecular systematics of the order Anaspidea based on mitochondrial DNA sequence (12S, 16S, and COI). *Mol Phylogenet Evol*. (2000) 15:41–58. doi: 10.1006/mpev.1999.0736
80. Paternina LE, Verbel Vergara D, Bejarano EE. Comparación y utilidad de las regiones mitocondriales de los genes 16S y *COX1* para los análisis genéticos en garrapatas (Acari: Ixodidae). *Biomedica*. (2016) 36:295–302. doi: 10.7705/biomedica.v36i2.3116
81. Chan AHE, Chaisiri K, Dusitsittipon S, Jakkul W, Charoennitiwat V, Komalamisra C, et al. Mitochondrial ribosomal genes as novel genetic markers for discrimination of closely related species in the *Angiostrongylus cantonensis* lineage. *Acta Trop*. (2020) 211:105645. doi: 10.1016/j.actatropica.2020.105645

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REVIEW
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Leishmaniasis Beyond East Africa

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Climate change is having a substantial impact on our environment and ecosystems and has altered the way humans live, access, and utilize resources with increased risk of zoonotic infectious disease encounters. As global temperatures continue to increase, they impact on public health, migration, food security and land conflict, and as new environments become favorable, exposure to disease carrying vectors. Increased forests or natural habitat clearance for land repurposing, urbanization, road building, and water management are related to an increase in emerging vector borne parasitic diseases. The East African region remains one of the most impacted regions globally for leishmaniasis, a vector borne disease that impacts significantly on the health, wellbeing and livelihoods of affected communities and for which a lack of reporting and control interventions hinder progress toward elimination of this neglected tropical disease. As our world continues to transform, both politically and climatically, it is essential that measures are put in place to improve surveillance and disease management with implementation of control measures, including vector control, especially in low- and middle-income countries that are expected to be most impacted by changes in climate. Only through effective management, now, can we be sufficiently resilient to preventing the inevitable spread of vectors into suitable habitat and expansion of the geographical range of leishmaniasis. This review offers a current perspective on Leishmaniasis as an endemic disease in East Africa and examines the potential of the recent emergence of *Leishmania* infection in hitherto unaffected regions to become a public health concern if no disease management is achieved.

Keywords: leishmaniasis, climate, epidemiology, Africa, visceral, cutaneous, vector

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BACKGROUND

Each year, neglected tropical diseases have a substantial impact on upwards of 1 billion people globally, contributing to the damage of economies and impairment of particularly vulnerable communities including those living in extreme poverty (1). The leishmaniases are estimated to affect 0.7–1 million people, with 350 million people at risk, globally (2, 3). Currently, 98 countries are endemic for leishmaniasis (2). Leishmaniasis occurs after the infection of a mammalian host with the obligate, intracellular parasite *Leishmania* (4). *Leishmania* species are categorized into either Old World or New World species, corresponding to the different geographical regions in which they are found (5). Old World (OW) species can be found in Asia, the Middle East, the Mediterranean basin and Africa, whereas New World (NW) species are found in the Americas (5). At least 20 *Leishmania* species are known to cause human disease, of the *Leishmania* genus and

Leishmania or *Viannia* subgenera (6, 7). The subgenera were distinguished in the 1980's based on parasite location and site of attachment within the sand-fly gut (7).

Leishmania parasites can infect over 90 sandfly species; the *Phlebotomus* and *Lutzomyia* species being most implicated in human leishmaniasis transmission (8). The manifestation of leishmanial disease can occur when parasites are transmitted to the mammalian host upon the infected female sandfly taking a blood meal, which provides the protein required for egg development (9). The dimorphic parasites are transmitted to the human host in their motile, metacyclic promastigote form, invade phagocytic cells—predominantly macrophages—and transform into their non-motile amastigote form (4). Within the cell the amastigotes replicate by binary fission until cell rupture and proceed to further invade healthy neighboring phagocytes (4).

Disease outcome is dependent on a number factors, including parasite species, host genetics and the host immune responses (10). Disease severity can range from cases being serologically positive yet asymptomatic, to cases of disfiguring and fatal infection (11). The three disease forms are characterized as cutaneous, mucocutaneous and visceral leishmaniasis (2). Several complications are also associated, such as post-kala-azar dermal leishmaniasis (PKDL), diffuse leishmaniasis, disseminated leishmaniasis and leishmaniasis recidivans (12). Cutaneous leishmaniasis (CL) is considered the mildest and most common form of the disease, and can present as painless nodules, macules, papules or ulcerative lesions on exposed areas of the body (13, 14).

There are more than 20 species that cause cutaneous leishmaniasis, most notably the OW species: *L. major*, *L. tropica*, *L. aethiopica*, and the NW species: *L. amazonesis*, *L. braziliensis*, *L. mexicana*, *L. panamensis*, and *L. guyanensis* (2, 15). Lesions are local to the site of the sandfly bite and can be self-healing, however the appearance and healing time varies between species (16). While cutaneous lesions due to *L. tropica* or *L. major* are likely to self-heal after 12 months, leaving significant scarring, *L. aethopica* lesions have been shown to become plaque-like and hyperkeratotic, taking many years to heal completely (17, 18). Although not fatal, cutaneous leishmaniasis can lead to serious scarring or disfigurement, which can result in significant psychological stress, including depression and anxiety and social stigmatization leading to a poor quality of life (19–21). Presently, there are 59 countries where the cutaneous form is endemic, 10 of which (Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, Iraq, Pakistan, the Syrian Arab Republic and Tunisia) are responsible for 85% of all recorded cases (22).

Mucocutaneous leishmaniasis (MCL) involves the destruction of mucus membranes and cartilage in the nose, mouth, upper respiratory tract and pharynx, often beginning at the lips and nostrils (23). Although the pathology of MCL is poorly understood, it is thought to be, in part, due to a strong host immunological response (23, 24). It has been suggested that parasites may reach mucosa either by haematic or lymphatic spread or are directly injected into the area by the sandfly (23). Symptoms of MCL can arise years after an initial cutaneous lesion has healed, include discharge, congestion, and acute hemorrhage of the nose (25, 26). This can be extremely disfiguring as well as life-threatening, potentially leading to complications such as aspiration pneumonia, starvation, sepsis due to secondary infection, and airway obstruction thereby resulting in asphyxia (27). Of all mucocutaneous cases, 90% are found in Bolivia caused by NW species, however *L. aethiopica* has also demonstrated the ability to cause MCL in Ethiopia, a country in the OW Region (2, 27).

Visceral leishmaniasis (VL) occurs after the systemic spread of *Leishmania* parasites and can be life-threatening (28, 29). VL is characterized by presence of an enlarged liver and spleen, anemia, weight loss, and irregular fevers (2). Patients with VL often die within 2 years of contracting the disease if left untreated, commonly from subsequent infections or severe anemia (30, 31). Although not seen in all VL species it is expected that darkening of the skin, particularly in India, is caused by the cytokine-induced production of adrenocorticotrophic hormone (32). Thus, VL was given the Hindu name kala-azar, meaning "black fever" (33). Two species are known to cause human VL, where *L. donovani* predominantly affects the OW and *L. infantum* (synonym *L. chagasi)* the NW regions (34).

There are 59 countries endemic for VL, although cases fall disproportionately on seven of these, with 90% of all cases found in Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan (22). There are an estimated 20,000–40,000 deaths annually due to VL, however this figure is likely to be higher as it is likely that the vast majority of VL deaths are not recognized as being caused by leishmaniasis or are not reported (35). Leishmaniasis is well-documented in several countries, including India, which carries one of the highest burdens of leishmaniasis globally, followed by Sudan (36). In other areas of the world, the epidemiology of this disease remains poorly understood.

LEISHMANIASIS, AN ENDEMIC PUBLIC HEALTH PROBLEM IN EAST AFRICA

In the East African region, cutaneous and visceral leishmaniasis have numerous strongholds across Ethiopia, Sudan, Uganda and Kenya, Somalia, and Eritrea (37).

ETHIOPIA

Ethiopia is a landlocked country found in the Horn of Africa peninsula (38), endemic for both CL and VL (39). Leishmaniasis has been long established in Ethiopia, with the first scientific reports of CL dating back to 1913 and VL in 1942 (40, 41). It is estimated that Ethiopia accounts for surplus of 30,000–50,000 cases of CL and 3,700–7,400 cases of VL annually (21, 42). In the East African region, Ethiopia has the second largest number of VL cases a year, behind Sudan (43). The incidence rate per 10,000 people in endemic areas is 6.28 for VL and 1.05 for CL (44). Nationally, over 28–30 million people are considered to live in areas that put them at risk of contracting leishmaniasis (45, 46). The major causative species in this region are *L. donovani* for VL (though *L. infantum* has been recorded) and *L. aethiopica* for CL

(*L. major* and *L. tropica* have also less frequently been reported) (35, 47, 48). An official country-wide or state level prevalence for either form is not reported, however a recent systematic review reported a national pooled prevalence of 19% (95% CI = 14–25%) (49).

Currently, there is no active case screening for leishmaniasis, only tracking of active cases, and so the basal pool of cases may be much greater than expected (38, 44). VL has been identified in eight states: the northern states of Tigray, Amhara, Afar, Benshangul-Gumuz and the southern states of Oromia, Gambella, Somali, and the Southern Nations and Nationalities People's Regional State (SNNPR) (49). There are several well-established endemic VL foci, including the Metema and Humera plains in the NW of the country which accounts for around 60% of cases, as well as the Omo plains and the Weyto Valley in the south (50–52). Approximately 20% of VL cases occur in the south western savannah and semi-arid lowlands in the SE (53). VL cases have also been recorded in the surrounding areas of Moyale and the Genale river basins in the south of Oromia, in the Afder and Liben zones in the south eastern Somali state and the Awash Valley in the northern state of Afar (43, 49, 54). Despite VL being predominantly found in the lowlands, there have been outbreaks recorded in the previous unaffected highland area of Libo Kemkem (altitude above 2,000 m) in 2005 and Belessa in 1970, both in Amhara (54, 55). In contrast to VL, the cutaneous form of disease is strongly associated with the highland regions of Ethiopia where altitudes are above 1,400 m (21, 51). It is expected that CL is present nationally, however well-recognized foci include Ochollo of the Rift Valley, Kutaber district in Amhara, Aleku, Sebeta, the Bale and Sidamo highlands of Oromia, and the Adi-grat and Saesie Tsaedaemba districts of Tigray (39, 56–59).

Transmission of *Leishmania* species that have the potential to cause VL or CL can be zoonotic or anthroponotic [49]. Transmission is not fully understood in Ethiopia, although *L. aethiopica*, which can cause CL, is thought to primarily be zoonotic [49, 56]. A reservoir for the CL causative agent *L. aethiopica* has been identified as the rock hyrax, with two species primarily involved: *Heterohyrax brucei* and *Procavia capensis* (60, 61). Transmission of VL caused by *L. donovani* is generally considered to be anthroponotic in the East African region, however, in Ethiopia it is thought to be partially zoonotic and partially anthroponotic, the nature of which varies with geographical areas (62). Animal reservoirs of *Leishmania* species causing VL have not been definitively identified, although dogs are likely candidates, as is the case in the neighboring country of Sudan (54, 63). Other potential reservoirs are squirrels, wild canids, rodents, reptiles and bats (39, 53, 64–66).

SUDAN

To the west of Ethiopia, Sudan is highly endemic for both CL and VL. Although initial reports of VL date back to 1904, it is thought that the *Leishmania* parasite has been present in Sudan for over 4,000 years, with parasite DNA having been detected in the bone marrow of mummies from this time (67, 68). Annually, Sudan accounts for roughly two thirds of all reported VL cases in East Africa (65). Since 2015, around 93% of the population of Sudan is considered at risk of CL and 25% at risk of VL (69), despite having incidence rates that are lower than that for Ethiopia in 2015 (3.25 per 10,000 for VL and 0.94 per 10,000 for CL) likely due to significant underreporting at all levels as a function of poor infrastructure (38) and the lack of ability to diagnose cases locally (38, 65, 69, 70). Additionally, this is mirrored in the number of new cases reported in 2015, with only 2,829 cases of VL and 3,503 of CL having been reported by the WHO (69). There is currently no active screening for the control of leishmaniasis in Sudan, but passive case detection and subsequent treatment (71).

The parasite species responsible for VL in Sudan is predominantly *L. donovani*, however, sporadic detection of *L. archibaldi* and *L. infantum* has occurred in humans and dogs in the State of Gedaref (72). In Sudan, *L. donovani* is transmitted to the human by the sandfly species *P. orientalis*, though the presence of *P. martini* has also been reported (72). Regions endemic for VL are found in the north and east of Sudan, particularly along the border with northern Ethiopia, including the Sudanese states of Gedaref, Senna, Al Qadarif, and Blue Nile (73). Villages close to the Ethiopian border, concentrated along the Atbarah and Rahad rivers are particularly affected by VL (73). In contrast, CL which is caused by *L. major* in this region, can be found in the central and western states of Sudan, including Northern and Southern Dafur, Northern and Southern Kordofan, Khartoum, and El Gezira (74). In Sudan, *L. major* is transmitted by the sandfly species *P. papatasi* (75, 76). A recent study utilizing surveillance data reported that the number of CL cases reported from these states in 2014–2017 increased annually, greatly exceeding the estimates reported by the WHO in 2014 (74). Whilst the aforementioned species causing visceral and cutaneous leishmaniasis in Sudan are considered anthroponotic, mammals including the Egyptian mongoose and dogs have also been investigated as parasite reservoir hosts (63, 77, 78).

KENYA

VL is endemic in the arid and semi-arid regions of Kenya including the Rift Valley and provinces in the east and north east of Kenya caused by *L. donovani* (79, 80). Confirmed VL endemic regions are focussed in the arid lowlands, and include Baringo, Turkana, Marakwet, Smaburu, Pokot, Laikipia and Kajiado, Machakos, Mwingi, Meru, Wajir, and Keiyo (79–81). Baringo County is a well-established focus for both CL and VL (82). The West Pokot focus stretches across the border into the Nakapiripirit district of Karamoja region, Uganda, where the most Ugandan cases are concentrated (83). Since its first report in 1935, there have been several epidemics of VL in Kenya, including in the previously unaffected areas of Wajir and Mandera during 2000–2001 (80). It is expected that there are around 4,000 cases of VL annually (84). Over 5 million people in Kenya are considered at risk of exposure to leishmaniasis, with an incidence rate of 2.96 per 10,000 people for VL, however, incidence rate for CL is not reported (84, 85).

In Kenya, the cutaneous form of the disease is deemed endemic by the WHO, however there are a lack of accurate data describing the true extent of disease or case numbers (85, 86). CL was first documented in 1969 and is caused by: *L. tropica*, predominantly found centrally and in the Rift Valley; *L. major*, reported in the lowlands of Kitui and Baringo (38, 82, 87); and *L. aethiopica,* detected in areas of high altitudes such as Mount Elgon (88). The vectors responsible for transmission in these regions are *Phlebotomus duboscqi*, *Phlebotomus guggisbergi*, and *Phlebotomus pedifer* (89). In some instances, there have been cases where individuals have been positive for both *L. major* and *L. tropica* (80). Transmission of *L. aethiopica, L. major* or *L. tropica* in Kenya is primarily thought to be zoonotic, with rodents, rock hyrax and dog being implicated in the transmission of parasites to humans (82, 90, 91).

UGANDA

In Uganda, the visceral form of disease is endemic and was first reported in 1951 in the north east region of Karamoja (92). Familiarity of VL within the community is evident as it is known vernacularly as "Termes" to the Pokot people of Uganda (93). Several foci of VL are located across the Amarut, Moroto, Kotido, and Nakapiripirit districts of the Karamoja region which lies on the Kenyan border, where residents freely move between Uganda and the West Pokot and Baringo counties of Kenya (93, 94). It is thought that the vector involved in VL transmission to humans in this region is *P. martini*, similarly to Kenya, and the infective parasite species is *L. donovani.* There is currently a lack of data describing any possible reservoir hosts for *L. donovani*, as well as prevalence, incidence rates or risk factors in these endemic areas (93).

SOMALIA

The WHO reports that VL, but not CL, is endemic in Somalia, where incidence is 4.98 per 10,000 of the population in endemic areas (95). The causative species of VL in Somalia is identified as the *L. donovani* complex (96). Despite no definitive data confirming the competent vector, it is suspected that *P. martini* is a likely candidate in endemic areas due to few studies identifying its presence (97). As with most East African countries, VL is likely to be anthroponotic in Somalia, with no definite animal reservoir reported. Somalia has suffered from conflict for many years, which has restricted access to health care and impeded efforts for control and surveillance of infectious diseases, with the only current conceivable method of control being active case treatment (98). Healthcare provided by non-governmental organizations such as Médecins Sans Frontières (MSF), was withdrawn in 2013 due to ongoing violence and attacks on MSF workers rendering up to 1.5 million locals without health care and vulnerable to infectious diseases (99). The availability of epidemiological data for leishmaniasis in Somalia is rare (98). A few reports have documented VL endemic areas in Baidoa, lower Juba and middle Shebelle river, one citing the prevalence of VL seropositivity as high as 23% in one village (98, 100, 101).

ERITREA

In Eritrea, there is a significant lack of data on the epidemiology of leishmaniasis. Few cases have been reported, mainly originating from the administrative regions on the Ethiopian and Sudanese side of the border, with a more recent case being described in the capital, Asmara (38, 102). Upon detection of leishmaniasis in Eritrea, the disease form is not distinguished and most commonly recorded as VL (103). Therefore, the overall extent of both disease forms is not clear (103). Currently, there are no control programmes, data defining vector species or reservoir hosts for *Leishmania* parasites in Eritrea (103).

RISK FACTORS OF DISEASE

Children and young adults are demographically most at risk of developing symptomatic leishmaniasis in endemic areas, potentially due to lack of protective immunity or due to them collecting water or playing in gorges close to sandfly habitat (48, 54, 60). In areas with active outbreaks, all ages are at risk, with risk increasing with exposure to the vector (104).

Males may be more exposed than females, since they are more involved in outdoor agriculture or activity (38). Agriculture workers commonly work at night which coincides with the peak vector activity (53). Seasonal agricultural work sees a large influx of people moving from unaffected areas to endemic areas, such as the Ethiopian Humera plains near the Sudanese border, putting individuals at risk of VL (105). Similarly, infected individuals migrating from VL hotspots back to their homes serve as reservoirs for establishing new disease foci in previously unaffected areas (106).

Incidence in the north of Ethiopia is associated with cracking black cotton soil and Acacia-Balanite trees and termite hills and reddish clay soil in the south—the variation being due to the breeding site preferences of varying sandfly species (64).

Civil unrest, such as that seen in Sudan, can result in forced migration from endemic areas to non-endemic, further spreading the disease (107, 108). From 1983 until 2005, civil war between Sudan and South Sudan resulted in the uprooting and displacement of over 4 million people, which coincided with VL outbreaks and consequently 100,000 deaths over the 22 year period (65, 109, 110). In subsequent years tension and unrest persisted, forcing millions of people to flee, with many immunologically naïve individuals migrating to endemic regions of neighboring countries such as the Omo plains or the Rift Valley (65). From the resumption of unrest in 2013, MSF reported that VL cases doubled in three endemic areas by 2014 (65).

Living near to, resting by or sleeping under Acacia-Balanite trees will increase the likelihood of exposure to the vector and thereby increase risk of disease in Ethiopia, Kenya, Somalia, and the Sudanese-Ethiopian borders (105). Additionally, proximity to dogs, cattle or termite hills increase the risk of exposure in endemic areas such as Libo Kemkem in Ethiopia (105).

Some species of sandfly are expected to be exophagic, and so sleeping outside increases the chance of sandfly bite (42, 111). Those subjected to living in poverty often own poor-quality housing (20). Houses with thatch walls or with no protective

measures in place, such as bed nets or curtains, are more likely to be exposed to the vector (105, 108). With poverty often comes malnutrition, including lack of protein, iron, vitamin A and zinc levels which has been linked to VL development (54, 112). HIV-VL co-endemicity in north-west Ethiopia has resulted in coinfection rates of 20–40%, where the associated immunosuppression aids VL development and HIV progression (113, 114). Other proposed risk factors include implementation of irrigation systems, the development of the sugar industry and forest clearance (45).

CLIMATE CHANGE AND LEISHMANIASIS

Increased global temperatures have resulted in increased flooding, droughts, land fires, and other destructive natural disasters. Since the 1880s, the temperature has been steadily rising in increments of 0.07°C, up until the past four decades, where this increased to 0.18°C (115). It is expected that climate warming will continue throughout the 21st century, with higher latitudes being more heavily impacted (116). Global warming has had a substantial impact on our environment and ecosystems and has altered the way humans live and move around. These two factors combined mean that human and infectious disease encounters will become more frequent and new environments will become more favorable to disease carrying vectors. Increased forest area clearance for land repurposing, urbanization, the building of roads, and water management structures are mirrored by an increase in emerging parasitic diseases (117).

The arthropod vectors responsible for transmitting the parasite, sandflies, are greatly impacted by changes to their ecology, resulting in changes in vector numbers, species present within an area, the mix of species within an area, and vector behaviors including, feeding, resting, or activity periods (117). In the OW, there are 31 proven sandfly species with the ability to transmit *Leishmania* parasites (6). Knowledge of vector behavior is a crucial part of understanding disease transmission and allows for the development of vector control strategies (118). Sandflies are commonly found in warm countries with tropical or subtropical climates, notably Australia, the Americas, Asia, Africa, and the south of Europe, between 50°N and 40°S (119). Sandflies are considered thermophilic and so necessitate an environment where the average temperature is consistently high, as temperatures below 15°C will reduce survival rates in several species (120, 121).

Parasite development within the sandfly is optimum at temperatures around 25°C, where higher temperatures decrease incubation time and permit faster transmission through increased reproduction in the midgut and movement through the sandfly (122). Heavy precipitation brought about by increased global temperatures will decrease flight ability, availability of landing surfaces, and can be fatal to larvae, however, some moisture within the soil of the sandfly environment is still required for survival. Despite this, as global warming intensifies, soil is likely to lack the required moisture due to fast evaporation rates as a result of long periods of sustained high temperatures and drought.

LEISHMANIASIS, AN EMERGING PUBLIC HEALTH PROBLEM

In the WHO European region, CL is endemic in Israel, Turkey, Uzbekistan, and Turkmenistan, whereas VL is much more widespread in the south-west of Europe, the Balkans, Turkey, Caucasus, and central Asia; with over 70% of VL cases in Italy, Spain, Albania, and Georgia (123). The parasites found in this region are *L. infantum*, *L. tropica*, and *L. major*, where CL is caused by all three species and VL by solely by *L. infantum* (123). The northern limit of sandfly distribution in Europe previously covered the south Mediterranean region and south eastern Europe (124). In recent years, there have been increases in the number of sporadic cases of leishmaniasis (cases identified by PCR as *L. infantum* or *L. donovani*/*L. infantum*) being reported from countries in central Europe including Germany, Austria, and in southern England (124–126). In southern European countries, there are several established sandfly species that are known to have the ability to host human-infective *Leishmania* parasites, including *P. sergenti*, *P. papatasi*, *P. alexandri*, *P. tobbi*, *P. perniciosus*, *P. ariasi*, *P. perfiliewi*, and *P. neglectus* (120). A rise in temperature by 1°C is suitable for survival of Phlebotomine sandflies in parts of Austria, such as the Slovenian border region, in which foci of leishmaniasis are likely to develop supported by canine reservoirs (124). Additionally, *L. infantum* DNA was detected from a caught sandfly (*P. mascittii*) in Austria (127). By 2040 more areas in the west and northwest of Germany are expected to become moderately or highly suitable for sandfly species survival (128). Sandflies were considered absent in Germany prior to 1999 but have now been found in Baden-Württemberg and were identified at 37 different sites in the Rhine Valley, Southwestern Germany (129, 130). Although the predominant species identified in this area is *P.mascittii*, in 2001 *P. perniciosus* sandflies were reported for the first time which have shown the ability to host *L. infantum* parasites (130). A recent ecological niche modeling study reported that the future climatic conditions could provide a suitable environment for sandfly species as far north as the UK and Scandinavia (*P. papatasi*, *P. tobbi*, *P. perniciosus*, *P.ariasi*, *P. perfiliewi*, *P. mascittii*, and *P. neglectus*) (120). This is supported by another bioclimatic envelope modelling study which found that the central European climate will become more suitable for sandfly species currently found in the south-west of Europe, however dispersal of sandflies may also be inhibited by geographical barriers such as the Alps and sandfly flight ability (131). Around 100,000 dogs in Germany are estimated to be carrying *Leishmania* parasites (*L. infantum*) which could, given that there are competent vectors, act as reservoirs across the country (130). In addition to this, studies have revealed high numbers of asymptomatic *L.infantum* carriers in endemic regions, which suggests that humans could also act as reservoirs in new European foci (132).

The World Health Assembly resolution WHA 60.13 and WHO expert committee brought attention to the necessity leishmaniasis for epidemiology research, particularly in the European region, so that efficient policies, guidelines, and control strategies could be implemented to prevent further disease dissemination (123, 133). The WHO aim to prevent

further spread of leishmaniasis by introducing surveillance, encouraging research on diagnostics and therapeutics, improving communication between collaborators and having epidemic management plans in place (123). Given the changes that are expected with climate change, it is fathomable that leishmaniasis could become a public health concern not only in the south of Europe, but central and north also. Suitable conditions for sandfly survival incorporated with factors such as increase in global travel and importation of reservoirs, such as dogs, from endemic countries where the disease is not efficiently managed could contribute to leishmaniasis outbreaks in these previously non-endemic countries. Similarly, in the Americas, with leishmaniasis already detected in Texas and Ohio, it has been predicted that the number of people exposed to leishmaniasis in North America will double by 2080 as leishmaniasis moves further north into the Americas, affecting east central North America and potentially southern Canada (134, 135). Ecological niche modeling of the impact climate change may have on both suspected vectors, *Lutzomyia diabolica* and *Lutzomyia anthophora*, and the woodrat reservoirs indicated their expansion of their habitats in North America, providing a wider environment suitable for *Leishmania mexicana* parasites (134). Although generally considered an imported disease in the US, either from travelers, the military or migrants, a study conducted in Texas demonstrated that 59% of identified leishmaniasis cases were autochthonous (32% of these cases were identified as *L. mexicana,* the other cases did not provide species information) (136). The underlying circulation of *Leishmania* parasites in animal reservoirs in this area may provide a starting point for expansion across North America as climate change ensues and vector and reservoir habitats expand. Despite this, in the US there is a possibility that the sandfly expansion can also be hindered, due to factors such as unsuitable landscape, species competition, and also geographical barriers (134).

CONCLUSION

Leishmaniasis remains a severe public health threat, particularly for those afflicted by poverty, war, living in conditions of poor nutrition and with impaired health systems. The East African region remains one of the most impacted regions globally and a lack of reporting with regards to the prevalence, distribution, social/risk factors, and transmission of leishmaniasis continues to be a constraint to disease management in most afflicted countries. As our world continues to transform, both politically and climatically, it is essential that we understand the current epidemiology of leishmaniasis in endemic regions, that we improve surveillance and disease management and implement vector control where appropriate, especially in the low- and middle-income countries that are expected to be most impacted by changes in climate. Sporadic cases and *Leishmania* parasites have already been identified in canines in non-endemic central European countries, as well as southern states of the US. Regions of the world previously considered unaffected by leishmaniasis are predicted to become suitable to vector and reservoir of *Leishmania* species. Without a proper understanding of the disease in the endemic setting, we lack the ability to plan and efficiently implement disease control. For the leishmaniases, as for other neglected zoonoses, intervention costs can seem high when compared to the public health benefits alone. However, these costs are easily outweighed when a full cross-sector analysis is carried out and the monetary and non-monetary benefits to all stakeholders are taken into account. Only through effective management now, can we be sufficiently resilient to prevent the inevitable spread of infection with increased suitable vector habitat and prevention of expansion of the geographical range for leishmaniasis.

AUTHOR CONTRIBUTIONS

CJ and SW were both involved in conception, design, and writing of this perspective. Both authors read and approved the final version of the manuscript.

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REFERENCES

1. WHO | World Health Organization. *Neglected Trop Diseases Q & A Detail*. (2012). Available online at: https://www.who.int/news-room/q-a-detail/neglected-tropical-diseases (accessed April 2, 2020).
2. WHO. *Leishmaniasis Fact Sheet*. World Health Organ (2020). Available online at: https://www.who.int/news-room/fact-sheets/detail/leishmaniasis (accessed April 4, 2020).
3. Arenas R, Torres-Guerrero E, Quintanilla-Cedillo M.R, Ruiz-Esmenjaud J. Leishmaniasis: a review. *F1000Research.* (2017) 6:750. doi: 10.12688/f1000research.11120.1
4. Walker DM, Oghumu S, Gupta G, McGwire BS, Drew ME, Satoskar AR. Mechanisms of cellular invasion by intracellular parasites. *Cell Mol Life Sci.* (2014) 71:1245–63. doi: 10.1007/s00018-013-1491-1
5. Kevric I, Cappel MA, Keeling JH. New world and old world leishmania infections. *Dermatol Clin.* (2015) 33:579–93. doi: 10.1016/j.det.2015.03.018
6. Akhoundi M, Kuhls K, Cannet A, Votypka J, Marty P, Delaunay P, et al. A historical overview of the classification, evolution, and dispersion of Leishmania parasites and sandflies. *PLoS Negl Trop Dis.* (2016) 10:e0004349. doi: 10.1371/journal.pntd.0004349
7. Lainson R, Ryan L, Shaw JJ. Infective stages of Leishmania in the sandfly vector and some observations on the mechanism of transmission. *Mem Inst Oswaldo Cruz.* (1987) 82:421–4. doi: 10.1590/S0074-02761987000300015
8. Bogitsh BJ, Carter CE, Oeltmann TN. Chapter 18: Arthropods as vectors. In: Bogitsh BJ, Carter CE, Oeltmann TN, editors. *Human Parasitology, 5th edn.* London: Academic Press (2019). p. 331–60.
9. Aklilu E, Abbasi I, Gebresilassie A, Yared S, Kindu M, Kirstein OD, et al. Some aspects of entomological determinants of *Phlebotomus orientalis* in

highland and lowland visceral leishmaniasis foci in northwestern Ethiopia. *PLoS One.* (2018) 13:e0192844. doi: 10.1371/journal.pone.0192844
10. Colmenares M, Kar S, Goldsmith-Pestana K, McMahon-Pratt D. Mechanisms of pathogenesis: differences amongst Leishmania species. *Trans R Soc Trop Med Hyg.* (2002) 96(Suppl 1):S3–7. doi: 10.1016/S0035-9203(02)90044-1
11. Singh OP, Hasker E, Sacks D, Boelaert M, Sundar S. Asymptomatic leishmania infection: a new challenge for leishmania control. *Clin Infect Dis Off Publ Infect Dis Soc Am.* (2014) 58:1424–9. doi: 10.1093/cid/ciu102
12. Burza S, Croft SL, Boelaert M. Leishmaniasis. *Lancet Lond Engl.* (2018) 392:951–70. doi: 10.1016/S0140-6736(18)31204-2
13. Bilgic-Temel A, Murrell DF, Uzun S. Cutaneous leishmaniasis: a neglected disfiguring disease for women. *Int J Womens Dermatol.* (2019) 5:158–65. doi: 10.1016/j.ijwd.2019.01.002
14. Aronson NE, Magill AJ. 104 – Leishmaniasis. In: Ryan ET, Hill DR, Solomon T, Aronson NE, Endy TP, editors. *Hunter's Tropical Medicine Emerging Infectious Diseases, 10th edn.* London: Content Repository Only (2020). p. 776–98.
15. Mans DR, Kent AD, VPF Hu R, Schallig HD. Epidemiological, biological and clinical aspects of leishmaniasis with special emphasis on busi yasi in suriname. *J Clin Exp Dermatol Res.* (2017) 08:2. doi: 10.4172/2155-9554.1000388
16. Juckett G. Chapter 33: Leishmaniasis. In: Walker PF, Barnett ED, editors. *Immigrant Medicine.* Edinburgh: W.B. Saunders (2007). p. 447–54.
17. Prieto-Granada CN, Lobo AZC, Mihm MC. Chapter 19: Skin infections. In: Kradin RL, editor. *Diagnostic Pathology of Infectious Disease.* New York, NY: W.B. Saunders (2010). p. 519–616.
18. Ryan ET, Hill DR, Solomon T, Endy TP, Aronson N. *Hunter's Tropical Medicine and Emerging Infectious Diseases E-Book.* Philadelphia, PA: Elsevier Health Sciences (2019).
19. Bennis I, Thys S, Filali H, De Brouwere V, Sahibi H, Boelaert M. Psychosocial impact of scars due to cutaneous leishmaniasis on high school students in Errachidia province, Morocco. *Infect Dis Poverty.* (2017) 6:46. doi: 10.1186/s40249-017-0267-5
20. Okwor I, Uzonna J. Social and economic burden of human leishmaniasis. *Am J Trop Med Hyg.* (2016) 94:489–93. doi: 10.4269/ajtmh.15-0408
21. Tamiru HF, Mashalla YJ, Mohammed R, Tshweneagae GT. Cutaneous leishmaniasis a neglected tropical disease: community knowledge, attitude and practices in an endemic area, Northwest Ethiopia. *BMC Infect Dis.* (2019) 19:855. doi: 10.1186/s12879-019-4506-1
22. WHO. *Global Health Observatory Data Leishmaniasis*. WHO. Available online at: http://www.who.int/gho/neglected_diseases/leishmaniasis/en/ (accessed April 2, 2020).
23. Strazzulla A, Cocuzza S, Pinzone MR, Postorino MC, Cosentino S, Serra A, et al. Mucosal leishmaniasis: an underestimated presentation of a neglected disease. *BioMed Res Int.* (2013) 2013:805108. doi: 10.1155/2013/805108
24. Scott P, Novais FO. Cutaneous leishmaniasis: immune responses in protection and pathogenesis. *Nat Rev Immunol.* (2016) 16:581–92. doi: 10.1038/nri.2016.72
25. Piscopo TV, Mallia Azzopardi C. Leishmaniasis. *Postgrad Med J.* (2007) 83:649–57. doi: 10.1136/pgmj.2006.047340corr1
26. Berkowitz FE. 267 - Leishmania Species (Leishmaniasis). In: Long SS, editor. *Principles Practice of Pediatric Infectious Diseases, 4th edn.* London: Content Repository Only (2012). p. 1285–91.e2.
27. van Griensven J, Gadisa E, Aseffa A, Hailu A, Beshah A.M, Diro E. Treatment of cutaneous leishmaniasis caused by leishmania aethiopica: a systematic review. *PLoS Negl Trop Dis.* (2016) 10:e0004495. doi: 10.1371/journal.pntd.0004495
28. Kumar R, Nylén S. Immunobiology of visceral leishmaniasis. *Front Immunol.* (2012) 3:251. doi: 10.3389/fimmu.2012.00251
29. Martins-Melo FR, Lima M da S, Ramos AN, Alencar CH, Heukelbach J. Mortality and case fatality due to visceral leishmaniasis in Brazil: a nationwide analysis of epidemiology, trends and spatial patterns. *PLoS One.* (2014) 9:e0093770. doi: 10.1371/journal.pone.0093770
30. Lewis MD, Paun A, Romano A, Langston H, Langner CA, Moore IN, et al. Fatal progression of experimental visceral leishmaniasis is associated with intestinal parasitism and secondary infection by commensal bacteria, and is delayed by antibiotic prophylaxis. *PLOS Pathog.* (2020) 16:e1008456. doi: 10.1371/journal.ppat.1008456
31. Goto Y, Cheng J, Omachi S, Morimoto A. Prevalence, severity, and pathogeneses of anemia in visceral leishmaniasis. *Parasitol Res.* (2017) 116:457–64. doi: 10.1007/s00436-016-5313-x
32. Mundkur S, Shashidhara S, Hebbar S, Kanaparthi S. Case 3: Hepatosplenomegaly with Hyperpigmentation in a 6-year-old Girl. *Pediatr Rev.* (2019) 40:145–7. doi: 10.1542/pir.2017-0040
33. Bleeker-Rovers CP, De Vries HJC. 13 - Dermatologic manifestations of systemic infections. In: Cohen J, Powderly WG, Opal SM, editors. *Infectious Diseases, 4th edn.* Saint Louis: Elsevier (2017). p. 113–21.e1.
34. Sundar S. Visceral leishmaniasis. *Trop Parasitol.* (2015) 5:83–5. doi: 10.4103/2229-5070.162487
35. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One.* (2012) 7:e35671. doi: 10.1371/journal.pone.0035671
36. Barley K, Mubayi A, Safan M, Castillo-Chavez C. A comparative assessment of visceral leishmaniasis burden in two eco-epidemiologically different countries, India and Sudan. *bioRxiv 592220*. (2019). doi: 10.1101/592220
37. Reithinger R, Brooker S, Kolaczinski JH. Visceral leishmaniasis in eastern Africa - current status. *Trans R Soc Trop Med Hyg.* (2007) 101:1169–70. doi: 10.1016/j.trstmh.2007.06.001
38. Malaria Consortium C. *Leishmaniasis Control in Eastern Africa: Past and Present Efforts and Fututre Needs*. Situation Gap Analysis (2010). Available online at: https://www.malariaconsortium.org/userfiles/file/NTD%20Resources/VL%20EA%20Situation%20Analysis%20Fina_Janl.pdf (accessed May 3, 2020).
39. Kassahun A, Sadlova J, Benda P, Kostalova T, Warburg A, Hailu A, et al. Natural infection of bats with Leishmania in Ethiopia. *Acta Trop.* (2015) 150:166–70. doi: 10.1016/j.actatropica.2015.07.024
40. Lemma A, Foster WA, Gemetchu T, Preston PM, Bryceson A, Minter DM. Studies on leishmaniasis in Ethiopia. I. Preliminary investigations into the epidemiology of cutaneous leishmaniasis in the highlands. *Ann Trop Med Parasitol.* (1969) 63:455–72. doi: 10.1080/00034983.1969.11686649
41. Cole ACE, Cosgrove PC, Robinson G. A preliminary report of an outbreak of kala-azar in a battalion of king's African rifles. *Trans R Soc Trop Med Hyg.* (1942) 36:25–34. doi: 10.1016/S0035-9203(42)90053-1
42. Aschale Y, Ayehu A, Worku L, Tesfa H, Birhanie M, Lemma W. Malaria-visceral leishmaniasis co-infection and associated factors among migrant laborers in West Armachiho district, North West Ethiopia: Community based cross-sectional study. *BMC Infect Dis.* (2019) 19:239. doi: 10.1186/s12879-019-3865-y
43. Hailu A, Gramiccia M, Kager PA. Visceral leishmaniasis in Aba-Roba, south-western Ethiopia: prevalence and incidence of active and subclinical infections. *Ann Trop Med Parasitol.* (2009) 103:659–70. doi: 10.1179/000349809X12554106963555
44. The World Health Organisation. *WHO - Leishmaniasis- Ethiopia-Country File 2015*. (2017). Available online at: https://www.who.int/leishmaniasis/burden/Ethiopia_2015-hl.pdf?ua=1 (accessed May 3, 2020).
45. Gebremichael D. Zoonotic impact and epidemiological changes of leishmaniasis in Ethiopia. *Open Vet J.* (2018) 8:432–40. doi: 10.4314/ovj.v8i4.13
46. Seid A, Gadisa E, Tsegaw T, Abera A, Teshome A, Mulugeta A, et al. Risk map for cutaneous leishmaniasis in Ethiopia based on environmental factors as revealed by geographical information systems and statistics. *Geospatial Health.* (2014) 8:377–87. doi: 10.4081/gh.2014.27
47. Hailu A, Di Muccio T, Abebe T, Hunegnaw M, Kager PA, Gramiccia M. Isolation of Leishmania tropica from an Ethiopian cutaneous leishmaniasis patient. *Trans R Soc Trop Med Hyg.* (2006) 100:53–8. doi: 10.1016/j.trstmh.2005.04.017
48. van Henten S, Adriaensen W, Fikre H, Akuffo H, Diro E, Hailu A, et al. Cutaneous leishmaniasis due to *Leishmania Aethiopica*. *EClinicalMedicine.* (2018) 6:69–81. doi: 10.1016/j.eclinm.2018.12.009
49. Assefa A. Leishmaniasis in Ethiopia: a systematic review and meta-analysis of prevalence in animals and humans. *Heliyon.* (2018) 4:e00723. doi: 10.1016/j.heliyon.2018.e00723
50. Bekele F, Belay T, Zeynudin A, Hailu A. Visceral leishmaniasis in selected communities of Hamar and Banna-Tsamai districts in Lower Omo Valley,

South West Ethiopia: Sero-epidemological and Leishmanin Skin Test Surveys. *PLoS One.* (2018) 13:e197430. doi: 10.1371/journal.pone.0197430
51. Gebremichael Tedla D, Bariagabr FH, Abreha HH. Incidence and trends of leishmaniasis and its risk factors in Humera, Western Tigray. *J Parasitol Res.* (2018) 2018:8463097. doi: 10.1155/2018/8463097
52. Dawit G, Shishay K. Epidemiology, public health impact and control methods of the most neglected parasite diseases in Ethiopia: a review. *World J Med Sci.* (2014) 10:94–102. doi: 10.5829/idosi.wjms.2014.10.2.81231
53. Gadisa E, Tsegaw T, Abera A, Elnaiem D.-E, Den Boer M, Aseffa A, et al. Eco-epidemiology of visceral leishmaniasis in Ethiopia. *Parasit Vectors.* (2015) 8:381. doi: 10.1186/s13071-015-0987-y
54. Leta S, Dao T.H.T, Mesele F, Alemayehu G. Visceral leishmaniasis in Ethiopia: an evolving disease. *PLoS Negl Trop Dis.* (2014) 8:e0003131. doi: 10.1371/journal.pntd.0003131
55. Alvar J, Bashaye S, Argaw D, Cruz I, Aparicio P, Kassa A, et al. Kala-azar outbreak in Libo Kemkem, Ethiopia: epidemiologic and parasitologic assessment. *Am J Trop Med Hyg.* (2007) 77:275–82. doi: 10.4269/ajtmh.2007.77.275
56. Bsrat A, Berhe N, Balkew M, Yohannes M, Teklu T, Gadisa E, et al. Epidemiological study of cutaneous leishmaniasis in Saesie Tsaeda-emba district, eastern Tigray, northern Ethiopia. *Parasit Vectors.* (2015) 8:149. doi: 10.1186/s13071-015-0758-9
57. Bugssa G. The current status of cutaneous leishmaniasis and the pattern of lesions in Ochollo Primary School Students, Ochollo, Southwestern Ethiopia. *Sci J Clin Med.* (2014) 3:111. doi: 10.11648/j.sjcm.20140306.13
58. G D. A review on biology, epidemiology and public health significance of leishmaniasis. *J Bacteriol Parasitol.* (2013) 04:2. doi: 10.4172/2155-9597.1000166
59. Pareyn M, Van Den Bosch E, Girma N, Van Houtte N, Van Dongen S, Van Der Auwera G, et al. Ecology and seasonality of sandflies and potential reservoirs of cutaneous leishmaniasis in Ochollo, a hotspot in southern Ethiopia. *PLoS Negl Trop Dis.* (2019) 13:e0007667. doi: 10.1371/journal.pntd.0007667
60. Ashford RW, Bray MA, Hutchinson MP, Bray RS. The epidemiology of cutaneous leishmaniasis in Ethiopia. *Trans R Soc Trop Med Hyg.* (1973) 67:568–601. doi: 10.1016/0035-9203(73)90088-6
61. Lemma W, Erenso G, Gadisa E, Balkew M, Gebre-Michael T, Hailu A. A zoonotic focus of cutaneous leishmaniasis in Addis Ababa, Ethiopia. *Parasit Vectors.* (2009) 2:60. doi: 10.1186/1756-3305-2-60
62. Bashaye S, Nombela N, Argaw D, Mulugeta A, Herrero M, Nieto J, et al. Risk factors for visceral leishmaniasis in a new epidemic site in Amhara region, Ethiopia. *Am J Trop Med Hyg.* (2009) 81:34–39. doi: 10.4269/ajtmh.2009.81.34
63. Hassan MM, Osman OF, El-Raba'a FMA, Schallig HDFH, Elnaiem DEA. Role of the domestic dog as a reservoir host of *Leishmania donovani* in eastern Sudan. *Parasit Vectors.* (2009) 2:26. doi: 10.1186/1756-3305-2-26
64. Gebresilassie A, Yared S, Aklilu E. Sandfly fauna and ecological analysis of *Phlebotomus orientalis* and *Phlebotomus martini* in the lowland foci of visceral leishmaniasis in Somali Regional State, southeast Ethiopia. *Asian Pac J Trop Med.* (2020) 13:31–7. doi: 10.4103/1995-7645.273572
65. Al-Salem W, Herricks JR, Hotez PJ. A review of visceral leishmaniasis during the conflict in South Sudan and the consequences for East African countries. *Parasit Vectors.* (2016) 9:460. doi: 10.1186/s13071-016-1743-7
66. Abebe A, Evans DA, Gemetchu T. The isolation of Leishmania aethiopica from the ground squirrel *Xerus rutilus*. *Trans R Soc Trop Med Hyg.* (1990) 84:691. doi: 10.1016/0035-9203(90)90147-7
67. Zink AR, Spigelman M, Schraut B, Greenblatt CL, Nerlich AG, Donoghue HD. Leishmaniasis in ancient Egypt and upper Nubia. *Emerg Infect Dis.* (2006) 12:1616–7. doi: 10.3201/eid1210.060169
68. Osman OF, Kager PA, Oskam L. Leishmaniasis in the Sudan: a literature review with emphasis on clinical aspects. *Trop Med Int Health.* (2000) 5:553–62. doi: 10.1046/j.1365-3156.2000.00598.x
69. The World Health Organisation. *WHO Leishmaniasis Country File - Sudan 2015*. (2015) Available online at: https://www.who.int/leishmaniasis/burden/Sudan_2015_updated.pdf?ua=1 (accessed October 13, 2020).
70. Charani E, Cunnington AJ, Yousif AHA, Ahmed MS, Ahmed AEM, Babiker S, et al. In transition: current health challenges and priorities in Sudan. *BMJ Glob Health.* (2019) 4:e001723. doi: 10.1136/bmjgh-2019-001723
71. Meheus F, Abuzaid AA, Baltussen R, Younis BM, Balasegaram M, Khalil EAG, et al. The economic burden of visceral leishmaniasis in Sudan: an assessment of provider and household costs. *Am J Trop Med Hyg.* (2013) 89:1146–53. doi: 10.4269/ajtmh.12-0585
72. Federal Ministry of Health. *Manual for the Diagnostics and Treatment of Leishmaniasis*. Republic of Sudan (2014).
73. Thomson MC, Bendall A, Osman ME, Schorscher J, Connor SJ, Obsomer V, et al. RISK MAPPING OF VISCERAL LEISHMANIASIS: THE ROLE OF LOCAL VARIATION IN RAINFALL AND ALTITUDE ON THE PRESENCE AND INCIDENCE OF KALA-AZAR IN EASTERN SUDAN. *Am J Trop Med Hyg.* (2003) 68:10–7. doi: 10.4269/ajtmh.2003.68.1.0680010
74. Collis S, El-Safi S, Atia AA, Tapan Bhattacharyya, Hammad A, den Boer M, et al. Epidemiological and molecular investigation of resurgent cutaneous leishmaniasis in Sudan. *Int J Infect Dis.* (2019) 88:14–20. doi: 10.1016/j.ijid.2019.08.018
75. Khalid NM, Aboud MA, Alrabba FM, Elnaiem DEA, Tripet F. Evidence for genetic differentiation at the microgeographic scale in *Phlebotomus papatasi* populations from Sudan. *Parasit Vectors.* (2012) 5:249. doi: 10.1186/1756-3305-5-249
76. Elaagip A, Ahmed A, Wilson MD, Boakye DA, Hamid MMA. Studies of host preferences of wild-caught *Phlebotomus orientalis* and Ph. papatasi vectors of leishmaniasis in Sudan. *PLoS One.* (2020) 15:e0236253. doi: 10.1371/journal.pone.0236253
77. Dereure J, El-Safi SH, Bucheton B, Boni M, Kheir MM, Davoust B, et al. Visceral leishmaniasis in eastern Sudan: parasite identification in humans and dogs; host-parasite relationships. *Microbes Infect.* (2003) 5:1103–8. doi: 10.1016/j.micinf.2003.07.003
78. Elnaiem DA, Hassan MM, Maingon R, Nureldin GH, Mekawi AM, Miles M, et al. The Egyptian mongoose, *Herpestes ichneumon*, is a possible reservoir host of visceral leishmaniasis in eastern Sudan. *Parasitology.* (2001) 122:531–6. doi: 10.1017/S0031182001007594
79. Okindo EG, Kutima HL, Mutai J, Kasili S. Attitude and practices of household heads towards leishmaniases infections in Marigat sub-county, Baringo County, Kenya. *East Afr Med J.* (2017) 94:86–94.
80. Ngure PK, Kimutai A, Ng'ang'a ZW, Rukunga G, Tonui WK. A review of Leishmaniasis in Eastern Africa. *J Nanjing Med Univ.* (2009) 23:79–86. doi: 10.1016/S1007-4376(09)60032-0
81. Kolaczinski J.H, Reithinger R, Worku D.T, Ocheng A, Kasimiro J, Kabatereine N, et al. Risk factors of visceral leishmaniasis in East Africa: a case-control study in Pokot territory of Kenya and Uganda. *Int J Epidemiol.* (2008) 37:344–52. doi: 10.1093/ije/dym275
82. Tonui WK. Situational analysis of leishmaniases research in Kenya. *Afr J Health Sci.* (2006) 13:7–21. doi: 10.4314/ajhs.v13i1.30812
83. Mutero CM, Mutinga MJ, Ngindu AM, Kenya PR, Amimo FA. Visceral leishmaniasis and malaria prevalence in West Pokot District, Kenya. *East Afr Med J.* (1992) 69:3–8. PMID:1628545.
84. Kanyina EW. Characterization of visceral leishmaniasis outbreak, Marsabit County, Kenya, 2014. *BMC Public Health.* (2020) 20:446. doi: 10.1186/s12889-020-08532-9
85. WHO. *Leishmaniasis Country Profile - Kenya 2015*. (2015). Available online at: https://www.who.int/leishmaniasis/burden/Kenya_2015-hl.pdf?ua=1 (accessed October 10, 2020).
86. Ngere I, Gufu Boru W, Isack A, Muiruri J, Obonyo M, Matendechero S, et al. Burden and risk factors of cutaneous leishmaniasis in a peri-urban settlement in Kenya, 2016. *PLoS One.* (2020) 15:e0227697. doi: 10.1371/journal.pone.0227697
87. Odiwuor S, Muia A, Magiri C, Maes I, Kirigi G, Dujardin JC, et al. Identification of Leishmania tropica from micro-foci of cutaneous leishmaniasis in the Kenyan Rift Valley. *Pathog Glob Health.* (2012) 106:159–65. doi: 10.1179/2047773212Y.0000000015
88. Sang DK, Okelo GBA, Chance ML. Cutaneous leishmaniasis due to *Leishmania aethiopica*, on Mount Elgon, Kenya. *Ann Trop Med Parasitol.* (1993) 87:349–57. doi: 10.1080/00034983.1993.11812778
89. Owino BO, Matoke-Muhia D, Alraey Y, Mwangi JM, Ingonga JM, Ngumbi PM, et al. Association of *Phlebotomus guggisbergi* with Leishmania major and Leishmania tropica in a complex transmission setting for cutaneous leishmaniasis in Gilgil, Nakuru county, Kenya. *PLoS Negl Trop Dis.* (2019) 13:e0007712. doi: 10.1371/journal.pntd.0007712

90. Mutinga MJ. The animal reservoir of cutaneous leishmaniasis on Mount Elgon, Kenya. *East Afr Med J.* (1975) 52:142–51.
91. The World Health Organisation. *Leishmaniasis Kenya WHO Resources.* Available online at: https://www.who.int/leishmaniasis/resources/KENYA.pdf?ua=1 (accessed December 12, 2020).
92. Mckinnon JA. Kala-azar in the Upper Rift Valley of Kenya. Part I. Background and discovery of the disease. *J Trop Med Hyg.* (1962) 65:51–63.
93. Ministry of Health Uganda. *Guidlines for the Diagnosis, Treatment and Prevention of Visceral Leishmaniasis in Uganda*. Ministry of Health Uganda (2019). Available online at: https://www.who.int/leishmaniasis/burden/MOH_Uganda_Guidelines_diagnosis_treatment_prevention_VL.pdf?ua=1 (accessed December 12, 2020).
94. Olobo-Okao J, Sagaki P. Leishmaniasis in Uganda: historical account and a review of the literature. *Pan Afr Med J.* (2014) 18:16. doi: 10.11604%2Fpamj.2014.18.16.1661
95. The World Health Organisation. *WHO Leishmaniasis Country Profile - Somalia 2015*. (2015) Available online at: https://www.who.int/leishmaniasis/burden/Somalia_2015-hl.pdf?ua=1 (accessed December 12, 2020).
96. Marlet MVL, Wuillaume F, Jacquet D, Quispe KW, Dujardin JC, Boelaert M. A neglected disease of humans: a new focus of visceral leishmaniasis in Bakool, Somalia. *Trans R Soc Trop Med Hyg.* (2003) 97:667–71. doi: 10.1016/S0035-9203(03)80099-8
97. Elnaiem D-EA. Ecology and control of the sand fly vectors of *Leishmania donovani* in East Africa, with special emphasis on Phlebotomus orientalis. *J Vector Ecol.* (2011) 36:S23–S31. doi: 10.1111/j.1948-7134.2011.00109.x
98. Sunyoto T, Potet J, Boelaert M. Visceral leishmaniasis in Somalia: a review of epidemiology and access to care. *PLoS Negl Trop Dis.* (2017) 11:e0005231. doi: 10.1371/journal.pntd.0005231
99. Burki TK. Somalia: a gathering storm? *The Lancet.* (2013) 382:1237–38. doi: 10.1016/S0140-6736(13)62083-8
100. Shiddo SA, Mohamed AA, Akuffo HO, Mohamud KA, Herzi AA, Mohamed HH, et al. Visceral leishmaniasis in Somalia: prevalence of markers of infection and disease manifestations in a village in an endemic area. *Trans R Soc Trop Med Hyg.* (1995) 89:361–5. doi: 10.1016/0035-9203(95)90008-X
101. Shiddo SA, Akuffo HO, Mohamed AA, Huldt G, Nilsson L-A, Ouchterlony Ö, et al. Visceral leishmaniasis in Somalia: prevalence of leishmanin-positive and seropositive inhabitants in an endemic area. *Trans R Soc Trop Med Hyg.* (1995) 89:21–4. doi: 10.1016/0035-9203(95)90640-1
102. Alamin AA. Visceral Leishmaniasis in a Non-endemic Region of Eritrea. *Cureus.* (2020) 12:e11318. doi: 10.7759/cureus.11318
103. The World Health Organisation. *Leishmaniasis Eritrea WHO Resources.* Available online at: https://www.who.int/leishmaniasis/resources/ERITREA.pdf (accessed December 12, 2020).
104. Ali A, Ashford R.W. Visceral leishmaniasis in Ethiopia. IV. Prevalence, incidence and relation of infection to disease in an endemic area. *Ann Trop Med Parasitol.* (1994) 88:289–93. doi: 10.1080/00034983.1994.11812869
105. Argaw D, Mulugeta A, Herrero M, Nombela N, Teklu T, Tefera T, et al. Risk factors for visceral Leishmaniasis among residents and migrants in Kafta-Humera, Ethiopia. *PLoS Negl Trop Dis.* (2013) 7:e2543. doi: 10.1371/journal.pntd.0002543
106. Ayehu A, Aschale Y, Lemma W, Alebel A, Worku L, Jejaw AG, et al. Seroprevalence of asymptomatic leishmania donovani among laborers and associated risk factors in agricultural camps of west Armachiho District, Northwest Ethiopia: a cross-sectional study. *J Parasitol Res.* (2018) 2018:5751743. doi: 10.1155/2018/5751743
107. World Health Organization. *Response*. WHO. Available online at: https://www.who.int/leishmaniasis/epidemic/response_more/en/ (accessed April 30, 2020).
108. Alvar J, Yactayo S, Bern C. Leishmaniasis and poverty. *Trends Parasitol.* (2006) 22:552–7. doi: 10.1016/j.pt.2006.09.004
109. Zijlstra EE, El-Hassan AM. 3. Visceral leishmaniasis. *Trans R Soc Trop Med Hyg.* (2001) 95:S27–S58. doi: 10.1016/S0035-9203(01)90218-4
110. Wakabi W. Peace has come to southern Sudan, but challenges remain. *Lancet.* (2006) 368:829–30. doi: 10.1016/S0140-6736(06)69307-0
111. Custodio E, Gadisa E, Sordo L, Cruz I, Moreno J, Nieto J, et al. Factors associated with leishmania asymptomatic infection: results from a cross-sectional survey in highland Northern Ethiopia. *PLoS Negl Trop Dis.* (2012) 6:e1813. doi: 10.1371/journal.pntd.0001813
112. Mengesha B, Endris M, Takele Y, Mekonnen K, Tadesse T, Feleke A, et al. Prevalence of malnutrition and associated risk factors among adult visceral leishmaniasis patients in Northwest Ethiopia: a cross sectional study. *BMC Res Notes.* (2014) 7:75. doi: 10.1186/1756-0500-7-75
113. Diro E, Blesson S, Edwards T, Ritmeijer K, Fikre H, Admassu H, et al. A randomized trial of AmBisome monotherapy and AmBisome and miltefosine combination to treat visceral leishmaniasis in HIV co-infected patients in Ethiopia. *PLoS Negl Trop Dis.* (2019) 13:e0006988. doi: 10.1371/journal.pntd.0006988
114. Diro E, Lynen L, Ritmeijer K, Boelaert M, Hailu A, van Griensven J. Visceral Leishmaniasis and HIV coinfection in East Africa. *PLoS Negl Trop Dis.* (2014) 8:e2869. doi: 10.1371/journal.pntd.0002869
115. National Centers for Environmental Information. *NOAA National Centers for Environmental Information, State of the Climate: Global Climate Report for Annual 2019*. (2020) Available online at: https://www.ncdc.noaa.gov/sotc/global/201913 (accessed October 10, 2020).
116. Carvalho BM, Rangel EF, Vale MM. Evaluation of the impacts of climate change on disease vectors through ecological niche modelling. *Bull Entomol Res.* (2017) 107:419–30. doi: 10.1017/S0007485316001097
117. Patz JA, Graczyk TK, Geller N, Vittor AY. Effects of environmental change on emerging parasitic diseases. *Int J Parasitol.* (2000) 30:1395–405. doi: 10.1016/S0020-7519(00)00141-7
118. Wilson AL, Courtenay O, Kelly-Hope LA, Scott TW, Takken W, Torr SJ, et al. The importance of vector control for the control and elimination of vector-borne diseases. *PLoS Negl Trop Dis.* (2020) 14:e0007831. doi: 10.1371/journal.pntd.0007831
119. Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. *Med Vet Entomol.* (2013) 27:123–47. doi: 10.1111/j.1365-2915.2012.01034.x
120. Koch LK, Kochmann J, Klimpel S, Cunze S. Modeling the climatic suitability of leishmaniasis vector species in Europe. *Sci Rep.* (2017) 7:13325. doi: 10.1038/s41598-017-13822-1
121. Kasap OE, Alten B. Comparative demography of the sand fly *Phlebotomus papatasi* (Diptera: Psychodidae) at constant temperatures. *J Vector Ecol.* (2006) 31:378–85. doi: 10.3376/1081-1710 (2006) 31[378:CDOTSF]2.0.CO;2
122. Rioux JA, Dereure J, Daoud W, el Kubati Y, Rageh HA, Moreno G, et al. Eco-epidemiology of visceral and cutaneous leishmaniasis in the Yemen Arab Republic. I. Presence, in sympatric condition, of Leishmania infantum and *Leishmania donovani* complexes. *Bull Soc Pathol Exot Filiales.* (1989) 82:658–64.
123. The World Health Organisation. *Leishmaniasis in the WHO European Region Fact Sheet*. Available online at: https://www.euro.who.int/__data/assets/pdf_file/0007/246166/Fact-sheet-Leishmaniasis-Eng.pdf (accessed December 12, 2020).
124. Aspöck H, Gerersdorfer T, Formayer H, Walochnik J. Sandflies and sandfly-borne infections of humans in Central Europe in the light of climate change. *Wien Klin Wochenschr.* (2008) 120:24–9. doi: 10.1007/s00508-008-1072-8
125. Darne S, Sinclair SA. A sandfly in Surrey? A case of cutaneous leishmaniasis in the United Kingdom without history of recent travel to an endemic area. *Clin Exp Dermatol.* (2006) 31:155–6. doi: 10.1111/j.1365-2230.2005.01984.x
126. Bogdan C, Schonian G, Banuls A-L, Hide M, Pratlong F, Lorenz E, et al. Visceral Leishmaniasis in a German child who had never entered a known endemic area: Case Report and Review of the Literature. *Clin Infect Dis.* (2001) 32:302–6. doi: 10.1086/318476
127. Obwaller AG, Karakus M, Poeppl W, Töz S, Özbel Y, Aspöck H, Walochnik J. Could *Phlebotomus mascittii* play a role as a natural vector for Leishmania infantum? New data. *Parasit Vectors.* (2016) 9:458. doi: 10.1186/s13071-016-1750-8
128. Haeberlein S, Fischer D, Thomas SM, Schleicher U, Beierkuhnlein C, Bogdan C. First assessment for the presence of phlebotomine vectors in Bavaria, Southern Germany, by Combined Distribution Modeling and Field Surveys. *PLoS One.* (2013) 8:e81088. doi: 10.1371/journal.pone.0081088

129. Naucke TJ, Pesson B. Presence of *Phlebotomus (Transphlebotomus) mascittii* Grassi, 1908 (Diptera: Psychodidae) in Germany. *Parasitol Res.* (2000) 86:335–6. doi: 10.1007/s004360050053
130. Oerther S, Jöst H, Heitmann A, Lühken R, Krüger A, Steinhausen I, et al. Phlebotomine sand flies in Southwest Germany: an update with records in new locations. *Parasit Vectors.* (2020) 13:173. doi: 10.1186/s13071-020-04058-6
131. Fischer D, Moeller P, Thomas SM, Naucke TJ, Beierkuhnlein C. Combining climatic projections and dispersal ability: a method for estimating the responses of sandfly vector species to climate change. *PLoS Negl Trop Dis.* (2011) 5:e1407. doi: 10.1371/journal.pntd.0001407
132. Michel G, Pomares C, Ferrua B, Marty P. Importance of worldwide asymptomatic carriers of *Leishmania infantum* (*L. chagasi*) in human. *Acta Trop.* (2011) 119:69–75. doi: 10.1016/j.actatropica.2011.05.012
133. WHO Expert Committee on the Control of the Leishmaniases & World Health Organization (2010). *Control of the Leishmaniases: Report of a Meeting of the WHO Expert Commitee on the Control of Leishmaniases.* Geneva: World Health Organization (2010). Available online at: https://apps.who.int/iris/handle/10665/44412 (accessed December 12, 2020).
134. González C, Wang O, Strutz SE, González-Salazar C, Sánchez-Cordero V, Sarkar S. Climate change and risk of leishmaniasis in North America: Predictions from ecological niche models of vector and reservoir species. *PLoS Negl Trop Dis.* (2010) 4:e585. doi: 10.1371/journal.pntd.0000585
135. Moo-Llanes D, Ibarra-Cerdeña CN, Rebollar-Téllez EA, Ibáñez-Bernal S, González C, Ramsey JM. Current and future niche of North and Central American Sand Flies (Diptera: Psychodidae) in climate change scenarios. *PLoS Negl Trop Dis.* (2013) 7:e2421. doi: 10.1371/journal.pntd.0002421
136. McIlwee BE, Weis SE, Hosier GA. Incidence of Endemic Human Cutaneous Leishmaniasis in the United States. *JAMA Dermatol.* (2018) 154:1032–9. doi: 10.1001/jamadermatol.2018.2133

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Global Goat! Is the Expanding Goat Population an Important Reservoir of *Cryptosporidium*?

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Goats are a primary or additional income source for many families in resource-poor areas. Although often considered inferior to other livestock, the resilience of goats and their ability to thrive in a range of environments means that that they are of particular value. Furthermore, goats emit less methane than other livestock species. In these same areas, it is well-documented that cryptosporidiosis has a substantial impact on infant morbidity and mortality, as well as reducing child growth and development. As *Cryptosporidium* also causes diarrheal disease in goats, the question arises whether goats may represent a reservoir of infection to humans. Epidemiological studies regarding the potential for transmission of *Cryptosporidium* between goats and humans have largely concluded that *Cryptosporidium* species infecting goats are not zoonotic. However, these studies are mostly from developed countries, where goat husbandry is smaller, management routines differ greatly from those of developing countries, contact between goats and their owners is more limited, and cryptosporidiosis has less impact on human health. In this article, background information on goat husbandry in different countries is provided, along with information on *Cryptosporidium* prevalence among goats, at both the species and sub-species levels, and the potential for zoonotic transmission. The intention is to indicate data gaps that should be filled and to increase awareness of the role of goats as providers for low-income families, often living in areas where cryptosporidiosis is endemic and where appropriate baseline interventions could have a positive impact, regardless of species of goat or parasite.

Keywords: ***Cryptosporidium*, goats (Capra aegagrus hircus), genotypes, One Health, zoonosis**

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INTRODUCTION

Goats are one of the species of livestock that were domesticated earliest, and are used worldwide for milk, meat, and hair/skin. Nowadays, goats are among the most popular and beneficial livestock for those with limited resources (1). Small-scale goat production is of considerable benefit to families and communities globally, in a variety of climates and conditions.

A landmark paper from 2005, "Goats – pathway out of poverty," argued that goats are worthy of serious investment, with the potential for transforming the lives of some of the world's poorest

people (2). Even under extreme climate conditions, goats have several characteristics that enable their capacity to convert feed into milk and meat (3).

In a world where our future is increasingly dominated by adaptation to climate change, goat-keeping is emerging as a truly important husbandry, not only for maintaining production levels, but also due to its relatively minor impact on climate as goats emit less methane than other livestock (4). There are about one billion goats worldwide, and the global goat population has more than doubled during the last four decades. According to the Food and Agriculture Organization, over 90% of goats are found in developing countries; Asia has the largest proportion of the world's goat population, followed by Africa (5).

Goats are traditionally managed differently to cattle, with flocks grazing in expansive enclosures or not enclosed at all, rather than being kept indoors. Goats are also popular as backyard livestock for hard-pressed families with few resources since livestock accounts for up to 60% of their income (1). In these settings, barriers against animal-human-animal transmission of zoonotic diseases are weakened. Thus, in promoting and supporting goat farming, it is important that efforts are also made to ensure that transfer of pathogens between goats and their owners is minimized.

WHERE ARE THE GOATS, AND WHO KEEPS THEM?

Over two-thirds of goats can be found in subtropical and tropical countries [(6); **Figure 1**].

In low-income countries of e.g., Asia, Africa, and Latin-America, locally adapted goat breeds are raised for milk and meat, and in dry and drought-prone areas, goat milk is often the only protein source in children's diets (7). In countries where the majority of goats are found, most goat owners belong to the lower socioeconomic strata (8–12), and, in rural areas, goats are largely managed by women and children (13, 14).

The International Livestock Research Institute recognized that goats are more important than cattle to the livelihoods of the rural poor, so investments in goat health, productivity, and sales may greatly assist with poverty alleviation.

The Cultures of Goat Keeping in Asia and Africa

Where extensive grazing is a main source of livelihood, goats have become an essential aspect of culture, social life, and even, in some places, religion, as goat meat is acceptable according to most scriptures.

Asia has identified the dairy-goat husbandry as especially sustainable in the face of climate change, and investments in several dairy-goat projects have been made during the past decade (7).

In India, for example, domesticated goats account for 20% of the global goat population (15) and goats remain a vital, but under-resourced and denigrated, part of the economy (7). Goats are an integral component in Indian livelihoods, contributing significantly to the income and socio-economic structure of rural farmers, and are often referred to as "the poor man's cow" (16). Goats are a reliable livelihood source in a range of Indian terrains, from deserts to coastal areas and high altitudes. However, unlike other sectors of Indian animal husbandry, the goat-meat industry is relatively disorganized, and abattoirs are usually unsuitable for goat slaughter.

Furthermore, goat husbandry in India takes place under federally unchecked conditions, particularly in rural areas. Regulatory bodies associated with commercial livestock rearing are lacking and most veterinary services inadequate, focusing on treatment rather than preventive measures (17), and gastrointestinal parasitism is prevalent in goats from all areas of India, representing a major health issue (18–20).

Africa holds over 40% of the global goat population, and over 60% are found in sub-Saharan countries. However, in contrast to Asia's relatively positive outlook on goat husbandry, goats are often associated with "backwardness" and "environmental destruction," by government officials in Africa, making it difficult to gain their investment support (7).

Nevertheless, goats play a major role as a source of food and income, accounting for 30% of Africa's ruminant livestock and producing 17 and 12% of its meat and milk (13). Production systems vary, including smallholder mixed crop-livestock systems, smallholder intensive systems, extensive pastoral and transhumance systems, and large-scale ranching systems (14, 21). Goats in Africa usually graze freely, scavenging feed resources where available, and, during the cropping season, forage for crop residues. The limited management and reliance on children for care and welfare probably exacerbates the low meat and milk production per goat. In urban areas, goats may graze common ground, which is often contaminated and used as a communal latrine, or may be held in stalls and fed at home (14). However, in some parts of East-Africa, there are extensive pastoral and transhumance systems, where goats are reared in large numbers and occupy 50% of the region (22).

CRYPTOSPORIDIUM: AN OVERVIEW

Cryptosporidium is an intestinal protozoan parasite with a worldwide distribution, a fecal-oral lifecycle, and is generally associated with diarrheal disease. It has a direct lifecycle in which the robust infectious oocyst stages are excreted with the feces into the environment and are immediately infectious for the next susceptible host.

Effects of *Cryptosporidium* on Goat Health

Cryptosporidium infection has an impact on growth and production in goats, and has been found to cause anorexia and diarrhea in goat kids, with morbidity and mortality reaching 50 and 100%, respectively (23–27), with accompanying economic consequences, impacting especially marginal farmers. Reduced growth, with and without diarrhea, has also been associated with *Cryptosporidium* infections in goats aged between 9 and 15 months, including in asymptomatic goats, raising further questions regarding long-term effects of apparently asymptomatic infections (28). Some studies have reported asymptomatic shedding of *Cryptosporidium*

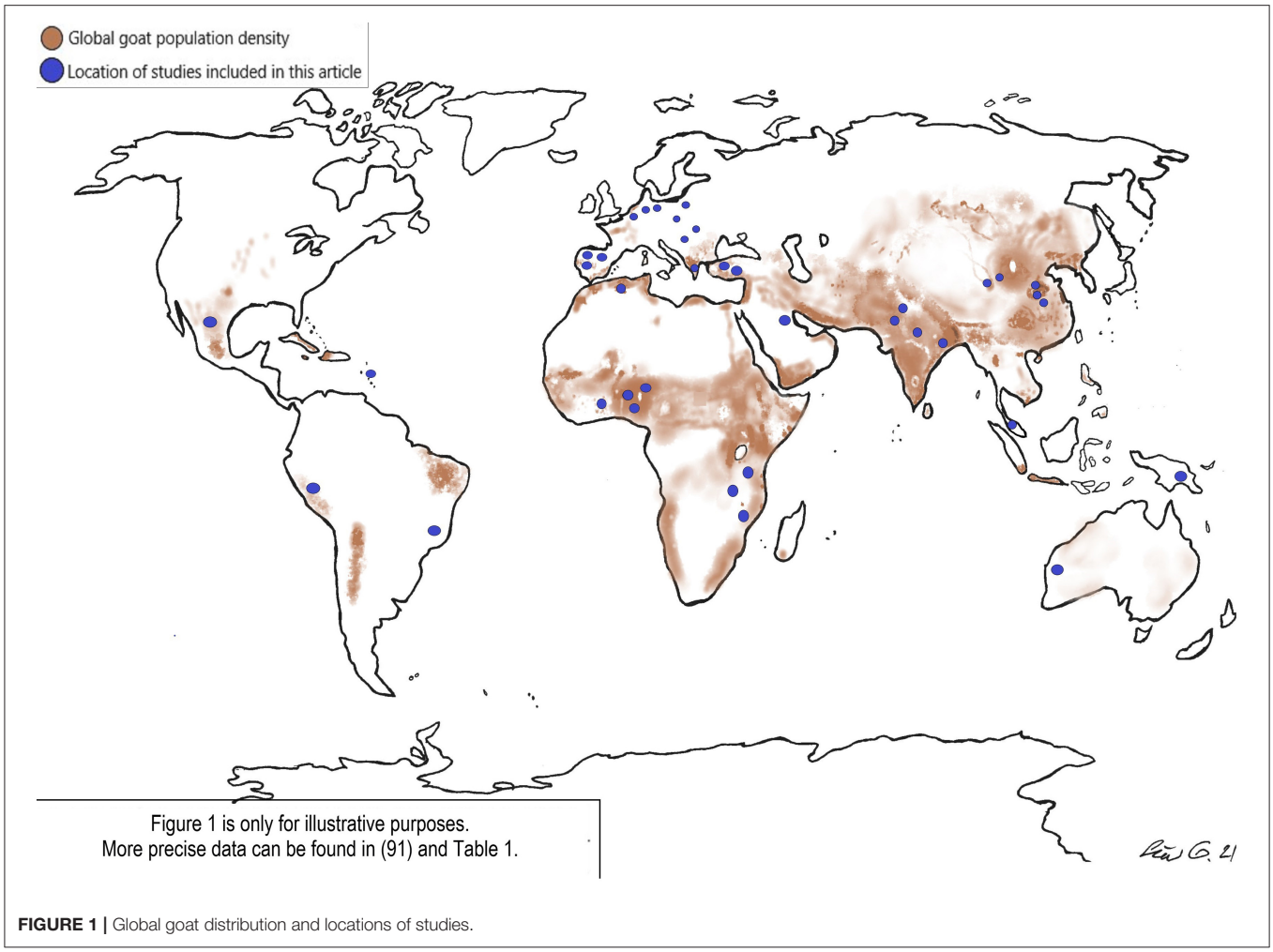

FIGURE 1 | Global goat distribution and locations of studies.

oocysts in adult goats (29, 30), but the long-term effects of chronic asymptomatic infections remain unclear, and goat health protocols recommended screening for *Cryptosporidium* infections after weaning, even in the absence of diarrhea (28).

Effects of *Cryptosporidium* on Human Health

Although *Cryptosporidium* has a global distribution, its impact on human health is greatest in developing countries where diarrheal disease exerts a huge health burden. Although global health is steadily improving, diarrheal disease remained the third most common cause of disability-adjusted life-years (DALYs) in the under-10 years age group in 2019 (31).

Given the high prevalence of cryptosporidiosis in people in resource-poor areas, this pathogen was included in the WHO "neglected disease initiative" in 2004 (32).

Cryptosporidium infection is particularly associated with pediatric diarrhea (33), but tends to be less important as a diarrheal pathogen in older age groups. A considerable mortality burden from cryptosporidiosis in children younger than 5-years (7.6 deaths per 100,000) has been reported (34), probably reflecting that cryptosporidiosis is acute and the explosive, voluminous diarrhea likely to have a major and immediate impact on infant survival. In addition, *Cryptosporidium* damages cells of the intestine and reduces absorption of nutrients. A meta-analysis suggested that the true burden of cryptosporidiosis was probably underestimated in previous reports, as effects subsequent to the acute phase of infection (decreased growth and enhanced risk of subsequent infections) were not included (35).

DIAGNOSTIC METHODS

There are no techniques particularly for diagnosis of *Cryptosporidium* infection in goats, although various procedures are available. Staining techniques are often applied in studies investigating prevalence, and molecular techniques provide information regarding species and subtype. Choice of diagnostic technique depends on available equipment and reagents, analyst experience, and time and cost of analysis. Molecular methods are usually not a routine diagnostic in resource-poor settings, but sensitive and specific diagnostic methods are important everywhere, particularly when positive findings result in

appropriate interventions such as improved hygiene and better farm management, both of which can be essential for disease control and prevention in both goats and humans. A recently published study indicated that auramine-phenol staining has high sensitivity and specificity for cryptosporidiosis and can be easily integrated with existing laboratory infrastructures in low-resource settings (36). Targeted sampling and preparation before diagnostics, along with dual application of staining and molecular techniques may provide the best possible results in terms of prevalence and epidemiology investigations.

MOLECULAR ASPECTS

Molecular tools have changed our understanding of *Cryptosporidium* spp. transmission. Genotyping and subtyping data have clearly demonstrated the presence of anthroponotic, as well as zoonotic, *Cryptosporidium* species in humans in industrialized nations. In contrast, transmission of cryptosporidiosis appears largely anthroponotic in some developing countries; for example, in Africa, despite frequent close contact between humans and animals, transmission appears to be mainly anthroponotic, and human *Cryptosporidium* infection is most often with *C. hominis* or *C. parvum anthroponosum* (37).

Nevertheless, as many *Cryptosporidium* species infect both humans and goats there is clearly the potential for transmission between the two host species. In the overview below, our focus remains on the most common zoonotic types. Details of studies are provided in **Table 1**, and the location of studies as related to goat distribution is shown in **Figure 1**.

C. parvum is perhaps the most studied zoonotic *Cryptosporidium* species. In studies from China in which *C. parvum* infectons from goats were diagnosed and the subtypes determined, the IId-subtype was found (not exclusively) in all investigations. *C. parvum* IId-subtypes seem to have a unique distribution in China, being predominant in *C. parvum* infections in humans, farm animals, and rodents (76–79). The IId-subtype has also been detected in goats in Europe, Asia, and Oceania (**Table 1**). However, the role of the rodent host, potentially an additional endemic amplifier, remains unknown in these areas.

In Africa, human *C. parvum* infections are dominated by the Iic-subtype, and the role of goats in transmission remains largely unknown. Although a study from Ghana reported finding the Iic-subtype in a goat, non-zoonotic, *C. xiaoi* dominated among goats kept in or around households (80). As far as we know, this is the only study where the Iic-subtype has been found in a goat.

The IIa-subtype seems to be present in *C. parvum* infections in goats in many parts of the world, having been reported from all continents except Africa, and, to date, publications investigating *C. parvum* subtypes in goats in North- and South-America are lacking.

C. ubiquitum has been detected in goats in studies from Europe, Asia, Africa, South America and Oceania (**Table 1**); in studies where subtyping has been conducted, only the subtype-XIIa was found. This subtype seems to predominate in ruminants, and humans are susceptible hosts for subtypes XIIa–XIId (81). *C. ubiquitum* is the most common species found in drinking water in rural USA, and human infections with this species has been detected mostly in developed countries, possibly due to the lower background of anthroponotic infections that predominate in developing countries (82), *C. ubiquitum* has been detected in feces from more animal species, and over a greater geographic range, than most *Cryptosporidium* species – with the exception of *C. parvum* (80). This distribution facilitates establishment of life cycles in extensive farming, where susceptible host animals are likely to be present and the infection barrier is weak. Data on clinical signs is scant, although this species has been identified in many cases of human cryptosporidiosis (81) and it has been isolated from diarrheic goat kids in Spain (65). A French study also found a periparturient rise in *C. ubiquitum* oocyst shedding from asymptomatic nanny goats (29). Although genotype analysis of *C. ubiquitum* has not been extensively performed, this species may represent a greater threat to both humans and animals given its ability to infect its next host, be it humans or their livestock.

Epidemiological Evidence for Sharing of *Cryptosporidium* Between Goats and Their Keepers

It is well known that younger animals, and people, are at greatest risk of *Cryptosporidium* infection, and are most likely to develop symptomatic disease if infected. Other epidemiological aspects are concerned with routes of exposure, and geographical, meteorological, cultural, and other environmental factors that may affect transmission patterns. Of interest regarding epidemiological pressures for interspecies transmission between goats and people, is looking at where zoonotic transmission from goats to humans has been documented. Although we know that the brunt of the global cryptosporidiosis burdens is borne by populations in Africa, Asia, and Latin America, it is difficult to recognize specific transmission occasions or outbreaks in these countries due to the high prevalence of infections. In other countries, however, outbreaks can be recognized, and some have been associated with direct or indirect contact with goats and their products. For example, an outbreak of cryptosporidiosis in USA was associated with consumption of unpasteurized goat milk (83) and an outbreak of cryptosporidiosis among school children in Norway was associated with contact with lambs and goat kids at a holiday farm, where the same sub-type of *C. parvum* (IIaA19G1R1) was found in both children, lambs, and goat kids (84). It is also noteworthy that in all studies from **Table 1** where the species of Cryptosporidium was identified, zoonotic species were detected in all investigations except two.

Of particular relevance regarding goats and *Cryptosporidium* regarding human health, is that in those countries where cryptosporidiosis exerts a particular burden, it is, as previously outlined, children who are most affected; and it is also children who most often have the job of looking after goats in these same regions of the world. The grazing habits of goats, generally browsing on woody shrubs and weeds rather than grazing grass, may indicate that they are less likely to ingest parasites (85).

TABLE 1 | Studies investigating Cryptosporidium in goats.

Occurrence of *Cryptosporidium* spp. in goats worldwide using different diagnostic techniques								Species and subtypes of *Cryptosporidium* in goats worldwide			
Continent and Country	**Study period**	**No. of goats**	**Goat age**	**Positive numbers of goats according to the diagnostic technique**				**Genes investigated**	***Cryptosporidium* species**	**gp60 genotype**	**References**
				List of tests used	**Microscopy**	**Immunological**	**Molecular**				
AFRICA											
Algeria	2012–2014	92	4 weeks or younger	PCR			8	SSU) rRNA gp60	*C. ubiquitum* *C. xiaoi*	XIIa	(38)
Ghana	NA	285	0–>24 months	PCR			95	(SSU) rRNA gp60	*C. parvum* *C. baileyi* *C. xiaoi*	IIcA5G3q	(39)
Mozambique	NA	60	Kids	ZN IFA	0						(40)
Nigeria	2013	98	Pre-weaned Post-weaned Adults	ELISA		28					(41)
Nigeria	NA	36	Pre-weaned	ELISA		30					(42)
Tanzania	2010–2011	56	NA	PCR-RFLP			5	SSU) rRNA gp60	C. xiaoi		(43)
Zambia	Na	17	NA	IFA		1					(44)
ASIA											
China	2014–2015	629	NA	PCR			104	(SSU) rRNA gp60	*C. parvum* *C. ubiquitum,* *C. xiaoi*	IIdA19G1, IIdA20G1	(45)
China	2007–2013	604	Pre-weaned to adults	PCR			69	(SSU) rRNA gp60	*C. parvum* *C. ubiquitum* *C. xiaoi*	IIaA14G2R1 IIaA15G1R1 IIaA15G2R1 IIaA17G2R1 XIIa	(46)
China	2011–2012	51	NA	IFA PCR		14	8	(SSU) rRNA	*C. parvum*		(47)
China	2006–2007, 2011	1256	Pre-weaned Post-weaned Adult Pregnant Postparturition nannies nannies	Modified acid-fast staining PCR-RFLP	44		44	(SSU) rRNA gp60	*C. ubiquitum* *C. andersoni* *C. xiaoi*	XIIa subtype 2	(48)
China	2006	42 goats + 1 ibex	NA	IFA PCR		15 + 1	2 + 1	(SSU) rRNA	*Cryptosporidium* sp. C. bovis-like genotype *Cryptosporidium* cervine genotype		(49)
India	2016	207	Adults	IFA PCR		1	1	(SSU) rRNA COWP Actin	*C. ubiquitum*		(50)

(Continued)

TABLE 1 | Continued

Occurrence of *Cryptosporidium* spp. in goats worldwide using different diagnostic techniques								Species and subtypes of *Cryptosporidium* in goats worldwide			
Continent and Country	**Study period**	**No. of goats**	**Goat age**	**Positive numbers of goats according to the diagnostic technique**				**Genes investigated**	***Cryptosporidium* species**	**gp60 genotype**	**References**
				List of tests used	**Microscopy**	**Immunological**	**Molecular**				
India	2009–2012	116	>3 months	ZN PCR-RFLP	4		4		*C. parvum*		(51)
India		57	>3 months	ZN PCR-RFLP	2	2	2	(SSU) rRNA Actin	*C. parvum*		(52)
India	NA	20 pooled samples (à 5)	NA	IFA PCR		35 (16–60)	0				(53)
Kuwait	2014–2015	222	Pre-weaned Post-weaned	ZN ELISA PCR-RFLP	22	54	10	SSU) rRNA gp60	*C. parvum* *C. ubiquitum* *C. xiaoi*	IIdA20G1 XIIa	(54)
Malaysia	2015	478	NA	ZN PCR	207		207	(SSU) rRNA	*C. parvum*		(55)
Turkey	2012–2016	NA	10–15 days old, symptomatic	Kinyoun Carbol Fuchsin staining PCR	9		9	(SSU) rRNA gp60	*C. parvum*	IIaA13G2R1 IIaA15G1R1 IIdA22G1 IIdA18G1 mixed subtypes	(56)
Turkey	2016	112	2–4 weeks	IFA PCR		76	73	(SSU) rRNA, gp60	*C. parvum* *C. xiaoi*	IIdA18G1 IIdA17G1 IIaA15G1R1 IIaA14G1R1	(57)
EUROPE											
Belgium	NA	148	1 day−10 weeks	IFA PCR		14	11	HSP-70 (SSU) rDNA gp60	*C. parvum*	IIdA22G1 IIdA15G2R1	(58)
Czech Republic	2005–2007	26	0.5–4 months	Milacek-Vitovec	2						(59)
France	2012	20 (longitudinal)	Adults	IFA PCR		16	12	(SSU) rRNA	*C. ubiquitum*		(28)
France	2011	35 animals (longitudinal), 254 samples	From birth to weaning	IFA PCR		61	19	(SSU) rRNA	*C. xiaoi* *C. parvum*		(60)
Greece	NA	255	na	IFA		18					(61)
Poland	NA	46	1–7 years old	ZN ELISA	0	0					(62)
Romania	NA	412	One day−6 weeks	ZN	99						(63)
Serbia	NA	88	Up to 90 days old		28						(64)

(Continued)

TABLE 1 | Continued

Occurrence of *Cryptosporidium* spp. in goats worldwide using different diagnostic techniques								Species and subtypes of *Cryptosporidium* in goats worldwide			
Continent and Country	**Study period**	**No. of goats**	**Goat age**	**Positive numbers of goats according to the diagnostic technique**				**Genes investigated**	***Cryptosporidium* species**	**gp60 genotype**	**References**
				List of tests used	**Microscopy**	**Immunological**	**Molecular**				
Spain	2008–2013	118	Up to 5 weeks old	Carbol fuchsin, auramine phenol PCR -RFLP	74		66	SSU rRNA gp60	*C. parvum* *C. ubiquitum* *C.xiaoi*	IIaA13G1R1 IIaA14G2R1 IIaA15G2R1 IIaA16G3R1 IIdA17G1	(65)
Spain	2004–2006	Na/sampled from symptomatic animals	Up to 21 days old	Carbol-fuchsin PCR RFLP	17		17	(SSU) rRNA gp60	*C. parvum*	IIdA17G1a IIdA19G1 IIdA25G1 IIdA26G1	(66)
Spain	2005	184	148 Adults, 36 kids	IFA		A:14 K:11					(67)
Spain	NA	116	Adults, asymptomatic	IFA		9		(SSU) rRNA hsp70	No positives	No positives	(68)
Spain	NA	5	<21 days	Carbol-fuchsin PCR RFLP	2		2	(SSU) rRNA Actin	*C. xiaoi*		(69)
North America											
Grenada	2011	202	All age groups	ELISA		45					(70)
South America											
Brazil	NA	105	56 > 12months 49 < 12months	Centrifuge-flotation Safranine Blue	CF:5 SB:2						(71)
Mexico	2014	80	>3 months	ZN	58						(72)
Peru	NA	402	NA	NA				NA	*C. ubiquitum*		(73)
OCEANIA											
Australia	NA	125 animals, 500 samples analyzed	9–12 months	PCR			36/500	(SSU) rRNA gp60	*C. ubiquitum* *C. parvum*	XIIa IIaA17G2R1 IIaA17G4R	(74)
Papua New Guinea	2011	228	Adults	PCR			10	(SSU) rRNA gp60	*C. hominis* *C. parvum* *C. xiaoi* *Cryptosporidium* rat-genotype II	IdA15G1 IIaA19G4R1 IIaA15G2R1	(75)

PCR, Polymerase Chain reaction; RFLP; Restriction Fragment Length Polymorphism; ZN, Ziehl-Nielsen; ELISA, Enzyme-Linked Immunosorbent Assay; IFA, Immunofluorescence Assays; NA, Not available.

However, in many settings, particularly poor urban or peri-urban areas, where shrubs are scant, they will be forced to search for nutrients closer to the ground. When foraging these scarce food resources on the ground, goats may be more likely to ingest *Cryptosporidium* spp. oocysts contaminating the environment, possibly shed by the human kid tending the goats, or from the goat kid foraging beside it. Similarly, children tending a flock of goat kids are likely to be exposed to parasite transmission stages in goat feces. In the cooler climates of temperate regions bovines, particularly calves, are often considered a source of zoonotic transmission of *Cryptosporidium*; in other global regions, it seems possible that goats may be an even more likely source.

PREVENTION AND CONTROL

Persistent diarrhea seriously affects nutritional status, growth, and intellectual function. Meeting these challenges is profoundly important, particularly in developing countries. *Cryptosporidium* oocysts have high infectivity, robustness, and resistance to disinfectants, which underscores the need for improved treatment options. No safe and effective treatment for cryptosporidiosis has been identified to date, although efforts to direct resources toward this objective continue to be made (86). Although *C. hominis* apparently still predominates in many settings, zoonotic transmission should not be neglected. In line with the One Health initiative, general rules of hygiene barriers between and among humans and animals in any setting should be implemented and thus reduce infection risks, not only of *Cryptosporidium*, but other zoonotic pathogens as well. As children and women are often responsible for tending backyard livestock, and also usually prepare food and/or fetch water, focusing on this group in hygiene training and information dissemination could improve the wellbeing of both them and their goats beyond their backyard. Studies that focus particularly on the likelihood of transmission of *Cryptosporidium* between goats and their keepers may provide more specific information on where interventions should be targeted, without losing the value from goat-keeping as an important resource for lifting families and communities out of poverty.

GOATS ARE SAVING THE WORLD

Organizations like Heifer International have helped small-scale farmers to obtain and benefit from goats in widely ranging situations, including in the dry forest areas of Peru, landless women in India, tropical forest areas of West-Africa, farmers in peri-urban areas St. Petersburg, the densely populated highlands of East-Africa, as well as the Sichuan province in China. Most of these goats are kept in small flocks of 3–10 animals, and are mainly cared for by children and women. Women have a significant role in goat-keeping in rural areas, enabling them to contribute substantially to the household economy (87).

In a resource-poor region of northern India, goat prices almost doubled when low-cost shelters, feeders, and water sheds were provided, in addition to improved breeding practices and prophylactic measures (7). Other development projects with goat interventions have given a positive return rate for both small- and large-scale goat-keepers in both Africa and South America, which, in turn, increased their income substantially (88). A zero-grazing management practice has often been introduced, which involves keeping goats in pens with limited outdoor space for exercise and all feed being brought to them. Manure is collected and either composted or applied to crops (89). This system has proven very successful in disease control, breeding management, and goat-rearing integration, including better protection of natural resources (90). However, the application of manure to crops might impose potential health risks and appropriate measures to protect both farmers and ensure safe produce should be taken into consideration.

The socio-economic status of farmers plays a major role in flock size and adoption of scientific management practices for goat rearing, which thereby raises income and socio-economic level of the owner, and particularly benefits socio-economically deprived women.

CONCLUSION

Cryptosporidiosis is an important diarrheal illness; in people in developing countries it exerts a substantial burden on child health, growth, and development (35) and in ruminant livestock, including goats, it affects growth and production (28). With goats an important livestock species for under-resourced communities, it is important to ensure that this potential reservoir of zoonotic *Cryptosporidium* is addressed and managed, and research needs to be conducted in the relevant regions.

The One Health initiative, focusing on reducing disease interface between humans and animals in areas where infection risk is greatest, could be harnessed to reduce health burdens and economic challenges where most needed. This depends largely on local endemic status and appropriate interventions. Studies on prevalence and species/genotypes of *Cryptosporidium* infecting people in developing countries are extensive, but there are considerably fewer of such investigations among domestic livestock. More information provided through further epidemiological studies on the species of *Cryptosporidium* infecting livestock and humans in these regions will fill data gaps and may assist in pinpointing relevant approaches to minimizing transmission. Goat-keeping is often a trade for the poorest in society, and awareness of proper hygienic routines and appropriate animal management strategies could benefit both human and animal health, as well as improving the economy and welfare of the goat-keepers and their herds.

AUTHOR CONTRIBUTIONS

KU conceived the study and wrote the main bulk of the manuscript. SC and TK contributed significantly

with local knowledge regarding both epidemiology and animal husbandry in the manuscript. LJR structured and contributed to all parts of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Devendra C. Small ruminants in Asia; contribution to food security, poverty alleviation and opportunities for productivity enhancement. In: *Proceeding of International Workshop on Small Ruminant Production and Development in South East Asia*. Vietnam: MEKARN, Nong Lam, HCMC (2005). p. 19–32.
2. Peacock C. Goats—A pathway out of poverty. *Small Rumin Res.* (2005) 60:179–86. doi: 10.1016/j.smallrumres.2005.06.011
3. Silanikove N. The physiological basis of adaptation in goats to harsh environments. *Small Rumin Res.* (2000) 35:181–93. doi: 10.1016/S0921-4488(99)00096-6
4. Darcan NK, Silanikove N. The advantages of goats for future adaptation to climate change: a conceptual overview. *Small Ruminant Research.* (2018) 163:34–8. doi: 10.1016/j.smallrumres.2017.04.013
5. *FAO Live Animals, Goats, World Total, Africa Total, America Total, Asia Total, Europe Total, Oceania Total, Year 1980, 1990, 2000, 2010 and 2018 QA, "Live Animals", "5000", "World", "5111", "Stocks", "1016", "Goats", "1990", "1990", "Head",' '588817562", "A", "Aggregate, may include official, semi-official, estimated or calculated data*". Available online at: http://www.fao.org/faostat/en/#data/QA (accessed December 31, 2020).
6. Späth H, Thume O. *Raising Goats*. Bucharest: M.A.S.T. Publishing (2008).
7. Miller BA, Lu CD. Current status of global dairy goat production: an overview. *Asian Austr J Anim Sci.* (2019) 32:1219–32. doi: 10.5713/ajas.19.0253
8. Singh M, Dixit A, Roy A, Singh S. Goat rearing: a pathway for sustainable livelihood security in Bundelkhand region. *Agric Econo Res Rev.* (2013) 26:79–88. doi: 10.22004/ag.econ.158491
9. Neupane N, Neupane H, Dhital B. A socioeconomic view of status and prospects of goat farming in rural areas of Nepal. *J Inst Agric Anim Sci.* (2018) 35:1–8. doi: 10.3126/jiaas.v35i1.22508
10. Ngambi JW, Alabi QJ, Norris D. Role of goats in food security; poverty alleviation and prosperity with special reference to sub-saharan africa: a review. *Indian J Anim Res.* (2013) 47:1–9.
11. Islam MA, Hossain MN, Chokraborti SS, Rahman S, Tasnim A, Al Zabir A. Socio-economic profile of goat rearing farmers and their management practices in Sylhet, Bangladesh. *J Agric Ecol Res Int.* (2018) 15:1–10. doi: 10.9734/JAERI/2018/43818
12. Byaruhanga C, Oluka J, Olinga S. Socio-economic aspects of goat production in a rural agro-pastoral system of Uganda. *Crops.* (2015) 114:105. doi: 10.13189/ujar.2015.030604
13. Lebbie SHB. Goats under household conditions. *Small Rumin Res.* (2004) 51:131–6. doi: 10.1016/j.smallrumres.2003.08.015
14. Muigai AWT, Okeyo AM, Ojango JMK. Goat production in eastern Africa: practices, breed characteristics, and opportunities for their sustainability. *In: Sustainable Goat Production in Adverse Environments: Volume I*. Cham: Springer International Publishing (2017). p. 31–57.
15. Joshi MB, Rout PK, Mandal AK, Tyler-Smith C, Singh L, Thangaraj K. Phylogeography and origin of Indian domestic goats. *Mol Biol Evol.* (2004) 21:454–62. doi: 10.1093/molbev/msh038
16. MacHugh DE, Bradley DG. Livestock genetic origins: goats buck the trend. *Proc Natl Acad Sci USA*. (2001) 98:5382–4. doi: 10.1073/pnas.111163198
17. Kumar S, Vihan VS, Deoghare PR. Economic implication of diseases in goats in India with reference to implementation of a health plan calendar. *Small Rumin Res.* (2003) 47:159–64. doi: 10.1016/S0921-4488(02)00237-7
18. Jithendran KP, Bhat TK. Epidemiology of parasitoses in dairy animals in the North West Humid Himalayan Region of India with particular reference to gastrointestinal nematodes. *Trop Anim Health Prod.* (1999) 31:205–14. doi: 10.1023/A:1005263009921
19. Choubisa SL, Jaroli VJ. Gastrointestinal parasitic infection in diverse species of domestic ruminants inhabiting tribal rural areas of southern Rajasthan, India. *J Parasit Dis.* (2013) 37:271–5. doi: 10.1007/s12639-012-0178-0
20. Singh AK, Das G, Roy B, Nath S, Naresh R, Kumar S. Prevalence of gastro-intestinal parasitic infections in goat of Madhya Pradesh, India. *J Parasit Dis.* (2015) 39:716–9. doi: 10.1007/s12639-014-0420-z
21. Pamo TE, Boukila B, Tendonkeng F. Goat production research in Africa: a sign post review for research in the new millennium. *Int J Biol Sci.* (2007) 1:76–89. doi: 10.4314/ijbcs.v1i1.39702
22. Muigai AW, Okeyo AM, Ojango JM. *Goat production in Eastern Africa: Practices, Breed Characteristics, and Opportunities for their Sustainability*. Cham: Springer (2017). p. 31–57.
23. Santín M. Clinical and subclinical infections with Cryptosporidium in animals. *N Z Vet J.* (2013) 61:1–10. doi: 10.1080/00480169.2012.731681
24. Thompson RCA, Palmer CS, O'Handley R. The public health and clinical significance of Giardia and Cryptosporidium in domestic animals. *Vet J.* (2008) 177:18–25. doi: 10.1016/j.tvjl.2007.09.022
25. Thamsborg SM, Jørgensen RJ, Henriksen SA. Cryptosporidiosis in kids of dairy goats. *Vet Rec.* (1990) 127:627–8.
26. Sevinç F, Simşek ATILLA, Uslu U. Massive *Cryptosporidium parvum* infection associated with an outbreak of diarrhoea in neonatal goat kids. *Turkish J Vet Anim Sci.* (2006) 29. 6:1317–20. Available online at: https://dergipark.org.tr/en/pub/tbtkveterinary
27. Vieira LS, Silva MB, Tolentino AC, Lima JD, Silva AC. Outbreak of cryptosporidiosis in dairy goats in Brazil. *Vet Rec.* (1997) 140:427–8. doi: 10.1136/vr.140.16.427
28. Jacobson C, Al-Habsi K, Ryan U, Williams A, Anderson F, Yang R, et al. Cryptosporidium infection is associated with reduced growth and diarrhoea in goats beyond weaning. *Vet Parasitol.* (2018) 260:30–7. doi: 10.1016/j.vetpar.2018.07.005
29. Paraud C, Pors I, Rieux A, Brunet S. High excretion of *Cryptosporidium ubiquitum* by peri-parturient goats in one flock in western France. *Vet Parasitol.* (2014) 202:301–4. doi: 10.1016/j.vetpar.2014.03.024
30. Castro-Hermida JA, Delafosse A, Pors I, Ares-Mazás E, Chartier C. Giardia duodenalis and *Cryptosporidium parvum* infections in adult goats and their implications for neonatal kids. *Vet Rec.* (2005) 157:623–7. doi: 10.1136/vr.157.20.623
31. Vos T, Lim SS, Abbafati C, Abbas KM, Abbasi M, Abbasifard M, et al. Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the global burden of disease study 2019. *Lancet.* (2020) 396:1204–22. doi: 10.1016/S0140-6736(20)30925-9
32. Savioli L, Smith H, Thompson A. Giardia and Cryptosporidium join the "Neglected diseases initiative." *Trends Parasitol.* (2006) 22:203–8. doi: 10.1016/j.pt.2006.02.015
33. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the global enteric multicenter study, GEMS): a prospective, case-control study. *Lancet.* (2013) 382:209–22. doi: 10.1016/S0140-6736(13)60844-2
34. GBD 2016 Diarrhoeal Disease Collaborators. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Infect Dis.* (2018) 18:1211–28. doi: 10.1016/S1473-3099(18)30362-1
35. Khalil IA, Troeger C, Rao PC, Blacker BF, Brown A, Brewer TG, et al. Morbidity, mortality, and long-term consequences associated with diarrhoea from Cryptosporidium infection in children younger than 5 years: a meta-analyses study. *Lancet Glob Health.* (2018) 6:e758–68. doi: 10.1016/S2214-109X(18)30283-3

36. Johansen ØH, Abdissa A, Zangenberg M, Mekonnen Z, Eshetu B, Bjørang O, et al. Performance and operational feasibility of two diagnostic tests for cryptosporidiosis in children (CRYPTO-POC): a clinical, prospective, diagnostic accuracy study. *Lancet Infect Dis.* (2020). doi: 10.1016/S1473-3099(20)30556-9. [Epub ahead of print].
37. Robertson LJ, Johansen ØH, Kifleyohannes T, Efunshile AM, Terefe G. Cryptosporidium infections in Africa—how important is zoonotic transmission? A review of the evidence. *Front Vet Sci.* (2020) 7:575881. doi: 10.3389/fvets.2020.575881
38. Baroudi D, Hakem A, Adamu H, Amer S, Khelef D, Adjou K, et al. Zoonotic Cryptosporidium species and subtypes in lambs and goat kids in Algeria. *Parasit Vectors.* (2018) 11:582. doi: 10.1186/s13071-018-3172-2
39. Squire SA, Yang R, Robertson I, Ayi I, Ryan U. Molecular characterization of Cryptosporidium and Giardia in farmers and their ruminant livestock from the Coastal Savannah zone of Ghana. *Infect Genet Evol.* (2017) 55:236–43. doi: 10.1016/j.meegid.2017.09.025
40. Miambo RD, Laitela B, Malatji MP, De Santana Afonso SM, Junior AP, Lindh J, et al. Prevalence of Giardia and Cryptosporidium in young livestock and dogs in Magude District of Maputo Province, Mozambique. *Onderstepoort J Vet Res.* (2019) 86:e1–6. doi: 10.4102/ojvr.v86i1.1709
41. Akinkuotu OA, Okwelum N, Famakinde SA, Akinkuotu AC. Prevalence of Cryptosporidium infection in recently acclimatized Kalahari red goats in Nigeria. *Vom J Vet Sci.* (2016) 11:112–116. Available online at: https://www.ejmanager.com/my/vjvs/submit.php?lng=
42. Ambrose AO, Olakunle FB. *Cryptosporidium Infection in Pre-Weaned Ruminants and Pigs in Southwestern Nigeria* (2014). Framingham, MA: Global Journal of Medical Research G: Veterinary Science & Veterinary Medicine.
43. PLOS Neglected Tropical Diseases Staff. Correction: epidemiology and molecular characterization of Cryptosporidium spp. in humans, wild primates, and domesticated animals in the Greater Gombe Ecosystem, Tanzania. *PLoS Negl Trop Dis.* (2015) 9:e0003650. doi: 10.1371/journal.pntd.0003650
44. Siwila J, Phiri IGK, Enemark HI, Nchito M, Olsen A. Occurrence of Cryptosporidium and Giardia in domestic animals in peri-urban communities of Kafue district, Zambia. *Tanzania Vet J.* (2013) 28:49–59. doi: 10.4314/tvj.v28i0
45. Peng X-Q, Tian G-R, Ren G-J, Yu Z-Q, Lok JB, Zhang L-X, et al. Infection rate of Giardia duodenalis, Cryptosporidium spp. and Enterocytozoon bieneusi in cashmere, dairy and meat goats in China. *Infect Genet Evol.* (2016) 41:26–31. doi: 10.1016/j.meegid.2016.03.021
46. Mi R, Wang X, Huang Y, Zhou P, Liu Y, Chen Y, et al. Prevalence and molecular characterization of Cryptosporidium in goats across four provincial level areas in China. *PLoS ONE.* (2014) 9:e111164. doi: 10.1371/journal.pone.0111164
47. Ma L, Sotiriadou I, Cai Q, Karanis G, Wang G, Wang G, et al. Detection of Cryptosporidium and Giardia in agricultural and water environments in the Qinghai area of China by IFT and PCR. *Parasitol Res.* (2014) 113:3177–84. doi: 10.1007/s00436-014-3979-5
48. Wang R, Li G, Cui B, Huang J, Cui Z, Zhang S, et al. Prevalence, molecular characterization and zoonotic potential of Cryptosporidium spp. in goats in Henan and Chongqing, China. *Exp Parasitol.* (2014) 142:11–6. doi: 10.1016/j.exppara.2014.04.001
49. Karanis P, Plutzer J, Halim NA, Igori K, Nagasawa H, Ongerth J, et al. Molecular characterization of Cryptosporidium from animal sources in Qinghai province of China. *Parasitol Res.* (2007) 101:1575–80. doi: 10.1007/s00436-007-0681-x
50. Utaaker KS, Myhr N, Bajwa RS, Joshi H, Kumar A, Robertson LJ. Correction to: goats in the city: prevalence of Giardia duodenalis and Cryptosporidium spp. in extensively reared goats in northern India. *Acta Vet Scand.* (2018) 60:52. doi: 10.1186/s13028-018-0402-8
51. Maurya PS, Rakesh RL, Pradeep B, Kumar S, Kundu K, Garg R, et al. Prevalence and risk factors associated with Cryptosporidium spp. infection in young domestic livestock in India. *Trop Anim Health Prod.* (2013) 45:941–6. doi: 10.1007/s11250-012-0311-1
52. Rakesh RL, Banerjee PS, Garg R, Maurya PS, Kundu K, Jacob SS, et al. Genotyping of Cryptosporidium spp. isolated from young domestic ruminants in some targeted areas of India. *Indian J Ani Sci.* (2014) 84:819–23.
53. Daniels ME, Shrivastava A, Smith WA, Sahu P, Odagiri M, Misra PR, et al. Cryptosporidium and Giardia in humans, domestic animals, and village water sources in rural India. *Am J Trop Med Hyg.* (2015) 93:596–600. doi: 10.4269/ajtmh.15-0111
54. Majeed QAH, El-Azazy OME, Abdou N-EMI, Al-Aal ZA, El-Kabbany AI, Tahrani LMA, et al. Epidemiological observations on cryptosporidiosis and molecular characterization of Cryptosporidium spp. in sheep and goats in Kuwait. *Parasitol Res.* (2018) 117:1631–6. doi: 10.1007/s00436-018-5847-1
55. Mat Yusof A, Hashim N, Md Isa ML. First molecular identification of Cryptosporidium by 18S rRNA in goats and association with farm management in Terengganu. *Asian Pac J Trop Biomed.* (2017) 7:385–8. doi: 10.1016/j.apjtb.2017.01.008
56. Taylan-Ozkan A, Yasa-Duru S, Usluca S, Lysen C, Ye J, Roellig DM, et al. Cryptosporidium species and Cryptosporidium parvum subtypes in dairy calves and goat kids reared under traditional farming systems in Turkey. *Exp Parasitol.* (2016) 170:16–20. doi: 10.1016/j.exppara.2016.06.014
57. Ipek DS. Prevalence and molecular characterisation of Cryptosporidium spp. in diarrhoeic pre-weaned goat kids reared under traditional farming system in Diyarbakir, Southeastern Anatolia City, Turkey. *Rev Med Vet.* (2017) 168:229–34. Available online at: https://www.scimagojr.com/journalsearch.php?tip=sid&q=18865
58. Geurden T, Thomas P, Casaert S, Vercruysse J, Claerebout E. Prevalence and molecular characterisation of Cryptosporidium and Giardia in lambs and goat kids in Belgium. *Vet Parasitol.* (2008) 155:142–5. doi: 10.1016/j.vetpar.2008.05.002
59. Strnadová P, Svobodová V, Vernerova E. Protozoální infekce jehnat a kuzlat na farmách v Ceské republice P, Protozoal infections occuring in farms in the Czech Republic (in Czech). *Veterinárství.* (2008) 58:451–8. Available online at: http://vetweb.cz/protozoalni-infekce-jehnat-a-kuzlat-na-farmach-v-ceske-republice/
60. Rieux A, Paraud C, Pors I, Chartier C. Molecular characterization of Cryptosporidium spp. in pre-weaned kids in a dairy goat farm in western France. *Vet Parasitol.* (2013) 192:268–72. doi: 10.1016/j.vetpar.2012.11.008
61. Tzanidakis N, Sotiraki S, Claerebout E, Ehsan A, Voutzourakis N, Kostopoulou D, et al. Occurrence and molecular characterization of Giardia duodenalis and Cryptosporidium spp. in sheep and goats reared under dairy husbandry systems in Greece. *Parasite.* (2014) 21:45. doi: 10.1051/parasite/2014048
62. Majewska AC, Werner A, Sulima P, Luty T. Prevalence of Cryptosporidium in sheep and goats bred on five farms in west-central region of Poland. *Vet Parasitol.* (2000) 89:269–75. doi: 10.1016/S0304-4017(00)00212-0
63. Bejan AL. *Criptosporidioza viteilor si izeilor: cercetari privind diagnosticul, epidemiologia si etiopatogeneza, Cryptosporidiosis in calves and goat kids: research concerning diagnosis, epidemiology, and etiopathogenesis (In Romanian)*. Dissertation. Universitatea de Ştiinţe Agricole i Medicină Veterinară Cluj-Napoca (2009). Available online at: http://usamvcluj.ro/files/teze/bejan.pdf (accessed February 18, 2021).
64. Mišić ZB, Katić-Radivojević SP, Kulišić Z. Cryptosporidium infection in lambs and goat kids in Serbia. *Acta Vet.* (2006) 56:49–54. doi: 10.2298/AVB0601049M
65. Díaz P, Quílez J, Prieto A, Navarro E, Pérez-Creo A, Fernández G, et al. Cryptosporidium species and subtype analysis in diarrhoeic pre-weaned lambs and goat kids from north-western Spain. *Parasitol Res.* (2015) 114:4099–105. doi: 10.1007/s00436-015-4639-0
66. Quílez J, Torres E, Chalmers RM, Hadfield SJ, Del Cacho E, Sánchez-Acedo C. Cryptosporidium genotypes and subtypes in lambs and goat kids in Spain. *Appl Environ Microbiol.* (2008) 74:6026–31. doi: 10.1128/AEM.00606-08
67. Castro-Hermida JA, González-Warleta M, Mezo M. Natural infection by Cryptosporidium parvum and Giardia duodenalis in sheep and goats in Galicia (NW Spain). *Small Rumin Res.* (2007) 72:96–100. doi: 10.1016/j.smallrumres.2006.08.008
68. Castro-Hermida JA, Almeida A, González-Warleta M, Correia da Costa JM, Rumbo-Lorenzo C, Mezo M. Occurrence of Cryptosporidium parvum and Giardia duodenalis in healthy adult domestic ruminants. *Parasitol Res.* (2007) 101:1443–8. doi: 10.1007/s00436-007-0624-6
69. Díaz P, Quílez J, Robinson G, Chalmers RM, Díez-Baños P, Morrondo P. Identification of Cryptosporidium xiaoi in diarrhoeic goat kids (Capra hircus) in Spain. *Vet Parasitol.* (2010) 172:132–4. doi: 10.1016/j.vetpar.2010.04.029

70. Chikweto A, Veytsman S, Tiwari K, Cash K, Stratton G, Thomas D, et al. Prevalence of Cryptosporidium spp. in asymptomatic small ruminants in Grenada, West Indies. *Vet Parasitol.* (2019) 15:100262. doi: 10.1016/j.vprsr.2019.100262
71. Bomfim TCB, Huber F, Gomes RS, Alves LL. Natural infection by Giardia sp. and Cryptosporidium sp. in dairy goats, associated with possible risk factors of the studied properties. *Vet Parasitol.* (2005) 134:9–13. doi: 10.1016/j.vetpar.2005.05.067
72. Romero-Salas D, Alvarado-Esquivel C, Cruz-Romero A, Aguilar-Domínguez M, Ibarra-Priego N, Merino-Charrez JO, et al. Prevalence of Cryptosporidium in small ruminants from Veracruz, Mexico. *BMC Vet Res.* (2016) 12:14. doi: 10.1186/s12917-016-0638-3
73. Xiao L. Overview of Cryptosporidium presentations at the 10th international workshops on opportunistic protists. *Eukaryot Cell.* (2009) 8:429–36. doi: 10.1128/EC.00295-08
74. Al-Habsi K, Yang R, Williams A, Miller D, Ryan U, Jacobson C. Zoonotic Cryptosporidium and Giardia shedding by captured rangeland goats. *Vet Parasitol.* (2017) 7:32–5. doi: 10.1016/j.vprsr.2016.11.006
75. Koinari M, Lymbery AJ, Ryan UM. Cryptosporidium species in sheep and goats from Papua New Guinea. *Exp Parasitol.* (2014) 141:134–7. doi: 10.1016/j.exppara.2014.03.021
76. Zhao GH, Du SZ, Wang HB, Hu XF, Deng MJ, Yu SK, et al. First report of zoonotic Cryptosporidium spp. Giardia intestinalis and Enterocytozoon bieneusi in golden takins (Budorcas taxicolor bedfordi. *Infect Genet Evol.* (2015) 34:394–401. doi: 10.1016/j.meegid.2015.07.016
77. Jian F, Liu A, Wang R, Zhang S, Qi M, Zhao W, et al. Common occurrence of *Cryptosporidium hominis* in horses and donkeys. *Infect Genet Evol.* (2016) 43:261–6. doi: 10.1016/j.meegid.2016.06.004
78. Zhao Z, Wang R, Zhao W, Qi M, Zhao J, Zhang L, et al. Genotyping and subtyping of Giardia and Cryptosporidium isolates from commensal rodents in China. *Parasitology.* (2015) 142:800–6. doi: 10.1017/S0031182014001929
79. Lv C, Zhang L, Wang R, Jian F, Zhang S, Ning C, et al. Cryptosporidium spp. in wild, laboratory, and pet rodents in china: prevalence and molecular characterization. *Appl Environ Microbiol.* (2009) 75:7692–9. doi: 10.1128/AEM.01386-09
80. Li N, Xiao L, Alderisio K, Elwin K, Cebelinski E, Chalmers R, et al. Subtyping *Cryptosporidium ubiquitum*,a zoonotic pathogen emerging in humans. *Emerg Infect Dis.* (2014) 20:217–24. doi: 10.3201/eid2002.121797
81. Fayer R, Santín M, Macarisin D. Cryptosporidium ubiquitum n. sp. in animals and humans. *Vet Parasitol.* (2010) 172:23–32. doi: 10.1016/j.vetpar.2010.04.028
82. Feng Y, Ryan UM, Xiao L. Genetic diversity and population structure of Cryptosporidium. *Trends Parasitol.* (2018) 34:997–1011. doi: 10.1016/j.pt.2018.07.009
83. Rosenthal M, Pedersen R, Leibsle S, Hill V, Carter K, Roellig DM. Cryptosporidiosis associated with consumption of unpasteurized goat milk—Idaho. *Morb Mortal Wkly Rep.* (2015) 64:194. Available online at: https://www.jstor.org/stable/24856365
84. Lange H, Johansen OH, Vold L, Robertson LJ, Anthonisen IL, Nygard K. Second outbreak of infection with a rare *Cryptosporidium parvum* genotype in schoolchildren associated with contact with lambs/goat kids at a holiday farm in Norway. *Epidemiol Infect.* (2014) 142:2105–13. doi: 10.1017/S0950268813003002
85. Robertson LJ. Giardia and Cryptosporidium infections in sheep and goats: a review of the potential for transmission to humans via environmental contamination. *Epidemiol Infect.* (2009) 137:913–21. doi: 10.1017/S0950268809002295
86. Choy RKM, Huston CD. Cryptosporidiosis should be designated as a tropical disease by the US Food and Drug Administration. *PLoS Negl Trop Dis.* (2020) 14:e0008252. doi: 10.1371/journal.pntd.0008252
87. Tudu NK, Roy DC. Socio-economic profile of women goat keepers and rearing challanges in goat in nadia district of West Bengal. *Int J Sci Environ Technol.* (2015) 4:331–6.
88. Skapetas B, Bampidis V. Goat production in the world: present situation and trends. *Livest Res Rural Dev.* (2016) 28:200. Available online at: http://www.lrrd.org/lrrd28/11/skap28200.html
89. De Vries J. Goats for the poor: some keys to successful promotion of goat production among the poor. *Small Rumin Res.* (2008) 77:221–4. doi: 10.1016/j.smallrumres.2008.03.006
90. Shirima E. Benefits from dual purpose goats for crop and livestock production under small-scale peasant systems in Kondoa eroded areas, Tanzania. *Livestock Res Rural Dev.* (2005) 17:138. Available online at: http://www.lrrd.org/lrrd17/12/shir17138.htm
91. Gilbert M, Nicolas G, Cinardi G, Van Boeckel TP, Vanwambeke SO, Wint GRW, et al. Global distribution data for cattle, buffaloes, horses, sheep, goats, pigs, chickens and ducks in 2010. *Sci Data.* (2018) 5:180227. doi: 10.1038/sdata.2018.227

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REVIEW
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Neurocysticercosis: Current Perspectives on Diagnosis and Management

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Porcine cysticercosis, human taeniasis, and (neuro)cysticercosis are endemic in many low- and middle-income countries (LMIC) where they present a significant health burden to affected communities. Neurocysticercosis (NCC) is one of the leading causes of human epilepsy in many hyperendemic regions in Latin America, Asia, and sub-Saharan Africa. The World Health Organization (WHO) reports an estimated 2.5–8.3 million cases of NCC annually with a disability-adjusted life year (DALY) burden of 2.8 million, but as for all neglected tropical diseases (NTDs), these values are likely to be underestimated. Diagnosis of NCC is complex and most accurately diagnosed using clinical neuroimaging that is unavailable in most hyperendemic regions in LMIC. On January 28, 2021, WHO will launch its road map for the NTDs' "Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021–2030." Taeniasis/cysticercosis is targeted for control success considered as steady increase in the number of countries with intensified control in hyperendemic areas [increasing from 2 (3%) in 2020 to 4 (6%) in 2023, to 9 (14%) by 2025, and to 17 (27%) by 2030]. Cross-cutting targets that include 100% access to at least basic water supply, sanitation, and hygiene in areas endemic for NTDs and 75% integrated treatment coverage for preventative chemotherapy will additionally impact on the taeniasis/cysticercosis/NCC complex. With no vaccine available for humans, prevention of infection depends on communication to the public of the life cycle of a complex zoonosis to promote behavior change, underpinned by practical control measures including treatment of human taeniasis and (neuro)cysticercosis with albendazole and praziquantel [widely used as part of the mass drug administration (MDA) deworming programs], surgery where appropriate, and effective vaccination and deworming for pigs supported by meat inspection. Here, we review recent advances in tools and implementation for *Taenia solium* taeniasis/(neuro)cysticercosis (TSTC) control and milestones on the onward path to elimination.

Keywords: neurocysticercosis, cysticercosis, epilepsy, mass drug administration, anthelmintics, drug resistance, *Taenia solium*, taeniasis

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INTRODUCTION

Taeniasis and (neuro)cysticercosis are caused by the cestode *Taenia solium* or pork tapeworm. *T. solium* is a multi-host parasite with a complex zoonotic transmission cycle, circulating between the intermediate pig host and the definitive or accidental intermediate human host (1, 2). *T. solium* infection arises from ingestion of contaminated food or water and ingestion of raw or undercooked pork and may result in taeniasis (caused by the adult tapeworm living in the small intestine) and/or cysticercosis or neurocysticercosis [NCC; caused by invasion of the larvae into the central nervous system (CNS)] in humans.

When humans consume pork containing live *T. solium* cysts, the cysticercus develops into a mature tapeworm in the human intestine, shedding eggs that are expelled in human feces.

Cysticercosis develops when, following ingestion of *T. solium* eggs, *T. solium* larvae migrate and become encysted, typically in the muscle tissue of the host. Pigs can harbor thousands of cysts. When *T. solium* cysticerci develop in the human brain, the condition is defined as NCC. NCC is the most common parasitic disease of the CNS in humans affecting between 2.5 and 8.3 million people annually, accounting for a global burden of 2.8 million disability-adjusted life years (DALYs) (1, 2). NCC is a major clinical consequence of *T. solium* infection and the dominant cause of global preventable epilepsy associated with morbidity and mortality from epileptic seizures and epilepsy related death; where *T. solium* is endemic, 30% of epilepsy cases are estimated to be caused by NCC (3, 4).

Taeniasis can cause abdominal pain, nausea, and diarrhea, although it is often asymptomatic, at around 8 weeks post-ingestion with symptoms persisting until treatment with anthelmintic drugs or for around 2–3 years (the lifespan of the adult tapeworm) if untreated (5, 6). Many carriers of *T. solium* (taeniasis and cysticercosis) are asymptomatic and become long-term carriers of infection through self-reinfection and re-infection from others within the household (6). *T. solium* infection in pigs can be detected by meat inspection by visual inspection of cut meat and by lingual examination of the live animal; this, however, has low sensitivity as cysts can be missed.

As for most of the neglected zoonoses, under-diagnosis and under-reporting of cysticercosis and NCC result in underestimation of case numbers and global burden (1, 7, 8). NCC is endemic in low- and middle-income countries (LMIC), where sanitation and clean water are substandard, and in communities where pig-keeping is an integral part of the local economy and often promoted as a route out of poverty (8, 9). NCC is becoming more prevalent in developed economies with increased immigration from regions endemic for *T. solium* (10).

NEUROCYSTICERCOSIS

In cases of NCC, *T. solium* larvae are found either in the brain tissue (parenchymal NCC) or in the intraventricular and subarachnoid spaces of the brain and spinal cord where the cerebrospinal fluid (CSF) circulates (extraparenchymal NCC) resulting in different clinical manifestations and prognoses. Parenchymal NCC manifests with seizures and headaches with psychiatric symptoms being rare and generally has a better prognosis since seizures tend to respond well to anti-seizure drug therapy (11–13). Extraparenchymal NCC may result in increased intracranial pressure and hydrocephalus, and patients show poorer prognosis, in part due to the growth (increase in size) of cysts in the subarachnoid space prior to symptoms becoming apparent and from late diagnosis (14).

The clinical presentation of NCC is similar to a wide range of neurological conditions making clinical diagnosis, especially in low-income country settings, difficult. Depending on the number, size, stage, and location of the cysts and the immune response of an individual patient, NCC presentation can vary from being asymptomatic to sudden death. A definitive clinical diagnosis is only made by visualization of cysts or larvae in the brain tissue *via* neuroimaging (15–17); in some cases, intracranial calcification of cysts is the only evidence of the disease (5).

Studies relating infection to mortality are rare. The limited number of hospital-based studies reporting deaths, in general, reports mortality from extraparenchymal NCC. In Brazil, endemic for *T. solium*, 1,570 NCC deaths were reported between 1985 and 2011, whereas in the United States (non-endemic), 221 NCC deaths were reported between 1985 and 2011 (14, 18, 19). These numbers represent deaths where NCC was considered the direct or an associated cause of death. In the absence of imaging or autopsy data, NCC-associated deaths are invariably under-reported.

DIAGNOSIS

Established methods for NCC diagnosis include a detailed clinical examination, serological testing, and neuroimaging. Each method has its benefits and drawbacks, some being more successful at diagnosing NCC infection at different stages (cysts, calcified cysts). Definitive classifications have been provided by Del Brutto et al. (20), revised in 2017 (**Table 1**), and Carpio et al. (**Table 2**) (20–22).

Serological Diagnostic Tests

Serological methods enable the detection of specific anti-*T. solium* antibodies or *T. solium* antigens in the blood, urine, and CNS (23, 24). Testing for *T. solium*-specific antibodies does not differentiate between an active infection or exposure from a previous infection (24, 25). Enzyme-linked immunoelectrotransfer blot (EITB) identifies specific antibodies to lentil lectin purified glycoprotein (LLGP-EITB) antigens of *T. solium*. In patients with multiple parenchymal cysts, or subarachnoid NCC, EITB has near 100% sensitivity (26, 27); however, in patients with only calcified cysts or single parenchymal lesions, the test reaches only 60–70% sensitivity (25, 27). Enzyme-linked immunosorbent assay (ELISA) detection of *T. solium* antibodies using crude or purified parasitic antigen extracts uses IgG as the target immunoglobulin; however, Ab-ELISAs generally have a lower specificity and sensitivity of EITB (28, 29). Despite this, specific ELISAs are useful in confirming diagnosis and evaluating treatment of extraparenchymal cysts (30). Detection of circulating cysticercus antigens can be done by

TABLE 1 | Diagnostic criteria [derived from (20, 21)].

Definitive
- Histological demonstration of the parasite from biopsy of a brain or spinal cord lesion
- Evidence of cystic lesions showing the scolex on neuroimaging studies
- Direct visualization of subretinal parasites by fundoscopic examination

Neuroimaging criteria:

Major neuroimaging criteria:
- Multilobulated cystic lesions in the subarachnoid space
- Typical parenchymal brain calcifications

Confirmative neuroimaging criteria
- Resolution of cystic lesions after cysticidal drug therapy
- Spontaneous resolution of single small enhancing lesions
- Migration of ventricular cysts documented on sequential neuroimaging studies

Minor neuroimaging criteria
- Obstructive hydrocephalus (symmetric or asymmetric) or abnormal enhancement of basal leptomeninges

Clinical/exposure criteria

Major
- Evidence of lesions highly suggestive of neurocysticercosis on neuroimaging studies
- Positive serum immunoblot for the detection of anticysticeral antibodies or cysticeral antigens by well-standardized immunological tests
- Resolution of intracranial cystic lesions after therapy with albendazole or praziquantel
- Spontaneous resolution of single small enhancing lesions
- Cysticercosis outside the central nervous system
- Evidence of contact with *T. solium* infection

Minor
- Evidence of lesions compatible with neurocysticercosis on neuroimaging
- Presence of clinical manifestations suggestive of neurocysticercosis
- Positive CSF ELISA for the detection of anticysticeral antibodies or cysticeral antigens
- Evidence of cysticercosis outside the central nervous system
- Individuals coming from or living in an area where cysticercosis is endemic

Epidemiological
- Individuals coming from or living in an area where cysticercosis is endemic
- History of travel to disease-endemic areas
- Evidence of household contact with *T. solium* infection

Degrees of diagnostic certainty

Definitive
- Presence of one absolute criterion
- Presence of two major plus one minor and one epidemiological criteria
- Two major neuroimaging criteria plus any clinical/exposure criteria
- One major and one confirmative neuroimaging criteria plus any clinical/exposure criteria
- One major criterion plus two clinical/exposure criteria (including at least one major clinical/exposure criterion) together with the exclusion of other pathologies producing similar neuroimaging findings

Probable
- Presence of one major neuroimaging plus two minor clinical/exposure criteria
- Presence of one major plus one minor and one epidemiological criterion
- Presence of three minor and one epidemiological criterion
- One minor neuroimaging criteria plus at least one major clinical/exposure criteria

Diagnostic criteria from 2001 (in black) and changes from Del Brutto et al. (21) (in red). Criteria moved or deleted from the original are in blue. CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay.

using monoclonal antibody-based antigen capturing ELISA (Ag-ELISA) (23, 31). These tests only detect the presence of active viable cysts. In combination with antibody-detecting tests, Ag-ELISA can be used to differentiate between live parasite infections and dead larvae in degenerating cysts (23, 31); high antigen levels are associated with extraparenchymal NCC, whereas low or undetectable antigen levels are associated with intraparenchymal NCC (23, 31).

In the absence of neuroimaging, serological tests can assist in making a diagnosis of extraparenchymal NCC or intraparenchymal NCC; this is critical in low-income country settings where neuroimaging is not readily available outside of major hospitals. However, lower-cost diagnostic tools are desperately needed for LMIC for which the infection is endemic. ELISA kits vary from US$5 to US$30 per sample, and cross-react with echinococcosis where the diseases are co-endemic (32, 33). EITB tests range from US$22 to US$100 but can cost as much as US$347 per sample (34).

Neuroimaging

Neuroimaging is the gold standard for NCC diagnosis, but in many areas endemic for NCC, this technology is either unavailable or prohibitively expensive. Magnetic resonance imaging (MRI) or computed tomography (CT) is used to visualize cysticerci in the CNS, providing evidence of the number of cysts, topography of lesions, stage of evolution of the cyst, and assessment of the level of the host's inflammatory reaction against parasites. Where available, CT scanning is the most common imaging tool used for diagnosis, especially in developing countries; however, CT is less effective than MRI at identifying intraventricular cysts, which comprise up to 22% of all NCC cases (35, 36).

In 2017, Del Brutto et al. revised their diagnostic criteria for NCC to include neuroimaging with a view to eliminating false-positive diagnoses in endemic areas (from serological examinations) and increase diagnosis in non-endemic areas where NCC is often overlooked (21). The revised diagnostic criteria determine that NCC cannot be definitively diagnosed without neuroimaging, and that for a definitive NCC diagnosis, the tapeworm scolex (head) should be visible on the scan (21, 37). However, neuroimaging is unavailable in many endemic areas, training of radiologists for correct interpretations of the scans can be problematic in developing countries, and the high cost of imaging precludes initial and sequential scans.

TREATMENT

Treatment options include destroying the cysts using chemotherapy, surgically removing the cysts, and/or application of symptomatic treatment (with or without removal of cysts). Normally, therapy involves the administration of a combination of cysticidal drugs and drugs to alleviate symptoms (38).

Chemotherapy

The anthelmintic drugs praziquantel and albendazole have been routinely used to control schistosomiasis, cysticercosis, and intestinal nematodes for over 30 years (39). NCC can be treated

TABLE 2 | Definitive diagnostic criteria for symptomatic neurocysticercosis by Carpio et al. (22).

Parenchymal neurocysticercosis
Definitive parenchymal neurocysticercosis, one of the following:
1. Parenchymal cyst with pathological diagnosis
2. Single or multiple active parenchymal cysts, with at least one cyst with scolex on CT or MRI
3. Multiple parenchymal vesicles without scolex associated with at least one of the following:
a. Seizures: focal or generalized tonic–clonic
b. Positive serum or CSF immunological test (ELISA, EITB)
4. Any combination of the parenchymal cysticercus in different evolutive stages: vesicular with or without scolex, degenerative (colloidal or nodular), and calcified
Probable parenchymal neurocysticercosis, one of the following:
1. Single parenchymal calcification or vesicle (without scolex) or degenerating cyst(s), establishing differential diagnoses with other etiologies, associated with at least two of the following:
a. Seizures: focal or generalized tonic–clonic
b. Subcutaneous or muscle cysts location confirmed by biopsy
c. Positive serum or CSF immunological test (ELISA, EITB)
d. Plain X-ray films showing "cigar-shaped" calcifications
e. Individual who lives or has lived in or has traveled frequently to endemic countries
2. Multiple parenchymal calcifications in an individual who lives or has lived in or has traveled frequently to endemic countries and in whom clinical state excludes other etiologies of calcifications
Extraparenchymal neurocysticercosis (intraventricular/basal subarachnoid)
Definitive extraparenchymal neurocysticercosis, one of the following:
1. Extraparenchymal cyst with pathological diagnosis
2. One or more extraparenchymal cysts on MRI special sequences with scolex in at least one of them
3. One or more extraparenchymal cysts on MRI special sequences without scolex associated with at least two of the following:
a. Hydrocephalus
b. Inflammatory CSF
c. Positive CSF immunological test (ELISA, EITB)
d. Presence of single or multiple calcifications or parenchymal vesicular or degenerative cyst
Parenchymal and extraparenchymal neurocysticercosis
Combination of the above definitive parenchymal and definitive extraparenchymal criteria

CT, computed tomography; MRI, magnetic resonance imaging; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; EITB, enzyme-linked immunoelectrotransfer blot.

using albendazole and/or praziquantel, and while there have been changes to recommended dosages, these remain the only drugs available for NCC treatment. Albendazole cannot be taken by pregnant women but can be given in smaller doses to children over the age of 2 (40). Praziquantel can be used by both pregnant women and children and is the preferred treatment for pregnant women (40). Neither are 100% effective due to poor absorption; praziquantel has an oral absorption rate of 80%, whereas albendazole has an oral absorption rate of less than 5% (although this increases to up to 25% if taken with a high-fat meal) (2, 9).

Praziquantel is commonly prescribed at a dosage of 50 mg/kg/day for 10–14 days (6); it is rapidly absorbed (26, 41). Albendazole is typically given as 15 mg/kg/day for 10–14 days (26). In the event of severe disease and for some parenchymal cases, an extended treatment of 30 days of albendazole may be required. In comparative clinical trials, albendazole was equivalent or superior to praziquantel in terms of reduction of live cysticerci (26). Treatment with albendazole at 15 mg/kg/day for 10 days, with 6 mg dose of dexamethasone to reduce inflammation, was shown to reduce the frequency of generalized seizures over 30 months following treatment (2).

Albendazole penetrates the CSF more efficiently than praziquantel (6) and is more effective against extraparenchymal forms and is prescribed more frequently. In some patients, these drugs may exacerbate the symptom of intracranial hypertension with cysticercotic encephalitis (42).

Treating with a combination of the two drugs may be optimal in some cases, since some patients respond better to one drug or the other (2, 43). Garcia and Del Brutto found that in patients with multiple brain cysts, treating with albendazole and praziquantel increased cysticidal effects without potentiating drug-induced side effects (44). Routine dual prescribing may, however, contribute to the risk of development of anthelmintic resistance (AR).

Complications of Chemotherapy

When a cyst is destroyed by cysticidal drugs, the resulting inflammatory reaction may be pathogenic, appearing acutely as a brain edema or chronically as a gliotic scar (12). To avoid complications from a rise in intracranial pressure, seizures, or epileptic scar, some clinicians argue against using cysticidal drugs and recommend symptomatic treatment (anti-epileptic drugs) and/or surgery to remove the cysts. Anti-epileptic drugs normally adequately control seizures in patients with calcified cysticerci, whereas mannitol can relieve intracranial pressure (12).

Steroids administered together with cysticidal drugs can suppress the inflammatory response associated with the destruction of viable cysts and control edema that is associated with the lesions (1, 12).

Surgery

Surgery is a recommended treatment for NCC in cases of intraventricular cysts, hydrocephalus, or when the diagnosis

is uncertain from neuroimaging (45). Calcified cysts can be removed by minimally invasive neuroendoscopy prior to the administration of cysticidal drugs as the drugs may cause the cysts to rupture and create an inflammatory response to impair removal (21), and/or a ventricular shunt can be inserted to reduce intracranial pressure (45, 46). Limited data exist as to the number of surgeries performed annually to remove the cysts; whether this is due to poor reporting record or a lack of emphasis to report remains unclear.

THE FUTURE FOR DIAGNOSIS, TREATMENT, AND PREVENTION

Diagnosis

Simple, cheap, and effective diagnostic tools are needed to identify infections and at-risk groups and communities. Toribio et al. have demonstrated the viability of extracting *T. solium* DNA in patients' urine, confirmed with positive EITB results for the presence of anti-*T. solium* antibodies in all subarachnoid and patients with viable parenchymal cysts. The sensitivity of the urine test is, however, dependent on infection load, and similar to all serological tests, it cannot determine whether the cysts are present in the CNS or elsewhere in the body (47).

Portable fluorescent sensors that can detect antibodies and enable results to be captured on a mobile device and reviewed later offer significant benefits for diagnosis and surveillance. These tools can enable the identification of hyperendemic areas to target for control. Being able to make a diagnosis while the patient is still in the vicinity and enabling data to be assimilated for prevalence and control studies would support control efforts (48).

A real-time quantitative polymerase chain reaction (qPCR) test to detect the repetitive Tsol13 sequence within the *T. solium* genome has been shown to be highly sensitive and specific for NCC and can be used as a marker for "cure" in the CSF and for the definitive diagnosis of NCC from plasma samples (49). Out of 18 CSF samples taken from patients with active NCC, all were found positive for *T. solium* DNA using TsolR13 qPCR (49).

Advances in neuroimaging will continue to improve the early diagnosis and treatment of NCC. In Mexico, a population study of 155 apparently asymptomatic, healthy patients underwent MRI scanning, and 9.1% were found to have calcified lesions (50). A new Food and Drug Administration (FDA) approved portable MRI machine offers the opportunity to make MRIs more accessible in hospitals and clinics in LMIC (51).

Treatment

A new delivery system for triclabendazole has been developed for the treatment of trematodes, promoting absorption by encapsulating triclabendazole into nanometer-sized capsules using nanoparticles to increase the drug dissolution rate (52). Similar approaches could be applied to albendazole and praziquantel (52).

A tumor necrosis factor alpha (TNF-α) inhibitor TNF etanercept (ETN) is being trialed to reduce inflammation resulting from TNF-α and other pro-inflammatory cytokines from the administration of cysticidal drugs in NCC patients resulting in fewer symptoms for the patients (12). Anecdotal success has been reported in 16 patients with reductions in corticosteroid usage and decreases in headaches and seizures (53). However, since most patients were taking methotrexate, it is unclear whether ETN alone or used with methotrexate is key to clinical improvement (53).

Avoidance of Drug Resistance

The World Health Organization (WHO) currently recommends the mass drug administration (MDA) of benzimidazoles (albendazole, mebendazole, pyrantel pamoate, and levamisole) for the treatment and control of soil-transmitted helminths (STH) (54). In 2012, a WHO strategic goal aimed to "eliminate soil-transmitted helminthiases as a public health problem in children" by 2020 (55). In STH-endemic countries, school-aged children were to receive treatment at 75% national coverage and 100% geographical coverage, treating children once or twice annually when STH prevalence is $\geq$20 and $<$50 or $\geq$50%, respectively (56), with a single dose of 400 mg of albendazole. Over 385 million school-aged children at risk of STH received treatment in 2016 alone (68% global coverage), double that of 5 years previously (57). Thirty-eight countries reached their target of 75% coverage (58).

Similarly, the MDA for control of human schistosomiasis aims to prevent morbidity by regular treatment with praziquantel, the only recommended drug for the treatment of human schistosomiasis. In 2018, over 95.3 million people, 87.6% of doses delivered in sub-Saharan Africa, were treated for schistosomiasis (59). Mass treatment is targeted at high-risk groups dependent on the prevalence of infection. Praziquantel is deemed safe in pregnancy, and it is recommended that women, and adolescent girls of child-bearing age, be included in public health interventions.

While the MDA has resulted in considerable progress in the control of STH and schistosomiasis, it has the possibility to drive the potential emergence of AR. While the development and spread of AR in human helminths and the loss of efficacy of albendazole and praziquantel have not yet been confirmed, AR in livestock helminths is widespread (60). Many factors have driven the emergence of AR in animals (**Table 3**) (61–68), and given the limited drugs available to treat tapeworms in humans, AR remains a potential risk for sustainable chemotherapy, with indefinite rollout of mass chemotherapy.

Prevention

Prevention of infection is key for success in the management of cysticercosis. Pig-keeping has been promoted as a route out of poverty in LMIC. The large increase in pigs being kept in rural communities has not been matched by efforts to help smallholder farmers keep their pigs healthy. In Tanzania, pig production is one of the fastest-growing livestock sectors, with more than 7% of smallholder famers keeping pigs (69). Pigs are considered by poor farmers in LMIC to require minimum inputs, and farmers see opportunity in keeping free-roaming pigs within the community. To raise healthy pigs, significant veterinary inputs are needed including anthelmintics to break the cycle of *T. solium* transmission between pigs and humans (70). Much can be achieved by cessation of open defecation and penning pigs to

TABLE 3 | Factors influencing the emergence of anthelmintic resistance in livestock.

Factor	Description	Livestock	Humans
Treatment frequency	The greater the frequency, the greater the drug pressure and risk for resistance	5–10 treatments per year (61)	1–3 treatments per year (62, 63). Selection for resistance in goats and sheep at these treatment frequencies (64, 65)
Refugia	Proportion of the parasite population free from being exposed to the drug (66)	Can delay emergence of anthelmintic resistance by leaving some animals untreated (1–4% of adult stock) (67)	MDA only targets children, with coverage less than 80%. Adults have the highest worm burdens. Treatment is given during dry seasons, when the proportion of living parasites in the soil is low, for logistical reasons, thereby reducing refugia. As treatment coverage increases for the 2020 goal, the proportion of children treated rises (68).
Under-dosing	Specific dose regimens have different effects on resistance allele frequency, depending on prior frequency of resistance allele in pre-drug parasite population.	Drug efficacy is very high (ERR >99%). Dosing is well-controlled.	Sub-optimal efficacy, never achieve 100% cure. Drugs are often shared among poor families, produced at substandard qualities, and even sold past their expiry date. This could either aid the development of AR (allow for the survival of resistant strains) or delay it (lower drug pressure) (68).

ERR, egg reduction rate; AR, anthelmintic resistance.

prevent them from eating feces, and with health education (70–72). Raising free-range pigs is linked to not only *T. solium* and cysticercosis but also other zoonotic diseases, such as ascariasis and hepatitis E, which trap poor populations in poverty (73).

Vaccination and Cysticidal Drugs for Pigs

A vaccine for use in pigs against *T. solium*, TSOL18 (Cysvax), has been registered for use in India since 2016 and is undergoing registration in Tanzania, Uganda, South Africa, West Africa, Kenya, Nepal, Philippines, Thailand, and Sri Lanka (73, 74). Cysvax can provide 99.5% protection against porcine cysticercosis, and when combined with the anthelmintic drug, oxfendazole to deworm the pigs, protection can be increased to 99.7% effectiveness (74–76). Importantly, this treatment does not make the pork unfit for human consumption (74, 77, 78). A second vaccine against *T. solium*, SP3VAC, and a modified parenterally administered SP3vac-phage version have undergone trials in Mexico but are yet not available for commercial use. These vaccines all require two doses, and since no vaccine currently treats existing cysts, they would require application of oxfendazole to have "viable cyst"-free pigs (79–81).

Meat Inspection

Lingual palpation (or tongue inspection) in pigs and meat inspection are used to detect cysticerci in pork, but only around 21% of infected pigs will be detected by lingual examination alone. Depending on infection load and inspection practice, infected pork can easily pass into the food chain. Home-slaughtered pigs and lack of farmer knowledge of the zoonotic risk from *T. solium* heighten and exacerbate the risk in the community. Meester et al. showed that home-slaughtered pigs were 13 times more likely to be contaminated than commercially slaughtered pigs, regardless of the country of origin (82).

Kenya has had a law in place for meat inspection since 1977; all meat must be inspected by ministry officials prior to leaving the slaughterhouse. If cysticerci are found, the pig carcass is condemned, and the meat cannot be sold (83). In one study, inspectors reported that all pigs leaving the slaughterhouse had been inspected; however, no inspectors were visible at the facility, and it was not possible to confirm any inspections having been completed (84). This example shows how contaminated meat can enter the food chain, putting consumers at risk despite legislation being in place.

Communication and Health Messaging

Most NTDs affect poor people in communities that are poorly served by both medical and veterinary services. Health messaging is challenging in resource-poor communities and particularly challenging for neglected zoonotic diseases. Control tools designed to prevent zoonotic disease transmission for uptake and adoption in the community demand a comprehensive understanding of how the affected community members perceive the disease. This does not necessarily require an explanation of the complex disease causation. Knowledge within communities is also patchy. Communities may erroneously relate epilepsy to witchcraft but correctly associate the presence of white nodules in pigs to bad practice in pig husbandry. Studies in Zambia showed that while some village inhabitants were aware that eating pork containing cysts was unhealthy and could cause disease, other individuals from the same villages saw nothing wrong with eating infected meat, arguing that the cysts gave a satisfying "burst in the mouth feel" (73). Focus group discussions with women in villages endemic for *T. solium* in Zambia showed that the women were aware that pigs brought diseases and worms and especially that pigs ate feces; however, despite this knowledge, pigs were allowed to be predominantly free ranging within the village (73, 85).

In contrast, studies in Mozambique showed that only 17.4% of households were aware how pigs acquired the *T. solium* infection (73, 85).

Indirect approaches to prevent open defecation have contributed to community approaches for control of *T. solium* infection. The Community Led Total Sanitation (CLTS) program (https://www.communityledtotalsanitation.org/) focuses on introducing behavioral change, essentially shocking communities into an awareness of fecal contamination in their environment, leading communities to a point where they decide freely that they want to become "Open Defecation Free" (86, 87). The CLTS as a standalone intervention, to prevent pigs from being able to eat human stools in the environment, was not particularly successful in Zambia. There was a significant increase in latrines (31%), but many villages failed to eliminate open defecation practices (86); the study focused on the prevalence of porcine cysticercosis before and after the CLTS implementation but did not address why villages in the study area continued the practice of open defecation. When combined with other interventions, the CLTS is likely to show benefits and is one piece in the elimination toolbox, in addition to improved pig husbandry, training and education programs, vaccines, and MDA. More anthropological studies will be needed to gain a comprehensive understanding of cultural taboos on latrine use and how to make interventions more appealing to communities (86).

For cysticercosis and NCC, educational messages and materials that explain that disease in pigs comes from humans and that preventing pigs from eating human feces can interrupt disease transmission are needed, rather than attempts to explain the complex life cycle and disease epidemiology of *T. solium*. Communication is key, and affected communities have been shown to understand and adopt the message that pigs eat stools and people eat pigs. The computer-based tale of the "The Vicious Worm" (https://theviciousworm.sites.ku.dk/) reinforces this very simple message and has been highly effective with individuals completing the program, achieving an average score of 71% in knowledge, after 1 year of follow-up (88).

Advocacy

Neglected zoonotic diseases are predominately diseases of the poorest populations, living in close contact with domestic animals, on which they are dependent, in communities often lacking adequate health care for humans and animals (8). In 2014, WHO's 4th annual meeting on Control of Neglected Zoonotic Disease stated that the tools to eliminate cysticercosis were in place; however, no country endemic for cysticercosis has been able to eliminate the disease (89). There has been some progress; in 2000, there were 3,362,000 DALYs for cysticercosis (including NCC), and by 2016, this had reduced to 1,912,000 (90). However, the ambitious targets set within the 2012 WHO NTD roadmap have, unfortunately, not been met.

An elimination study in Peru showed the efficacy of the MDA in both humans and pigs for elimination of taeniasis/cysticercosis/NCC but also showed that 90% coverage was needed in both pigs and humans to prevent transmission (91). The MDA narrative offers policy makers a relatively straightforward solution to a complex zoonotic disease that requires addressing issues of clean water, adequate latrines, and pig husbandry.

Addressing NTDs has contributed to alleviating the human and economic burden they impose on the world's poorest communities. NTD interventions offer one of the best buys in global public health, and NTDs serve as an important indicator for identifying disparities in progress toward both universal health coverage and equitable access to high-quality health services. Against a backdrop of large investments in de-worming for NTDs in humans (schistosomiasis, filariasis, and STH) (38, 52), the lack of advocacy to support the prioritization of vaccination of pigs is of concern if cysticercosis and NCC are to be eliminated. Albendazole and praziquantel have been extensively applied for the MDA programs for NTD control for schistosomiasis and STH (36, 50). Deworm the World (https://www.evidenceaction.org/dewormtheworld/), Children without Worms (http://www.childrenwithoutworms.org), Schistosomiasis Control Initiative (https://schistosomiasiscontrolinitiative.org), and Global Programme to Eliminate Lymphatic Filariasis (GPELF) (https://www.who.int/lymphatic_filariasis/elimination-programme/en/) are just a few of the examples of the many deworming programs being run around the world.

On January 28, 2021, WHO will launch its road map for the NTDs' "Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021–2030" (92). The road map sets global targets for 2030 and includes milestones and strategies for prevention, control, elimination, and eradication of 20 diseases and disease groups and cross-cutting targets broadly aligned to the Sustainable Development Goals (SDG's). Taeniasis/cysticercosis has been targeted for control; success is defined as a steady increase in the number of countries with intensified control in hyperendemic areas, increasing from 2 (3%) in 2020 to 4 (6%) in 2023, to 9 (14%) by 2025, and to 17 (27%) by 2030. Cross-cutting targets that will impact on taeniasis/cysticercosis include the target for 100% access to at least basic water supply, sanitation, and hygiene in areas endemic for NTDs and 75% integrated treatment coverage for preventative chemotherapy. The goals also seek to achieve 90% of countries reporting on all their relevant NTDs. By moving from single-disease vertical programs to integrated approaches, it aims to promote improved coordination and collaboration. The overarching 2030 global targets are to reduce by 90% the number of people requiring treatment for NTDs, eliminate at least one NTD in 100 countries, and reduce by 75% the DALYs related to NTDs (90).

Control of taeniasis/cysticercosis/NCC demands a One Health approach from multiple stakeholders, in that the MDA in both humans and pigs, vaccination of pigs, and clean water, latrines, and community education will all be needed to effectively eliminate the infection. *T. solium* transmission dynamics models can contribute to this process including CystiSim and EPICYST (93). For the ambitious goals for 2030 to be met, there is a need for greater understanding of the underlying spatial epidemiology, the socio-economic drivers for pig-keeping, and

social, individual, behavioral, and community perception of these neglected infections.

AUTHOR CONTRIBUTIONS

CB was responsible for conception, assimilation of works, and drafting of the paper. TB was responsible for examination of anthelmintic resistance. AM and SW were involved in the conception and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Gripper LB, Welburn SC. Neurocysticercosis infection and disease–A review. *Acta Trop*. (2017) 166:218–24. doi: 10.1016/j.actatropica.2016.11.015
2. Prevention C-C for DC. *CDC - Cysticercosis - Resources for Health Professionals*. (2019). Available online at: https://www.cdc.gov/parasites/cysticercosis/health_professionals/index.html
3. Ndimubanzi PC, Carabin H, Budke CM, Nguyen H, Qian Y-J, Rainwater E, et al. A systematic review of the frequency of neurocyticercosis with a focus on people with epilepsy. *PLoS Negl Trop Dis*. (2010) 4:e870. doi: 10.1371/journal.pntd.0000870
4. Gripper LB, Welburn SC. The causal relationship between neurocysticercosis infection and the development of epilepsy - a systematic review. *Infect Dis Poverty*. (2017) 6:31. doi: 10.1186/s40249-017-0245-y
5. Lè Ne Carabin H, Ndimubanzi PC, Budke CM, Nguyen H, Qian Y, Cowan LD, et al. Clinical manifestations associated with neurocysticercosis: a systematic review. *PLoS Negl Trop Dis*. (2011) 5:e1152. doi: 10.1371/journal.pntd.0001152
6. Matthaiou DK, Panos G, Adamidi ES, Falagas ME. Albendazole versus praziquantel in the treatment of neurocysticercosis: a meta-analysis of comparative trials. *PLoS Negl Trop Dis*. (2008) 2:e194. doi: 10.1371/journal.pntd.0000194
7. Maudlin I, Eisler MC, Welburn SC. Neglected endemic zoonoses. *Philos Trans R Soc B Biol Sci*. (2009) 364:2777–87. doi: 10.1098/rstb.2009.0067
8. Welburn SC, Beange I, Ducrotoy MJ, Okello AL. The neglected zoonoses-the case for integrated control and advocacy. *Clin Microbiol Infect*. (2015) 21:433–43. doi: 10.1016/j.cmi.2015.04.011
9. Fogang YF, Savadogo AA, Camara M, Toffa DH, Basse A, Sow AD, et al. Managing neurocysticercosis: challenges and solutions. *Int J Gen Med*. (2015) 8:333–44. doi: 10.2147/IJGM.S73249
10. Singh BBB, Khatkar MSS, Gill JPSPS, Dhand NKK. Estimation of the health and economic burden of neurocysticercosis in India. *Acta Trop*. (2017) 165:161–9. doi: 10.1016/j.actatropica.2016.01.017
11. Praet N, Speybroeck N, Manzanedo R, Berkvens D, Nsame Nforninwe D, Zoli A, et al. The disease burden of *Taenia solium* cysticercosis in Cameroon. *PLoS Negl Trop Dis*. (2009) 3:e406. doi: 10.1371/journal.pntd.0000406
12. Garcia HH, Nash TE, Del Brutto OH. Clinical symptoms, diagnosis, and treatment of neurocysticercosis. *Lancet Neurol*. (2014) 13:1202–15. doi: 10.1016/S1474-4422(14)70094-8
13. Vos T, Barber RM, Bell B, Bertozzi-Villa A, Biryukov S, Bolliger I, et al. Global, regional, national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet*. (2015) 386:743–800. doi: 10.1016/S0140-6736(15)60692-4
14. Sierra MM, Arroyo M, Torres MC, Ramírez Cruz N, García Herná Ndez F, Taboada D, et al. Extraparenchymal neurocysticercosis: demographic, clinicoradiological, inflammatory features. *PLoS Negl Trop Dis* 11:e0005646. doi: 10.1371/journal.pntd.0005646
15. Del Brutto OH. Neurocysticercosis. *Handb Clin Neurol*. (2014) 121:1445–59. doi: 10.1016/B978-0-7020-4088-7.00097-3
16. Del Brutto OH. Twenty-five years of evolution of standard diagnostic criteria for neurocysticercosis. How have they impacted diagnosis and patient outcomes? *Expert Rev Neurother*. (2020) 20:147–55. doi: 10.1080/14737175.2020.1707667
17. Del Brutto OH. Current approaches to cysticidal drug therapy for neurocysticercosis. *Expert Rev Anti Infect Ther*. (2020) 18:789–98. doi: 10.1080/14787210.2020.1761332
18. Santo AH. Cysticercosis-related mortality in the State of São Paulo, Brazil, 1985-2004: a study using multiple causes of death. *Cad Saude Publ*. (2007) 23:2917–27. doi: 10.1590/S0102-311X2007001200013
19. Sorvillo FJ, DeGiorgio C, Waterman SH. Deaths from cysticercosis, United States. *Emerg Infect Dis*. (2007) 13:230–5. doi: 10.3201/eid1302.060527
20. Del Brutto O, Rajshekhar V, White A Jr, Tsang V, Nash T, Takayanagui O, et al. Proposed diagnostic criteria for neurocysticercosis Europe PMC Funders Group. *Neurology*. (2001) 57:177–83. doi: 10.1212/WNL.57.2.177
21. Del Brutto OH, Nash TE, White AC, Rajshekhar V, Wilkins PP, Singh G, et al. Revised diagnostic criteria for neurocysticercosis. *J Neurol Sci*. (2017) 372:202–10. doi: 10.1016/j.jns.2016.11.045
22. Carpio A, Fleury A, Romo ML, Abraham R, Fandiño J, Durán JC, et al. New diagnostic criteria for neurocysticercosis: reliability and validity. *Ann Neurol*. (2016) 80:434–42. doi: 10.1002/ana.24732
23. Rodriguez S, Dorny P, Tsang VCW, Pretell EJ, Brandt J, Lescano AG, et al. Detection of *Taenia solium* antigens and anti-*T. solium* antibodies in paired serum and cerebrospinal fluid samples from patients with intraparenchymal or extraparenchymal neurocysticercosis. *J Infect Dis*. (2009) 199:1345–52. doi: 10.1086/597757
24. Bustos JA, Rodriguez S, Jimenez JA, Moyano LM, Castillo Y, Ayvar V, et al. Detection of *Taenia solium* taeniasis coproantigen is an early indicator of treatment failure for taeniasis. *Clin Vaccine Immunol*. (2012) 19:570–3. doi: 10.1128/CVI.05428-11
25. Tsang VCW, Brand JA, Boyer AE. An enzyme-linked immunoelectrotransfer blot assay and glycoprotein antigens for diagnosing human cysticercosis (*Taenia solium*). *J Infect Dis*. (1989) 159:50–9. doi: 10.1093/infdis/159.1.50
26. White AC, Coyle CM, Rajshekhar V, Singh G, Hauser WA, Mohanty A, et al. Diagnosis and treatment of neurocysticercosis: 2017 clinical practice guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). *Am J Trop Med Hyg*. (2018) 98:945–66. doi: 10.1093/cid/cix1084
27. Arroyo G, Rodriguez S, Lescano AG, Alroy KA, Bustos JA, Santivañez S, et al. Antibody banding patterns of the enzyme-linked immunoelectrotransfer blot and brain imaging findings in patients with neurocysticercosis. *Clin Infect Dis*. (2018) 66:282–8. doi: 10.1093/cid/cix774
28. Carod JF, Randrianarison M, Razafimahefa J, Ramahefarisoa RM, Rakotondrazaka M, Debruyne M, et al. Evaluation of the performance of 5 commercialized enzyme immunoassays for the detection of *Taenia solium* antibodies and for the diagnosis of neurocysticercosis. *Diagn Microbiol Infect Dis*. (2012) 72:85–9. doi: 10.1016/j.diagmicrobio.2011.09.014
29. Brandt JRA, Geerts S, Deken RD, Kumar V, Ceulemans F, Brijs L, et al. A monoclonal antibody-based ELISA for the detection of circulating excretory-secretory antigens in *Taenia saginata* cysticercosis. *Int J Parasitol*. (1992) 22:471–7. doi: 10.1016/0020-7519(92)90148-E
30. Fleury A, Garcia E, Hernández M, Carrillo R, Govezensky T, Fragoso G, et al. Neurocysticercosis: HP10 antigen detection is useful for

the follow-up of the severe patients. *PLoS Negl Trop Dis*. (2013) 7:e2096. doi: 10.1371/journal.pntd.0002096
31. Bustos JA, Ninaquispe BE, Rodriguez S, Castillo Y, Yang SY, Gilman RH, et al. Performance of a sandwich antigen-detection ELISA for the diagnosis of Porcine *Taenia solium* cysticercosis. *Am J Trop Med Hyg*. (2019) 100:604–8. doi: 10.4269/ajtmh.18-0697
32. Muro C, Gomez-Puerta LA, Flecker RH, Gamboa R, Barreto PV, Dorny P, et al. Porcine cysticercosis: possible cross-reactivity of *Taenia hydatigena* to GP50 antigen in the enzyme-linked immunoelectrotransfer blot assay. *Am J Trop Med Hyg*. (2017) 97:1830–2. doi: 10.4269/ajtmh.17-0378
33. *Human TNF alpha Antibody Pair - BSA and Azide free (ab241791) | Abcam*. (2019). Available online at: https://www.abcam.com/human-tnf-alpha-antibody-pair-bsa-and-azide-free-ab241791.html
34. Lightowlers MW, Garcia HH, Gauci CG, Donadeu M, Abela-Ridder B. Monitoring the outcomes of interventions against *Taenia solium*: options and suggestions. *Parasite Immunol*. (2016) 38:158–69. doi: 10.1111/pim.12291
35. Zhao J-L, Lerner A, Shu Z, Gao X-J, Zee C-S. Imaging spectrum of neurocysticercosis. *Radiol Infect Dis*. (2015) 1:94–102. doi: 10.1016/j.jrid.2014.12.001
36. Lerner A, Shiroishi MS, Zee CS, Law M, Go JL. Imaging of neurocysticercosis. *Neuroimaging Clin N Am*. (2012) 22:659–76. doi: 10.1016/j.nic.2012.05.004
37. Carpio A, Romo ML. State of the art in neurocysticercosis: imaging and epidemiology. *Asian Pac J Trop Med*. (2016) 9:821–2. doi: 10.1016/j.apjtm.2016.06.010
38. Wang W, Garcia HH, Gonzales I, Bustos JA, Saavedra H, Gavidia M, et al. Combined antiparasitic treatment for neurocysticercosis. *Lancet Infect Dis*. (2015) 15:265. doi: 10.1016/S1473-3099(15)70047-2
39. Albanese G, Venturi C. Albendazole: a new drug for human parasitoses. *Dermatol Clin*. (2003) 21:283–90. doi: 10.1016/S0733-8635(02)00085-2
40. Merck Manuals. (2019). Available online at: https://www.merckmanuals.com/professional/resources/brand-names-of-some-commonly-used-drugs
41. Osorio R, Carrillo-Mezo R, Romo ML, Toledo A, Matus C, González-Hernández I, et al. Factors associated with cysticidal treatment response in extraparenchymal neurocysticercosis. *J Clin Pharmacol*. (2019) 59:548–56. doi: 10.1002/jcph.1346
42. Papich MG. Albendazole. In: *Saunders Handbook of Veterinary Drugs* Elsevier. (2016). p. 12–4. Available online at: https://linkinghub.elsevier.com/retrieve/pii/B9780323244855000656
43. Garcia HH, Gonzales I, Lescano AG, Bustos JA, Zimic M, Escalante D, et al. Efficacy of combined antiparasitic therapy with praziquantel and albendazole for neurocysticercosis: a double-blind, randomised controlled trial. *Lancet Infect Dis*. (2014) 14:687–95. doi: 10.1016/S1473-3099(14)70779-0
44. Garcia HH, Del Brutto OH, Cysticercosis Working Group in Peru. Antiparasitic treatment of neurocysticercosis - The effect of cyst destruction in seizure evolution. *Epilepsy Behav*. (2017) 76:158–62. doi: 10.1016/j.yebeh.2017.03.013
45. Rajshekhar V. Surgical management of neurocysticercosis. *Int J Surg*. (2010) 8:100–4. doi: 10.1016/j.ijsu.2009.12.006
46. Torres-Corzo JG, Islas-Aguilar MA, Cervantes DS, Chalita-Williams JC. The role of flexible neuroendoscopy in spinal neurocysticercosis: technical note and report of 3 cases. *World Neurosurg*. (2019) 130:77–83. doi: 10.1016/j.wneu.2019.06.194
47. Toribio L, Romano M, Scott AL, Gonzales I, Saavedra H, Garcia HH, et al. Detection of *Taenia solium* DNA in the urine of neurocysticercosis patients. *Am J Trop Med Hyg*. (2019) 100:327–9. doi: 10.4269/ajtmh.18-0706
48. Lee C, Noh J, O'Neal SE, Gonzalez AE, Garcia HH, Handali S. Feasibility of a point-of-care test based on quantum dots with a mobile phone reader for detection of antibody responses. *PLoS Negl Trop Dis*. (2019) 13:e0007746. doi: 10.1371/journal.pntd.0007746
49. O'Connell EM, Harrison S, Dahlstrom E, Nash T, Nutman TB. A novel, highly sensitive qPCR assay for the diagnosis of subarachnoid and ventricular neurocysticercosis and for assessing response to treatment. *Clin Infect Dis*. (2019) 70:1875–81. doi: 10.1093/cid/ciz541
50. Fleury A, Gomez T, Alvarez I, Meza D, Huerta M, Chavarria A, et al. High prevalence of calcified silent neurocysticercosis in a rural village of Mexico. *Neuroepidemiology*. (2003) 22:139–45. doi: 10.1159/000068748
51. Wald LL, McDaniel PC, Witzel T, Stockmann JP, Cooley CZ. Low-cost portable MRI. *J Magn Reson Imaging*. (2020) 52:686–96. doi: 10.1002/jmri.26942
52. Bergquist R, Utzinger J, Keiser J. Controlling schistosomiasis with praziquantel: how much longer without a viable alternative? *Infect Dis Poverty*. (2017) 6:74. doi: 10.1186/s40249-017-0286-2
53. Nash TE, Ware JM, Coyle CM, Mahanty S. Etanercept to control inflammation in the treatment of complicated neurocysticercosis. *Am J Trop Med Hyg*. (2018) 100:609–16. doi: 10.4269/ajtmh.18-0795
54. World Health Organization. *19th WHO Model List of Essential Medicines*. (2015). Available online at: http://WwwWhoInt/Medicines/Publications/Essentialmedicines/En
55. World Health Organization. Soil-transmitted helminthiases: eliminating soil-transmitted helmnthiases as a public health problem in children. *Prog Rep*. (2012) 1–90.
56. World Health Organization. Prevention and control of schistosomiasis and soil-transmitted helminthiasis. *World Health Organ Tech Rep Ser*. (2002) 912:i–vi, 1–57.
57. World Health Organization. *Integrating Neglected Tropical Diseases Into Global Health and Development : Fourth WHO Report on Neglected Tropical Diseases*. Department of Control of Neglected Tropical Diseases. (2017) p. 270.
58. WHO. *Soil-Transmitted Helminthiases*. WHO. (2017). Available online at: https://www.who.int/gho/neglected_diseases/soil_transmitted_helminthiases/en/
59. WHO. *Schistosomiasis and soil-Transmitted Helminthiases: Number of People Treated in 2018*. Available online at: https://www.who.int/publications/i/item/who-wer9450
60. Sutherland IA, Leathwick DM. Anthelmintic resistance in nematode parasites of cattle: a global issue? *Trends Parasitol*. (2011) 27:176–81. doi: 10.1016/j.pt.2010.11.008
61. Dorny P, Claerebout E, Vercruysse J, Sani R, Jalila A. Anthelmintic resistance in goats in peninsular Malaysia. *Vet Parasitol*. (1994) 55:327–42. doi: 10.1016/0304-4017(94)90073-6
62. Warren K, Bundy DAP, Anderson RM, Davis AR, Henderson DA, Jamison DT, et al. Helminth infection. In: Jamison D, Mosley W, Measham AR, Bobadilla J, editors. *Disease Control Priorities in Developing Countries*. New York, NY: Oxford University Press (1993). p. 131–60.
63. Renganathan E, Ercole E, Albonico M, De Gregorio G, Alawi KS, Kisumku UM, et al. Evolution of operational research studies and development of a national control strategy against intestinal helminths in Pemba Island, 1988-92. *Bull World Health Organ*. (1995) 73:183–90.
64. Coles GC. Chemotherapy of human nematodes: learning from the problems in sheep. *J R Soc Med*. (1995) 88:649–51.
65. Boudsocq A, Chartier C, Cabaret J. Breeding management and development of benzimidazole resistance on goat nematode species diversity. In: *WAAVP 17th International Conference*. Copenhagen: Abstract Book, A107 (1999).
66. Van Wyk JA. Refugia–overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. *Onderstpoort J Vet Res*. (2001) 68:55–67.
67. Dobson R, Barnes E, Tyrrell K, Hosking B, Larsen J, Besier R, et al. A multi-species model to assess the effect of refugia on worm control and anthelmintic resistance in sheep grazing systems. *Aust Vet J*. (2011) 89:200–8. doi: 10.1111/j.1751-0813.2011.00719.x
68. Vercruysse J, Albonico M, Behnke JM, Kotze AC, Prichard RK, McCarthy JS, et al. Is anthelmintic resistance a concern for the control of human soil-transmitted helminths? *Int J Parasitol Drugs Drug Resist*. (2011) 1:14–27. doi: 10.1016/j.ijpddr.2011.09.002
69. Trevisan C, Devleesschauwer B, Schmidt V, Winkler AS, Harrison W, Johansen MV, et al. The societal cost of *Taenia solium* cysticercosis in Tanzania. *Acta Trop*. (2017) 165:141–54. doi: 10.1016/j.actatropica.2015.12.021
70. Hobbs EC, Mwape KE, Phiri AM, Mambwe M, Mambo R, Thys S, et al. Perceptions and acceptability of piloted *Taenia solium* control and elimination interventions in two endemic communities in eastern Zambia. *Transbound Emerg Dis*. (2019) 67(Suppl. 2):69–81. doi: 10.1111/tbed.13214
71. Mwanjali G, Kihamia C, Kakoko DVC, Lekule F, Ngowi H, Johansen MV, et al. Prevalence and risk factors associated with human *Taenia solium* infections

in Mbozi District, Mbeya Region, Tanzania. *PLoS Negl Trop Dis*. (2013) 7:e2102. doi: 10.1371/journal.pntd.0002102
72. Okello AL, Gibbs EPJ, Vandersmissen A, Welburn SC. One Health and the neglected zoonoses: turning rhetoric into reality. *Vet Rec*. (2011) 169:281–5. doi: 10.1136/vr.d5378
73. Thys S, Mwape KE, Lefèvre P, Dorny P, Phiri AM, Marcotty T, et al. Why pigs are free-roaming: Communities' perceptions, knowledge and practices regarding pig management and taeniosis/cysticercosis in a *Taenia solium* endemic rural area in Eastern Zambia. *Vet Parasitol*. (2016) 225:33–42. doi: 10.1016/j.vetpar.2016.05.029
74. WHO. *First Ever Licensed Vaccine and Anthelmintic Against the Major Cause of Epilepsy in the Developing World*. WHO (2017). Available online at: https://www.galvmed.org/news/first-ever-licenced-vaccine-major-cause-epilepsy-developing-world/
75. Gilman RH, Bernal T, Tsang VC, Gonzalez AE, Gavidia C, Garcia HH, et al. Time-response curve of oxfendazole in the treatment of swine cysticercosis. *Am J Trop Med Hyg*. (1998) 59:832–6. doi: 10.4269/ajtmh.1998.59.832
76. Sánchez-Torres NY, Bobadilla JR, Laclette JP, José MV. How to eliminate taeniasis/cysticercosis: porcine vaccination and human chemotherapy (Part 2). *Theor Biol Med Model*. (2019) 16:4. doi: 10.1186/s12976-019-0100-x
77. Jayashi CM, Kyngdon CT, Gauci CG, Gonzalez AE, Lightowlers MW. Successful immunization of naturally reared pigs against porcine cysticercosis with a recombinant oncosphere antigen vaccine. *Vet Parasitol*. (2012) 188:261–7. doi: 10.1016/j.vetpar.2012.03.055
78. Flisser A, Gauci CG, Zoli A, Martinez-Ocaña J, Garza-Rodriguez A, Dominguez-Alpizar JL, et al. Induction of protection against porcine cysticercosis by vaccination with recombinant oncosphere antigens. *Infect Immun*. (2004) 72:5292–7. doi: 10.1128/IAI.72.9.5292-5297.2004
79. Dorny P, Phiri I, Vercruysse J, Gabriel S, Willingham A, Brandt J, et al. A Bayesian approach for estimating values for prevalence and diagnostic test characteristics of porcine cysticercosis. *Int J Parasitol*. (2004) 34:569–76. doi: 10.1016/j.ijpara.2003.11.014
80. Sciutto E, Rosas G, Hernández M, Morales J, Cruz-Revilla C, Toledo A, et al. Improvement of the synthetic tri-peptide vaccine (S3Pvac) against porcine *Taenia solium* cysticercosis in search of a more effective, inexpensive and manageable vaccine. *Vaccine*. (2007) 25:1368–78. doi: 10.1016/j.vaccine.2006.10.018
81. Morales J, Martínez JJ, Manoutcharian K, Hernández M, Fleury A, Gevorkian G, et al. Inexpensive anti-cysticercosis vaccine: S3Pvac expressed in heat inactivated M13 filamentous phage proves effective against naturally acquired *Taenia solium* porcine cysticercosis. *Vaccine*. (2008) 26:2899–905. doi: 10.1016/j.vaccine.2008.03.042
82. Meester M, Swart A, Deng H, Van Roon A, Trevisan C, Dorny P, et al. A quantitative risk assessment for human *Taenia solium* exposure from home slaughtered pigs in European countries. *Parasit Vectors*. (2019) 12:82. doi: 10.1186/s13071-019-3320-3
83. Thomas LF, Harrison LJS, Toye P, de Glanville WA, Cook EAJ, Wamae CN, et al. Prevalence of *Taenia solium* cysticercosis in pigs entering the food chain in western Kenya. *Trop Anim Health Prod*. (2016) 48:233–8. doi: 10.1007/s11250-015-0949-6
84. Adesokan HK, Adeoye FA. Porcine cysticercosis in slaughtered pigs and factors related to *Taenia solium* transmission amongst abattoir workers in Ibadan, Nigeria. *Pan Afr Med J*. (2019) 32:145. doi: 10.11604/pamj.2019.32.145.10695
85. Mosler H-J, Mosch S, Harter M. Is Community-Led Total Sanitation connected to the rebuilding of latrines? Quantitative evidence from Mozambique. *PLoS ONE*. (2018) 13:e0197483. doi: 10.1371/journal.pone.0197483
86. Bulaya C, Mwape KE, Michelo C, Sikasunge CS, Makungu C, Gabriel S, et al. Preliminary evaluation of Community-Led Total Sanitation for the control of *Taenia solium* cysticercosis in Katete District of Zambia. *Vet Parasitol*. (2015) 207:241–8. doi: 10.1016/j.vetpar.2014.12.030
87. Gebremariam B, Hagos G, Abay M. Assessment of community led total sanitation and hygiene approach on improvement of latrine utilization in Laelay Maichew District, North Ethiopia. A comparative cross-sectional study. *PLoS ONE*. (2018) 13:e0203458. doi: 10.1371/journal.pone.0203458
88. Hobbs EC, Mwape KE, Devleesschauwer B, Van Damme I, Krit M, Berkvens D, et al. Effects of 'The Vicious Worm' educational tool on *Taenia solium* knowledge retention in Zambian primary school students after one year. *PLoS Negl Trop Dis*. (2019) 13:e0007336. doi: 10.1371/journal.pntd.0007336
89. *The Control of Neglected Zoonotic Diseases From Advocacy to Action*. Available online at: https://apps.who.int/iris/bitstream/handle/10665/183458/9789241508568_eng.pdf;jsessionid=A5DDAB6830DD61A746EE223F29FF763F?sequence=1
90. World Health Organization, Food and Agriculture Organization of the United Nations, International Office of Epizootics. *The Control of Neglected Zoonotic Diseases : From Advocacy to Action : Report of the Fourth International Meeting Held at WHO Headquarters*. Geneva (2014). p. 44.
91. Garcia HH, Gonzalez AE, Tsang VCW, O'Neal SE, Llanos-Zavalaga F, Gonzalvez G, et al. Elimination of *Taenia solium* transmission in Northern Peru. *N Engl J Med*. (2016) 374:2335–44. doi: 10.1056/NEJMoa1515520
92. Malecela MN, Ducker C. A road map for neglected tropical diseases 2021–2030. *Trans R Soc Trop Med Hyg*. (2021) 115:121–3. doi: 10.1093/trstmh/trab002
93. Winskill P, Harrison WE, French MD, Dixon MA, Abela-Ridder B, Basáñez MG. Assessing the impact of intervention strategies against *Taenia solium* cysticercosis using the EPICYST transmission model. *Parasit Vectors*. (2017) 10:73. doi: 10.1186/s13071-017-1988-9

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