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Special Issue Reprint

Parasitic Zoonoses

From a Public Health Perspective

Edited by
Márius Vicent Fuentes i Ferrer, María Trelis Villanueva and Sandra Sáez Durán

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Guest Editors

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Preface

Human beings coexist in a close relationship with numerous animals, and this interrelation can be a source of diseases with a marked impact on public health and the social and economic well-being of the world population. These diseases, transmissible from animals to humans, either by direct contact or through contamination of water, food or the environment, are known as zoonoses. In humans, more than 1400 pathogens are known, of which about 900 species are zoonotic, with more than 200 of them being parasitic zoonoses caused by protists, helminths and arthropods; these not only have impacts on health, but also have socioeconomic repercussions, since they also cause diseases in animals. Consequently, the World Health Organization (WHO), in collaboration with the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (WOAH), has established the concept of "One Health" to promote multisectoral responses to food safety hazards, zoonosis risks and other threats to public health stemming from interactions between humans, animals and the ecosystem, and to provide guidance on how to reduce these risks.

This reprint is a compilation of articles published in the Special Issue "Parasitic Zoonoses: from a Public Health Perspective", of the MDPI journal *Animals*, which have focused on several aspects (genetics, molecular biology, histopathology, epidemiology, etc.) of parasitic zoonoses, both protists (*Blastocystis*, *Giardia*, *Toxoplasma* and *Cryptosporidiidum*, etc.) and helminths (*Taenia*, *Rodentolepis*, *Hymenolepis*, *Calodium*, *Gongylonema*, *Dirofilaria*, *Moniliformis*, etc.), in several kinds of animals (rodents, cats, dogs, cattle, sheep, pigs, wild boars, non-human primates, etc.), from various cities and countries (Spain, Italy, the Azores islands, Algeria, Egypt, Mexico, China, etc.), including, also, zoological institutions.

Màrius Vicent Fuentes i Ferrer, María Trelis Villanueva, and Sandra Sáez Durán
Guest Editors

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Article

The Role of the Norway Rat, *Rattus norvegicus*, as a Reservoir of Zoonotic Helminth Species in the City of Barcelona (Spain)

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Simple Summary: Urban rat species, Norway and black rats, are involved in the transmission of several zoonotic pathogens to humans, such as helminth parasites. As part of a multidisciplinary study concerning the rodent population in Barcelona (Spain), 300 specimens of the Norway rat were analyzed to elucidate their role in the transmission of zoonotic helminths. The sample included 263 specimens from the sewage system and 37 from public gardens. A total of 84.3% of the rats analyzed were found parasitized, and 206 (68.7%) harbored zoonotic species. Adult rats were found to be more heavily parasitized with zoonotic helminths than juveniles, but sex and site of capture had no influence. Six zoonotic helminths were identified: *Hydatigera taeniaeformis* larvae, *Rodentolepis nana*, *Hymenolepis diminuta*, *Calodium hepaticum*, *Gongylonema neoplasticum*, and *Moniliformis moniliformis*. Human zoonotic helminth infections often go unreported, so the role of *R. norvegicus* in their transmission is unknown. According to our results, it is advisable to monitor and control rodent populations in deprived settlements close to cities and in cities themselves, and to promote good hygienic and sanitary practices, especially among vulnerable populations and workers with high exposure such as sewage workers.

Abstract: Synanthropic rodents are involved in the transmission of several parasitic zoonoses to humans, such as helminth parasites. As part of a multidisciplinary study concerning the rodent population in Barcelona (Spain), 300 specimens of the Norway rat, *Rattus norvegicus*, were analyzed to elucidate their helminth community, mainly species with a zoonotic potential. The sample included 263 specimens from the sewage system and 37 from public gardens. A total of 253 (84.3%) rats were found to be parasitized, and 206 (68.7%) harbored zoonotic species. Adult rats were found to be more heavily parasitized with zoonotic helminths than juveniles, but the sex and site of capture had no influence. Six zoonotic helminths were identified: *Hydatigera taeniaeformis* larvae (1.7%), *Rodentolepis nana* (8.0%), *Hymenolepis diminuta* (21.3%), *Calodium hepaticum* (46.3%), *Gongylonema neoplasticum* (36.7%), and *Moniliformis moniliformis* (2.3%). Human zoonotic helminth infections often go unreported, so that the role of *R. norvegicus* in their transmission is unknown. According to our results, it is advisable to monitor and control rodent populations in deprived settlements close to cities and in cities themselves, and to promote good hygienic and sanitary

practices, especially among vulnerable populations and workers with high exposure such as sewage workers.

Keywords: *Rattus norvegicus*; reservoir; helminths; zoonoses; Barcelona; Spain

1. Introduction

The World Health Organization (WHO), in collaboration with the Food and Agriculture Organization of the United Nations (FAO), the United Nations Environment Programme (UNEP), and the World Organization for Animal Health (WOAH), established the concept of One Health to promote multi-sector responses to food safety hazards, zoonotic risks and other threats to public health in the interaction between humans, animals and the environment, and provide guidance on how to reduce these risks (https://www.who.int/news-room/questions-and-answers/item/one-health, accessed on 2 September 2024).

Human beings coexist in a close relationship with various animals and this interrelation can be a source of diseases with a marked impact on public health and the social and economic well-being of the world population. These diseases, transmissible from animals to humans, either by direct contact or through contamination of water, food or the environment, are known as zoonoses. In humans, more than 1400 pathogens are known, of which approximately 900 species are zoonotic (>60%), and 80% of the latter have the capacity to affect different animal species [1]. Zoonoses have certain characteristics that favor their spread, complicate their control and eventual eradication, and have a severe impact on human and animal health [2].

Humans are affected by more than 100 parasitic zoonoses [3]. In urban areas, humans live with both synanthropic and domestic animals, usually small animals and pets, susceptible to the transmission of various protozoa and helminths, causing, almost always, non-occupational diseases and with a higher incidence in children. Among synanthropic animals, which take advantage of human habitats and food sources, rodents are the most common, together with insects, bats, pigeons and sparrows.

In recent decades, there has been an increase in diseases associated with small mammals that act as reservoirs [4]. Rodents are currently the most abundant and diverse order of mammals, representing 43% of the total number of mammalian species [5]. In peri-urban environments, rodents are a link between wild animals and humans, exposing the latter, even to zoonoses that usually tend to circulate in natural ecosystems. Rodents are reservoirs for a large number of infectious organisms that can be transmitted to humans and cause outbreaks of various zoonotic diseases with high morbidity and some mortality.

Synanthropic rodents of urban areas, *Rattus norvegicus* (Berkenhouth, 1769) (Norway rat) and *Rattus rattus* (Linnaeus, 1758) (black rat), act as active reservoirs (carriers and spreaders) of numerous pathogens (viruses, bacteria, and parasites) that can affect human health and therefore cause serious public health problems [6–8]. Human beings share cities with hundreds of thousands of rats and this contact between rats and humans can lead to the effective transmission of zoonotic diseases. Given the high rates of global urbanization [9], up-to-date knowledge of urban parasitic zoonoses is needed. It will thus be possible: (1) to evaluate the presence, magnitude, and nature of urban parasitic zoonoses in cities on a global scale; (2) to monitor and mitigate the emergency risk of urban parasitic zoonoses now and in the future; (3) to make health professionals aware of the presence and health risks of urban parasitic zoonoses and avert misdiagnosis of these diseases; and (4) to develop an effective rat population control strategy and other public health measures to

reduce and prevent rat-human disease transmission [10]. The role of rodents as parasite reservoirs for protozoa and helminths is well known [4,11].

There are two transmission routes of zoonotic parasites from rodents in urban areas. In the first route of transmission, the rodent transmits the infective parasitic form directly to the human being, either cysts/oocysts, or eggs (for example, the protists *Giardia*, *Entamoeba*, *Blastocystis*, *Cryptosporidium*; the cestode *Rodentolepis nana* (Siebold, 1852), formerly *Hymenolepis nana*), which are, at the same time, the cause of self-infection of the rodent in monoxenous cycles in which the parasitic form is already infective. In the second route of transmission, the rodent acts as an indirect reservoir, since, in this case, it does not transmit the parasite directly to the human being, but rather reaches the human being, generally, via an arthropod, either by bite (such as the hemotissular protozoan *Leishmania*) or accidental ingestion of the same (for example, cockroaches or beetles in the cases of the tapeworm *Hymenolepis diminuta* (Rudolphi, 1819), the nematode *Gongylonema neoplasticum* (Fibiger et Ditlevsen, 1914) and the acanthocephalan *Moniliformis moniliformis* Bremser, 1811), or there are carriers, which are those hosts that directly transmit the parasite to humans. The carriers are both the predators of the rat as well as those that become infected with the parasitic forms emitted by the rat and are part of the human diet. In the first case, the carriers are the emitters of infectious forms for human beings, as in the case of the cat in the protozoan *Toxoplasma gondii* (Nicolle et Manceaux, 1909), or they become infected through the ingestion of the parasitized carrier which is part of the human diet, e.g., pigs in trichinosis (less important in urban settings) and snails in the case of angiostrongyliasis caused by *Angiostrongylus cantonensis* (Chen, 1935).

As Himsworth et al. [10] pointed out in their review, the role of rats as zoonotic parasite reservoirs has not been adequately analyzed in the most important cities of the world, with a few exceptions.

Concerning zoonotic parasitic helminths, in addition to *A. cantonensis*, detected in *R. norvegicus* practically at a cosmopolitan level, but also in *R. rattus*, other helminths were detected in urban rats in most of the studies carried out, and reported in the above-mentioned review: *Hymenolepis* spp. (*H. nana* and *H. diminuta*) and *Calodium hepaticum* (Bancroft, 1893) in *R. norvegicus*.

Regarding other zoonotic helminths in urban rats, various studies have been carried out in some European cities: Marseille (France) [12], Milan (Italy) [13], London (England) [14], Palermo (Italy) [15], Belgrade (Serbia) [16], Liverpool (England) [17], Hauts-de-Seine (France) [18], Helsinki (Finland) [19], and Budapest (Hungary) [20]; but also studies in cities in other countries around the word, such as Malaysia [21], Peru [22], Brazil [23], and South Africa [24].

In the case of Spain, studies of parasites in rodents of the genus *Rattus* have been carried out almost exclusively in rural and unpopulated areas, while studies in urban or peri-urban areas are scarce. Regarding helminthiases, these studies concern the cities of Granada [25], La Coruña and Santiago de Compostela [26] and Barcelona [27], and recently in Valencia [28], where, among other helminths and zoonotic parasites, *A. cantonensis* has been reported for the first time in continental Europe parasitizing *R. norvegicus* and *R. rattus* [29]. To sum up, the following zoonotic parasites have been reported in the urban sewer rat: *Brachylaima* sp. (Trematoda), *Hydatigera (=Taenia) taeniaeformis* (Batsch, 1786) larvae, *R. nana*, *H. diminuta* (Cestoda), *C. hepaticum*, *A. cantonensis*, *G. neoplasticum* (Nematoda) and *M. moniliformis* (Acanthocephala).

Previous results, published by our research group concerning the city of Barcelona [30], after the helminthological analysis of the first 100 rats in this city, showed the presence of 5 zoonotic helminth species, specifically the cestodes *R. nana* and *H. diminuta*, the nematodes *C. hepaticum* and *G. neoplasticum*, and the acanthocephalan *M. moniliformis*, which provides

a glimpse into the spreading capacity and reservoir role of synanthropic rodents. These results should prompt public health authorities to address the situation within the concept of One Health, in which, disciplines such as public health in general, and the prevention of occupational risks in particular, must be involved.

Consequently, by updating the data on zoonotic helminth species in the Norway rat in the city of Barcelona, this research aims to shed light on the role *R. norvegicus* plays as a reservoir of zoonotic helminths, and warn the health authorities of parasites found in rats that can cause diseases in humans.

2. Materials and Methods

2.1. Study Area and Animals

To trap and dissect the rats, permission was granted by the Department of Territory and Sustainability of the regional government of Catalonia (reference number: SF/044), according to Directive 2010/63/EU of the European Parliament and Council decision of 22 September 2010 on the protection of animals used for scientific purposes. Then, to collect the rat individuals, snap traps were placed in the sewage system and rat cages in public gardens, always at night, when human access was restricted. All animals captured alive were euthanized by exposure to CO_2-saturated atmosphere.

A total of 300 *R. norvegicus* caught from December 2016 to November 2017 at 35 different locations in the sewage system (n = 263) and in 10 different public gardens (n = 37) (Table 1), placed in populated residential neighborhoods, covering the 10 different districts of the city of Barcelona was helminthologically analyzed.

Table 1. Selected characteristics of the 300 *Rattus norvegicus* analyzed.

Host Sex *	**Host Age ***	**Season of Capture**				
Males		Autumn	Winter	Spring	Summer	TOTAL
	Juveniles	1	33	--	11	45
	Adults	15	58	19	16	108
	TOTAL	16	91	19	27	153
Females		Autumn	Winter	Spring	Summer	TOTAL
	Juveniles	3	37	3	9	52
	Adults	7	48	13	20	88
	TOTAL	10	85	16	29	140

* In a total of 7 rat individuals, neither sex nor age could be determined.

Rat individuals collected were dissected at the Pest Surveillance and Control Agency of Barcelona, and identified at a specific level, their sex determined, and weight and morphometry data were also collected and introduced in a database, together with data concerning the date and place of capture of each rat. Moreover, each rat was assigned to an age group according to their external morphometry, reproductive status and body weight as juveniles (<150 g) and adults (≥150 g) [31]. After dissection, the organs of each specimen were preserved in 95% ethanol. The helminthological analysis of preserved organs was carried out at the laboratory of the Parasites and Health Research Group of the Department of Parasitology of the University of Valencia.

2.2. Helminthological Procedures

All organs of each rat individual preserved in 95% ethanol, including the entire gastrointestinal tract, lungs, heart, pancreas, spleen, liver, kidneys and urinary bladder, were placed in Petri dishes containing saline and examined under a stereomicroscope. All helminths were collected and preserved in 70% ethanol. Then, all helminths were

identified at a specific level based on their morphology and morphometry and according to the most relevant descriptions and findings of Cestoda [32–34], Nematoda [35–41] and Acanthocephala [42]. To do so, cestodes were stained with alcoholic hydrochloric carmine, differentiated with acidified ethanol, dehydrated in an alcohol series, cleared with xylene and mounted in Canada balsam, and nematodes were cleared in Amann lactophenol in non-permanent whole mounts.

2.3. Statistical Analysis

The number of parasitized hosts as well as the prevalence, mean abundance and range [43] of the helminth species found were analyzed.

Binary Logistic Regression (BRL) was applied to analyze the influence of the origin of capture, and the host sex and age on the prevalence of the zoonotic helminth species found. In the season-of-capture analyses, the four seasons were grouped in two groups, autumn–winter and spring–summer, due to the small number of captures, mainly during autumn and spring. Statistical significance was established at $p < 0.05$. The IBM SPSS Statistics 28.0 for Windows software package was used for statistical analysis.

3. Results

3.1. Helminth Species Found

The helminthological analysis of 300 *R. norvegicus* specimens from the city of Barcelona revealed that a total of 253 (84.3% [95% CI: 79.9–88.1]) rats were parasitized by at least one helminth species. Of these, 206 were carriers of at least one zoonotic helminth species (68.7% of the total rats analyzed [95% CI: 63.3–73.7]; 81.4% of the total parasitized rats [95% CI: 76.3–85.8]). The helminth species found (Table 2) were: 3 zoonotic cestodes, *H. taeniaeformis* larvae, *R. nana* and *H. diminuta*; 7 nematodes, 2 of them zoonotic, *C. hepaticum* and *G. neoplasticum*, and 5 non-zoonotic, *Eucoleus gastricus* (Baylis, 1926), *Aonchotheca annulosa* (Dujardin, 1843), *Trichosomoides crassicauda* (Bellinham, 1845), *Nippostrongylus brasiliensis* (Travassos, 1914) and *Heterakis spumosa* Schneider, 1866; and 1 zoonotic acanthocephalan, *M. moniliformis*. In addition, it should be noted that a total of 245 (81.7% [95% CI: 77.0–85.7]) rats were parasitized by helminth species with a monoxenous cycle (direct cycle, without intervention of intermediate hosts), and 94 rats (31.3% [95% CI: 26.3–36.7]) carried monoxenous helminth species only; furthermore a total of 159 (53.0% [95% CI: 47.3–58.6]) rats were parasitized by helminth species with a heteroxenous cycle (indirect cycle, with the intervention of a definitive host, and, at least, one intermediate host), but merely 8 rats (2.7% [95% CI: 1.3–5.0]) carried heteroxenous helminth species only. Moreover, 151 rats (50.3% [95% CI: 44.7–56.0] carried monoxenous and heteroxenous helminth species simultaneously.

The species with the highest percentage of parasitation (prevalence) was *H. spumosa*, with 63.7%, and the most abundant was *N. brasiliensis* (13.26). Among zoonotic helminths, *C. hepaticum* was the most prevalent species with 46.3% and *G. neoplasticum* was the most abundant (3.96). The most notable species in terms of the number of helminths of the same species found in an individual rat were *R. nana* with 500 helminths, *N. brasiliensis* with 423 and *G. neoplasticum* with 111 (Table 2).

Table 2. Selected characteristics of the helminth community of 300 *Rattus norvegicus* analyzed.

Helminth Species	Site	LC	Zoonotic	n	Prevalence (%) (95% CI)	Mean Abundance (SE)	Median Intensity (Range)
CESTODA							
Taenia taeniaeformis larvae	liver	DH	yes	5	1.7 (0.6–3.6)	0.02 (0.01)	1 (1)
Rodentolepis nana	small intestine	MX/DH	yes	24	8.0 (5.3–11.5)	2.36 (1.70)	29.5 (1–500)
Hymenolepis diminuta	small intestine	DH	yes	64	21.3 (17.0–26.2)	0.33 (0.05)	1.5 (1–12)
NEMATODA							
Eucoleus gastricus	stomach	MX	no	87	29.0 (24.1–34.3)	1.48 (0.19)	5.1 (1–25)
Aonchotheca annulosa	small intestine	DH	no	13	4.3 (2.5–7.1)	0.44 (0.18)	10.2 (1–32)
Calodium hepaticum *	liver	MH	yes	139	46.3 (40.7–52.0)	---	---
Trichosomoides crassicauda **	urinary bladder	MH	no	10	6.6 (3.4–11.4)	0.23 (0.09)	3.5 (1–10)
Nippostrongylus brasiliensis	small intestine	MH	no	110	36.7 (31.4–42.2)	13.26 (2.84)	36.2 (1–423)
Heterakis spumosa	large intestine	MH	no	191	63.7 (58.1–69.0)	11.87 (1.06)	18.6 (1–91)
Gongylonema neoplasticum	esophagus	DH	yes	110	36.7 (31.4–42.2)	3.96 (0.67)	10.8 (1–111)
ACANTHOCEPHALA							
Moniliformis moniliformis	small intestine	DH	yes	7	2.3 (1.0–4.5)	0.08 (0.04)	3.6 (1–9)

LC—life cycle, DH—diheteroxenous life cycle, MX—monoxenous life cycle, n—number of infested hosts, CI—confidence interval, and SE—standard error. * In the case of *Calodium hepaticum*, quantitative data were not recorded as it was impossible to determine the number of adults of this helminth in each liver. ** In the case of *Trichosomoides crassicauda*, the number of urinary bladders analyzed was 152 only; the results for this helminth take this fact into account.

3.2. Helminth Life Cycles

3.2.1. *Hydatigera taeniaeformis* larvae

The larval stage of the zoonotic taenid cestode *H. taeniaeformis* inhabits the liver surface of rats, their intermediate host. The life cycle of this tapeworm [44] is diheteroxenous (Figure S1), with cats and other felines acting as the definitive host carrying the adult stage in the small intestine. Eggs with or from the gravid proglottids are released in the feces of felines which infect rats directly. Eggs develop the larval stage, the strobilocercus in their abdominal cavity, on the liver surface. Finally, felines will be infected after ingestion of an infected rat. The most probable route of infection of humans, although not the only one, seems to be the ingestion of eggs released by felines, which develop into the larval stage.

The prevalence of this tapeworm larva was 1.7%, with only five rats infected, and the presence of one cyst in each one (Table 2).

3.2.2. *Rodentolepis nana*

The adult of the zoonotic hymenolepidid *R. nana*, the dwarf tapeworm, inhabits the small intestine of humans and rats, which act as its only hosts when the cycle [45] is monoxenous (Figure S2), the more common of the two possible kinds of biological cycles

of this helminth. The infection of the host occurs after the ingestion of eggs released by another infected host. Then, after emerging from the eggs, the oncospheres develop into cysticercoid-type larval stages in the intestinal mucosa which turn into adult tapeworms in the intestinal lumen. However, much less common, this tapeworm presents a diheteroxenous life cycle, with some arthropods (mainly beetles and fleas) acting as intermediate hosts harboring the cysticercoid. Rats and humans become parasitized after ingesting infected arthropods, accidentally in the case of humans.

The prevalence of this monexenous hymenolepidid was 8.0%, with a median intensity of 29.5 individuals in the parasitized rats (Table 2).

3.2.3. *Hymenolepis diminuta*

The adult of the other zoonotic hymenolepidid tapeworm, *H. diminuta*, the rat tapeworm, also inhabits the small intestine of humans and rats. However, in this case, its life cycle [46] is only diheteroxenous (Figure S3), with some arthropods (also mainly beetles and fleas) acting as intermediate hosts which after ingesting the eggs develop the cysticercoids in their internal cavity. The same as in the case of *R. nana*, rats and humans become parasitized after ingesting infected arthropods, accidentally in the case of humans.

The prevalence of this diheteroxenous hymenolepidid was 21.3%, with a low median intensity of 1.5 individuals only (Table 2).

3.2.4. *Eucoleus gastricus*

The non-zoonotic capillarid nematode *E. gastricus* inhabits the stomach mucosa of rats, causing, sometimes, a visible pathology. Its life cycle [47] seems to be monoxenous (Figure S4). Rats release unembryonated eggs in feces, which under favorable environmental conditions, embryonate in the soil, then being infective to rats. Alternatively, the participation of earthworms acting as paratenic hosts after the ingestion of embryonated eggs and subsequent development of the larval stage seems possible. Rats become infected after ingesting these earthworms.

The prevalence of this monoxenous stomachal capillarid was 29.0%, with a discrete median intensity of 5.1 individuals (Table 2).

3.2.5. *Aonchotheca annulosa*

The other non-zoonotic capillarid nematode *A. annulosa* inhabits the small intestine of rats. Its life cycle [47] is diheteroxenous (Figure S5). Rats release unembryonated eggs in feces, which under favorable environmental conditions, embryonate in the soil. These eggs are ingested by the intermediate host, earthworms, in which the larval stage develops. Rats will be infected after ingesting these earthworms.

The prevalence of this diheteroxenous intestinal capillarid was 4.3% only, but with a non-negligible median intensity of 10.3 individuals (Table 2).

3.2.6. *Calodium hepaticum*

The zoonotic capillarin nematode *C. hepaticum*, also known as *Capillaria hepatica*, typically inhabits the liver parenchyma of rats, but also of humans and other mammals. Its life cycle [47], although being a typically monoxenous cycle (Figure S6), has some particularities. The adults inhabit the liver parenchyma where gravid females lay the unembryonated eggs. As these eggs are trapped in the liver parenchyma, they reach the environment only after the death of their hosts. Then, when a dead infected rat is ingested by a predator or scavenger, the unembryonated eggs pass through the gastrointestinal tract to be released into the environment. Once these unembryonated eggs reach the soil, the embryonation process takes several weeks or months, under favorable conditions. The

embryonated eggs are infective to rats or any other mammal, including humans, who accidentally ingest them in contaminated water and food.

The prevalence of this hepatic capillarid was 46.3%, the highest among the zoonotic helminths found (Table 2). However, its median intensity and abundance cannot be recorded due to the limitation of assessing the number of adults of this helminth in each liver. The adults were unrecognizable as individual specimens, with liver parasitism having been detected by the presence of eggs in the liver parenchyma and remains of adult stages and, generally, also by the typical pathology produced by them.

3.2.7. *Trichosomoides crassicauda*

This non-zoonotic trichosomoid nematode inhabits the urinary bladder of rats and has a monoxenous life cycle [36,48] (Figure S7). Males live inside the female reproductive tract. In the urinary bladder the females shed embryonated eggs which are released in urine, in turn, infecting other rats after their ingestion. The eggs hatch in the stomach and the larvae penetrate the gastric wall to initiate, via the portal vein, an intraorganic migration to the urinary bladder, where the adults mature and mate.

The prevalence of this nematode was 6.9%, with a low median intensity of 3.5 individuals (Table 2).

3.2.8. *Nippostrongylus brasiliensis*

The non-zoonotic heligmosomid nematode *N. brasiliensis*, also known as the rat hookworm, inhabits the small intestine of rodents (mainly rats) and presents a monoxenous life cycle [49] (Figure S8). Unembryonated eggs released by females embryonate in the soil, where they hatch and, after two moults, the filariform L_3 larvae infect other rats transcutaneously, with the oral route of infection also being possible. The L_3 larvae undergo an intraorganic migration to the lungs and, after ingestion, the young adults reach the small intestine.

The prevalence of the rat hookworm was 36.7%, with the highest median intensity, 36.2 individuals per parasitized rat (Table 2).

3.2.9. *Heterakis spumosa*

The non-zoonotic ascaridid nematode *H. spumosa* inhabits the large intestine of rats. Females release unembryonated eggs, which are shed in rat feces. The eggs, under favorable environmental conditions, embryonate in the soil, being infective to other rats which become infected after their ingestion, thus completing the monoxenous biological cycle [36] (Figure S9).

The prevalence of this monoxenous ascaridid was 63.7%, the highest among all the helminth species found, with a median intensity of 18.6 individuals (Table 2).

3.2.10. *Gongylonema neoplasticum*

The zoonotic spirurid nematode *G. neoplasticum* inhabits the esophagus mucosa of rats, where the female lays embryonated eggs which are released in feces. Its life cycle [50] is diheteroxenous (Figure S10), with some arthropods, mainly cockroaches and beetles, acting as intermediate hosts, which ingest the embryonated eggs. In the hemocoel of the arthropod larval stages develop until the infective L_3, which encysts in the musculature. Rats and humans become parasitized after ingesting infected arthropods, accidentally in human cases.

The prevalence of this zoonotic spirurid was 36.7%, with a non-negligible median intensity of 10.8 individuals (Table 2).

3.2.11. *Moniliformis moniliformis*

The zoonotic acanthocephalan *M. moniliformis* inhabits the small intestine of rats, where the female lays embryonated eggs which are released in feces. Its life cycle [51] is diheteroxenous (Figure S11), with some arthropods, mainly cockroaches and beetles, acting as intermediate hosts. Once ingested, the acanthor that emerges from the embryonated eggs moults into a second larval stage, the acanthella in the hemocoel of the arthropod, and subsequently turns into an infective cystacanth. Rats and humans become parasitized after ingesting infected arthropods, accidentally in the case of humans.

The prevalence of this zoonotic acanthocephalan was 2.3%, with 7 infected rats, and with a median intensity was 3.7 individuals (Table 2).

3.3. Analysis of the Zoonotic Helminth Species

A total of 110 individual rats carried more than 1 zoonotic helminth species (Table 3), including 79 of them carrying 2 zoonotic helminths, 29 with 3 species, and 2 with 4 species. Among these associations, the most frequent is *C. hepaticum* and *G. neoplasticum*. Moreover, the presence of the triple association between *H. diminuta–C. hepaticum–G. neoplasticum* in a total of 17 rats is also worth highlighting. Three of the associations reported are statistically significant: *R. nana–M. moniliformis* (χ^2 = 11.832; p = 0.0006); *C. hepaticum–G. neoplasticum* (χ^2 = 36.175; $p < 0.0001$); and *G. neoplasticum–M. moniliformis* (χ^2 = 7.425; p = 0.0064).

Table 3. Co-infections of two, three or four zoonotic helminth species detected in the analyzed rats.

Zoonotic Helminth Species	Number of Rats
H. taeniaeformis–H. diminuta	1
H. taeniaeformis–C. hepaticum	1
R. nana–H. diminuta	1
R. nana–C. hepaticum	5
R. nana–G. neoplasticum	2
H. diminuta–C. hepaticum	14
H. diminuta–G. neoplasticum	3
C. hepaticum–G. neoplasticum	52
H. taeniaeformis–R. nana–C. hepaticum	1
R. nana–H. diminuta–C. hepaticum	1
R. nana–H. diminuta–G. neoplasticum	1
R. nana–C. hepaticum–G. neoplasticum	4
R. nana–G. neoplasticum–M. moniliformis	2
H. diminuta–C. hepaticum–G. neoplasticum	17
H. diminuta–G. neoplasticum–M. moniliformis	2
C. hepaticum–G. neoplasticum–M. moniliformis	1
H. taeniaeformis–H. diminuta–C. hepaticum–G. neoplasticum	1
R. nana–C. hepaticum–G. neoplasticum–M. moniliformis	1

Prevalences of zoonotic helminth species by host sex and age and by the place and season of capture of hosts are shown in Tables 4 and 5, respectively. Of note is the higher prevalence observed in adults vs. juveniles for most zoonotic helminth species. However, the differences observed based on sex, place and season of capture are different depending on the helminth species considered.

Table 4. Prevalence of parasitation of the zoonotic helminth species found in *Rattus norvegicus* by host sex and age.

		Hydatigera taeniaeformis l.	*Rodentolepis nana*	*Hymenolepis diminuta*	*Calodium hepaticum*	*Gongylonema neoplasticum*	*Moniliformis moniliformis*	**Total of Zoonotic Helminth Species**
Place of capture								
Sewage system	n (prevalence)	2 (0.8%)	16 (6.1%)	55 (20.9%)	126 (47.9%)	106 (40.3%)	7 (2.7%)	182 (69.2%)
	95% CI	0.2–2.4	3.7–9.5	16.3–26.1	41.9–53.9	34.5–46.3	1.2–5.2	63.4–74.5
Public gardens	n (prevalence)	3 (8.1%)	8 (21.6%)	9 (24.3%)	13 (35.1%)	4 (10.8%)	---	24 (64.9%)
	95% CI	2.3–20.1	10.8–36.7	12.8–39.7	21.3–51.2	3.8–23.7	---	48.8–78.7
Season of capture								
Autumn–Winter	n (prevalence)	5 (2.4%)	23 (11.0%)	43 (20.6%)	69 (33.0%)	58 (27.8%)	6 (2.9%)	130 (62.2%)
	95% CI	0.9–5.2	7.3–15.8	15.5–26.4	26.9–39.6	22.0–34.1	1.2–5.8	55.5–68.6
Spring–Summer	n (prevalence)	---	1 (1.1%)	21 (23.1%)	70 (76.9%)	52 (57.1%)	1 (1.1%)	76 (83.5%)
	95% CI	---	0.1–5.0	15.3–32.5	67.5–84.7	46.9–67.0	0.1–5.0	74.9–90.0

Table 5. Prevalence of parasitation of the zoonotic helminth species found in *Rattus norvegicus* by place and season of capture.

		Hydatigera taeniaeformis l.	*Rodentolepis nana*	*Hymenolepis diminuta*	*Calodium hepaticum*	*Gongylonema neoplasticum*	*Moniliformis moniliformis*	**Total of Zoonotic Helminth Species**
Place of capture								
Sewage system	n (prevalence)	2 (0.8%)	16 (6.1%)	55 (20.9%)	126 (47.9%)	106 (40.3%)	7 (2.7%)	182 (69.2%)
	95% CI	0.2–2.4	3.7–9.5	16.3–26.1	41.9–53.9	34.5–46.3	1.2–5.2	63.4–74.5
Public gardens	n (prevalence)	3 (8.1%)	8 (21.6%)	9 (24.3%)	13 (35.1%)	4 (10.8%)	---	24 (64.9%)
	95% CI	2.3–20.1	10.8–36.7	12.8–39.7	21.3–51.2	3.8–23.7	---	48.8–78.7
Season of capture								
Autumn–Winter	n (prevalence)	5 (2.4%)	23 (11.0%)	43 (20.6%)	69 (33.0%)	58 (27.8%)	6 (2.9%)	130 (62.2%)
	95% CI	0.9–5.2	7.3–15.8	15.5–26.4	26.9–39.6	22.0–34.1	1.2–5.8	55.5–68.6
Spring–Summer	n (prevalence)	---	1 (1.1%)	21 (23.1%)	70 (76.9%)	52 (57.1%)	1 (1.1%)	76 (83.5%)
	95% CI	---	0.1–5.0	15.3–32.5	67.5–84.7	46.9–67.0	0.1–5.0	74.9–90.0

Adult rats have a higher prevalence of helminth species parasitation than juveniles (odds ratio = 13.4 [95% CI: 6.1–28.3], monoxenous (odds ratio = 7.6 [95% CI: 3.9–14.5] and heteroxenous (odds ratio = 6.8 [95% CI: 3.9–11.8] species. Moreover, the combination of age, sex and the place of capture of the host has a statistically significant influence on the total parasitation of rats by helminths ($\chi^2 = 68.857$; df = 3; $p < 0.0001$) and the parasitation only by monoxenous helminths ($\chi^2 = 52.198$; df = 3; $p < 0.0001$), with the highest value being obtained for adult females captured in the sewage system. Likewise, the age and place of capture have a statistically significant influence on the parasitation by heteroxenous helminths ($\chi^2 = 63.612$; df = 2; $p < 0.0001$), with the highest value being obtained for adults captured in the sewage system.

The binary logistic regression models which best represent the influence that host sex and age, as well as place and season of capture have on the prevalences of the zoonotic

helminth species are shown in Table 6. All in all, adult rats have a higher prevalence of zoonotic helminth species parasitation than juveniles (odds ratio = 6.1 [95% CI: 3.6–10.5], and the combination of host age and season of capture has a statistically significant influence on this prevalence. The prevalence of all zoonotic helminth species, with the exception of the acanthocephalan *M. moniliformis*, was statistically influence by at least one of the host factors analyzed.

Table 6. Binary logistic regression models for the prevalences of the zoonotic helminth species of *Rattus norvegicus* by host sex and age, place and season of capture (*) by χ^2 values with associated probabilities (*p)* for the model created including independent variables. Only statistically significant models are reported.

Zoonotic Helminth Species/Independent Variables	df	χ^2	*p*
Hydatigera taeniaeformis larvae			
Place of capture	1	6.561	0.010
Rodentolepis nana			
Place of capture	1	8.724	0.03
Place and season of capture	2	16.928	<0.0001
Hymenolepis diminuta			
Host age	1	4.921	0.027
Calodium hepaticum			
Season of capture	1	55.876	<0.0001
Season of capture and host age	2	62.754	<0.0001
Gongylonema neoplasticum			
Host age	1	48.551	<0.0001
Host age and place of capture	2	72.525	<0.0001
Host age and place and season of capture	3	84.877	<0.0001
Host age and sex and place and season of capture	4	89.227	<0.0001
Total of helminth zoonotic species			
Host age	1	46.329	<0.0001
Host age and season of capture	2	58.624	<0.0001

df—degree of freedom. * Season of capture was grouped in two periods, autumn–winter and spring–summer, due to the small number of rats in autumn and spring.

As shown in Table 5, rats captured in public gardens have a higher prevalence of parasitation by the larva of *H. taeniaeformis* than rats captured in the sewage system (odds ratio = 11.6 [95% CI: 1.9–71.9]). In the case of *R. nana*, rats captured in public gardens also have a higher prevalence of parasitation (odds ratio = 4.6 [95% CI: 1.8–11.8]), and the combination of place and season of host capture (Table 6) has a statistically significant influence on this prevalence, with the highest value being obtained for rats captured in public gardens during the autumn–winter period.

Adult rats have a higher prevalence of parasitation by *H. diminuta* than juveniles of the tapeworm (odds ratio = 2.0 [95% CI: 1.1–3.9]), while for *C. hepaticum* rats captured in the sewage system have a higher prevalence of parasitation by this nematode (Table 5) (odds ratio = 7.5 [95% CI: 4.2–13.3]). Likewise, the combination of place of capture and host age has a statistically significant influence on the *C. hepaticum* prevalence (Table 6), with the highest value being obtained for adults captured in the sewage system.

Finally, adult rats have a higher prevalence of parasitation by *G. neoplasticum* than juveniles (Table 5) (odds ratio = 8.5 [95% CI: 4.2–17.4]), and the combination of place and season of capture as well as host sex has a statistically significant influence on this prevalence (Table 6), with the highest value being obtained for adult males captured in the sewage system during the spring–summer period.

4. Discussion

Although the present study has been carried out on 300 specimens of *R. norvegicus* in the city of Barcelona, the fact that only one year period has been analysed should be

considered a limitation of the study. Future research on new annual periods may serve to confirm the current results.

Two noteworthy facts make the results presented in this study significant: the results presented are among the few reports of helminths of the urban Norway rat in an important, touristic and sustainable Western European city, as is the case of Barcelona; and the detection of 11 helminth species parasitizing *R. norvegicus*, although they are parasites usually reported in previous studies of this rodent around the world. Moreover, the finding of the six zoonotic helminth species should be highlighted. The helminth species found include: three zoonotic cestodes, the larval stage of a taenid, *H. taeniaeformis*, and the adults of two hymenolepidids *R. nana* and *H. diminuta*; seven nematodes, among them, the zoonotic *C. hepaticum* and *G. neoplasticum*; and the zoonotic acanthocephalan *M. moniliformis*.

In the preliminary study that we previously carried out in that city [30], after the analysis of 100 rat individuals, 5 of the 6 zoonotic helminths had already been detected, with *H. diminuta* being the most prevalent, having a parasitation rate of 33%. However, in the current study, *C. hepaticum* and *G. neoplasticum* are the most prevalent zoonotic helminths. In addition, the larval stage of *H. taeniaeformis* is reported for the first time in Barcelona.

Concerning the zoonotic helminth species only, the analyses of *R. norvegicus* in other European cities [12–18,28,52,53] revealed the presence of almost the same species, with some specific exceptions. In Spain, the acanthocephalan *M. moniliformis* was reported in Barcelona and also recently in Valencia [28], as well as in several other studies around the world (Malaysia [21]; Peru [22]; Brazil [23]; and South Africa [24]), but not in other European cities. The trematode *Brachylaima* spp. has also been reported in Valencia [28], and previously in Hauts-de-Seine (France) [18] and Palermo (Italy) [15], but so far not in Barcelona. Moreover, the spirurid *Gongylonema* was only found in Palermo (Italy) [15], although reported as *Gongylonema* sp. *Gongylonema neoplasticum* was previously reported in Barcelona [30] and more recently in Valencia [28].

In addition to the studies carried out by our research group in Barcelona and Valencia, *R. nana*, *H. diminuta* and *C. hepaticum* have also been reported in Norway rats in other Spanish locations, such as Granada and Catalonia [25,54]. Likewise, *Trichinella spiralis* (Owen, 1935) has been reported in wild *R. norvegicus* in the Ebro Delta [54].

There is only 1 study carried out 3 decades ago in urban areas of Galicia in which 16 and 19 specimens of *R. norvegicus* were analyzed in the cities of La Coruña and Santiago de Compostela, respectively. However, article only provides information on helminths of the Capillariinae family, highlighting the presence of the zoonotic nematode *C. hepaticum* in the Norway rat [26]. More recently, another study also reported the presence of *C. hepaticum* in a specimen of the Norway rat captured in a peri-urban area of Barcelona [27].

In addition to all the zoonotic helminths mentioned above, a notable absence in the 300 individuals of *R. norvegicus* studied in Barcelona is that of the rat lungworm *A. cantonensis*, already found in Valencia [29] located 300 km south of Barcelona on the Spanish Mediterranean coast.

4.1. Zoonotic Helminth Species and Public Health Risk to Humans

Among the six zoonotic species found, *R. nana* is the only one to which urban rats transmit the infective parasitic form, the egg, directly to the human being. In the case of *H. diminuta*, *G. neoplasticum* and *M. moniliformis*, urban rats act as an indirect reservoir (maintaining the biological cycle of the helminth in nature) for humans, who are infected after the accidental ingestion of the intermediate host, an arthropod. Moreover, in the case of *C. hepaticum*, and *H. taeniaeformis*, there are carriers, which are those hosts that directly

transmit the parasite to humans; these carriers are the predators of the rat, including, in the case of *C. hepaticum*, other rats.

It is remarkable that a total of 110 of the 206 (53.4%) rats parasitized with zoonotic helminths are carriers of more than 1 of these species. This fact represents an additional risk with regard to potential human infections acquired from *R. norvegicus* in the city of Barcelona. Many of the associations detected are due to two possible factors: these are the zoonotic species with the highest prevalence (*C. hepaticum*, *G. neoplasticum* and *H. diminuta*); they share the same intermediate host (*H. diminuta*, *G. neoplasticum* and *M. moniliformis*, or even *R. nana*).

It is noteworthy that the statistically significant positive association between *G. neoplasticum* and *M. moniliformis* is repeated in six of the seven rats parasitized by this acanthocephalan, underlining the fact that they share the same intermediate host, cockroaches. However, the association between the most prevalent zoonotic species, *C. hepaticum*, and *M. moniliformis* is only repeated on two occasions. On the other hand, the positive association, also statistically significant, between the 2 most prevalent species seems to be due to the high number of rats parasitized by both helminths, which coincide on 76 occasions, taking into account both the associations of 2 or more helminth species.

4.1.1. *Hydatigera taeniaeformis* larvae

Rats play an indirect role in the transmission of this tapeworm to humans, acting as reservoirs of the helminth but not transmitting it directly to humans (Section 3.2.1).

As already mentioned, humans become infected with this zoonotic tapeworm after the accidental ingestion of the eggs released by felines which contaminate water, vegetables and other food. Cases of strobilocercus larvae in humans are rare [55,56], although there are several published reports, genuine or spurious, of human parasitation with the adult tapeworm in Argentina, Japan and Sri Lanka [57].

The higher prevalence of this larval stage in rats captured in public gardens than in in the sewage system might be related to the presence of infected cats, not in sewers, but on the ground in the city. Cats release the eggs in their feces, particularly in sandy soil and/or grass, making infestation of rats in these environments more likely. This fact also puts humans at risk, mainly workers and users of public parks and gardens, and therefore, preventive measures ought to be taken to diminish the risk of parasitation.

4.1.2. *Rodentolepis nana*

The direct life cycle of this zoonotic tapeworm is more frequent than the indirect life cycle (Section 3.2.2). Therefore, rats play a significant role in the infection of humans as the tapeworm eggs, directly infective to humans, are released in rat feces, contaminating water, vegetables, dirty hands, etc. In addition, rats act at the same time as reservoirs of this helminth in the less frequent indirect life cycle, i.e., with the participation of beetles and other insects as intermediate hosts.

The dwarf tapeworm is a neglected zoonotic parasite affecting more than 75 million people worldwide, mainly in Asia, Central and South America, Africa and Southern and Eastern Europe [58,59]. Although this hymenolepiasis is usually asymptomatic in humans, the parasitation can sometimes cause abdominal pain, diarrhea and anorexia, among other symptoms [60].

The highest prevalence of this tapeworm was found in rats captured in public gardens, in which humans could become parasitized by the infective eggs released in rat feces. Therefore, as in the case of *H. taeniaeformis* larvae, it is necessary to take preventive measures to diminish the risk of parasitation.

4.1.3. *Hymenolepis diminuta*

Hymenolepis diminuta has an indirect life cycle, with rats acting as reservoirs of this tapeworm. Therefore, humans can only become infected after the ingestion of infected intermediate hosts (Section 3.2.3).

This diheteroxenous tapeworm is more prevalent in adult rats than in juveniles, probably related to the diet of the rats, i.e., in older rats the diet presumably includes a greater number of arthropods [31], and, in turn, older rats have had a greater risk of becoming infected than juveniles.

Cases of human parasitism by *H. diminuta* are rare due to the route of infection. In fact, in the last 2 centuries, only approximately 1600 human cases have been reported worldwide, most of them in the Americas, Southeast Asia and the eastern Mediterranean [61]. Cases have also been reported in various European countries, such as Spain [62], Italy [63,64], Poland [65] and Romania [66]. Symptomatic infections in humans can mainly cause intestinal symptoms, such as abdominal pain, diarrhea, fever and anorexia [61].

Although human infestations appear to be incidental, given that the rat tapeworm prevalence is >20%, users of public gardens, especially children, should be made aware of the risk of the accidental ingestion of arthropods, intermediate hosts of *H. diminuta* and other zoonotic helminths.

4.1.4. *Calodium hepaticum*

The hepatic nematode *C. hepaticum* is a common parasite of rats. However, due to its low specificity, other mammals, including humans, can also become infected [67]. Parasitized rats play a reservoir role (Section 3.2.6).

Human infections (due to the accidental ingestion of infective eggs contaminating water and food) are rather rare, with approximately 200 cases worldwide. The most common symptoms include hepatomegaly, leukocytosis with eosinophilia and fever, being fatal without adequate and early diagnosis in more than 50% of cases [67].

The highest prevalences of this monoxenous nematode found in our study were in adult rats captured during the spring–summer period. Older rats have had a greater risk of becoming infected than juveniles which could be related to the higher mobility of older rat individuals, increasing the exposure time and in turn the likelihood of being infected, in this case, ingesting an infective egg. Moreover, the eggs embryonate in the soil to become infective, a development that is presumably more effective in the spring–summer period than in colder months. Therefore, the increased presence in the environment of infective eggs during the spring–summer period must be considered to minimize the risk to humans, mainly concerning sewage system workers as well as users and workers of public parks and gardens.

4.1.5. *Gongylonema neoplasticum*

In the case of this esophageal nematode, and as in the case of *H. diminuta,* humans become infected by accidentally ingesting the arthropod intermediate hosts, mainly cockroaches in the case of *G. neoplasticum*, (Section 3.2.10).

The highest prevalence of *G. neoplasticum* was found in adult male rats captured in the sewage system in the spring–summer period. This is presumably related to the diet of the older rats, the higher mobility of males, and the higher population of the main intermediate host, cockroaches, in the sewage system during the spring–summer period.

More than 50 human cases of *Gongylonema* sp. infections, mainly in the esophagus or the oral cavity, have been reported worldwide, including European countries, such as Italy, the former Soviet Union, Bulgaria, Austria, Germany and Hungary [68,69], and more recently Spain [70], France [71,72], Slovenia [73] and again Bulgaria [74]. The most common

symptoms in humans can be strange sensations in the oral cavity and/or symptoms related to reflux, indicating a possible esophageal condition [69].

Although human cases have been reported as caused by *Gongylonema pulchrum* Molin, 1857, this species presents a wide spectrum of definitive hosts, mainly domestic and wild pigs, while *G. neoplasticum* is an exclusive parasite of murids of the genus *Rattus*, mainly *R. norvegicus* and *R. rattus*. This reason could explain the low incidence of *G. pulchrum* in urban areas, with *G. neoplasticum*, probably being the predominant zoonotic species in urban areas where its definitive hosts, synanthropic rodents, are found [75].

As in the case of *H. diminuta*, the risk of human infestation appears to be rare, but the prevalence of *G. neoplasticum* observed in the rats studied is close to 37% (Table 5). Therefore, clinicians should consider gongylonemiasis in the differential diagnosis of clinical cases in which the patient experiences the above-mentioned symptoms, particularly in those cities where the parasite has already been found.

4.1.6. *Moniliformis moniliformis*

As in the cases of *H. diminuta* and *G. neoplasticum*, this zoonotic acanthocephalan has an indirect life cycle, and consequently rats act only as reservoirs of this helminth, with humans being infected when they accidentally ingest infected cockroaches, the main intermediate host of *M. moniliformis*.

Among the zoonotic helminths reported with an indirect life cycle, *M. moniliformis* has the lowest prevalence of parasitation in the rats in Barcelona, which could explain the absence of statistically significant influences of the factors analyzed on its prevalence. However, as expected, the highest prevalence corresponds to adult male rats living in the sewage system, which is consistent with the fact, above-mentioned for *G. neoplasticum*, that its main intermediate host is the cockroach.

Human cases of this intestinal acanthocephalan are rare and have occurred in several countries around the world, mostly in children, presenting with non-specific gastrointestinal symptoms such as diarrhea, abdominal pain and vomiting [76,77]. Therefore, as in the case of *G. neoplasticum*, health professionals, mainly pediatricians, should consider human acanthocephaliasis in the differential diagnosis if the patient experiences the above-mentioned symptoms.

5. Conclusions

The results obtained show that urban rats are carriers of zoonotic helminth parasites (at least five species in the case of Barcelona), for which rats can act, depending on the helminth species, transmitting the infective parasitic form directly to the human being, or acting as an indirect reservoir.

As rodents are present in all urban areas, the entire society can be exposed to these parasitic zoonoses. However, due to their proximity to the most important transmission sites, the groups most at risk of acquiring rat-borne helminth zoonosis are maintenance workers of the sewage system, parks and gardens, wastewater treatment and disposal units. Furthermore, visitors to green spaces, especially children and elderly people, should also be considered population groups at risk.

Consequently, health services as well as occupational risk prevention services should be aware of zoonotic helminths transmitted by urban rats in their city in order to provide, from a One Health approach, adequate information and implement preventive measures to protect the general public and the workers involved in particular.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani15030298/s1, Figure S1. Life cycle of *Hydatigera taeniaeformis* (illustration by A. L. Debenedetti); Figure S2. Life cycle of *Rodentolepis nana* (illustration by A. L. Debenedetti); Figure S3. Life cycle of *Hymenolepis diminuta* (illustration by A. L. Debenedetti); Figure S4. Life cycle of *Eucoleus gastricus* (illustration by A. L. Debenedetti); Figure S5. Life cycle of *Aonchotheca annulosa* (illustration by A. L. Debenedetti); Figure S6. Life cycle of *Calodium hepaticum* (illustration by A. L. Debenedetti); Figure S7. Life cycle of *Trichosomoides crassicauda* (illustration by A. L. Debenedetti); Figure S8. Life cycle of *Nippostrongylus brasiliensis* (illustration by A. L. Debenedetti); Figure S9. Life cycle of *Heterakis spumosa* (illustration by A. L. Debenedetti); Figure S10. Life cycle of *Gongylonema neoplasticum* (illustration by A. L. Debenedetti); Figure S11. Life cycle of *Moniliformis moniliformis* (illustration by A. L. Debenedetti).

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animals

MDPI

Communication

First Report on the Frequency and Subtype Distribution of *Blastocystis* sp. in Extensively Reared Holstein-Friesian Cattle from Terceira Island, Azores Archipelago, Portugal

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Simple Summary: This study reports the first detection of *Blastocystis* in dairy cattle on Terceira Island, highlighting its genetic diversity and zoonotic potential. The findings suggest a low risk of transmission from cattle to humans due to the region's grazing practices. Further research is needed to better understand the role of *Blastocystis* in livestock and its implications for public health.

Abstract: This study is the first to report *Blastocystis* sp. in dairy cattle from Terceira Island, part of the Azores Archipelago. *Blastocystis* sp. is an enteric protozoan with high genetic diversity and is associated with both zoonotic and non-zoonotic subtypes (STs). The present survey aimed to fill the gap in knowledge regarding the occurrence and genetic characterization of *Blastocystis* sp. isolates in Holstein-Friesian cattle in this geographical area. A total of 116 stool samples were thus collected from dairy cows, and *Blastocystis* sp. DNA was detected using qPCR. The results revealed a 14.7% occurrence rate (17/116), with seven STs identified including ST3, ST5, ST7, ST10, ST14, ST25, and ST42, with variable frequency. Some of these STs (ST5, ST7, ST10, and ST14) are potentially zoonotic, underscoring their potential public health significance. However, the low frequency of *Blastocystis* sp. in this animal cohort possibly attributed to the extensive grazing system practiced on Terceira Island and the limited presence of zoonotic isolates, suggests a minimal risk of transmission from cattle to humans. This study highlights the importance of further research on the transmission dynamics of *Blastocystis* sp., particularly in regions with varying farming practices, to better understand its epidemiology and zoonotic potential.

Keywords: *Blastocystis* qPCR; zoonotic subtypes; diary cows

1. Introduction

Blastocystis sp. is a stramenopile protozoa with significant genetic diversity and an uncertain pathogenicity. It is likely the most common enteric parasite inhabiting the human gut, with over one billion infections worldwide [1]. Its prevalence often exceeds 50% in

developing countries, with an average infection rate of 20% in developed nations [2]. The primary transmission route for this protozoan is the fecal-oral route, where *Blastocystis* sp. can be transmitted through the accidental ingestion of environmentally resistant cysts. This can occur via direct contact with infected individuals or through indirect contact with contaminated food or water sources [3]. Although the presence of *Blastocystis* sp. is frequently asymptomatic, its infection has been associated with gastrointestinal illnesses and/or urticaria in numerous clinical cases [4].

In addition to humans, *Blastocystis* sp. has been reported in various animal hosts including non-human primates, birds, rodents, reptiles, amphibians, and other mammals [5]. Phylogenetic analysis based on small-subunit ribosomal RNA (SSU rRNA) gene sequences has evidenced at least 44 legitimate STs, with 17 of these (ST1–ST10, ST12, ST14, ST16, ST23, ST26, ST35, and ST41) capable of infecting humans [6–9]. ST1–ST4 are the most common in humans worldwide and are considered as anthroponotic, accounting for approximately 90% of infections, despite the presence of other STs [10].

Blastocystis sp. is also commonly found in cattle, where at least 16 STs (ST1-ST7, ST10, ST12, ST14, ST17, ST21, ST23-ST26) have been identified, some of which are zoonotic [11]. The latest global systematic review and meta-analysis revealed that the most prevalent *Blastocystis* sp. STs in bovids are ST10 and ST14, with prevalence rates of 32.3% and 22.1%, respectively, which suggests that both STs are well adapted to cattle [11]. The zoonotic importance of these STs are supported as the recent identification of both STs in diverse human cohorts worldwide [12–14]. This supports the hypothesis that ruminants, particularly cattle, could serve as significant zoonotic reservoirs for humans, posing a risk to individuals with frequent contact, such as farmers and livestock breeders [15].

Close human–animal interactions increase the risk of zoonotic transmission. In particular, livestock breeders, veterinarians, and individuals in rural agricultural settings are more likely to acquire infections due to their prolonged and frequent exposure to animals, namely ST10 and ST14 associated and prevalent within cattle. [9,16–18]. Despite the zoonotic potential of *Blastocystis*, infections in cattle have not been linked to signs of disease, with most cases reported in healthy animals [4,19]. A study by Lee et al. (2018) found a higher prevalence of *Blastocystis* in cattle with normal feces (24.4%) compared to those with diarrhea (5.4%) [20].

Terceira Island, part of the Azores Archipelago in Portugal, covers an area of around 400 km² and operates as an autonomous region with its own political and administrative structures [21]. The Azores Archipelago experiences a stable climate, with temperatures typically ranging from 16 to 25 °C throughout most of the year [22]. Despite its temperate geographical location, this climate is characterized by consistently high relative humidity, resembling conditions found in tropical and subtropical regions [23]. This climate makes this island particularly well-suited for pasture-based dairy production, with milk and dairy products being the primary agricultural outputs. In contrast, the Azores climate differs from the Mediterranean climate that dominates the Portuguese mainland [23].

In 2023, milk production in Portugal totaled 1.996 billion liters, of which 603.43 million liters (30.4%) originated from the Azores Archipelago [24]. Within the archipelago, the islands of São Miguel and Terceira are the leading contributors, producing 405.67 million liters (20.3%) and 149.71 million liters (7.5%) of the total milk production in Portugal, respectively [25].

Despite the importance of the dairy industry in Azores, no studies have been conducted on the presence of *Blastocystis* sp. in cattle from the region, including Terceira Island, the second-largest producer of dairy products in the archipelago. Given the predominance of family-owned farms in the Azores, where close contact between farmers and cattle is common, there is an increasing risk of transmission from cattle harboring potentially

zoonotic *Blastocystis* sp. STs. To address this gap, the present study aims to perform the first molecular identification of *Blastocystis* sp. isolates in dairy cattle from Terceira Island.

2. Material and Methods

2.1. Sampling

A total of 116 stool samples were collected from healthy (non-diarreic) adult female Holstein-Friesian dairy cows between October and December 2023. The samples were obtained from 24 cattle farms located in the municipalities of Angra do Heroísmo (70 samples) and Praia da Vitória (46 samples) (Figure 1 and Table 1). The samples were collected either directly from the cows' recta following transrectal palpation or immediately after defecation. After collection, the samples were refrigerated and transported at 4 °C to the laboratory, where they were stored at −20 °C until DNA extraction.

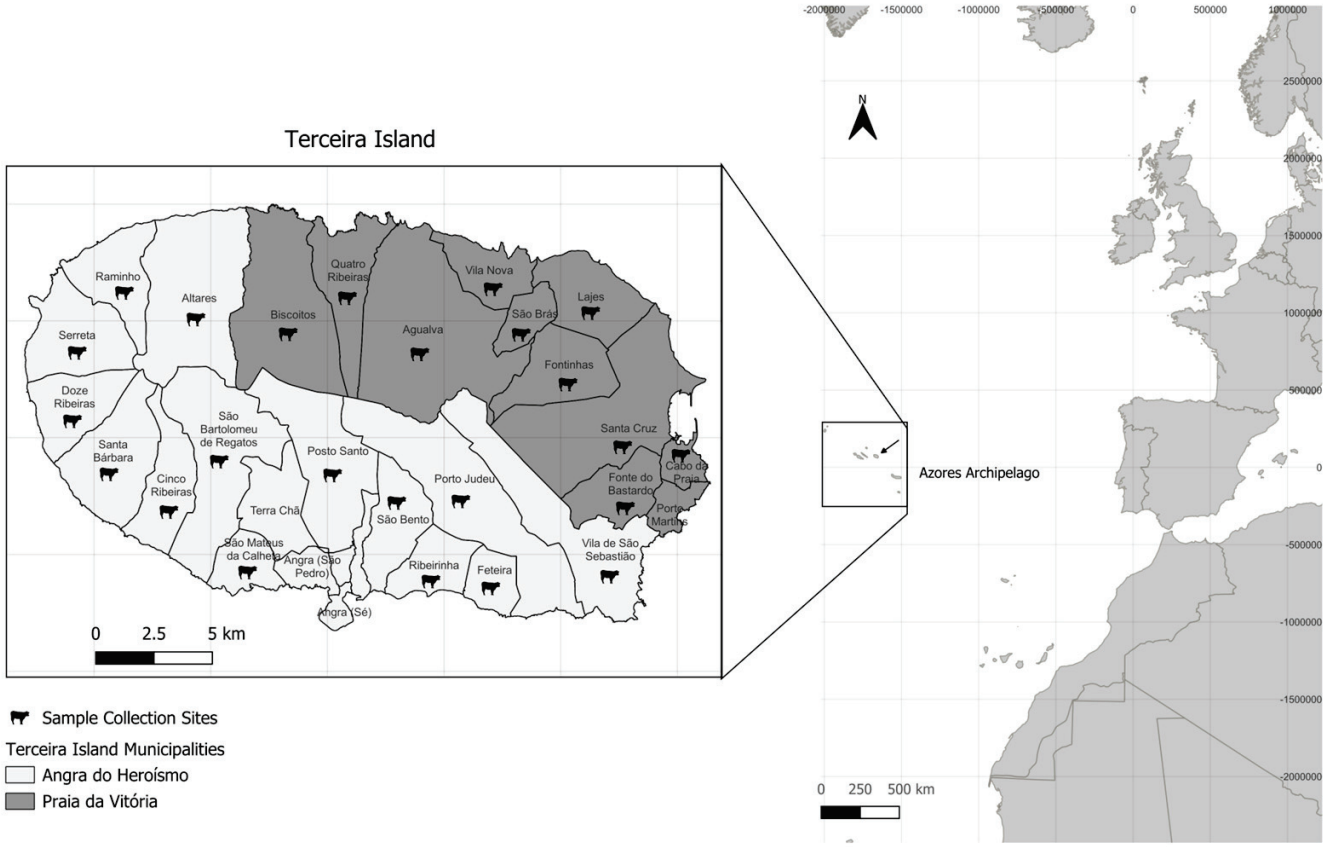

Figure 1. Detailed map of Terceira Island, part of the Azores Archipelago, showing the boundaries of its civil parishes, each labeled with their respective name. The municipalities are shaded in two different tones of grey: The lighter shade represents Angra do Heroísmo, while the darker shade represents Praia da Vitória. Cow icons indicate the locations of the sampling sites for this study. The map scale and coordinates are provided for spatial reference. The construction of the map was performed using QGIS software version 3.36.3.

2.2. DNA Extraction and Molecular Detection of Blastocystis sp.

DNA was extracted from approximately 200 mg of stool samples using the NucleoSpin 96 Soil Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. The extracted DNA was eluted in 100 µL of the supplied elution buffer and stored at −20 °C until further analysis. For amplification, 2 µL of purified DNA was subjected to real-time PCR (qPCR) targeting a ~300 bp region of the SSU rRNA gene, employing the primer pair BL18SPPF1 (forward) and BL18SR2PP (reverse), which is specific to the *Blastocystis* genus, as outlined in previous studies [26]. Sequencing was performed

on both strands using the same primer pair, by Genoscreen (Lille, France) using the 3730XL DNA Analyzer. The sequences obtained were processed, aligned, and examined using the BioEdit Sequence Alignment Editor software V7.2.5 [27]. The resulting sequences were deposited in the GenBank database with unique accession numbers PQ423101–PQ423109.

Table 1. Distribution of the number of samples (*n* = 116) collected from adult female Holstein-Friesian dairy cows across different civil parishes within the municipalities of Angra do Heroísmo and Praia da Vitória. Each entry provides the name of the civil parish and the corresponding number of samples collected from that area.

Municipality	Civil Parish	Number of Samples (*n*)
Angra do Heroísmo	Doze Ribeiras	2
	Cinco Ribeiras	6
	Altares	1
	Feteira	10
	Posto Santo	4
	Porto Judeu	7
	Raminho	5
	Ribeirinha	1
	Santa Bárbara	3
	São Bartolomeu dos Regatos	7
	São Bento	5
	São Mateus da Calheta	5
	São Sebastião	7
	Serreta	7
Praia da Vitória	Agualva	5
	Biscoitos	2
	Cabo da Praia	2
	Fonte do Bastardo	2
	Fontinhas	9
	Lajes	6
	Santa Cruz	7
	São Brás	6
	Vila Nova	5
	Quatro Ribeiras	2
Total	-	116

2.3. Statistical Analysis

The occurrence of *Blastocystis* sp. was assessed by calculating the proportion of positive samples relative to the total number of samples analyzed, along with the corresponding 95% confidence interval (95% CI). Data processing and preliminary analysis were carried out using Microsoft Excel® for Microsoft 365 MSO (Redmond, WA, USA) (version 2312 Build 16.0.17126.20132, 64-bit).

2.4. Phylogenetic Analysis and Subtyping of Blastocystis sp. Isolates

Full-length SSU rRNA gene sequences from representatives of the various *Blastocystis* sp. subtypes (STs) and subgroups available at the time of the analysis were retrieved from the GenBank database, serving as the primary reference framework. To improve the phylogenetic resolution for certain isolates, additional sequences were incorporated for ST30, ST21, ST25, and ST14 (5, 6, 8, and 8 additional sequences, respectively). Sequence alignment was performed using MAFFT v7.490, applying the L-INS-i method due to its robustness in aligning diverse sequences. While initial trimming using TrimAl at a threshold of 0.7 was explored, untrimmed alignments were ultimately preferred for

maximum-likelihood phylogenetic analysis, as they provided better tree resolution and sample placements. The Maximum Likelihood tree was constructed using IQ-TREE v2.3.6 with 1000 bootstrap replicates, selecting the K2P + I + G4 substitution model to ensure consistency with prior studies [6,7,28]. Moreover, *Proteromonas lacertae*, a commensal flagellate found in reptiles and amphibians, was used as the outgroup due to its close phylogenetic relationship with *Blastocystis* in earlier studies [9,29,30]. The resulting phylogenetic tree was annotated and visualized using the Interactive Tree of Life (iTOL) platform, enabling in-depth representation of phylogenetic relationships [31].

3. Results

In the present study, 17 out of 116 samples tested by qPCR were found to be positive for *Blastocystis* sp., of those eight were mixed infection resulting in an overall occurrence of 14.7% (17/116; 95% CI: 8.78–22.42). Except for one sample (PQ423109) from Praia da Vitória (Santa Cruz), all positive samples identified as single infection (n = 9) were collected from animals of the Angra do Heroísmo municipality. BLASTn analysis of sequences from these nine samples confirmed the presence of *Blastocystis* sp. and the highest hits are displayed in Table 2.

Table 2. Summary of the *Blastocystis* sp. sequences obtained in this study, including the accession numbers from this study, the highest-hit accession numbers from reference BLAST database, the country of origin of the highest hit, percentage identity (%), and the host organism.

Accession Number (This Study)	Accession Number Highest Hit	Country	Perc. Identity (%)	Host
PQ423101	MN326608	China	98.95	Human
PQ423102	OL981854	China	100	Cattle
PQ423103	OP716163	Vietnam	100	Human
PQ423104	MN326608	China	100	Human
PQ423105	OM522176	France	100	Cattle
PQ423106	OM522189	France	99.66	Cattle
PQ423107	PP320637	Egypt	100	Sheep
PQ423108	OL981921	China	100	Cattle
PQ423109	MH883053	Lebanon	100	Cattle

The phylogenetic analysis including homologous sequences of all known STs of *Blastocystis* sp., enabled the unambiguous subtyping of 8 out of the 9 sequences obtained in the present study (Figure 2). Seven distinct subtypes were detected: ST3 (two sequences from Altares and Feteira), ST5 (one sequence from Santa Cruz), ST7 (one sequence from Feteira), ST10 (one sequence from Posto Santo), ST14 (one sequence from Feteira), ST25 (one sequence from Feteira), and ST42 (one sequence from São Mateus da Calheta). One sample (sequence PQ423107 from Feteira) could not be assigned to a specific ST but showed some similarity to ST30 and ST21. Most of the identified STs (ST5, ST7, ST10, and ST14) are considered zoonotic and have been reported in humans.

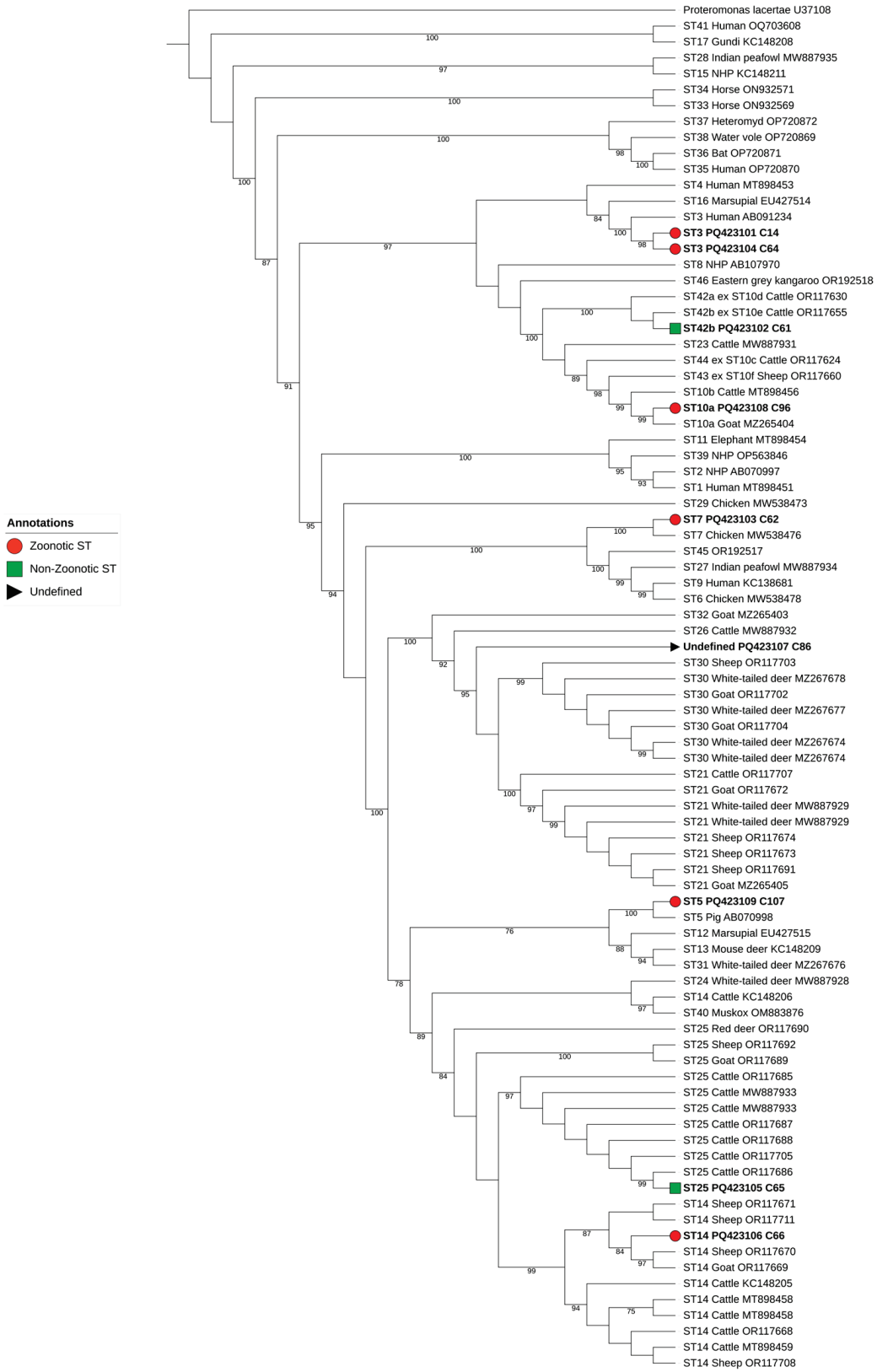

Figure 2. Maximum-likelihood phylogenetic tree of *Blastocystis* sp. STs based on the comparison of SSU rRNA gene sequences. The tree was generated using IQ-TREE with the K2P + I + G4 substitution model and 1000 bootstrap replicates. Zoonotic, non-zoonotic, and undefined isolates are represented by red circles, green squares, and gray triangles, respectively, as indicated in the legend. *Proteromonas lacertae* was used as an outgroup, and bootstraps lower than 75% are not displayed.

4. Discussion

This study represents the first molecular identification and ST distribution of *Blastocystis* sp. isolates colonizing Holstein-Friesian dairy cattle from Terceira Island, part of the Azores Archipelago. It provides new insights into the parasite's occurrence, genetic diversity, and potential zoonotic risk within this geographically isolated region. Although a diverse range of *Blastocystis* sp. STs was identified, the overall occurrence rate of 14.7% is significantly lower than the global prevalence rate of 24.4% determined worldwide from cattle cohorts [11]. This comparatively low prevalence is also evident when compared to rates reported in other European countries, including Denmark, the UK, Spain, France, and mainland Portugal, as summarized in Table 3.

Table 3. Prevalence of *Blastocystis* sp. and ST distribution in cattle cohorts from European countries. The same STs as those reported in the present study are highlighted in bold.

Country	% of *Blastocystis* sp. (no. pos/Total no.)	% *Blastocystis* ST (no. pos)	Method	Reference
Italy	7.69% (1/13)	NA (no ST reported)	PCR	[32]
Turkey	11.3% (9/80)	**ST10**: 22.2% (2), **ST14**: 77.8% (7)	PCR	[33]
Denmark	100% (25/25)	**ST5**: 12% (3/25) **ST10**: 48% (12/25)	PCR	[34]
UK	22.6% (7/31),	**ST1**: 3.23% (1/31), **ST5**: 3.23% (1/31) **ST10**: 9.68% (3/31) MI: 6.45% (2/31)	PCR	[35]
Spain	32.14% (108/336)	ST1: 0.30% (1), **ST3**: 0.30% (1), **ST5**: 2.98% (10), **ST10**: 31.55% (106), **ST14**: 19.94% (67), ST21: 20.83% (70), ST23: 11.61% (39), ST24: 8.33% (28), **ST25**: 27.38% (92), ST26: 30.06% (101)	PCR and NGS	[36]
France	54.79% (866/1581)	ST2: 0.13% (2), **ST10**: 2.28% (36), **ST14**: 4.05% (64), Indefinite MI: 3.61% (57)	qPCR	[37]
Portugal	32.18% (28/87)	**ST1**: 1.15% (1), **ST5**: 6.90% (6), **ST10a**: 17.24% (15), ST10b: 2.30% (2), ST13: 1.15% (1), **ST14**: 5.75% (5), ST21: 18.39% (16), ST23: 3.45% (3), ST24a: 2.30% (2), ST24b: 1.15% (1), ST24c: 1.15% (1), **ST25**: 29.89% (26), ST26: 28.74% (25), ST30: 1.15% (1), ST42a: 27.59% (24), **ST42b**: 25.29% (22), ST43: 1.15% (1), ST44: 4.60% (4)	PCR and NGS	[38]

PCR—Conventional polymerase chain reaction; qPCR—Real-time polymerase chain reaction; NGS—Next Generation Sequencing, MI—Mixed Infection.

Notably, with the exception of Italy and Turkey, where lower occurrences were observed, epidemiological surveys conducted in cattle from other European regions reported higher occurrence rates of *Blastocystis* sp. [32–38]. This lower occurrence on Terceira Island suggests that factors unique to the region, such as environmental conditions and farming practices, may be limiting the transmission of the protozoan, in contrast to the higher occurrence observed on mainland Portugal [38], where environmental conditions and farming practices may be more conducive to its transmission. However, caution is needed when comparing these results with other studies, as differences in sample sizes and methodologies may influence the findings. Regarding the STs identified herein, all sequences corresponding to single infection were successfully classified except for one, which did not cluster with any reference sequence of known ST and was therefore assigned as undefined, as it does not fulfill the criteria for designation as a new ST, as outlined in the proposed guidelines by Maloney, J.G., & Santin, M. (2021) [30]. These criteria include having an almost complete SSU rRNA gene sequence ($\geq$80% of the approximately 1800 bp) and a sequence divergence greater than 4% from any existing ST. In addition to subtype diversity, common mutations, such as single nucleotide polymorphisms (SNPs) and insertions within the SSU rRNA gene, are frequently observed [30]. These variations play a role in defining subtypes and may influence host specificity, zoonotic potential, and virulence, although these relationships are still not fully understood. Some subtypes appear to be more commonly associated with particular hosts, such as ST1–ST4 with humans,

ST5 with pigs, ST6 and ST7 with birds, and ST10 and ST14 with cattle [2,4,39]. Subtypes can also modulate the immune response in distinct ways, as exemplified by ST7 and ST1. ST7 has been shown to provoke a pro-inflammatory environment through its interaction with epithelial and dendritic cells, leading to a reduction in beneficial gut bacteria such as *Bifidobacterium longum* and *Lactobacillus brevis* [40–42]. Additionally, ST7 increases the production of proteases that can compromise the intestinal barrier, potentially contributing to gut dysfunction [43,44]. In contrast, ST1 is associated with an increased diversity of the gut microbiome and the promotion of an anti-inflammatory state in the intestinal mucosa, highlighting its potential role in maintaining intestinal homeostasis [43,45]. The lower-than-expected occurrence of *Blastocystis* sp. in cattle from Terceira Island contrasts with initial expectations. Indeed, cattle are commonly considered potential reservoirs for zoonotic *Blastocystis* sp. STs, such as ST10 and ST14, which are frequently observed in livestock populations [2]. However, in our study, the predominance of ST3, typically considered an anthroponotic (human-associated) ST, raises interesting possibilities about transmission dynamics. The slightly higher occurrence of ST3 suggests that there may be a greater degree of human-to-animal transmission than initially anticipated, particularly in settings like family-owned farms where closer interaction between humans and their cattle is more common. In such environments, the direct handling of cattle by farmers may contribute to the transmission of anthroponotic STs, such as ST3, from humans to animals. The presence of ST3 in cattle could thus reflect this close, potentially frequent contact, leading to a shift in the assumed source of the infection.

The detection of ST7 in our cohort of bovids further complicates the scenario, as its presence was not found in previous studies in European countries. ST7 is typically associated with birds [2], and its presence in cattle may be associated with possible transmission from ducks, hens, or other avian species. This likely implies a possible cross-species transmission pathway, where birds are contributing to the presence of this avian-adapted ST in the cattle population.

Furthermore, the identification of ST5, a zoonotic subtype commonly found in both humans and animals, adds another layer of complexity to understanding the potential zoonotic risk of *Blastocystis* on Terceira Island. While ST5 was found in this study, its low prevalence suggests a relatively minimal risk of zoonotic transmission from cattle to humans. Nevertheless, it highlights the need for further research into its distribution and zoonotic potential.

However, the overall low occurrence of *Blastocystis* sp., including zoonotic STs, could be attributed to the extensive grazing system practiced on Terceira Island. In this traditional system, cattle are widely dispersed across large pastures, which minimizes close contact between animals and, in turn, lowers the likelihood of parasite transmission within the cattle population. This finding is consistent with a study that reported higher occurrences of *Blastocystis* sp. in backyard cattle compared to those in more extensive farming systems [46], suggesting that farming practices significantly influence transmission rates. The traditional extensive grazing method on Terceira Island likely serves as a natural barrier to the spread of *Blastocystis* sp. whereas more intensive farming systems, in which cattle are confined and kept in closer proximity, tend to facilitate greater transmission of pathogens, including *Blastocystis* sp.

Although the pathogenic role of *Blastocystis* sp. in domestic and wild animals remains unclear, primarily because most studies have focused on non-diarrheic individuals, the primary concern lies in its potential zoonotic risk to humans [38]. In this study, zoonotic ST5, ST7, ST10, and ST14 were detected, but their low occurrence suggests a minimal risk of transmission from cattle to humans. Moreover, the identification of the two non-zoonotic

ST25 and ST42b further supports the conclusion that *Blastocystis* sp. poses a limited threat to human health on Terceira Island.

5. Conclusions

This study presents the first molecular identification of *Blastocystis* sp. in dairy cattle from Terceira Island. The low prevalence of the parasite, coupled with the limited presence of zoonotic STs, indicates a minimal risk of transmission from cattle to humans in the region. Local farming practices, particularly the extensive grazing systems, are likely to mitigate both the spread of *Blastocystis* among cattle and the potential for human exposure. However, the study is subject to several limitations, including a small sample size, data collection confined to a single season, and the absence of a comparison of different farming practices. These limitations underscore the need for further research on the transmission dynamics of *Blastocystis* sp., to address these gaps and provide a more comprehensive understanding of its zoonotic risks.

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Gastrointestinal Parasites in Non-Human Primates in Zoological Gardens in Northern Italy

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Simple Summary: Non-human primates, due to their close taxonomic relationship with humans, host the highest diversity of parasites with zoonotic potential. In zoological gardens, the presence of helminths and protist parasites with a direct lifecycle is virtually unavoidable; biosecurity measures are of the utmost importance to control their spread and environmental load and avoid transmission to staff and visitors. In this study, we investigated the population of non-human primates in two zoos in Northern Italy to evaluate gastrointestinal parasite diversity and their zoonotic potential. The highest prevalence was registered for protist taxa, i.e., *Giardia* and *Blastocystis*. Proof for the transmission of parasites from synanthropic rats to the animals in the collection was also provided.

Abstract: Non-human primates (NHPs) host a variety of helminth and protist parasites that are able to cause infection in humans. Gastrointestinal parasites in NHPs living in two zoological gardens of Northern Italy were studied. An total of 96 faecal pools were collected from 26 groups of NHPs. The mini-Flotac method was applied to fecal samples to detect gastrointestinal helminthiases, while the detection of the protists *Cryptosporidium* spp., *Blastocystis* sp. and *Giardia duodenalis* was performed by targeting SSU rRNA through nested PCR and real-time PCR; they were further studied by sequencing the same gene for *Blastocystis* and βgiardine and triosephosphate isomerase (TPI) genes for *Giardia*. Twenty-two out of the 96 examined fecal pools (22.9%) were positive for one or more helminth species, including *Hymenolepis diminuta*, Trichurid, Capillariid and Strongylid eggs. All samples were negative for *Cryptosporidium* spp., while 16/26 (61.5%) animals were positive for *G. duodenalis* in the real-time PCR; the sequences obtained assigned them all to sub-assemblage BIV. *Blastocystis* sp. was detected in 22/26 of the NHPs (84.6%); molecular analyses attributed the isolates to ST 4, allele 92. Analyses of the feces of sympatric rats revealed the presence of the same allele, as well as of *Hymenolepis diminuta* eggs, raising concern about their role as parasite reservoirs in the facilities.

Keywords: non-human primates; *Giardia*; *Blastocystis*; helminths; zoological gardens

1. Introduction

The close phylogenetic relationship between humans and non-human primates (NHPs) is the basis of the potential transmission of all classes of pathogens among the two. Biosecurity measures are of the utmost importance in zoological gardens to avoid the spreading of pathogens with zoonotic potential to the environment, given the close proximity between the animal collection and their human caregivers, and potentially, visitors.

Monoxenous parasites, here including both helminthic and protozoan species, greatly prevail in zoological gardens, facilitated by fecal–oral transmission and the accumulation of their infective forms in the environment of the enclosure. The spreading of protozoan infections is facilitated by the lower infecting dose and brief prepatent period. An assessment of the zoonotic risk coming from the contact between NHPs and humans has been

possible with the development of fine molecular tools that allow the definition of isolates beyond species, assemblages or subtypes, the identification of zoonotic and non-zoonotic strains, and the determination of the likelihood of transmission from animals to humans or vice versa. To date, four species of *Cryptosporidium*, including *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium felis* and *Cryptosporidium muris*, have been detected in NHPs in captive or wild conditions [1–4], with the bulk of the isolates from captive NHPs belonging to zoonotic strains of *C. hominis* [3–5]. *Giardia duodenalis* is considered a species complex with at least eight distinct genotypes/assemblages, designated as A–H. The couple A+B assemblages make up 95% of all isolates in both NHPs and humans, while assemblage E is less frequent but not rare in both groups [6–8]. The study of human and animal isolates through multilocus analysis has defined the existence of subtypes within assemblage A and B that are more adapted to one of the two groups, but the great bulk of the strains are shared between humans and NHPs, demonstrating possible transmission between the two [8–10]. *Blastocystis* is the most commonly found eukaryotic protist in the intestinal tract of humans and captive NHPs in surveys worldwide. At least 28 subtypes, named ST1-ST17, ST21 and ST23-32, have been recognized within the genus, based on polymorphism at the small subunit ribosomal RNA gene. A different host tropism is typical to each subtype, with a relevant overlap in the host spectrum among subtypes, including humans among animal hosts. Though it is now quite clear that certain subtypes are more often associated with disease in humans, causing IBS and urticaria, *Blastocystis* in wildlife and captive animals is often characterized as asymptomatic [11].

Strongylid nematodes are among the most commonly reported gastrointestinal parasites found in wild primates [12]. Primate species can be infected with several nematodes of the suborder Strongylida, such as hookworms (*Ancylostoma*, *Necator*), *Trichostrongylus*, *Ternidens*, and *Oesophagostomum*, whose eggs cannot be distinguished with certainty by microscopy alone.

The available data on the parasitofauna of NHPs in Italy are mainly related to zoological gardens located in central and southern Italy. With this survey, the prevalence of gastrointestinal parasites in captive NHPs in two zoological gardens in Northern Italy is evaluated, focusing on helminthic and protist taxa, with the aim of evaluating potential zoonotic transmission and informing correct prophylactic measures. The occurrence of parasitic taxa in rats living in sympatry in the zoological gardens was also investigated to reveal their eventual epidemiologic role in parasite transmission to NHPs.

2. Materials and Methods

2.1. Sampling

Fecal samples of non-human primates were collected from two zoological gardens (zoological garden 1 = ZG1; zoological garden 2 = ZG2) in Northern Italy. The species of NHPs included in the sampling belonged to the four major taxonomic groups, namely New World Monkeys (Plathyrrini, n = 11), Lemurs (Lemuridae, n = 8), Old World monkeys (Cercopithecidae, n = 6 groups) and apes (Hominoidea, n = 1) (overall animal groups n = 26) (Table S1). Each group was hosted in the same enclosure for the entire duration of the project, with both indoor and outdoor accommodations available. The sampling was repeated in each enclosure four times in a one-year period, precisely in the months of April (n = 24 samples), July (n = 24), October 2021 (n = 23) and January 2022 (n = 25), finally collecting 96 samples overall from 26 animals or groups of animals hosted in the same enclosure (11 from ZG1 and 15 from ZG2, respectively). Eight samples were missed due to logistic constraints. Sampling was conducted for 3 consecutive days in each enclosure to overcome the intermittent excretion of *Giardia* and *Cryptosporidium* sp. and the three-day samples were pooled in a unique sample.

Treatments were carried out on the animals as follows: ZG1: fenbendazole or albendazole after the 1st, 2nd and 3rd sampling; ZG2: ivermectin and fenbendazole after the 3rd sampling.

Additionally, the feces of free-living sympatric rats were also collected from the two zoos, in the close vicinity of cages or within them, at the end of the sampling period. Twelve samples, representing different areas of the two zoos (9 for ZG1 and 3 for ZG2), were overall collected.

After collection, the stool samples were stored at +4 °C before analyses and transferred to the Laboratory of Parasitology and Parasitic Diseases of the Department of Animal Medicine, Production and Health, Padova University.

2.2. Traditional Copromicroscopic Analyses

A quali-quantitative copromicroscopic analysis was performed on a 2 g aliquot from each sample, targeting helminth eggs and *Giardia* spp. cysts, by means of the MiniFlotac technique [13], using a 1350 high-gravity solution (sodium nitrate and sucrose). For the research of larvae of bronchopulmonary nematodes, a Baermann technique was additionally employed on all samples, using a further 4–5-g aliquot and allowing the sedimentation of eventually present larvae for 24 h [14]. The eggs and cysts were searched using an optic microscope at 100× and 400×, respectively, identified following guidelines in the literature to the lowest taxonomic level possible [15,16]; these were counted and quantified as eggs/cysts per gram of feces (EPG/OPG). A 50 g aliquot of each fecal sample was then subject to centrifugation to determine the concentration of protozoan elements [17]; this was to increase the sensitivity of traditional and molecular methods targeting *Cryptosporidium* spp., *Giardia* and *Blastocystis* spp. More in detail, each stool specimen was homogenized in distilled water, filtered through gauzes into a 50 mL tube and centrifuged at 900× *g* for 30 min. A faecal smear was obtained from the sediment, stained with the modified Ziehl–Neelsen method [18] and observed under a light microscope at 1000× magnification for the research of *Cryptosporidium* spp. oocysts. Additionally, a 2–5-g aliquot of the sediment was frozen at −20 °C for the molecular research of protist parasites.

2.3. Molecular Analyses

Molecular research of *Cryptosporidium* spp., *Giardia* and *Blastocystis* was performed on each sample. All primers and probes used are reported below in Tables 1 and 2. DNA from the stool specimens was extracted using a QIAampDNA Stool Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions.

Nested PCR targeting the 18S rDNA gene was used for the detection of *Cryptosporidium* spp. following Miller et al. [19]. A qPCR assay was applied for the detection of *Giardia duodenalis*, following Verweij et al. [20]. Positive samples were further studied for their genotype by amplifying fragments covering β-giardine (BG) and the Triophosphate Isomerase (TPI) genes by nested PCR following Lalle et al., 2005 and Sulaiman et al., 2003 [21,22]. A TaqMan qPCR assay was used for detecting *Blastocystis* STs targeting the rDNA 18S gene, following Stensvold et al., 2012 [23]. Positive samples were further characterized into subtypes using the PCR protocol outlined by Scicluna et al., 2006 [24], using the pan-*Blastocystis* primer RD5 and the universal eukaryotic primer BhRDr targeting the barcode region of the 18S rRNA gene.

The PCR products were visualised using UV light on a SYBR Safe DNA-stained 1% agarose gel. Subsequently, all the secondary PCR products were purified by ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, Thermo Fisher Scientific, Milan, Italy) and sent for sequencing at Macrogen Europe (Milan, Italy). The consensus sequences were assembled with the software ChromasPro version 2.4.3 (Technelysium Pty Ltd., South Brisbane, Australia). The sequences were compared with the non-redundant database available in the GeneBank® database using the software BLAST (Blast+2.14.0) [25] and PubMLST (www.pubmlst.org/species-id, accessed on 10 August 2023) [26].

The sequences of *Giardia duodenalis* were attributed to their subassemblages using previously defined references (TPI gene: AF069561—sub-assemblage BIII; AF069560—sub-assemblage BIV; BG gene: AY072726—sub-assemblage BIII; AY072726—sub-assemblage BIV) [8].

The sequences obtained from the selected strains isolated in this study have been deposited in the NCBI database.

Table 1. Primers used in PCR reactions targeting *Cryptosporidium* spp., *Giardia* spp. and *Blastocystis* sp.

		Primer F (5′-3′)	Primer R (5′-3′)	Amplicon Size (bp)
***Cryptosporidium* spp. [16]**				
18S	1st round	C1F TTCTAGAGCTAATACATGCG	C1R CCC TAA TCT TTC GAA ACA GGA	1325
	2nd round	C2F GGAAGGGTTGTATTTATTAGATAAAG	C2R AAGGAGTAAGGAACAACCTCCA	850
***Giardia* spp. [18,19]**				
β-giardine		G7 AAGCCCGACGACCTCACCCGCAGTGC	G759 GAGGCCGCCCTGGATCTTCGAGACGAC	753
		βGiar-F GAACGAACGAGATCGAGGTCCG	βGiar-R CTCGACGAGCTTCGTGTT	511
TPI		F1 AAATIATGCCTGCTCGTCG	R1 CAAACCTTITCCGCAAACC	605
		F2 CCCTTCATCGGIGGTAACTT	R2 GTGGCCACCACICCCGTGCC	530
***Blastocystis* [21]**				
18S		RD5 ATCTGGTTGATCCTGCCAGT	BhRDr GAGCTTTTTAACTGCAACAACG	600

Table 2. Primers used in qPCR reactions targeting *Giardia* spp. and *Blastocystis*.

	Primer F (5′-3′)	Primer R (5′-3′)	Probe (FAM-5′- 3′-TAMRA)
***Giardia* spp. [17]**			
18S	*Giardia* F GACGGCTCAGGACAACGGTT	*Giardia* R TTGCCAGCGGTGTCCG-	CCCGCGGCGGTCCCTGCTAG
***Blastocystis hominis* [20]**			
18S	Blasto FWD F5	Blasto R F2	Probe (FAM-5′-MGBNFQ)
	GGTCCGGTGAACACTTTGGATTT	CCTACGGAAACCTTGTTACGACTTCA	TCGTGTAAATCTTACCATTTAGAGGA

2.4. Statistical Analyses

Differences in the occurrence of parasite taxa along the seasons were tested using the using the $\chi 2$ test, setting significance at $p > 0.05$. Statistical analyses were performed using the online software EpiTools (AusVet2024), available from AusVet Animal Health Services https://epitools.ausvet.com.au/ (accessed on 28 August 2024).

3. Results

3.1. Copromicroscopic Analyses

Twenty-two of the 96 fecal samples (22.9%), belonging to 11 NHPs, were positive for helminth eggs; seven animal groups showed more than one positive sample throughout the sampling period. Eight animals had a co-infection with two or more parasitic taxa. Six helminths and one protozoan parasite were identified, with a higher prevalence of nematode eggs (*Trichuris* spp.: $p = 10.4\%$, *Capillariinae* Gen. spp.: $p = 9.3\%$, strongylids: $p = 6.2\%$, *Strongyloides* spp.: $p = 2.1\%$), followed by cestode eggs (*Hymenolepis diminuta*: $p = 2.1\%$) and cysts of *Giardia* spp. (1.04%) (Table 3, Figure 1).

Table 3. Parasites in the animal feces, as detected by copromicroscopy and molecular analyses.

Host		Tested Samples	Copromicroscopic Analyses					Molecular Analyses			
			Tric.	Cap.	Str.	H.d.	*Giardia* spp.	*Giardia duodenalis*	*Giardia* Assemblages	*Blastocystis* sp.	*Blastocystis* Subtypes
NWM	animal groups	12	1	6	3	1	Neg	9	B	11	ST4, ST8
	feces	42	1	6	3	1	Neg	17		16	
OWM	animal groups	5	2	1	Neg	1	Neg	3	B	1	nd
	feces	20	5	3	Neg	1	Neg	4		3	
Lemurs	animal groups	8	3	Neg	2	Neg	1	4	B	8	ST4
	feces	30	6	Neg	5	Neg	1	11		22	
Apes	animal groups	1	Neg	Neg	Neg	Neg	Neg	Neg	-	1	nd
	feces	4	Neg	Neg	Neg	Neg	Neg	Neg		1	

NWM = New World Monkeys; OWM = Old World Monkeys; Tric. = *Trichuris* spp.; Cap. = *Capillariinae* Gen. spp. sp.; Str. = strongylid eggs, *Strongyloides* spp.; H.d. = *Hymenolepis diminuta*; Neg = negative; nd = not determinated.

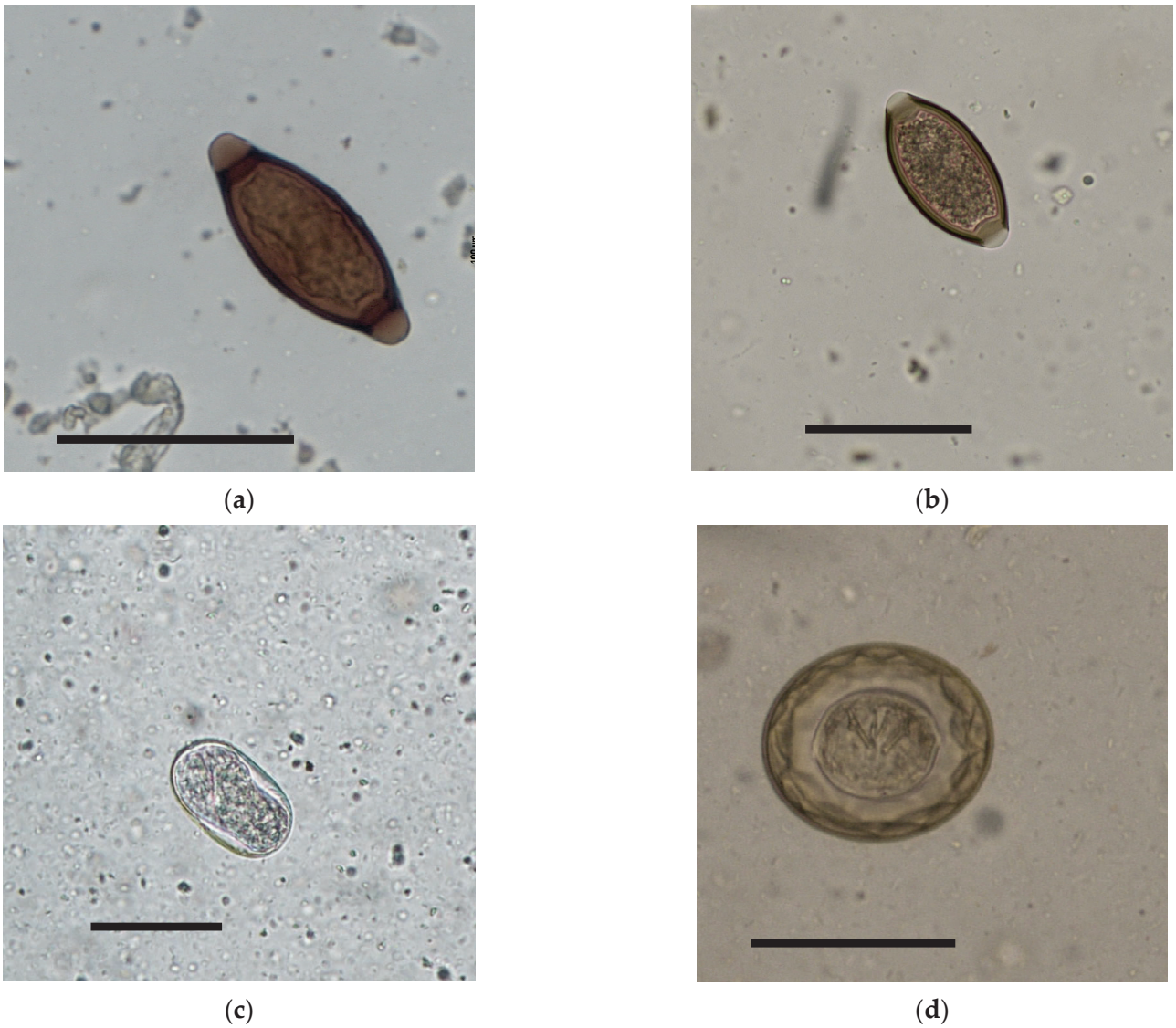

Figure 1. Light microscope pictures of parasite eggs recovered from fecal samples of NHPs: *Trichuris* spp. (**a**) *Lemur catta*, Capillarinae Gen. spp. (**b**) *Colubus guereza*, *Strongyloides* spp. (**c**) *Lemur catta* and *Hymenolepis diminuta*. (**d**) *Callithrix argentata*. Scale bar = 50 ηm.

Helminth eggs were observed in the feces of New World Monkeys (6/11), Old World monkeys (2/6) and Lemurs (2/8), while no parasitic elements were detected in the stool of apes. Low parasitic burdens were observed, with the exception of a few samples that were highly positive for strongylid eggs (*Capillariinae* Gen. spp. EPG: 5–90; *Trichuris* spp. EPG: 5–170; strongylid EPG: 10–1120; *Strongyloides* spp. EPG: 35–185; *H. diminuta*: 30–170).

No gastrointestinal symptoms were observed in parasitized animals during the investigation period. All samples of rat feces were positive for at least one helminth species, namely *Capillariinae* Gen. spp. (12/12, $p = 100\%$, EPG: 15–225) and *H. diminuta* (7/12, 58.3%; EPG: 15–800).

3.2. Molecular Analyses

Overall, 32/96 samples from NHPs (33.3%) tested positive for *Giardia duodenalis* when the qPCR assay was performed; these belonged to 16/26 animal groups, 10 of them showing more than one positive sample throughout the sampling period. Clear sequences of β-giardine (BG) and triosephosphate isomerase (TPI) genes were retrieved from three and four samples, respectively (BG accession numbers: PQ007548—PQ007549—PQ007552; TPI accession numbers: PQ007553—PQ007555—PQ007557—PQ007558), overall belonging to isolates from six animal groups belonging to four species (3 *Lemur catta*, 1 *Colobus guereza*, 1 *Saguinus oedipus*, 1 *Callithrix jacchus*); none of the isolates could be sequenced at both loci. All isolates were classified as *G. duodenalis* sub-assemblage BIV from alignment with reference sequences (all identities > 99.7%). *Blastocystis* sp. was detected by qPCR in 42/96 samples (43.7%), belonging to 21/26 animal groups, of which 10 had repeated positivity throughout the sampling period. Eight positive samples were suitable and submitted for sequencing, but only eight sequences of good quality were retrieved; these were from *Saguinus tripartitus* and *Varecia rubra*, respectively (Table 3) (accession numbers: PP952159—PP952160). A comparison with the public database PubMLST assigned both the isolates to ST4, allele 92 (length of nucleotidic region: 546 and 615 bp, query coverage 99.9% and 100%).

One of the rat fecal samples tested positive for *Blastocystis* with PCR, and sequences of good quality were obtained (accession number: PP952161); a comparison with the PubMLST database attributed the isolate to ST4, allele 92 (length of nucleotidic region 615 bp, query coverage 100%).

A higher prevalence of helminthiases was observed in spring and autumn, while the prevalence of *Giardia* and *Blastocystis* spp. was higher in summer and autumn, respectively. Nevertheless, no statistically significant difference in prevalence was found among the four seasons, neither for helminthic infections nor for *Giardia* or *Blastocystis* ($p > 0.05$).

4. Discussion

The permanence of specific animal groups in a given enclosure, sometimes for life-long periods, may lead to the accumulation of the infective forms of parasites in the environment, facilitating the spread of parasites, especially for monoxenous species. In this survey, eggs attributable to four helminth taxa (*Capillariinae* Gen. spp., *Trichuris* spp., strongylids, *H. diminuta*) and two protist genera (e.g., *Giardia* and *Blastocystis*) were isolated from captive non-human primates in two zoological gardens in Northeastern Italy, representing the first report of its kind in the area.

The prevalence of helmithiases in this survey is lower compared with other studies in zoological gardens of Europe [14,27] and Italy [28]. Among the parasite taxa reported, all of them, except the cestode *H. diminuta*, present a direct life cycle. Fecal–oral transmission parasites are among the most common found in wild animals kept in confined environments. Considering also the social habits of NHPs [29], despite the use of control and treatments, the radical elimination of parasitic elements excreted by the feces is challenging, if not impossible [14,30,31]. In this research, treatments with albendazole/fenbendazole/ivermectin were not efficient in the complete eradication of helminth infection in the enclosures, with the treated animals being positive again at the successive samplings. Despite the associated

daily cleaning routine, the resistance of envinronmental parasitic forms might account for the persistence of helminthiases in the captive NHPs.

The cestode *H. diminuta* is primarily a rodent parasite, with occasional reports in other mammals, including NHPs and humans [32,33]. In Italy, it is rarely reported in humans, dogs and pet squirrels [34–36]. In this study, *Trichuris* spp. and *Capillariinae* Gen. spp. were the most frequently detected helminths in copromicroscopic analyses. *Trichuris* spp. inhabits the cecum and colon of a broad range of mammals and has a cosmopolitan distribution. In zoological gardens of Europe, *Trichuris* is also one of the most common parasite genera detected in NHPs [31]; in Italy, it was reported in *L. catta*, *Papio cynocephalus*, *Eulemur albifrons, Macaca fuscata* and *Chlorocebus aethiops* in captivity [28,31]. Recent molecular and morphological studies suggest that *T. trichiura*, the only species traditionally thought to infect primates, may actually be considered as a species complex with several sibling/cryptic species, showing different host specificities [37,38]. The phylogenetic analysis performed on both mitochondrial and nuclear markers discriminated between two clades within *T. trichiura*, with some subclades including isolates from both humans and NHPs [38–40]. In this study, molecular characterization was not attempted on helminth eggs. As morphological discrimination among clades is unfeasible, at least from eggs, zoonotic risk cannot be excluded in this context.

Capillariinae eggs, observed in this survey in feces of OWM, NWM and rats, had previously been reported via copromicroscopic exam in capuchin monkeys in South America [41] and recently in *Sapajus paella* in a zoological garden in central Italy [31]. *Capillaria brochieri* n. sp. was described in the intestine of *Pan paniscus* with diarrhea in Zaire [42]. *Capillaria hepatica* (syn. *Calodium hepaticum*), a potentially zoonotic species, was histologically described in a retrospective study in the liver samples of primates from a zoological collection in the UK [43] and previously in wild *Gorilla gorilla* in Rwanda; in both cases, the most likely source of infection was judged to be rodents, such as rats, representing the most typical hosts for this species [44]. Despite the difficulties associated with DNA isolation and/or PCR inhibitors, the use of molecular testing could be useful and desirable for identifying species and elucidating the zoonotic potential [31].

Such a high prevelance of members of Trichuroidea in this and other surveys may be partially explained by the inefficiency of commonly used anthelminthic drugs against the two genera *Trichuris* and *Capillaria*, as well as by the high resistance of their eggs in the environment.

In this survey, strongylid and *Strongyloides* eggs were observed only in NWM and lemurs. *Oesophagostomum* sp. eggs, strongylid and Trichostrongylid eggs and strongyliform larvae were observed in copromicroscopic analyses of NHPs in zoological gardens in Italy [28,31,45]. Gastrointestinal strongylids are among the most commonly reported parasitic infections in NHPs all over the world. The strongylid species are indistinguishable through traditional microscopic approaches; molecular analyses have recently led to the identification, at the species level, of *Trichostrongylus colubriformis* larvae/eggs from ring-tailed lemurs in captivity [45]). DNA metabarconding has been used for studying strongylid community diversity in the wild [46], assuming the presence of multi-species infections in that context. A similar approach may also find application in captive NHPs in the future, overcoming the limitations of classic PCRs when dealing with a mixture of different DNAs.

Strongyloides spp. infections are commonly reported in free-ranging and captive NHPs in many countries [47]. Three *Strongyloides* species have been described in NHPs, two of which are potentially zoonotic (*S. stercoralis* and *S. fuelleborni*). Strongyloidiasis by *S. stercoralis* is present in humans and dogs in Italy [48–50] and *S. fuelleborni* eggs were described by copromicroscopic exam in the feces of baboons in a zoo in southern Italy [28]. The molecular approach is important for distinguishing species with and without zoonotic potential, when NHPs and humans share the same environment such as in captive and semi-captive settings [47,51].

To date, four species of *Cryptosporidium* have been detected in NHPs, with the bulk of the isolates from captive NHPs belonging to zoonotic genotypes of *C. hominis* [2–4]. The

prevalence of *Cryptosporidium* spp. seems to be low in zoological gardens, with values varying from 0% in zoos in Cordoba and Almuñecar in Spain [52,53] to 1% in French zoos [54]. A much higher prevalence was previously reported in a Barcelona zoo, with 27.6-44.4% of animals positive over a ten-year period [55,56]. A high prevalence (66.7%) of *Cryptosporidium* sp. was also reported in the fecal samples of NHPs from two zoo in Southern Italy [28]. High hygiene standards and the management of water sources in zoos probably account for the control of *Cryptosporidium* in modern zoos.

Giardia was reported to have a moderate prevalence among all investigated groups except apes, with a prevalence varying from 20% in samples from OWM to 40.5% in NWM. Apes are actually the only group of animals spatially isolated from other NHPs. Wether this is due to casualty, given the low number of sampled animals, or to a minor transmission risk could be a matter of investigation. A moderate to high prevalence of *Giardia* infections has been reported in NHPs in European zoos, varying from 28% at Zagreb zoo [57] to 47% of lemurs kept in Rome zoo [30] and 70% in two Spanish zoos [58]. A more recent, comprehensive survey on protist infections in Cordoba zoo reported that 22% of NHPs were infected with *Giardia* [53]. All surveys report asymptomatic infections, in agreement with this survey. The presence of visible *Giardia* spp. cysts at copromicroscopic examination only in one sample and the high number of qPCR-positive samples with ct > 30 may indicate infections with a low burden in the animals. Alternatively, chronic or repeated infections, as reflected by several positive results throughout the year in the same enclosure, may explain immune system activation towards the protozoan, resulting in asymptomatic infections [59].

Sequences of good quality could be retrieved only from seven samples, and none could be sequenced at more than one locus. An analysis of triosephosphate isomerase (TPI) or β-giardine (BG) genes attributed all isolates to subassemblage BIV. The dominance of assemblage B in NHPs has already been reported [6], and this assemblage seems to be well-adapted to all primates [8]. Sub-assemblage BIV has been widely reported in NHPs in captivity in Europe [30,57,58,60] and outside Europe [61]. With the exception of subassemblage AIII, assemblage A and B are all considered zoonotic, and the BIV sub-assemblage in particular is widespread among humans also in Europe [8,10]. Transmission from humans to animals in a zoological facility seems a quite unrealistic eventuality, but common hygienic procedures followed in zoos do not exclude the opposite route. The sanitary risk should thus be taken into account by keepers and zoo staff and quantified by performing regular testing for infections with this protozoan in NHPs.

In total, 80.7% of animal groups tested positive for *Blastocystis* at least once throughout the sampling period, confirming this as the most common eukaryotic protist in the intestinal tract of captive NHPs, with the reported prevalence reaching 45.5% in Cordoba zoo, 66.6% in Almuneçar zoo, and 20.3% in a survey among six European zoos [52,53,62]. Molecular studies of NHP isolates are often carried out to trace, in some cases, the sources of infection, and especially to investigate the eventuality of a human-to-animal or animal-to-human transmission. Indeed, not surprisingly, NHPs and humans are most often infected by the same subtypes, with ST1-3 being the most frequent in both [63,64]. The identification of the same alleles within subtypes in NHPs and their animal handlers has demonstrated that zoonotic transmission between the two is possible in zoos, in both directions [64]. In this survey, we did not have access to human samples, but another interesting pathway of *Blastocystis* transmission involving synantropic rodents can be hypothesized. ST4 is indeed considered rare in NHPs [63,65], being isolated in a few studies in *Varecia rubra* and captive lemurs [63,65,66], but it is the most frequent in rodents [50,61]; in particular, the allelic combination shared by both NHPs and rats in this study has been repeatedly isolated from rodents [53,67–70]. Because ST4, with the same allelic composition, has also been isolated from humans [71], and because ST4 has been demonstrated to have a higher correlation with the occurrence of symptomatic infections in humans [72], the zoonotic transmission of *Blastocystis* isolates from animals to zoo keepers in the context of zoos should not be overlooked.

5. Conclusions

In conclusion, this parasitological survey showed a generally low prevalence and burden of gastrointestinal helminth species in captive NHPs and more widespread infections by protist species in both facilities.

Rodents were found to represent a source of environmental fecal contamination, likely responsible for the transmission of both helminthic (*H. diminuta*) and protist taxa (*Blastocystis* ST4) to NHPs, different from what was recently reported in another survey in Spain [53]. Rodent control thus becomes pivotal in avoiding parasitic disease spread in NHP collections.

Given that the zoonotic potential of the isolated taxa has been proven or not ruled out, it is critical that zoo staff caring for primates maintain good hygiene practices. Routine copromicroscopic testing and targeted treatments are recommended to keep a low parasite load in the collections, reducing the impact of parasites on the health of captive NHPs, as well as the risk of zoonotic transmission to the zoo staff.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani14172607/s1, Table S1: List of captive primates species included in the study.

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animals

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Article

Dirofilaria immitis and *Dirofilaria repens*: Investigating the Prevalence of Zoonotic Parasites in Dogs and Humans in a Hyperenzootic Area

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Simple Summary: *Dirofilaria immitis* and *Dirofilaria repens* are mosquito-borne zoonotic parasites that primarily infect dogs. In recent years, the distribution of these parasites has expanded into previously non-enzootic areas. This study aimed to investigate the infection rates in dogs and humans within a hyperenzootic region of Europe, and to estimate the proportional relationship between infection prevalence in dogs and humans. Blood samples from 604 dogs and serum samples from 625 humans residing in Thrace, in northeastern Greece, were collected. The dog samples were examined for *Dirofilaria* spp. microfilariae using Kott's test and for *D. immitis* antigen using a commercial serological test. The human sera were analyzed by Western blot for both parasites. Overall, 177 (29.3%) dogs were found to be infected, with 173 (28.6%) positive for *D. immitis* and 7 (1.2%) for *D. repens*, including 6 (1%) dogs with mixed infections. Specific IgG was detected in 42 (6.7%) human samples, with 24 (3.8%) positive for *D. immitis* and 18 (2.9%) for *D. repens*. The proportion of infection in humans was 23.4% of the corresponding canine infections in the study area, indicating a high risk of human infection in this hyperenzootic region.

Abstract: The mosquito-borne zoonotic nematode parasites *Dirofilaria immitis* and *Dirofilaria repens* primarily affect dogs. In recent years, their distribution has expanded due to various factors influencing vector-borne pathogens. This study aimed to investigate the comparative prevalence of infection in dogs and humans within a hyperenzootic region of Europe, and to estimate the proportional relationship between infection prevalence in dogs and humans, within the concept of "One Health". To this end, 604 blood samples from dogs and 625 serum samples from humans living in the Thrace region of northeastern Greece were collected. The dog samples were examined for *Dirofilaria* spp. microfilariae using Kott's test and for *D. immitis* antigen using a commercial serological test. The human sera were analyzed for both parasites by Western blot. The overall prevalence of infection in dogs was 177 (29.3%), with 173 (28.6%) testing positive for *D. immitis* and 7 (1.2%) for *D. repens*, including 6 (1%) cases of mixed infection. Specific IgG antibodies were detected in 42 (6.7%) human samples, with 24 (3.8%) positive for *D. immitis* and 18 (2.9%) for *D. repens*. The infection proportion in humans was 23.4% of the corresponding canine infections, indicating a high risk of human infection in this hyperenzootic region.

Keywords: comparative epidemiology; dog; Greece; heartworm; human dirofilariosis; human; One Health; prevalence; pulmonary dirofilariosis; subcutaneous dirofilariosis; zoonosis

1. Introduction

Dirofilaria immitis and *Dirofilaria repens* (Rhabditida: Onchocercidae) are nematode parasites transmitted by the bites of infected culicid mosquitoes. While both primarily affect domestic dogs (*Canis lupus familiaris*), other animals, particularly cats and wild carnivores, can also be infected [1]. *Dirofilaria immitis*, which has a worldwide distribution, parasitizes the pulmonary artery and the right chambers of the heart, leading to canine cardiopulmonary dirofilariosis, commonly known as heartworm disease. In contrast, *Dirofilaria repens* is found in subcutaneous tissues, causing subcutaneous dirofilariosis, and is endemic in Europe, Asia, and Africa, but not in the Americas [2]. Regardless of the adult parasites' location, their offspring, the microfilariae, circulate in the bloodstream of competent hosts and can be transmitted to mosquito vectors, where they develop into the infective stage [2].

Both parasites have important zoonotic implications, as they may infect humans, especially in hyperenzootic areas, causing pulmonary, subcutaneous, or ocular dirofilariosis [3]. Although humans are accidental and in principle dead-end hosts for these parasites, these infections are considered emerging, as cases have increased in numbers in the last few decades [1].

The distribution of *Dirofilaria* species in Europe is expanding from the southern endemic areas to central and northern countries that were, until recently, considered free of infection [4]. In Greece, the prevalence of infection in dogs is higher in the northern regions and appears to be increasing in the south, where it was, until a few years ago, very low [5–9]. It has been documented that the prevalence of infection in dogs in a given area may predict the rate of infection of other permissive hosts, e.g., cats [6,10,11]. Furthermore, seroepidemiological studies of humans living in endemic areas have revealed seroprevalence rates similar to those recorded in dogs from the same regions [1].

As expected, most human cases in Europe have been recorded in Mediterranean regions; nonetheless, a few cases have originated in central and northern countries, e.g., Austria, Germany, Czechia, Slovakia, Poland, Belgium, and Finland [2,7]. Several human cases have been reported in Greece, involving pulmonary, ocular, and subcutaneous parasite localizations [12,13]. Other than the occasionally reported cases, no epidemiological study (e.g., serological surveys) of the population has been ever conducted to elucidate the epidemiology of human dirofilariosis in Greece. In this context, and within the collaborative and interdisciplinary-approach concept of One Health, the present study aimed to record, for the first time, the prevalence of dirofilariosis in dogs and humans living in Thrace, a hyperenzootic region in northeastern Greece [8,14].

2. Materials and Methods

2.1. Study Area

The geographical region of Thrace in northeastern Greece (Figure 1) was selected for this comparative study of *Dirofilaria* spp. infection in dogs and humans, based on previous data showing it had the highest prevalence of heartworm infection in dogs in the country [8,14]. Thrace (41°74′ N–40°73′ N, 26°63′ E–24°46′ E) is divided into three regional units, i.e., Xanthi, Rodopi, and Evros (Figure 1), and is characterized by extensive mountain ranges to the north, lowlands in the coastal zone, and the flow of two large rivers, Nestos and Evros. Approximately 31.3% of Thrace is covered by forests, while its main geological characteristic is wide wetland complexes of freshwater lakes and coastal lagoons, most of which are protected at national and European levels, as they are included in the Ramsar Convention on Wetlands [15,16]. In the lowlands and coastal areas, the climate is

Mediterranean and temperate, with milder weather than in the mountains, and with high humidity due to frequent rainfall. The average temperature in the region is 14–16 °C [16].

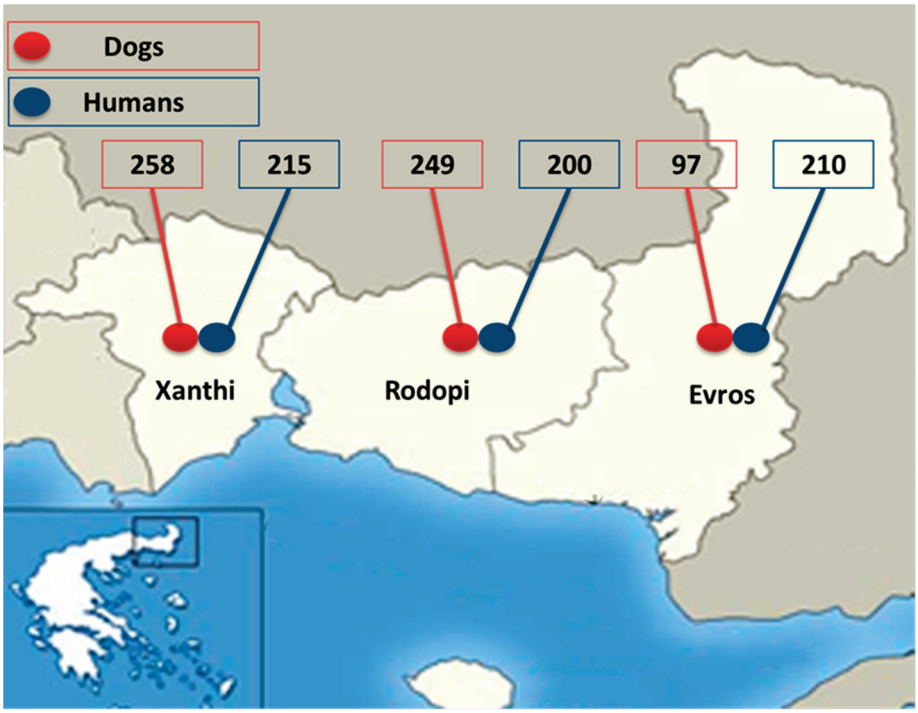

Figure 1. The geographic area of Thrace and the number of dog and human samples examined for *Dirofilaria* infection from the regional units, i.e., Xanthi, Rodopi, and Evros.

2.2. Dog Samples

Dog blood samples were collected during routine clinical examinations. The animals included in this study fulfilled the following criteria: (i) they were living permanently in one of the regional units of Thrace, (ii) they did not receive regular preventative treatment with macrocyclic lactones against dirofilariosis, (iii) they had lived through at least one full mosquito activity period (MAP, May to October), and (iv) they were older than 12 months and at least 9 months had passed since the end of the MAP, if they had lived through only one. The written informed consent of the dog owner or the licensing of the municipal authorities, in the case of stray dogs living in shelters, was also a prerequisite.

Overall, 604 dogs were examined, specifically 258, 249, and 97, from the regional units of Xanthi, Rodopi, and Evros, respectively (Figure 1). A questionnaire was filled out for each dog, including information regarding the region of residency, lifestyle (outdoor/indoor/indoor–allowed outdoor), sex, age, and hair length. For each subject, 2 mL of blood was collected in an EDTA tube from a peripheral vein (cephalic or jugular) and kept refrigerated (4 °C) until examination.

2.3. Human Samples

A total of 625 human samples were collected from residents of the three regional units of Thrace, i.e., 215, 200, and 210 samples from Xanthi, Rodopi, and Evros, respectively (Figure 1). The human sera were obtained from blood samples collected with the individual's written consent, from subjects living in one of the three regional units of Thrace. Humans of all ages and genders who were clinically healthy and who came to the General University Hospital of Alexandroupolis for a regular health check or as blood donors were included in the survey. After collection, the sera were stored in an Eppendorf tube at −20 °C until examination. Each study participant filled out a questionnaire, including information regarding their age, sex, and residency area. The collected human data were coded and stored anonymously, according to data protection legislation.

2.4. Sample Analysis

Each dog blood sample was examined by the modified Knott's method for the detection of microfilariae [17]. The identification of microfilariae was performed under a light microscope at 100× and 400× magnification, on the basis of morphometric and morphological features [17,18]. The detection of the *D. immitis* antigen was performed by the commercial serological test DiroCHECK® (Zoetis, Parsippany, NJ, USA).

The serological examination of the human blood samples was performed by Western blot analysis, separately for *D. immitis* and *D. repens* antigens, using parasites collected from canine cases of cardiopulmonary and subcutaneous dirofilariosis, respectively. For the development and standardization of the method, positive human sera from confirmed cases and negative control sera were used.

The parasites were homogenized per species in PBS supplemented with protease inhibitors (PMSF 100 μg/mL, Leupeptin 0.5 μg/mL, Aprotonin 0.5 μg/mL, and Pepstatin 1 μg/mL; Sigma Aldrich, St. Louis, MO, USA). Following homogenization, Triton X-100 at a 1% final concentration was added, and the samples were rapidly frozen at −80 °C, thawed, centrifuged at 10,000× *g* for five minutes, and the supernatant was collected and kept at −80 °C. The protein concentration of the lysates was calculated with a BCA assay kit (23225, Thermo Scientific, Waltham, MA, USA). The quality and quantity of the isolated proteins were estimated by loading 5 μg, 10 μg, and 20 μg from each species sample onto a 10% Tris-glycine gel (Figure 2). For the detection of *Dirofilaria*-specific antibodies, a quantity of 20 μg of protein from each parasite was selected and separated into 12% gels. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Darmstadt, Germany), and 5% non-fat dry milk was used to block non-specific binding. The sera were diluted 1:50 in a blocking buffer and incubated overnight at 4 °C. Following three washing steps, the membranes were incubated with an anti-human IgG HRP-conjugated secondary antibody (1:5000, 32935S, Cell Signaling, Danvers, MA, USA) and once again washed three times. Protein bands were detected using an ECL chemiluminescent substrate (34577, Thermo Scientific, Waltham, MA, USA) and a ChemiDoc MP Imaging System (BioRad, Hercules, CA, USA). The sera were considered positive for each parasite species when specific bands were detected, at 17–22 kDa for *D. immitis* and at 43–70 kDa for *D. repens*, respectively [19,20].

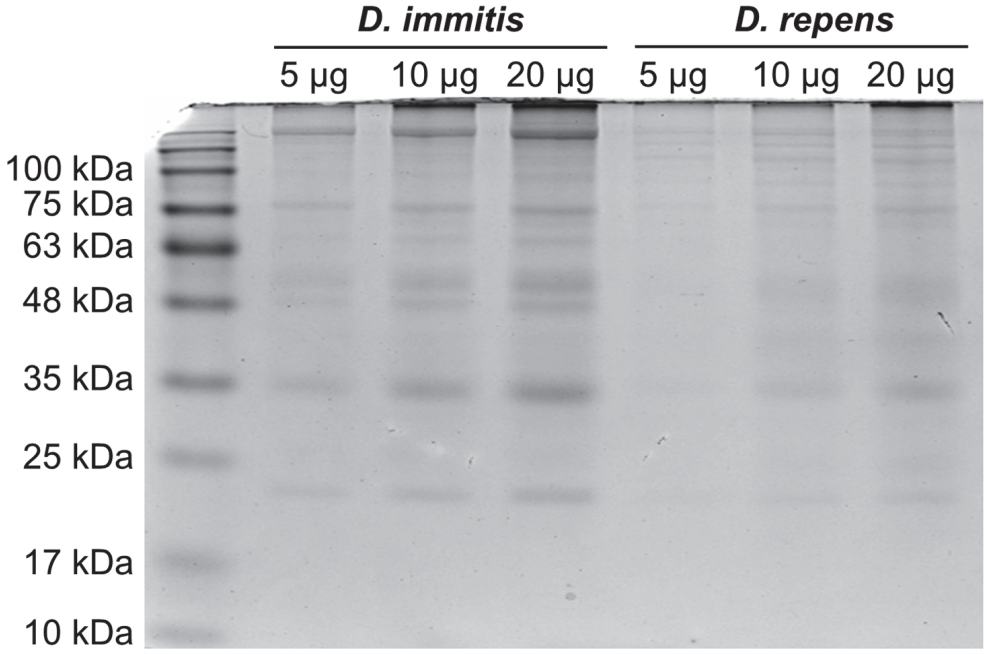

Figure 2. *Dirofilaria immitis* and *Dirofilaria repens* proteins' quality and quantity estimation by analysis of 5 μg, 10 μg, and 20 μg in 10% Tris-glycine gel.

2.5. Statistical Analysis

The sample sizes for both the dog and human samples met the recommended threshold, indicating that to detect the difference in proportions with 95% confidence and 80% power, 91 samples were required [21]. The statistical analysis was performed using the chi-square test of independence between the infection status for *Dirofilaria* spp. (positive or negative result for any of the *Dirofilaria* species examined) and a variety of variables [22]. Specifically, for dogs, the variables examined were breed, gender, age, hair length, lifestyle, and area of residency; for humans, the variables examined were sex, age, and area of residency. The level of significance was set at $p < 0.05$.

3. Results

3.1. Dog Samples

Overall, 177 (29.3%) dogs were found to be infected with at least one species of *Dirofilaria*. Of the 604 dogs examined, 173 (28.6%) were positive for *D. immitis* by at least one of the examination methods (serology and Knott's test). More specifically, 87 (50.3%) samples were positive for both methods, while 84 (48.6%) were positive for serology only, and 2 (1.2%) samples for Knott's test only. Moreover, in seven (1.2%) samples, *D. repens* microfilariae were found in the Knott's test. A mixed infection (*D. immitis* antigen or/and microfilariae and *D. repens* microfilariae) was found in six dogs. The results of the Knott's tests and the serological examinations of the dog samples are presented in detail in Table 1.

Table 1. Dogs found positive by different examination methods for *Dirofilaria immitis* and *Dirofilaria repens* in the geographical region of Thrace, and in each separate regional unit within Thrace.

	Thrace (n = 604)		**Xanthi (n = 258)**		**Rodopi (n = 249)**		**Evros (n = 97)**	
Examination Method	**D.i.**	**D.r.**	**D.i.**	**D.r.**	**D.i.**	**D.r.**	**D.i.**	**D.r.**
Knott	86 (14.2%) *	7 (1.2%) *	45 (17.4%)	1 (0.4%)	20 (8%)	0	21 (21.6%) *	6 (6.2%) *
Serology	171 (28.3%)	-	80 (31%)	-	56 (22.5%)	-	35 (36.1%)	-
Knott or/and Serology	173 (28.6%) *	7 (1.3%) *	81 (31.4%)	1 (0.4%)	57 (22.9%)	0	35 (36.1%) *	6 (6.2%) *

D.i. = *Dirofilaria immitis*; D.r.= *Dirofilaria repens*; * mixed infection in 3 animals.

At the regional unit level, the number of dogs found positive for *D. immitis* by serology and/or Knott's test were 81 (31.4%), 57 (22.9%), and 35 (36.1%), in Xanthi, Rodopi, and Evros, respectively. Furthermore, *D. repens* microfilariae were detected in one (0.4%) dog in Xanthi and in six (6.2%) in Evros (Table 1).

3.2. Human Samples

Based on the results from the method standardization using positive and negative control samples, and in accordance with the literature, human sera were considered positive for *D. immitis* when bands between 17 and 22 kDa were detected, and positive for *D. repens* when bands between 43 and 70 kDa were observed (Figure 3) [19,20,23].

Overall, 42 (6.7%) samples were positive on the Western blot analysis for *Dirofilaria* spp.-specific IgG antibodies, i.e., 24 (3.8%) for *D. immitis* and 18 (2.9%) for *D. repens*. In more detail, 5 (2.3%) samples were positive for *D. immitis* and 10 (4.7%) samples were positive for *D. repens* in Xanthi, 9 (4.5%) and 6 (3%) in Rodopi, and 10 (4.7%) and 2 (1%) in Evros, respectively (Table 2).

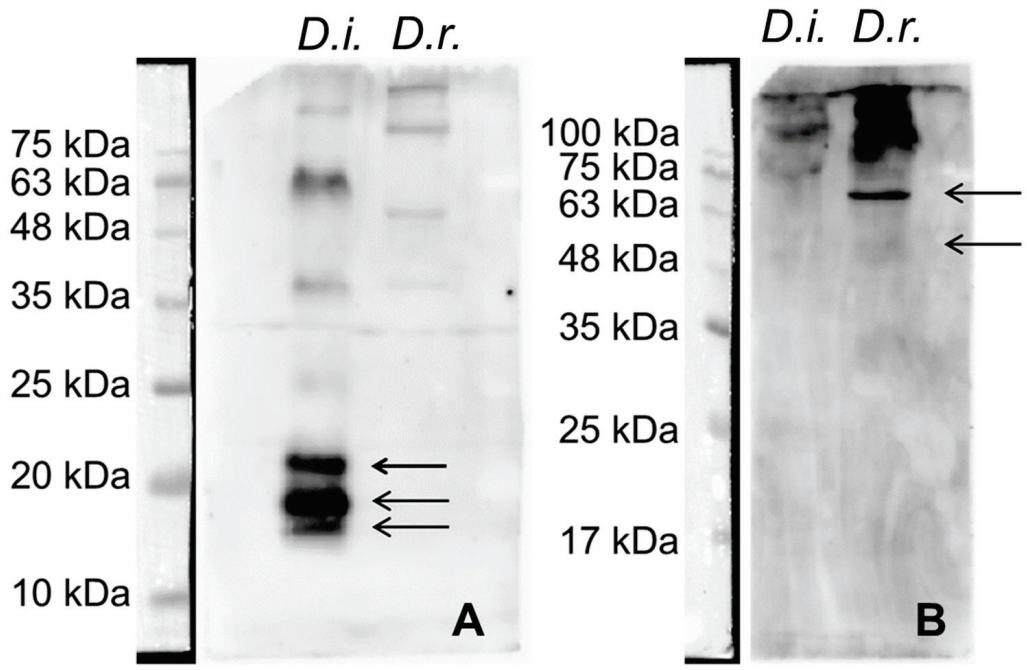

Figure 3. Western blot analysis of human sera showing bands between 17 and 22 KDa (indicative of *Dirofilaria immitis*, panel (**A**)) and between 43 and 70 kDa (indicative of *Dirofilaria repens*, panel (**B**)). The respective bands are indicated by arrows.

Table 2. Seropositive (IgG) human samples by Western blot analysis for *Dirofilaria* spp.

Regional Unit	Seropositive Samples Per Parasite		
	***Dirofilaria* spp.**	***D. immitis***	***D. repens***
Thrace (n = 625)	42 (6.7%)	24 (3.8%)	18 (2.9%)
Xanthi (n = 215)	15 (7%)	5 (2.3%)	10 (4.7%)
Rodopi (n = 200)	15 (7.5%)	9 (4.5%)	6 (3%)
Evros (n = 210)	12 (5.7%)	10 (4.7%)	2 (1%)

3.3. Statistical Analysis

The statistical analysis of the dogs' results included 546 of the total 604 animals, due to insufficient data for 58 of them. Factors, such as gender [$X\chi^2$ (1, N = 546) = 5.21, p = 0.0022], lifestyle [χ^2 (2, N = 546) = 18.23, $p < 0.001$], and area of residency [χ^2 (2, N = 546) = 10.57, p = 0.005], were associated with *Dirofilaria* spp. infection, while hair length and age were not (p-value > 0.05) (Table 3). Male dogs were more likely (OR = 1.55) to be infected than female dogs, and dogs living in the prefecture of Evros were more likely to be infected than dogs living in Rodopi (OR = 2.03) or Xanthi (OR = 1.15). Finally, dogs living outdoors had an increased risk of infection compared to dogs living indoors (OR = 2.4) or to indoors–allowed outdoors (OR = 2.27) (Table 3).

The statistical analysis of the human samples revealed no significant statistical correlation between demographic factors (sex, age, and area of residency) and the risk of infection (p-value $\geq$ 0.05).

Table 3. Statistical analysis of the variables recorded for the dogs examined for *Dirofilaria* spp. in the hyperenzootic area of Thrace, Greece.

Variable	*Dirofilaria* Positive	*Dirofilaria* Negative	χ^2 Test/Fisher Test (*p*-Value) Odds Ratio
Sex			
Male (199)	70 (35.2%)	129 (64.8%)	5.21 (**0.022**)
Female (347)	90 (25.9%)	257 (74.1%)	Odds ratio = 1.55
Age *			
≤3 (255)	67 (26.3%)	188 (73.7%)	4.92 (0.089)
3−7 (215)	63 (29.3%)	152 (70.7%)	
>7 (76)	30 (39.5%)	46 (60.5%)	
R.U. **			
Evros (97)	36 (37.1%)	61 (62.9%)	10.57 (**0.005**)
Rodopi (249)	56 (22.5%)	193 (77.5%)	Odds ratio = 2.03
Xanthi (200)	68 (34.0%)	132 (66.0%)	Odds ratio = 1.15
Lifestyle			
Outside (272)	102 (37.5%)	170 (62.5%)	18.23 (**<0.001**)
Inside (10)	2 (20.0%)	8 (80.0%)	Odds ratio = 2.40
In and out (264)	56 (21.2%)	208 (78.8%)	Odds ratio = 2.27
Hair length			
Short (243)	79 (32.5%)	164 (67.5%)	3.00 (0.223)
Medium (225)	57 (25.3%)	168 (74.7%)	
Long (78)	24 (30.8%)	54 (69.2%)	

* Age group (age in years); ** R.U.: regional unit; *p*-value in bold: statistically significant.

4. Discussion

This study is the first to comparatively investigate the prevalence of *Dirofilaria* spp. infection in both dogs and humans in a hyperenzootic area of Greece. Additionally, it represents the first serological screening for *Dirofilaria* spp. infection in the human population of Greece.

The hyperenzootic profile of the selected study area was confirmed by the present results, with an overall infection rate in dogs of 28.6% and the highest prevalence percentage of 36.1% recorded for the regional unit of Evros. On this basis, the results of the present study provide evidence regarding the rate of human contact with parasites of the genus *Dirofilaria* in an area of high infection pressure.

The hyperenzootic character of northern Greece for *D. immitis* has repeatedly been demonstrated by surveys of dog populations. The percentage of infection in dogs varies from 6.1% up to an extreme of 68%, which was found in dogs from the easternmost town of Didimoticho in Evros [5,14,24–27]. This epizootiological status is associated with the geomorphology of northern Greece and the agricultural profile of the area. Indeed, northern Greece holds most of the wetlands of the country, 91% of the total rice fields, and 68.5% of the farm cattle population at the national level [5,28,29]. These conditions may favor mosquito development and their populations' abundance and stability [30,31]. This hypothesis is confirmed by entomological surveys that have demonstrated that the *Aedes* spp. population in eastern Macedonia and Thrace is 10 times larger than the corresponding population in southern or in western Greece [32]. Furthermore, the mosquito species *Culex pipiens* and *Aedes caspius*, proven vectors of canine dirofilariosis, are the dominant mosquito species in northern Greece [32–35].

None of the dogs examined herein presented with clinical evidence of heartworm disease or subcutaneous dirofilariosis at the routine clinical examination. However, heartworm disease is one of the most severe parasitic diseases in dogs and can be fatal. The pathogenesis of *D. immitis* is mainly related to pulmonary hypertension that leads to congestive heart failure [2]. Common clinical signs include chronic cough, respiratory distress, weakness, ascites, abnormal cardiac sounds, and sudden death [2,36,37]. *Dirofilaria repens*

infection is de facto less severe. When clinical sings occur, they usually manifest as skin nodules that may develop in various sites, ranging from 0.5 to 3 cm in diameter [36].

Among the factors examined herein, the sex, lifestyle, and area of residency were found to be associated with a higher risk of infection in dogs. Although it has been observed that in hyperenzootic areas the sex and activity level of dogs do not play a role in the risk of infection [2], male dogs are used more often as guards than female dogs, increasing the time spent outdoors under the risk of mosquito bites. Indeed, dogs living exclusively outdoors were found to have an increased risk of infection compared to dogs living indoors or indoors with outdoor access. Dogs living in the regional unit of Evros had the highest probability of infection compared to the other two regional units of this study. This is in accordance with previous data indicating Evros as the area with the highest prevalence of dog infection in Greece [8,14]. Although age was not found to be a statistically significant factor for the risk of infection, dogs over 7 years of age showed a higher percentage of infection. This observation is common in canine dirofilariosis, which is a chronic condition, thus occurring more prevalently in older dogs as they accumulate exposure to infection over the years.

Human dirofilariosis is considered a sporadic zoonotic disease. However, in the last decades, the number of reported human cases have increased [1]. This may be attributed to a factual increase in incidences of human infection, due to various factors that promote the spreading of vector-borne diseases (VBDs), but also to the enhancement of awareness in the medical community. The factors that are linked to the expansion and increase in VBDs [38], primarily among natural hosts and subsequently among accidental hosts, in this case, dogs and humans, respectively, are i. climate change, especially global warming and extreme meteorological phenomena (e.g., floods), ii. land use changes, which influence vectors and reservoir hosts' biology and behaviors, and iii. the intensified movement of humans, animals, and goods that promote vector and host spreading [39,40].

In Europe, most human cases are reported in Italy, followed by France, and then Greece [1]. However, cases in central and northern European counties have also received publicity in the recent years [3,4]. *Dirofilaria repens* is the primary agent of human dirofilariosis in Europe and has been found in various sites of the human body, with the predominant region being the head, and particularly the subcutaneous tissues and the eye (eyelid, periorbital region, and subconjunctiva) [1,3]. Although humans are not the preferred host for *D. repens*, there have been several cases where the parasites fully matured and produced microfilariae within a human host. To date, there are at least 24 cases of *D. repens* microfilaremia in humans [41–43].

On the other hand, *D. immitis* infections in humans are less common in Europe [1,3]. *Dirofilaria immitis* typically migrates to the pulmonary arteries, where it is usually destroyed by the host's immune system, generating a granulomatous nodular lesion in the lung parenchyma, known as "coin lesion". This condition is usually asymptomatic and often discovered incidentally during imaging examinations. However, in some cases, the presence of the parasite can cause thoracic pain, cough, hemoptysis, low fever, and malaise [44]. Although the condition is generally self-limiting, the surgical removal of the lesion is the standard treatment, as malignancy is considered in the differential diagnosis [3]. *Dirofilaria immitis* has never been reported to have fully developed in humans, and there is no case of recorded microfilaremia.

The higher frequency of human *D. repens* infections compared to *D. immitis* infections may reflect the parasite's better adaptation to human host, as evidenced by the occasional reproductive maturity that the parasites reach in humans, resulting in microfilaremia. In addition, the subcutaneous and ocular localization commonly associated with *D. repens* infections facilitates its diagnosis compared to the parenchymal and most often pulmonary localization of *D. immitis* [3].

Despite the notion that in humans *D. repens* is related to subcutaneous nodules and ocular localizations while *D. immitis* causes pulmonary nodules, both parasites have been found in many different localizations within the human body; thus, the localization of the

parasite cannot by any means imply its species [1]. Furthermore, although the morphology of the cuticle of *Dirofilaria* may indicate the species, as *D. repens* displays a longitudinally striated cuticle while *D. immitis* does not, the parasites, when they are extracted from the organ of parasitism, are often already dead and destroyed to an extent, and thus morphologically altered. In many cases, the only reliable identification method is via molecular techniques (PCR) [3,13]. In fact, as reviewed by Pampiglione et al. [45], the morphological misidentification of parasites at the species level is rather common in the literature.

Human dirofilariosis, due to both *D. immitis* and *D. repens*, has been documented in Greece on several occasions [12,13]. However, in only a few instances of ocular and one case of subcutaneous dirofilariosis were the parasites unequivocally characterized by molecular means as *D. repens* [46] and *D. immitis* [47], respectively.

In contrast to the relatively straightforward diagnosis of *Dirofilaria* infections in dogs, diagnosis in humans is more complex. Indeed, in dogs, *D. immitis* infection is diagnosed by the combination of Knott's method and a serological test. This is the recommended laboratory diagnostic procedure, providing very high diagnostic accuracy, as it covers cases of occult infection (infection in the absence of circulating microfilariae) and false-negative serological results (low levels of circulating antigens) [36,37]. Notably, both scenarios were encountered in the present study, as an occult infection was detected in 84 out of the 173 infected animals, and two animals had circulating *D. immitis* microfilariae with a negative serological test. For *D. repens*, there is no serological test, so Knott's method is the only laboratory diagnostic approach, revealing an infection in cases with microfilariae circulation but missing occult infections [36]. On the other hand, diagnosis in humans, other than the surgical removal and identification of a parasite when detected, is not easy, as microfilaremia is very rare and has only been observed in some cases of *D. repens* infection. Similarly, due to the rarity of infections, there is no commercial serological test available for humans. Therefore, in-house ELISA and Western blot assays have been developed and applied in seroepidemiological surveys. In fact, serology is the only method by which to identify individuals who have recently been exposed to the parasites.

ELISAs developed for the detection of specific anti-*Dirofilaria* spp. antibodies have employed the somatic and excretory/secretory proteins of the parasites, as well as the proteins of their bacterial endosymbiont *Wolbachia*, as antigens [48–51]. The crude somatic or excretory/secretory antigens used in ELISA have the drawback of cross-reactions with other parasites that may infect humans, especially *Toxocara canis*, a canine nematode causing "visceral larva migrans", one of the most common zoonotic infections in the world [1,52]. Western blot analysis, on the other hand, provides a reliable serological tool, with specific molecular weight bands indicating seropositivity for *D. immitis* and *D. repens* separately [3].

The seroprevalence in humans in Europe has been investigated for a few cases. In western Spain and in the Canary Islands, 9.3% and 6.4% of the population examined by ELISA (somatic antigen) were *D. immitis* positive, respectively [49,53]. In Portugal, a serosurvey by ELISA using a *D. immitis* somatic antigen and a *Wolbachia* surface protein as antigens showed a prevalence of 6.1% [54]. In Romania, Moldova, and Serbia, an ELISA with a somatic antigen was used, coupled with the detection of an anti-*Wolbachia* surface protein and Western blot. Taking into account the mixed infections, 7.4%, 14.8%, and 2.6% were positive for *D. immitis,* and 0.5%, 1.5%, and 2.3% showed *D. repens* seropositivity in the three countries, respectively [19,20]. These percentages are close to the prevalence found in the present study, i.e., 3.8% for *D. immitis* and 2.9% for *D. repens*. Interestingly, the percentage of *D. repens*-seropositive humans was higher than that of *D. repens*-microfilaremic dogs (1.2%), suggesting that the actual infection rate in dogs is much higher than what is revealed by the presence of circulating microfilariae. The age, sex, and area of residency of the individuals included in the present study were not associated with a higher risk of human dirofilariosis. Age in humans, as in dogs, is associated with a higher infection rate, due to repeated exposure to the parasite in an enzootic area [55]. However, the short lifespan of the parasites in a non-natural host clears the infection much sooner than in dogs,

preventing chronic infections and, thus, restricting the time during which antibodies can be detected.

5. Conclusions

Dirofilaria spp. infection in dogs living in the geographic region of Thrace remains highly prevalent. The detected proportion of infection in humans was 23.4% of the respective canine infection in the same area. Knowing the rate of human contact with parasites in areas of high infection pressure is essential for implementing preventive measures, raising awareness among the medical community, and providing available data to promote and support the prompt and accurate diagnosis of human infections. By incorporating the "One Health" concept, the present study emphasizes the interconnectedness of human, animal, and environmental health.

Timely diagnosis and, more importantly, effective prevention in dogs are crucial for protecting the health of both animals and humans. This is especially vital given the anticipated rise in cases and the spread of parasites due to climate change, increased animal travel, and the presence of invasive mosquito species. The monitoring and control of *Dirofilaria* infections have acquired additional significance recently, due to the development of resistant strains of *D. immitis* to macrocyclic lactones, i.e., the only drug molecules used for heartworm prevention [56,57]. Furthermore, medical doctors should be adequately informed about *Dirofilaria* infections in humans for its accurate and prompt diagnosis that, in some cases, may prevent unnecessary medical procedures (e.g., surgeries) for patients. In this context, sensitive and easy-to-perform routine-level serological methods should be developed for diagnosis in humans. Furthermore, these infections should become notifiable diseases in all enzootic/endemic countries, as is currently the practice in some eastern European countries, such as Ukraine, Belarus, and Russia, where a high number of cases have been reported in recent years [58]. This measure, in combination with regular epizootiological/epidemiological screenings of the dog and human populations for *Dirofilaria* spp., is imperative for the surveillance of these important zoonotic infections.

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Informed Consent Statement: Informed consent was obtained from all the subjects involved in this study and from the owners or authorized agents of the animals.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

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Seroprevalence Assessment and Risk Factor Analysis of *Toxoplasma gondii* Infection in Goats from Northeastern Algeria

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Simple Summary: Toxoplasmosis, a zoonotic protozoan disease caused by the pathogen *Toxoplasma gondii*, can infect almost all homoeothermic animals including humans. *Toxoplasma gondii* is responsible for reproductive disorders in ruminants such as goats which could have an important role as a source of parasite transmission to humans. Data on seroepidemiology of *T. gondii* in Algeria is scarce, and almost null in the rural areas. This study was carried out to determine seroprevalence and to identify the associated risk factors for *T. gondii* infection in goats from four provinces in northeastern Algeria. A total of 460 serum samples were tested from 72 herds. A total of 245 samples showed the presence of anti-*T. gondii* IgG antibodies, representing 53.26% of the goats and 94.44% of herds, indicating they were positive for *T. gondii*. Factors such as the presence of a water source in the pasture, number of cats, and number of abortions were significantly linked to the high seropositivity of *T. gondii*. This investigation suggests that *T. gondii* is widespread in the goat population in Algeria and the transmission of the parasite to humans could potentially increase via meat consumption.

Abstract: *T. gondii* is the causal agent of toxoplasmosis, a worldwide zoonotic disease relevant in human and veterinary medicine. In Algeria, few reports focused on the presence and circulation of this parasite in the local goat population. The aim of the survey was to evaluate toxoplasmosis seroprevalence and associated risk factors. Sera from 460 goats reared on 72 farms in northeastern Algeria were collected and tested for IgG antibodies to *T. gondii* by an indirect ELISA. To identify risk factors, a linear regression analysis of the variables was performed. Anti-*T. gondii* antibodies were found in 94.44% (68/72; 95% CI: 73.34–119.73) of goat farms and in 53.26% (245/460; 95% CI: 46.80–60.36) at the individual level. The multivariable analysis showed that seasonal pasture (OR = 3.804; 95% CI: 3.321–4.358; $p = 0.003$), presence of water source in pasture area (OR = 4.844; 95% CI: 1.942–7.789; $p = 0.0004$), use of anthelminthics (OR = 2.640; 95% CI: 1.592–3.146; $p = 0.036$), number of cats, hygiene, proportion of abortions, number of abortions in the last year, year of sampling, region, and season were the variables significantly associated with *T. gondii* seropositivity. Abortions in goat herds seem to be related to *T. gondii* exposure, thus it is crucial to undertake measures and strategies to reduce, control, and prevent toxoplasmosis infection in goats, and thereby in humans, from Algeria.

Keywords: *Toxoplasma gondii*; caprine; Algeria; antibody detection; epidemiology

1. Introduction

Toxoplasma gondii is an obligate intracellular Apicomplexa parasite that can infect almost all warm-blooded animals, including farm animals, birds, sea mammals, and humans, with a variety of clinical manifestations [1,2]. The life cycle of this important zoonotic protozoan implies domestic cats, as well as other felids, as definitive hosts, and a wide range of mammals, including mainly herbivorous ones, and humans as intermediate hosts. Domestic cats and other felidae excrete via feces non-sporulated oocysts which are environmentally resilient [1]. The oocysts become infectious after sporulation, occurring when contacting the environment for 1 to 5 days [3]. Tachyzoites, the rapid-growing life stage of the parasite, and bradyzoites, which are the life stage present in tissue cysts, are two further infectious forms of *T. gondii* other than sporulated oocysts [4,5]. Generally, intermediate hosts acquire infection through ingestion of food or water contaminated by sporulated oocysts. Furthermore, ingestion of uncooked or undercooked meat as well as other animal products containing infectious parasitic stages (tissue bradyzoites) represents an important mode of transmission, specifically in humans [3].

Toxoplasmosis is considered one of the major constraints on livestock production worldwide, causing serious reproductive losses such as abortion, stillbirth, and a fall in milk production [6]. Furthermore, toxoplasmosis is of considerable public health importance, making it one of the most common zoonotic parasitic infections in humans worldwide [7]. Mostly, the infection seems to be asymptomatic in immunocompetent individuals, while causing serious problems in others, specifically in neonates and immunocompromised persons [1].

Of particular interest, toxoplasmosis represents a serious problem in the breeding of goats. It has been reported that 3.3% to 27.2% of goat abortions around the world were associated with *T. gondii* infection [8]. Fetal and newborn mortality rates in infected flocks can exceed 50%, with only minor losses in non-clinical cases [9]. Goats are most likely affected when they consume feed or water that has been contaminated with cat feces [8]. In this regard, *T. gondii* infection is a significant water-borne disease, and it may be influenced by ecosystem fragmentation, poor water quality, and changes in water flow [7,10].

Goats are one of the intermediate host species where *T. gondii* is vertically transmitted, with tachyzoites contributing to transplacental transmission typically during the acute phase of infection or after a reactivated chronic infection [1]. *Toxoplasma gondii* infection in goats was mostly investigated by the assessment of seroprevalence as compared to the detection of the parasite or its DNA in infected tissues as well as in blood and milk. *T. gondii* DNA has been recovered in goats that were slaughtered across the world, and living bradyzoites have also been found in the muscles of naturally infected goats [8,11]. The consumption of raw goat milk containing tachyzoites as well as undercooked meat containing bradyzoites is thought to be a significant source of infection in humans, with particular impact on pregnant women [12,13]. The role of goat's raw milk as a source of human infections remains uncertain due to the absence of solid evidence showing the presence of viable *T. gondii* [8].

In most developing nations, at least 30% of the population has *T. gondii* IgG antibodies [1]. High prevalence rates of human infection have been documented in recent years in North Africa, including Morocco (51%) [14] and Algeria (40.97% to 47.8% in pregnant women) [15,16].

Toxoplasma gondii is a widespread parasite that affects small ruminants all over the world. Several investigations have investigated the seroprevalence of *T. gondii* antibodies in goats and the potential associated risk factors in various countries, indicating infection rates ranging from 4.6 to 73.3% depending on the geographical area, the diagnostic approach, and the requested cut-off titer [8,17,18].

In different countries, breeding of goats is important for the economy, specifically for milk and meat production [19]. In Algeria, livestock husbandry, particularly small ruminant breeding, represents an important agricultural activity in the rural high plateaus and steppe areas. One of the most important areas for livestock production and agriculture is in northeastern Algeria, where goat farming is an additional economic activity that is primarily characterized by small, non-racial herds and tried-and-true specialties in an often semi-extensive system. The role of small ruminants, including goats, as a significant reservoir of zoonotic parasites such as *T. gondii* in rural areas of Algeria is poorly documented. Relatively limited epidemiological data are available in Algeria on toxoplasmosis in goats as well as in other animal species and humans [20,21]. The frequency of toxoplasmosis in goats in the northeastern region of Algeria has been reported in only one study so far [20]. To support current available data, the present study was conducted to provide the latest information on the seroprevalence of *T. gondii* infection in goats in northeastern Algeria and offer an up-to-date summary of potential associated risk factors using detailed statistical analysis.

2. Materials and Methods

2.1. Study Area and Environment

The present study was carried out in four provinces including Mila, Constantine, Guelma, and El Taref, which are located in northeastern Algeria (Figure 1).

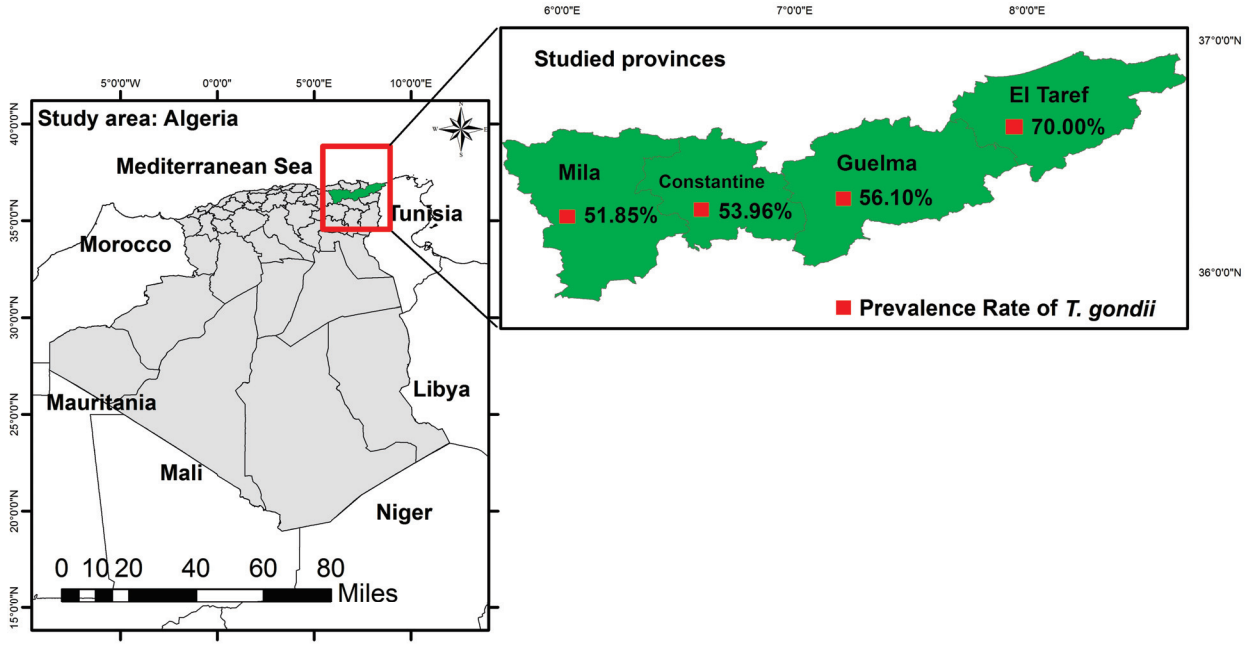

Figure 1. Map of the study area and prevalence rate of *Toxoplasma gondii* in goats submitted to the epidemiological investigation by province.

Mila lies inland, about 82 km from the Mediterranean coast. The district is characterized by a varied relief and presents two large, distinct zones: to the north, the mountains, and to the south, the plains and highlands, with an area of 3481 km^2. The region has a humid Mediterranean climate, with hot, dry summers and cold, wet winters. The annual rainfall is approximately 550 mm with a relative humidity of 70%, and the annual temperature varies between 8 °C and 24 °C [22]. Constantine is situated on a plateau at 698 m above sea level; the area has a humid climate with hot, dry summers and cold, moist winters and is characterized by an annual rainfall of 600 mm, an annual temperature range of 10 °C to 25 °C, and 60% annual relative humidity [22]. The territory of Guelma province is characterized by a sub-humid climate in the center and the north and a semi-arid climate towards the south. This climate is mild and rainy in the winter and warm in the summer.

Thus, the annual temperature varies between 12.5 °C and 25.5 °C, accompanied by 750 mm of annual rainfall and a relative humidity of 58.3%. El Taref province is located in the far northeast of Algeria, close to the Tunisian border. The climate is generally humid, with an important annual rainfall of 1000 mm, a high annual relative humidity of 77%, and an annual temperature ranging from 4 °C to 23 °C [22]. Livestock husbandry in these four provinces consists of a mixture of breeding cattle and small ruminants. According to data from the last years (2019–2020) from the Algerian Ministry of Agriculture, approximatively 73,658, 34,623, 35,136, and 11,365 goat heads are raised in Guelma, El Taref, Mila, and Constantine, respectively [23].

2.2. Study Design and Target Population

A cross-sectional study was conducted. An appropriate number of goats were sampled by a simple random sampling method. In selecting the municipalities and properties that participated in the study, the division of the state, ease of access, convenience, and availability of producers were taken into account. The goats were randomly selected from males and females, apparently healthy with different production patterns, and aged over three (03) months. The number of goats to be taken from each farm was defined based on the total number of animals. A representative sample of at least 10% of all individuals on each farm visited was achieved.

The required sample size was calculated according to the following formula [24] with an expected prevalence of 63% [20], an expected error of 5%, and assuming a 95% confidence interval:

$$N = [Z2 \times P\,(1 - P)]/d2$$

where:

N is the number of samples to be collected in the study;
Z is the value of the normal distribution for the confidence interval of 95% [Z= 1.96];
P is the expected prevalence;
d is the absolute error or required precision of ±5% for a 95% confidence interval (0.05).

A minimum of 358 samples was required. Seventy two herds were randomly selected and the herd sizes ranged from 5 to 50 heads.

At the individual level, the sample size was determined for each flock for eventual detection of *T. gondii* antibodies. The calculations were performed according to the formula commonly used in veterinary epidemiological surveys [24]:

$$n = ([1 - (1 - p)\,1/d] \times [N - (d/2)]) + 1$$

where:

n is the size of the sample in each flock;
p is the probability of detection of at least one seropositive goat in a herd determined at 95%;
N is the size of the flock;
d is the number of seropositive goats in the herd (it was calculated assuming that within-herd prevalence equals 10%).

2.3. Data Collection and Sampling

During visits to the properties, a structured questionnaire was administered to each farmer under the supervision of the principal investigator to assess the risk factors associated with *T. gondii* infection. The questionnaires consisted of several closed questions about the gender, age, characteristics of the herd, breeding purpose, management system, reproductive disorders such as abortion and stillbirth, and cat-related factors [8,9,17].

Blood samples were collected from September 2020 to March 2023, totaling 460 goats from about 72 farms in fifteen municipalities in four provinces (Mila, Constantine, Guelma, and El Taref). Blood samples (2–3 mL) were collected from the venipuncture of the jugular

with sterile 40 × 12 needles in a vacuum tube (Vacutainer®, VacuTube, Algiers, Algeria), without anticoagulant, and transported under refrigeration to the laboratory. They were centrifuged at 2000× *g* for 10 min to obtain serum, stored in Eppendorf tubes, and frozen at −20 °C until serological analysis.

2.4. Serological Analysis

All sera samples (n = 460) were tested for *T. gondii* IgG antibody detection using the ID Screen Toxoplasmosis Indirect ELISA Multi-species kit (ID Screen, ID.VET. Innovative Diagnostics, Montpellier, France), according to the manufacturer's instructions. This is an indirect ELISA that uses the native P30 (SAG1) antigen and the anti-multi-species conjugate as the secondary antibody. This configuration is effective for identifying *T. gondii*-specific antibodies present in the serum of ruminants, pigs, dogs, and cats, as well as in milk and meat juice [25]. Moreover, the use of the SAG1 antigen in this kit might provide greater specificity than tests using a whole tachyzoite antigen [26]. Positive and negative controls (supplied in the kit) were tested in duplicate, as per instructions. Results were expressed as a percentage of the optical density (OD) reading of the test, calculated as %OD = 100 × (OD sample − OD Negative Control)/(OD Positive Control − OD Negative Control). The samples were considered positive if they had a value ≥50%, doubtful for values between 40% and 50%, and negative if ≤40%.

2.5. Statistical Analysis

Data obtained from the questionnaire survey and ELISA analysis were recorded and coded in a Microsoft Excel spreadsheet (version 16.0) (Microsoft Corporation, Washington, USA), and were used to calculate seroprevalence values specific to the population and geographic locations (provinces). Descriptive analysis was applied to describe the study population in relation to risk factors and variables. We evaluated 139 variables of plausible risk factors (57), including gender of the animals, age, contact with other animal species, water source, management practices, presence of cats, number of cats, flock size, history of abortions, and proportion of abortions in the flock. The association of the assumed risk factors for *T. gondii* seropositivity was analyzed by univariate analysis that was performed using Pearson's chi-square test and, when necessary, Fisher's exact test. Variables with a p-value < 0.05 in univariate analysis were offered to the multivariate model analysis via generalized linear mixed model fit by maximum likelihood, considering the herd as the random effect depending on the result of the ELISA test. The explanatory variables considered fixed effects in the model were those presenting statistical significance. The full model was reduced by automatic model selection based on a finite sample AIC. The process was performed using R software (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria) via RStudio (version 1.1.383, RStudio Inc., Boston, MA, USA). In the final multivariate linear regression analysis, variables with a $p < 0.05$ were considered significant. The confidence interval was established at 95%.

3. Results

A total of 460 serum samples from goats were collected in different agroecological areas of northeastern Algeria and tested by indirect ELISA to detect IgG antibodies against *T. gondii*. Out of the 460 samples, 245 samples were found to be positive, making the overall prevalence in domestic goats 53.26% (245/460; 95% CI: 46.80–60.36) as shown in Table 1. At the herd level, 68/72 (94.44%; 95% CI: 73.34–119.73) had at least one animal serologically positive for *T. gondii*.

Table 1. Seroprevalence and univariate analysis of explanatory risk factors associated with *T. gondii* infection in the local goat population from northeastern Algeria.

Variable	Categories	Negative	Positive (%)	OR (95% CI)	*p*-Value
Individual-related factors					
Physiology status of male	Breeder	24	43 (64.18)	2.0903 (1.0939–3.9941)	0.024
	Non-breeder	49	42 (46.15)		
Physiology status of female	Pregnant	46	59 (56.19)	0.2599 (0.0786–0.859)	0.020
	Non-pregnant	12	4 (25)		
Herd-related factors					
Pasture	No	7	20 (74.07)	0.3786 (0.1569–0.9138)	0.025
	Yes	208	225 (51.96)		
Pasture frequency	Never	7	20 (74.07)	Ref	0.001
	Sporadic	57	93 (62.00)	1.7512 (0.6967–4.4018)	
	Seasonal	124	113 (47.68)	1.7904 (1.1802–2.7161)	
	Frequent	27	19 (41.30)	1.295 (0.6829–2.4556)	
Transhumance	No	206	242 (54.02)	0.2837 (0.0758–1.062)	0.046
	Yes	9	3 (25)		
Presence of water source in pasture	No	163	139 (46.03)	2.2411 (1.4641–3.4304)	0.0001
	Yes	45	86 (65.65)		
Presence of dogs	No	27	50 (64.94)	0.5601 (0.3366–0.932)	0.024
	Yes	188	195 (50.91)		
Cat-related factors					
Number of cats	1–2	10	03 (23.08)	Ref	0.031
	3–4	94	125 (57.08)	4.4326 (1.1869–16.5548)	
	>5	64	66 (50.77)	0.7755 (0.5016–1.199)	
Hygiene-related factors					
Hygiene	Bad	53	49 (48.08)	Ref	0.004
	Good	39	23 (37.10)	1.5677 (0.8223–2.9889)	
	Medium	123	173 (58.45)	2.3849 (1.3558–4.1952)	
Disease- and herd-health-related factors					
Use of anthelminthics	No	133	178 (57.23)	0.6105 (0.412–0.9047)	0.013
	Yes	82	67 (44.97)		
Vaccination of goats against other pathogens	No	98	88 (47.31)	1.4944 (1.0277–2.1729)	0.035
	Yes	117	157 (57.3)		
Reproduction-related factors					
Proportion of abortions	0%	33	19 (36.54)	Ref	0.003
	1–20%	130	136 (51.13)	0.5504 (0.298–1.0165)	
	21–50%	49	83 (62.88)	0.6176 (0.4029–0.9467)	
Number of abortions in the last year	0	33	19 (36.54)	Ref	0.022
	1–5	162	205 (55.86)	0.455 (0.2495–0.8298)	
	6–10	17	14 (45.16)	1.5366 (0.7355–3.2102)	
Spatio-temporal-related factors					
Year of sampling	2020	29	57 (66.28)	Ref	0.004
	2021	81	76 (48.41)	2.0948 (1.2138–3.6155)	
	2022	94	109 (53.69)	0.8092 (0.5331–1.2281)	
	2023	11	3 (21.43)	4.2518 (1.1517–15.6964)	
Region	Coastal	3	7 (70.00)	Ref	0.031
	Plateau	109	149 (57.75)	1.7069 (0.4316–6.7504)	
	Mountain	103	89 (46.35)	1.582 (1.086–2.3044)	
Season	Autumn	14	41 (74.55)	Ref	0.005
	Winter	54	52 (49.06)	3.0412 (1.4858–6.225)	
	Spring	104	98 (48.51)	1.0219 (0.6385–1.6355)	
	Summer	43	54 (55.67)	0.7504 (0.4613–1.2205)	

Ref.—reference value; OR—odds ratio; CI—confidence interval.

The results regarding the general characteristics of goats exhibited the fact that the rate of seropositive goats with toxoplasmosis was not affected by factors such as gender and age ($p > 0.05$), and showed that animals with different body conditions were equally

infected by *T. gondii* ($p = 0.59$). These results are available in the Supplementary Table S1. The seroprevalence of toxoplasmosis in pregnant goats (56.19%; 95% CI: 42.77–72.48) was found to be double that in non-pregnant goats (25%; 95% CI: 06.81–64.01), and that finding was statistically significant ($p = 0.02$). Pregnancy was observed to be a significant risk factor, and exhibited a significant increase in the risk of suffering from the disease as compared to a non-pregnant animal. A pregnant animal was 0.25 times more likely to be infected with the parasite than a non-pregnant animal. Physiology status of male goats seems to impact the risk of exposure to the parasite, which was more prevalent in breeder males (64.18%; 95% CI: 46.45–86.45) than non-breeder males (46.15%; 95% CI: 33.26–62.39) with a *p*-value equal to 0.02.

Univariate analysis showed that most of the factors related to the management system and herd characteristics were statistically non-significant ($p > 0.05$) and did not influence the risk to *T. gondii* exposure. Pasture (No/Yes) had a negative correlation with *T. gondii* prevalence, where goats raised permanently within farms were significantly more seropositive and more exposed by 0.37 times then the ones grazing in pasture ($p = 0.02$). In addition, pasture frequency highly impacted the exposure rate to the parasite with significantly association ($p = 0.001$). Regarding transhumance as a variable, the results revealed that it was a risk factor ($p = 0.04$) but with a limited effect on exposure to *T. gondii* related to a low value of OR (OR = 0.28) between categories of animals with and without transhumance practice. Few goats were submitted to transhumance, and only 25% (95% CI: 05.16–73.06) of them had antibodies against *T. gondii* and appeared to be less exposed.

Water- and feed-related factors had no significant chance to be positively correlated with seroprevalence variations regarding toxoplasmosis. A factor that appeared to be potentially implicated in the increasing of animals' exposure to *T. gondii* was the presence of a water source in the pasture area ($p < 0.001$). Goats grazing in areas associated with a water source showed a high risk of being seropositive (OR= 2.24 times and $p < 0.001$). The seroprevalence of goats was also influenced by the presence of dogs in herds, where goats were less exposed to the parasite (50.91%; 95% CI: 44.02–58.58) than those living in herds without dog cohabitation (64.94%; 95% CI: 48.20–85.61), and that was significantly different ($p = 0.02$). The presence of cats on farms was not considered as a risk factor until *p*-value was higher than 0.05, and under the category of cat-related factors, only the number of cats seemed to significantly impact the rate of seropositivity against *T. gondii* ($p = 0.031$). In herds where the presence of cats was recorded, the presence of three to four cats in the herd increased the risk of the disease to 4.43 times that of the situation where only one to two cats were present. Furthermore, herd areas where more than five cats were being raised had OR = 0.77 compared to cases where only three or four cats were being raised.

The use of anthelminthic drugs notably decreased the percentage of *T. gondii* infection in goats ($p = 0.01$) by 0.61 times. Application of vaccines against other pathogens was associated with a high prevalence of toxoplasmosis (OR = 1.49), the difference being statistically significant ($p = 0.03$). Hygiene was strongly associated with the prevalence of anti-*T. gondii* IgG antibodies, as the rate was 48.08% (49/102; 95% CI: 35.54–63.51) in farms with bad hygiene conditions and 37.10% (23/62; 95% CI: 23.52–55.66) in those with good hygiene ($p = 0.004$). Goats living in good hygiene conditions had reduced risk of infection by the parasite with OR = 1.56. Similarly, goats in herds with a different range of abortion proportions had notably significantly different rate of exposure ($p = 0.003$). The rate of seropositivity highly increased with the proportion of abortions, presenting a positive correlation with a value of odds ratio ranging from 0.55 to 0.61. The link between *T. gondii* prevalence rate and the number of abortions in the last year was assessed and revealed to be significant ($p = 0.02$). These findings show that *T. gondii* should be considered in control measures for any outbreak or sporadic cases of abortions in goat herds.

The role of sampling time and season was also found to be significantly related to the occurrence of *T. gondii* in goats ($p = 0.004$ and $p = 0.005$, respectively). Region was also significantly linked to a higher prevalence of goat anti-*T. gondii* IgG antibodies, where goats in coastal regions were 1.7 times more exposed to the parasite than goats in plateau and

mountain regions ($p = 0.03$). *T. gondii* seroprevalence in goats was higher in autumn (74.55%; 95% CI: 53.50–101.13), followed by summer (55.67%; 95% CI: 41.82–72.64), and closely similar in winter (49.06%; 95% CI: 36.64–64.33) and spring (48.51%; 95% CI: 39.39–59.12), as shown in Table 1. The difference between these rates was highly significant ($p = 0.005$). Considering the prevalence of anti-*T. gondii* IgG in different provinces, the highest rate was recorded in El Taref (70%; 95% CI: 28.14–144.23), followed by Guelma (56.10%; 95% CI: 35.56–84.17) and Constantine (53.96%; 95% CI: 42.44–667.64), and the lowest was detected in Mila (51.85%; 95% CI: 43.62–61.19) (Figure 1), which showed more than half of the animals tested in each province had anti-*T. gondii* antibodies without significant difference.

Goats included in the present study were intended for milk, meat, and mixed production. The seroprevalence was 54.8% (95% CI: 44.44–66.85), 54.41% (95% CI: 38.31–75.00), and 51.63% (95% CI: 42.47–62.17) in farms for milk, meat, and mixed production, respectively. Statistically, a non-significant association ($p > 0.05$) was recorded between the type of production and the seroprevalence variations. Furthermore, the results provided in Table 1 showed that herd size, size of pasture area, type of pasture area, common pastures, use of concentrate feed, watering type, location of the water trough, species on the farm, presence of other farm animals, presence of cattle, equids, poultry, rodents, and other factors had no association with disease exposure.

Regarding multivariate analysis, no significance was found for the variables comprising physiology status of males and females, transhumance, pasture, presence of dogs, and vaccination of goats against other pathogens, showing no significant association to the occurrence of anti-*T. gondii* IgG antibodies. In addition, the generalized linear mixed model analysis showed that the main risk factors potentially associated with *T. gondii* infection in goats were pasture frequency, presence of water source in pasture, number of cats, hygiene, use of anthelminthics, proportion of abortions, number of abortions in the last year, year of sampling, region, and season (Table 2).

Table 2. Seroprevalence of *T. gondii* antibodies and multivariate linear regression analysis of potential risk factors for local goat populations from northeastern Algeria.

Variable	Category	SE	OR	95% CI	*p*-Value
Pasture frequency	Never		Ref		
	Sporadic	0.983	1.806	1.241–2.630	0.547
	Seasonal	1.105	3.804	3.321–4.358	0.003
	Frequent	1.203	2.756	2.320–3.149	0.036
Presence of water source in pasture	Yes		Ref.		
	No	0.466	4.844	1.942–7.789	0.0004
Number of cats	1–2		Ref.		
	3–4	1.613	2.364	1.000–4.237	0.004
	>5	1.968	3.059	1.450–6.879	0.031
Hygiene	Bad	0.620	4.902	1.653–5.574	0.003
	Good	-	Ref		-
	Medium	0.665	4.289	1.581–5.829	0.035
Use of anthelminthics	Yes	0.638	2.640	1.592–3.146	0.036
	No		Ref		
Proportion of abortions	0%	-	Ref		
	1–20%	0.703	0.891	0.647–1.265	0.025
	21–50%	1.027	1.450	1.023–2.845	0.004
Number of abortions in the last year	0		Ref		
	1–5	2.392	4.641	4.181–6.142	0.005
	6–10	2.654	4.964	3.552–5.797	0.008

Table 2. *Cont.*

Variable	Category	SE	OR	95% CI	*p*-Value
Year of sampling	2020		Ref		
	2021	0.740	1.814	0.740–3.264	0.046
	2022	0.892	1.036	0.843–2.631	0.038
	2023	1.082	1.914	1.237–3.816	0.022
Region	Costal		Ref		
	Plateau	0.721	2.157	1.450–3.641	0.0015
	Mountain	0.569	3.658	1.197–4.919	0.0001
Season	Autumn		Ref		
	Winter	1.158	2.120	1.814–3.761	0.023
	Spring	1.648	1.846	1.023–3.460	0.005
	Summer	1.271	0.951	0.698–2.024	0.020

SE: standard error; Ref—reference value; OR—odds ratio; CI—confidence interval.

4. Discussion

The current research is the first report of *T. gondii* seroprevalence and risk factor relationships in goats from Algeria's northeastern regions. The seroprevalence found in this investigation (53.26%) was greater than that identified in central Algeria (11.92%) and Djelfa province (13.21%), as determined by indirect ELISA and indirect fluorescent anti-body tests (IFATs), respectively [27,28]. The rate of anti-*T. gondii* antibody prevalence in goats in this study was found to be lower than that (71.74%) reported in the humid areas of Mila province using an ELISA test [20], but higher than the rate of infection (35.37%) revealed in sheep in the arid and semiarid regions of northeastern Algeria using an ELISA test [29]. According to multiple studies that have employed diverse serological tests such as ELISA, IFAT, and LAT, the overall prevalence of *T. gondii* in goats in Algeria has been shown to range from 11.92% to 71.74% with an average rate of 33.61% [21]. The current rate of *T. gondii* in this study was higher than the average prevalence in Algerian goats. These variations might be explained by the serological assays applied in these investigations, as the modified agglutination test (MAT) and ELISA showed significant agreement, and almost perfect concordance between IFAT and ELISA was observed [30]. The comparison of research is challenging in this context. Our results imply that the prevalence identified in this study may be associated with suitable climatic conditions, because the four study provinces (Mila, Constantine, Guelma, and El Taref) have similar ecological patterns characterized by a wet period during most of the year [22] that are favorable for *T. gondii* oocyst formation and survival [5]. As a result, this region in Algeria has the greatest prevalence rate of the parasite in animals [21]. In different regions of the world, prevalence rates ranging from 0% to 100% have been recorded [31]. The variations were attributed to regional customs, local traditions, residents' lifestyles, and meteorological conditions [32]. Given the various epidemiological settings, research designs, number of samples studied, diagnostic procedures, and cut-off points used, differences between studies should be carefully assessed.

In the current study, the goat population from northeastern Algeria had a high individual prevalence rate *T. gondii* (53.26%), and 94.44% of goat herds had at least one seropositive animal, showing a widespread distribution of this parasite, as also reported in other Mediterranean countries [33–35]. This individual rate was higher than that documented by many researchers from different areas worldwide: 38.28% in Egypt [36], 8.5% in Morocco [37], and 34.4% in Tunisia [38]. Al-Mabruk [39] reported an exposure percentage of 71% in Libyan sheep, which is higher than the current study's results. A total of 53.15% of goats' blood sera were found to be positive in Faisalabad, Pakistan [40]; that was in close correlation with what we found. Gazzonis et al. [41] showed that 41.7% of tested goats in northern Italy were *T. gondii* seropositive with a comparable herd level (96.6%). Additionally, in comparison to the present data, goats showed lower herd seroprevalences of 60% and 72.2% in Morocco [37] and southern Spain [42], respectively. The seropositivity

variance between these studies might be related to differences in management strategies, biosecurity measures, and climatic conditions at each goat farm [7].

The findings of this study contribute to the body of information about the epidemiology of *T. gondii* on a global scale. In many regions of the world, a few studies have looked into seroprevalence and associated risk factors in traditional husbandry systems [43]. In the current study, gender was not a notable significant risk factor for exposure to *T. gondii*, consistent with findings from Martínez-Rodriguez et al. [43] and Jilo et al. [44], which showed goats of both genders were equally likely to contract a *T. gondii* infection. The increased incidence of *T. gondii* antibodies in female goats in this study might be attributable to the fact that there were more females studied than males. Our findings revealed that animals of all ages had been exposed to the parasite in the same way, according to what was reported in previous studies [45,46] showing that age had no relationship with *T. gondii* exposure in goats. The variable "geographic areas" has a considerable influence on *T. gondii* prevalence, particularly in regions with ecological patterns typified by a long wet period of the year, which is compatible with what was reported previously [28,43,47]. This state might be due to topography and climatic differences related to geographical characteristics, rainfall, and yearly average temperatures that can promote the survival and sporulation of *T. gondii* oocysts [28]; thus, their transmission to goats in pastures is enhanced [48,49].

Different breeding strategies had no effect on *T. gondii* seroprevalence levels registered in the present investigation. These findings corroborated those published by Mohamed-Cherif et al. [28] and Rizzo et al. [50]. The current investigation confirmed the findings of Rêgo et al. [51] showing that production of milk and/or meat from goat husbandry did not positively correlate with the prevalence rate of *T. gondii* infection. Contrary to what we found, the positive rate of this parasitic infection was significantly impacted by more dairy and meat purposes in goat farming [52]. Most studies found that semi-intensive and extensive practices make it easier for goats to be exposed to *T. gondii* oocysts in the environment [38,51]. Dahmane et al. [20] reported that the degree of exposure to the parasite in Mila province, northeastern Algeria, was unaffected by herd practices. Traditional husbandry is still the most common way of raising goats in northeastern Algeria, and it might be a significant risk factor for *T. gondii* infection. A few goats are often kept by each rural family as an alternative form of animal husbandry. Early in the morning, these animals are moved from the settlements to their natural pastures, and they return in the late afternoon. The surroundings are home to a large number of stray and domestic cats; thus, there is a higher chance that these animals will contract oocysts from cat feces.

Several reports have shown a negative correlation between flock size and *T. gondii* seroprevalence [34,41], and that was not recorded in the present study. The same outcome of *T. gondii* infection in herds with small and large numbers of animals was reported in other studies [29,42,53]. In addition, common pastures did not appear as a risk factor; thus, contact with other herds of goats or other livestock animals had no effect on *T. gondii* exposure. A non-significant correlation was observed between the *T. gondii* seroprevalence and the presence of other animal species, including sheep, cattle, poultry, and cats, which had been in close contact with goat flocks. In contrast, Udonsom et al. [45] reported that the presence of other domestic animals on a goat farm increased by 1.69 times the risk of exposure to this parasite. According to the current results, the presence of dogs appeared to be related to an increased risk of *T. gondii* exposure, and dogs may be a possible contamination source for grazing pastures [43,54]. However, other studies did not find an association between *T. gondii* infection and the presence of dogs and wild dogs [34,51]. Similarly to our findings, the presence of equines and cattle on the same farms as goats was regarded as a protective factor, decreasing goat exposure to *T. gondii* [43]. Contact with rodents was considered a potential risk factor in various studies [17]. However, this was not consistent with our findings. Overall, the presence of other animal species may have a direct effect on the risk of infection for livestock animals, including goats, where they may serve as *T. gondii* reservoirs and play a role in the expansion within the farm [41,51].

Poor management and hygienic standards characterizing goat herds in the studied locations promote the exposure of goats to *T. gondii*, particularly where herds had poor hygienic status, which was also reported previously [50,53]. Measures of hygiene and regimes of cleaning and disinfection applied at the farms may play an important role in reducing oocyst contamination [17]. Biosecurity measures such as quarantine and having parturition space for goat females had no statistically significant effect on *T. gondii* seropositivity in the present study, and these variables could be considered protective factors, which was consistent with findings from other studies [34,50]. The absence of facilities and supervised areas for delivery of females made goats more exposed to the parasite [43]. In addition, in farms where elimination of placental tissues and abortion products was not applied correctly, the risk of exposure to *T. gondii* was notably high, which might be related to the easy eating of the aborted fetuses by domestic and wild cats [51]. In our study, no association was found between *T. gondii* seropositivity and abortion tissue elimination, which might be related to the limited access of the cats to abortion products that were mostly disposed of by owners or consumed by dogs.

Consistent with previous studies [51,55], anthelmintic treatment and deworming were shown to reduce the rate of *T. gondii* infection in goat herds and protect against toxoplasmosis. In addition, the use of antiparasitic drugs against ectoparasites such as ticks and the use of antibiotics did not have a correlation with the high prevalence of *T. gondii*. However, vaccination of goats against some microbial diseases, such as enterotoxemia and brucellosis, could affect the level of exposure to the parasite because animals could become more susceptible to the infection when the immune system is in a critical state and producing antibodies against vaccines and other pathogens, such as *T. gondii*, at the same time. Stress on the immune system caused by various states of illness may be a contributing factor to the greater seroprevalence of *T. gondii* [56].

The prevalence of *T. gondii* in our study was insignificantly higher in farms with a history of reproductive disorders, as recorded by other authors [33,34,42]. It seems there was a direct relationship between the history of abortion and the seropositivity rate of *T. gondii* [29,57]. Moreover, the proportion of abortions and number of aborted fetuses in the last year were in significant correlation with the occurrence of *T. gondii*, consistently with the findings of Benlakehal et al. [29] and Gharekhani et al. [58], whereas an insignificant association was reported previously [34,42,54]. Several studies have used serological methods to identify reproductive losses associated with *T. gondii* [8]. It would be important to use a combination of diagnostic methods, such as histopathological and molecular assays, to identify any association between *T. gondii* and placental lesions, and to rule out other infectious causes of reproductive disorders [59,60]. In a recent study, the occurrence of *Coxiella burnetii* in the local goat population in the same study areas was reported [61], which reflects an exposure of this population to various abortifacient agents, and it should be taken into consideration when applying strategies to prevent and reduce the rate of abortions in goat herds.

The presence of cats on goat farms/flocks appeared to have a considerable influence on *T. gondii* prevalence and transmission [44,45], contrary to the current findings and to what was recorded in other investigations [34,41,42]. According to our results, the risk of contracting the infection increased not only with the presence of cats but mostly with their number on goat farms or in neighboring areas, which played an important role in maintaining parasite dissemination [43,62]. Interestingly, the presence of cats on grazing sites, which allows a contamination of pastures or water sources, might be responsible for the high prevalence rate, which can also be related to transhumance and rearing frequency. It was estimated that at any given time and in a given cat population, 1% to 2% of cats excrete millions of oocysts over a short period of 1 to 2 weeks throughout their lives, which ensures major contamination of the environment [1,3]. In addition, the presence of wild felids had no notable influence on *T. gondii* seropositivity, and this might be related to the limited presence of wild felids or the fact that their presence was not correctly noticed by farmers. The main cat-related risk factor identified was the number of cats. Unexpectedly,

Rêgo et al. [51] found that the proximity of cats to a water source had a statistically beneficial impact. The possibility of cats contaminating cropland, feed, or water delivered to livestock appeared to be a potential risk factor that requires more investigation [17]. Cat-related factors, such as the number of cats on the farm, the contact of animals with cats or cat feces, and the contact of cats with water or water sources, promote the exposure of goats to *T. gondii* oocysts. Multivariate analysis revealed that the probability of contracting a *T. gondii* infection increased with the number of cats or their density, which was similar to a previous report [63]. In addition, the absence of feline population control programs, especially in the studied rural areas, could result in more cases of infection on goat farms [64]. Similarly to our findings, Rizzo et al. [50] showed a non-significant association between cats' access to food storage and the rate of anti-*T. gondii* antibodies. On the other hand, access of cats to exterior water sources significantly increased oocyst exposure, and water from rivers and the public supply enhanced the probability of exposing animals to the parasite [34,41]. Furthermore, the use of water from a source (weir or dam) in containers (inside and outside the farms) did not affect the rate of *T. gondii* seropositivity [45,50], which was also reported in our study. In the study areas, the high rate of goat toxoplasmosis might be attributed to the presence of water sources in pastures such as stagnant water or lakes, which are able to be accessed by cats and eventually become contaminated with *T. gondii* oocysts. The use of stagnant water was documented as a major risk factor [62]. Other authors reported that the use of a lake's water or surface waters appears to be more susceptible to easily transmitting the parasite compared with other water sources [28,45,65]. However, drinking water from cisterns and taps seems less able to enhance the risk of *T. gondii* transmission compared with the use of aqueduct water [43,45]. We suggested that cats can contaminate unprotected water sources such as artesian wells and small lakes in pastures, especially in rainy periods, and that makes the parasite able to complete their biological cycle in the intermediate hosts, including goats [1]. Additionally, water sources found in pastures could be contaminated by feces containing *T. gondii* oocysts excreted by pet cats in rearing areas near farms or by feral cats in forest areas far from the farm location. Overall, it is hard to quantify the risk of infection of the animals through contaminated water [17].

5. Conclusions

For the first time, our study showed a considerable seroprevalence of *T. gondii* infection in goats across different agro-geographical areas in northeastern Algeria, reflecting a large distribution of the parasite, which was related to factors significantly impacting the infection rate, such as the number of cats, seasonal pastures, presence of water sources in these pastures, and hygiene conditions. Control measures to prevent and reduce the transmission of *T. gondii*, including preventing access of cats and other biological or mechanical vectors for this pathogen to water sources and feed storage facilities, need to be planned. A significant association between abortions and *T. gondii* seropositivity was reported, which makes it important to apply strategies for reducing abortion rates in goat herds.

The high prevalence of *T. gondii* in goats showed the zoonotic importance of this parasite through consumption of their meat, raw milk, and milk products; thus, it is necessary to sensitize goat owners and other peoples, through education, on the zoonotic transmission of *T. gondii*. Further epidemiological investigations based on serological and molecular assays and genotypic characterization of the parasite in goat meat designed for human consumption should be conducted to better understand the impact of caprine toxoplasmosis on food production and public health in Algeria.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani14060883/s1, Table S1: Univariate analysis of explanatory risk factors associated to *T. gondii* infection in goat population from Northeastern Algeria, with p-value ≥ 0.05; Table S2: Sampling information of the 460 goat serum samples collected from Algeria.

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Informed Consent Statement: Oral consent was obtained from farmers.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to [confidentiality agreements with participants/subjects involved in the research].

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Article

Wild Animals in Captivity: An Analysis of Parasite Biodiversity and Transmission among Animals at Two Zoological Institutions with Different Typologies

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Simple Summary: We have conducted a 10-year coprological study of animals housed in two zoological institutions with different housing conditions to assess parasite biodiversity and prevalence, their relationship with host class (mammal/bird), diet (carnivorous/omnivorous/herbivorous), and enclosure characteristics (soil type, isolation from wild fauna), and evaluated the risk of transmission to humans. A total of 4476 faecal samples from 132 mammal species and 951 samples from 86 avian species were examined, with 62.1% of mammal species and 12.8% of avian species testing positive. Statistically significant differences were found based on diet type; few carnivorous species were detected infected, primarily by nematodes, while many herbivorous and omnivorous species were primarily infected by protists. No statistically significant differences were observed based on soil type (artificial, natural, mixed) and isolation level (isolated/accessible). Several parasite species found in the study (*Entamoeba* spp., *Giardia* spp., *Balantioides coli*, *Trichuris* spp.) could potentially be transmitted between housed animals, wild fauna, and humans. Regular analyses of the animals and implementation and follow-up of health programs would minimise transmission risks between housed animals, wild fauna, and humans.

Abstract: We have conducted a 10-year-long coprological study of the animals housed in two zoological institutions (ZooAquarium and Faunia, Madrid, Spain) to assess the parasite biodiversity, prevalence, and their relation with host class, diet, and enclosure type (soil type and level of isolation from wild fauna). A total of 4476 faecal samples from 132 mammal species and 951 samples from 86 avian species were examined. The results indicated that only 12.8% of avian species had parasites at least once during the study period, whereas 62.1% of mammal species tested positive. Predominantly, protists (*Entamoeba*, flagellates, and ciliates) and nematodes (mainly *Trichuris*) were identified in the findings. Carnivorous species were primarily infected by nematodes, while herbivorous and omnivorous species were mainly infected by protists. The number of infected herbivorous and omnivorous species was significantly greater than carnivorous species. Differences were observed based on soil type (artificial, natural, mixed) and isolation level (isolated/accessible), but these differences were not statistically significant. Several parasites (*Entamoeba* spp., *Giardia* spp., *Balantidoides coli*, *Trichuris* spp.) could potentially be transmitted between humans and some mammals and birds. Regular animal analyses and a personnel health program in the institutions would minimise transmission risks between zoo animals, wildlife, and humans.

Keywords: intestinal parasites; Protista; helminths; captive wild mammals; captive wild birds; zoological gardens; epidemiology; transmission risk

1. Introduction

Parasites can affect their hosts both at an individual level (even causing their death) and at a population level, potentially affecting biodiversity by interfering in species competition, migration, and ecosystem stability [1]. The importance of parasites in the conservation of endangered host species is due to two circumstances: habitat degradation leads to increased contact between populations or species that are usually separated, easing the cross-transmission of pathogens, and the distribution of species populations in fragmented habitats leads to increased animal density, favouring disease outbreaks [2–4].

One of the most important objectives of zoological gardens is to contribute to the conservation of wild species, with special attention to those threatened or endangered in their natural habitats. Specific programs, such as the European Association of Zoos and Aquaria (EAZA) Ex situ programmes, are currently ongoing [5]. In the European Union, the importance of zoological gardens in education and species conservation is regulated by Directive 1999/22/EC, which, in the particular case of Spain, was transposed in 2003 into national law (31/2003). However, zoos could inadvertently serve as an opportunity for pathogens to be transmitted between individuals and species, given that the conditions mentioned above are present in zoo facilities: closer contact between different species and increased animal density. The occurrence of parasites in zoo animals could vary according to environmental conditions, management practices, disease prophylaxis, and treatment protocols [6,7]. Moreover, the physical characteristics of the facilities and the physiological status of the animals induced by captivity could contribute to the transmission of pathogens [8–10].

Despite the veterinary regulations in importing countries, zoo animals could be parasitised by co-imported parasites as well as other autochthonous species, and in some cases, cross-transmission with zoo personnel could occur [11–15]. However, although there are many studies on the prevalence of gastrointestinal parasites in zoo animals, only a few deal with the possible cross-transmission between housed and free-ranging animals or with the characteristics of the facilities [9,14,16–18]. In the present study, we investigated the biodiversity and host range of parasites infecting non-aquatic mammals and birds in two zoological institutions with different housing conditions during a 10-year period (2013–2022), compared the results between them, and evaluated the possibility of transmission between animals and humans.

2. Materials and Methods

2.1. Study Location and Host Species

This study was conducted from 2013 to 2022 in two zoos located in Madrid city (Spain): the ZooAquarium, situated in the Casa de Campo urban park, and Faunia Park, located within an urban area.

The ZooAquarium is organised into five main zones corresponding to different continents. In each region, animals are kept in groups or isolated by species based on their compatibility within enclosures of suitable size relative to the number of individuals. There is no crowding, and there are feeders, water sources, and hidden areas for resting. Flying birds are housed in open-air enclosures of adequate size. Mammals are in open-air natural areas delimited by water bodies and/or wood and metal fences; only a few species are animals in partially or totally enclosed installations limited by glass or metal fences and nets. The soil is natural and has grass in most sections; only in the case of large herbivores and some carnivores is there almost no grass. In some cases (i.e., the aoudads), the soil is concrete. The enclosures are encircled by paved pathways to accommodate visitor passage. In total, there are over 6000 animals of about 500 species from the 5 continents; the numbers vary over time depending on new acquisitions, deaths, and interchanges with other zoos.

Faunia Park is organised in ecosystems mostly recreated in closed installations; only in some cases are animals in open-air facilities. Depending on the zone and the species compatibility, animals are in open areas in direct contact with visitors' pathways, in enclosures with wood or metal fences and nets, or in closed, isolated ambients recreating

their natural habitat under controlled light and humidity conditions. There are more than 1200 animals of 152 species from 4 different ecosystems.

2.2. Sample Collection and Processing

Fresh faecal material was obtained from 83 species of terrestrial mammals and 64 species of birds at ZooAquarium and from 68 species of terrestrial mammals and 40 species of birds at Faunia Park. Nineteen mammals and eighteen bird species were housed at both zoos (for the purpose of this study, the Iberian eagle-owl, *Bubo bubo hispanus*, and the western Siberian eagle-owl, *Bubo bubo sibiricus*, are treated separately). They were classified as carnivores (including insectivores and scavengers), herbivores, or omnivores according to their main diet range. Mammal and bird scientific names follow the Mammal Diversity Database [19] and the International Ornithological Committee (IOC) World Bird List v.14.1 [20].

The samples were collected by the zookeepers early in the morning and kept in clean, new plastic recipients; they were transported to the laboratory 1–3 h after collection. Samples were usually processed upon arrival or kept at 4 °C until processed (maximum delay, 24 h). Individual samples were collected in some cases (i.e., when only one or a few individuals were in the group, from large animals, or in symptomatic or quarantined ones). All animals from the same species were sampled at the same time or in a two-week interval. In animals from large groups, faecal pools were collected. The results of the analyses were communicated to the zoo veterinarians, and they decided upon the correct treatment; in these cases, new samples were analysed after treatment to confirm their efficacy. Samples taken from animals that had received an antiparasitic treatment within the month before sampling were not included in this study.

Once in the laboratory, a macroscopic analysis of each sample was made, searching for the presence of parasitic structures. Faecal concentrates (following the formalin–ethyl acetate stool concentration technique) [21] were made, and the sediments were examined on temporary slides stained with Lugol's iodine. Morphological features were measured and photographed with Olympus DP20 or Olympus DP23 cameras on an Olympus BX51 microscope (Olympus, Tokyo, Japan).

2.3. Parasite Biodiversity, Housing Conditions, and Feeding Habits

The possible relationship between the type of parasite life cycle (direct/indirect life cycle) and frequency of findings in each host species was investigated, taking into account the zoological institution, housing conditions, vertebrate class, and feeding habits as independent variables. The parasitological analyses were conducted for diagnostic purposes only, and a multifactorial analysis was not designed. Therefore, other environmental variables such as temperature, sunlight exposure, air or soil humidity, or rain were not considered during samplings. A time analysis was not performed as the samples from each host species were irregularly spaced over time, ranging from some weeks to more than one year between sampling a given species. Statistical comparisons were made using the IBM SPSS Statistics ver. 29 software (IBM Inc., New York, NY, USA).

Binary logistic regressions were conducted, considering the host species as "at least once infected" or "never infected" as the dependent variable. Housing conditions were considered according to the type of soil and the level of isolation. The types of soil were categorised as natural (with/without natural vegetation; with natural drainage), artificial (cement base, with/without sand or wood shavings covering; without drainage or with drainage through artificial systems), or mixed (animals spending time in both natural and artificial soils, e.g., animals for exhibitions or with periods outdoors for environmental enrichment). In terms of isolation level, the animals were considered "isolated" when housed in enclosed spaces where access by wild fauna (small mammals like rodents or birds) was not possible or as "accessible" when uncontrolled access by wild fauna to the facilities was feasible. Regarding feeding habits, the host species analysed were classified as herbivorous, carnivorous (including ichthyophagous, insectivorous, and scavengers), or omnivorous, depending on their main diet type. For example, animals like lar gibbon

(*Hylobates lar*) that may sporadically feed on animals but usually consume vegetables were considered herbivorous, while predators like wolf (*Canis lupus*) that may, in some instances, feed on vegetables were considered carnivorous.

3. Results

3.1. Overall Parasite Biodiversity and Prevalence

A total of 4476 faecal samples from mammals and 951 from birds (excluding those from repetitions after treatment courses) were collected and analysed from both zoos. Among them, 1333 samples from 82 mammal species and 63 samples from 11 avian species were found positive (Table 1). Parasites were found in 62.1% of the mammal species (82/132), while only in 12.8% of the avian species (11/86). The parasites found in mammals included protists (protozoa and chromists), trematodes, cestodes, and nematodes, while only one protozoan, one cestode, and several nematodes were found in birds (Table 2). In mammals, the higher number of host species found infected (mainly by protists) were herbivorous animals, while carnivorous hosts are the group with a lower number of species infected (Tables 1 and 2). The morphological characteristics of eggs/cysts/oocysts often do not allow for differentiation between species. In cases where morphologically similar genera or species infect the same or related host species, the parasites were identified using group names (e.g., trichomonads, trichostrongylids) or as spp. (e.g., *Trichuris* spp.). Findings resembling the amoebae species *Entamoeba bovis* Liebetanz 1905, *Entamoeba polecki* Prowazek 1912, *Entamoeba coli* (Grassi 1879), and *Entamoeba muris* (Grassi 1879); the ciliate *Balantioides coli* (Malmstem 1857); and the cestode genus *Raillietina* Fuhrmann 1920, were identified as taxon-like.

Table 1. Total number of mammalian and avian species analysed at ZooAquarium and Faunia zoological parks, Madrid, Spain.

Zoo	Hosts	Diet Type	Animal Species Studied	Hosts Infected	Samples Analysed	Positive Samples
ZooAquarium	Mammals	Herbivores	55	49 (89.1%)	1891	956 (50.6%)
		Omnivores	17	9 (53.0%)	455	164 (36.0%)
		Carnivores	11	2 (18.2%)	254	27 (10.6%)
		Total	83	60 (72.3%)	2600	1147 (44.1%)
	Birds	Herbivores	17	0 (0.0%)	127	0 (0.0%)
		Omnivores	15	3 (20.0%)	127	8 (6.3%)
		Carnivores	32	4 (12.5%)	285	26 (9.1%)
		Total	64	7 (11.0%)	539	34 (6.3%)
Faunia	Mammals	Herbivores	29	17 (58.6%)	783	136 (17.4%)
		Omnivores	23	7 (30.4%)	671	46 (6.9%)
		Carnivores	16	1 (6.3%)	422	2 (0.5%)
		Total	68	25 (36.8%)	1876	184 (9.8%)
	Birds	Herbivores	14	0 (0.0%)	130	0 (0.0%)
		Omnivores	12	5 (41.7%)	188	29 (15.4%)
		Carnivores	14	0 (0.0%)	94	0 (0.0%)
		Total	40	5 (12.5%)	412	29 (7.0%)

Table 1. *Cont.*

Zoo	Hosts	Diet Type	Animal Species Studied	Hosts Infected	Samples Analysed	Positive Samples
Total results *	Mammals	Herbivores	73	62 (84.9%)	2674	1092 (40.8%)
		Omnivores	34	16 (47.1%)	1126	211 (18.7%)
		Carnivores	25	4 (16.0%)	676	30 (4.4%)
		Total	132	82 (62.1%)	4476	1333 (29.8%)
	Birds	Herbivores	24	0 (0.0%)	257	0 (0.0%)
		Omnivores	22	7 (31.8%)	315	37 (11.8%)
		Carnivores	40	4 (10.0%)	379	26 (6.9%)
		Total	86	11 (12.8%)	951	63 (6.6%)

* The number of host species does not correspond to the direct sum of species from both zoos, as there are 19 species of mammals and 18 of birds housed in both centres.

Table 2. Number of mammal and avian species found infected by different parasites in each zoological centre. Codes: C—carnivorous, O—omnivorous, H—herbivorous. The number in the parenthesis under the code indicates the number of host species for each classification.

	ZooAquarium Infected Host Species						Faunia Infected Host Species					
	Mammals			Birds			Mammals			Birds		
	C (2)	O (9)	H (49)	C (4)	O (3)	H (0)	C (1)	O (7)	H (17)	C (0)	O (5)	H (0)
Amoebae												
Entamoeba Casagrandi and Barbagallo 1897	1	6	34		3			3	9		1	
Endolimax Kuenen and Swellengrebel 1913		2	6						2			
Flagellates												
Giardia Künstler 1882		1	4					1	3			
Chilomastix Aléxéieff 1910		3	8					2	2			
Trichomonads	1		1									
Coccidia												
Eimeria Schneider 1875			5						2			
Toxoplasma Nicolle and Manceaux 1909/*Neospora* Dubey et al. 1988	1											
Ciliates												
Balantioides Alexeieff 1931		5	8		1			2	1			
Buxtonella Jameson 1926			5									
Troglodytella (Brumpt and Joyeux 1912)			1									
Endosymbiotic ciliates			6						2			
Trematodes												
Unidentified eggs		1										
Cestodes												
Unidentified eggs		1	5									1
Nematodes												
Trichuris Roederer 1761		3	10	1			1	1	6			
Capillaria Zeder 1800/capillariids	1	1	2	3					1		3	
Nematodirus Ransom 1907			2									
Trichostrongylids			1									
Baylisascaris Sprent 1968		1						1				
Parascaris Yorke and Maplestone 1926			2									
Porrocaecum Railliet and Henry 1912				1								
Ascaridia Dujardin 1845/*Heterakis* Schrank 1790											2	
Ascarid (unidentified)							1					

The biodiversity and prevalence of the parasites found in each host species are given in Tables 3–6. All parasites found were of the direct life cycle, except for the unidentified

trematode eggs found in bears and the cestodes found in several mammal species in the ZooAquarium and in one bird in Faunia (Figure 1).

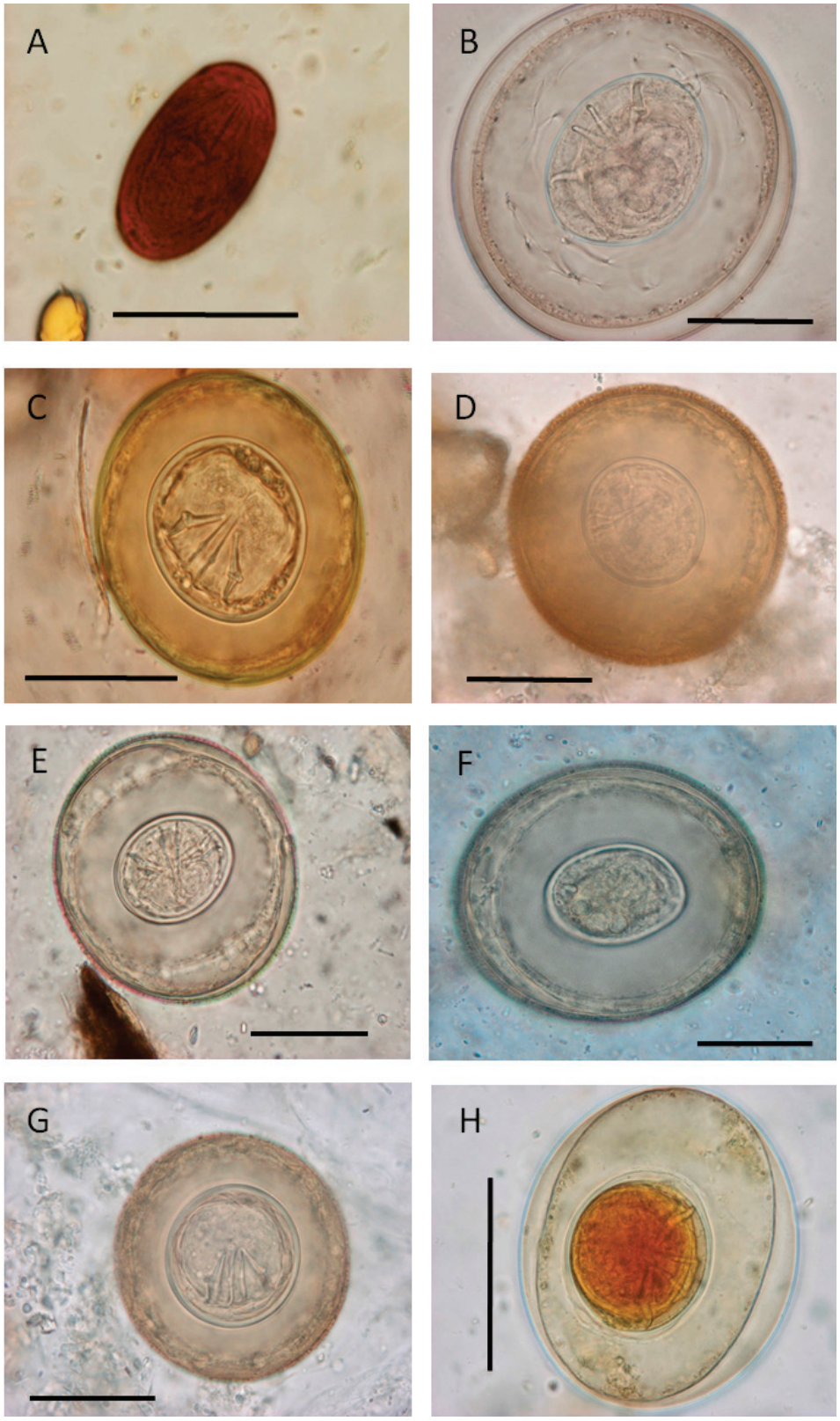

Figure 1. Eggs from indirect life-cycle parasite species found in the samplings. (**A**) Unidentified trematode egg resembling *Dicrocoelium* (Dujarding 1845) egg from the sun bear (*Helarctos malayanus*).

(**B**–**H**) Cestode eggs. (**B**) *Raillietina*-like eggs from the helmeted guineafowl (*Numida meleagris*). (**C**) Unidentified egg from the red deer (*Cervus elaphus*); (**D**) unidentified egg from the yak (*Bos grunniens*); (**E**) unidentified egg from the South American tapir (*Tapirus terrestris*); (**F**) unidentified egg from the hippopotamus (*Hippopotamus amphibious*); (**G**) unidentified egg from common brown lemur (*Eulemur fulvus*); (**H**) unidentified egg from the mandrill (*Mandrillus sphinx*). Scale bars: 30 μm.

Table 3. List of parasites found in the mammal hosts at ZooAquarium. Species in bold are also housed at Faunia Park.

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
Artiodactyla	Bovidae	*Ammotragus lervia* (Pallas 1777)	21/14	*Entamoeba bovis*-like (66.7%)
		Antilope cervicapra (Linnaeus 1758)	18/9	*Entamoeba bovis*-like (50.0%)
		Bison bison (Linnaeus 1758)	37/15	*Entamoeba bovis*-like (40.5%)
		Bison bonasus (Linnaeus 1758)	27/19	*Entamoeba bovis*-like (70.4%), *Buxtonella sulcata* Jameson 1926 (3.7%)
		Bos grunniens (Linnaeus 1766)	17/12	*Entamoeba bovis*-like (52.9%), *Buxtonella sulcata* (41.2%), unidentified cestode eggs (5.9%)
		Bos taurus (Linnaeus 1758)	4/1	*Entamoeba bovis*-like (25.0%)
		Boselaphus tragocamelus (Pallas 1766)	13/12	*Entamoeba bovis*-like (92.3%)
		Budorcas taxicolor Hodgson 1850	39/26	*Entamoeba bovis*-like (66.7%)
		***Capra hircus* Linnaeus 1758**	37/31	*Entamoeba bovis*-like (81.1%), *Eimeria* spp. (5.4%), *Trichuris* spp. (5.4%)
		Capra pyrenaica Schinz 1838	30/20	*Entamoeba bovis*-like (66.7%)
		Connochaetes gnou (Zimmermann 1780)	23/14	*Entamoeba bovis*-like (60.9%)
		Gazella dorcas osiris Blaine 1913	127/106	*Entamoeba bovis*-like (70.1%), *Eimeria* spp. (1.6%), *Trichuris* spp. (16.5%), *Nematodirus* spp. (21.3%)
		Nanger dama mhorr (Bennett 1833)	99/85	*Entamoeba bovis*-like (74.7%), *Entamoeba* spp. (8-nucleated) (2.0%), *Giardia* spp. (2.0%), *Chilomastix* spp. (1.0%), *Eimeria* spp. (2.0%), *Trichuris* spp. (15.2%), *Nematodirus* spp. (8.1%), Trichostrongylids (10.1%)
		***Ovis aries* Linnaeus 1758**	39/13	*Entamoeba bovis*-like (25.6%), *Chilomastix* spp. (2.6%), *Eimeria* spp. (5.1%)
		Ovis gmelinii Blyth 1841	11/6	*Entamoeba bovis*-like (54.6%)
		Syncerus caffer nanus Boddaert 1785	59/54	*Entamoeba bovis*-like (89.8%), trichomonads (3.4%), *Buxtonella sulcata* (44.1%)
		Tragelaphus eurycerus (Ogilby 1837)	17/15	*Entamoeba bovis*-like (88.2%)
		Tragelaphus spekii gratus Sclater 1880	40/26	*Entamoeba bovis*-like (60.0%), *Chilomastix* spp. (2.5%), *Balantioides coli*-like (15.0%)
	Camelidae	*Camelus bactrianus* Linnaeus 1758	40/19	*Entamoeba bovis*-like (20.0%), *Buxtonella cameli* (Boschenko 1925) (25.0%), *Trichuris* spp. (5.0%)
		***Camelus dromedarius* Linnaeus 1758**	34/7	*Entamoeba bovis*-like (5.9%), *Buxtonella cameli* (20.6%)

Table 3. *Cont.*

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
		***Lama guanicoe* (Müller 1776)**	19/8	*Entamoeba bovis*-like (42.1%)
	Cervidae	*Alces alces* (Linnaeus 1758)	16/11	*Balantioides coli*-like (43.8%), *Trichuris* spp. (56.3%)
		Capreolus capreolus (Linnaeus 1758)	6/5	*Entamoeba bovis*-like (83.3%)
		***Cervus elaphus* Linnaeus 1758**	34/30	*Entamoeba bovis*-like (85.3%), unidentified cestode eggs (2.9%)
		Dama dama (Linnaeus 1758)	40/35	*Entamoeba bovis*-like (87.5%), *Capillaria* spp. (5.0%)
		Elaphurus davidianus Milne-Edwards 1866	29/20	*Entamoeba bovis*-like (69.0%)
		Muntiacus reevesi (Ogilby 1839)	21/18	*Entamoeba bovis*-like (85.7%), *Chilomastix* spp. (4.8%)
		Rangifer tarandus (Linnaeus 1758)	23/4	*Entamoeba bovis*-like (4.4%), *Trichuris* spp. (13.0%)
	Giraffidae	*Giraffa camelopardalis* (Linnaeus 1758)	40/14	*Entamoeba bovis*-like (35.0%), *Trichuris* spp. (2.5%)
	Hippopotamidae	*Hippopotamus amphibius* Linnaeus 1758	11/1	Unidentified cestode eggs (9.1%)
	Suidae	*Potamochoerus porcus* (Linnaeus 1758)	37/14	*Entamoeba polecki*-like (29.7%), *Giardia* spp. (2.7%), *Chilomastix* spp. (8.1%), *Balantioides coli* (24.3%)
		***Sus scrofa* Linnaeus 1758**	59/43	*Entamoeba polecki*-like (52.5%), *Chilomastix* spp. (8.5%), *Balantioides coli* (42.4%)
	Tayassuidae	***Dicotyles tajacu* (Linnaeus 1758)**	1/0	
Carnivora	Ailuridae	***Ailurus fulgens* Cuvier 1825**	33/2	*Capillaria* spp. (6.1%)
	Canidae	*Canis lupus occidentalis* Linnaeus 1758	15/0	
		Speothos venaticus (Lund 1842)	45/1	*Toxoplasma/Neospora* (2.2%)
	Felidae	*Lynx lynx* (Linnaeus 1758)	13/0	
		Lynx pardinus (Temminck 1827)	20/0	
		Panthera leo (Linnaeus 1758)	16/0	
		Panthera pardus saxicolor (Linnaeus 1758)	22/0	
		Panthera tigris (Linnaeus 1758)	16/0	
	Herpestidae	***Suricata suricatta* (Schreber 1776)**	10/0	
	Mustelidae	***Mustela lutreola* (Linnaeus 1761)**	30/0	
		Pteronura brasiliensis (Zimmermann 1780)	20/0	
	Procyonidae	***Nasua nasua* (Linnaeus 1766)**	9/0	
		***Procyon lotor* (Linnaeus 1758)**	18/2	*Capillaria* spp. (11.1%)
	Ursidae	*Ailuropoda melanoleuca* (David 1869)	36/0	
		Helarctos malayanus (Raffles 1822)	35/1	Trematoda (2.9%)
		Tremarctos ornatus (Cuvier 1825)	1/0	

Table 3. *Cont.*

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
		Ursus americanus Pallas 1780	16/0	
		Ursus arctos Linnaeus 1758	63/7	*Baylisascaris* spp. (11.1%)
		Ursus thibetanus Cuvier 1823	37/0	
	Viverridae	***Arctictis binturong* (Raffles 1822)**	32/0	
Diprotodontia	Macropodidae	***Notamacropus rufogriseus* (Desmarest 1817)**	6/0	
		Petrogale xanthopus Gray 1855	20/5	*Entamoeba bovis*-like (25.0%)
	Phascolarctidae	*Phascolarctos cinereus* (Goldfuss 1817)	35/0	
Lagomorpha	Leporidae	***Oryctolagus cuniculus* (Linnaeus 1758)**	65/4	*Eimeria* spp. (6.2%)
Perissodactyla	Equidae	*Equus quaga* Boddaert 1785	159/9	Endosymbiotic ciliates (4.4%), *Parascaris equorum* (Goeze 1782) (1.3%)
		Equus asinus Linnaeus 1758	25/21	Endosymbiotic ciliates (84.0%), *Parascaris equorum* (8.0%)
		***Equus caballus* Linnaeus 1758**	44/28	Endosymbiotic ciliates (63.6%)
	Rhinocerotidae	*Ceratotherium simum* (Burchell 1817)	35/18	Endosymbiotic ciliates (51.4%)
		Rhinoceros unicornis Linnaeus 1758	32/27	Endosymbiotic ciliates (84.4%)
	Tapiridae	*Tapirus indicus* (Desmarest 1819)	31/1	*Chilomastix* spp. (3.2%)
		Tapirus terrestris (Linnaeus 1758)	12/2	*Balantioides coli* (8.3%), unidentified cestode eggs (8.3%)
Pilosa	Myrmecophagidae	*Myrmecophaga tridactyla* Linnaeus 1758	47/26	*Entamoeba* spp. (4-nucleated) (2.1%), *Tetratrichomonas* spp. Parisi 1910 (25.5%), *Capillaria*-like eggs (36.2%)
Primates	Cebidae	***Sapajus apella* (Linnaeus 1758)**	26/0	
	Cercopithecidae	*Colobus guereza* Rüppell 1835	45/40	*Entamoeba coli*-like (24.4%), *Entamoeba polecki*-like (22.2%), *Balantioides coli*-like (2.2%), *Trichuris* spp. (84.4%)
		Macaca spp. Lacepede 1799	1/0	
		Mandrillus sphinx (Linnaeus 1758)	47/47	*Entamoeba polecki*-like (76.6%), *Entamoeba coli*-like (10.6%), *Chilomastix* spp. (10.6%), *Balantioides coli*-like (66.0%), *Trichuris* spp. (2.1%), unidentified cestode eggs (2.1%)
		Papio spp. Erxleben 1777	17/17	*Entamoeba coli*-like (94.1%), *Endolimax* spp. (5.9%), *Trichuris* spp. (82.4%)
	Hominidae	*Pongo pygmaeus* (Linnaeus 1760)	103/79	*Balantioides coli*-like (76.7%)
		Gorilla gorilla (Savage 1847)	29/17	*Entamoeba coli*-like (3.5%), *Balantioides coli*-like (51.7%), *Troglodytella abrassarti* (Brumpt and Joyeux 1912) (10.3%)
		Pan troglodytes (Blumenbach 1775)	28/21	*Entamoeba coli*-like (39.3%), *Entamoeba polecki*-like (14.3%), *Endolimax* spp. (3.6%), *Balantioides coli*-like (39.3%)
	Hylobatidae	*Hylobates lar* (Linnaeus 1771)	25/9	*Entamoeba coli*-like (4.0%), *Entamoeba polecki*-like (4.0%), *Balantioides coli*-like (28.0%)
		Hylobates muelleri (Martin 1841)	28/12	*Entamoeba coli*-like (28.6%), *Balantioides coli*-like (14.3%), *Trichuris* spp. (3.6%)

Table 3. *Cont.*

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
	Lemuridae	*Eulemur fulvus* (Geoffroy 1796)	14/2	*Giardia* spp. (7.1%), unidentified cestode eggs (7.1%)
		Lemur catta Linnaeus 1758	16/2	*Entamoeba polecki*-like (6.3%), *Giardia* spp. (6.3%), *Trichuris* spp. (6.3%)
		***Varecia variegata* (Kerr 1792)**	40/0	
Proboscidea	Elephantidae	*Elephas maximus* Linnaeus 1758	55/30	*Chilomastix* spp. (1.8%), endosymbiotic ciliates (54.5%)
Rodentia	Caviidae	***Cavia porcellus*** (Linnaeus 1758)	17/0	
		***Dolichotis patagonum* (Zimmermann 1780)**	27/8	*Entamoeba muris*-like (3.7%), *Giardia* spp. (14.8%), *Chilomastix* spp. (7.4%), *Trichuris* spp. (3.7%)
		***Hydrochoerus hydrochaeris* (Linnaeus 1766)**	15/2	*Chilomastix* spp. (6.7%), *Balantioides coli*-like (6.7%)
	Chinchillidae	*Chinchilla* spp. Bennett 1829	1/0	

Table 4. List of parasites found in the avian hosts at ZooAquarium. Species in bold are also housed at Faunia Park.

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
Accipitriformes	Accipitridae	***Aegypius monachus* (Linnaeus 1766)**	7/0	
		Aquila adalberti Brehm 1861	13/0	
		Aquila verreauxii Lesson 1831	3/0	
		Buteo buteo (Linnaeus 1758)	13/0	
		***Geranoaetus melanoleucus* (Vieillot 1819)**	3/0	
		Gypohierax angolensis (Gmelin 1788)	2/0	
		***Gyps fulvus* (Hablizl 1783)**	23/0	
		Haliaeetus albicilla (Linnaeus 1758)	3/0	
		***Haliaeetus leucocephalus* (Linnaeus 1766)**	3/0	
		***Haliaeetus pelagicus* (Pallas 1811)**	10/3	Capillariids (34.3%)
		Ichtyophaga vocifer (Daudin 1800)	3/0	
		Milvus migrans (Boddaert 1783)	36/21	Capillariids (38.9%), *Porrocaecum* spp. (33.3%)
		Neophron percnopterus (Linneaus 1758)	3/0	
		***Parabuteo unicinctus* (Temminck 1824)**	28/1	*Trichuris* spp. (3.6%)
Anseriformes	Anatidae	*Alopochen aegyptiaca* (Linnaeus 1766)	5/0	
		Aythya nyroca (Güldenstädt 1770)	1/0	
		Cairina moschata (Linnaeus 1758)	8/0	
		***Cygnus atratus* (Latham 1790)**	1/0	

Table 4. *Cont.*

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
Bucerotiformes	Bucerotidae	*Tadorna ferruginea* (Pallas 1764)	4/0	
		Tadorna tadorna (Linnaeus 1758)	3/0	
		Bycanistes brevis Friedmann 1929	5/0	
		Bycanistes bucinator (Temminck 1824)	8/0	
	Bucorvidae	*Bucorvus leadbeateri* (Vigors 1825)	5/0	
Cathartiformes	Cathartidae	*Sarcoramphus papa* (Linnaeus 1758)	10/0	
		Vultur gryphus Linnaeus 1758	5/0	
Ciconiiformes	Ciconiidae	*Ciconia ciconia* (Linnaeus 1758)	10/0	
		Leptoptilos crumenifer (Lesson 1831)	3/0	
Columbiformes	Columbidae	*Columba livia* Gmelin 1789	11/0	
Coraciiformes	Alcedinidae	*Dacelo novaeguineae* Hermann 1783	2/0	
Falconiformes	Falconidae	*Falco naumanni* Fleischer 1818	13/0	
		Caracara plancus (Miller 1777)	1/0	
Galliformes	Numididae	***Numida meleagris* (Linnaeus 1758)**	4/0	
	Phasianidae	*Gallus gallus* (Linnaeus 1758)	39/1	*Entamoeba gallinarum* Tyzzer 1920 (2.6%)
Gruiformes	Gruidae	*Balearica regulorum* (Bennett 1834)	4/0	
Musophagiformes	Musophagidae	*Tauraco erythrolophus* (Vieillot 1819)	8/0	
Passeriformes	Corvidae	***Menelikornis leucotis* (Rüppell 1835)**	5/0	
		Corvus corax Linnaeus 1758	3/0	
Pelecaniformes	Pelecanidae	***Pelecanus rufescens*** Gmelin 1789	8/0	
	Threskiornithidae	*Eudocimus ruber* (Linnaeus 1758)	11/0	
		Threskiornis aethiopicus (Latham 1790)	23/0	
Phoenicopteriformes	Phoenicopteridae	***Phoenicopterus ruber* Linnaeus 1758**	7/0	
Piciformes	Ramphastidae	***Ramphastos toco* Müller 1776**	5/0	
Psittaciformes	Cacatuidae	*Cacatua alba* (Müller 1776)	6/0	
	Psittacidae	*Cacatua galerita* (Latham 1790)	11/0	
		Cacatua goffiniana Roselaar and Michels 2004	5/0	
		Cacatua pastinator (Gould 1841)	8/0	
		Cacatua sulphurea (Gmelin 1788)	5/0	
		***Amazona aestiva* (Linnaeus 1758)**	8/0	
		***Ara ararauna* (Linnaeus 1758)**	22/0	

Table 4. *Cont.*

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
		***Ara chloropterus* Gray 1859**	8/0	
		***Ara rubrogenys* Lafresnaye 1847**	1/0	
		***Aratinga solstitialis* (Linnaeus 1758)**	15/0	
		Myiopsitta monachus Boddaert 1783	1/0	
		Psittacus erithacus Linnaeus 1758	7/0	
		Trichoglossus haematodus (Linnaeus 1771)	4/0	
	Psittaculidae	***Eclectus roratus* (Müller 1776)**	2/0	
Strigiformes	Strigidae	***Bubo bubo hispanus* Rothschild and Hartert 1910**	15/1	Capillariids (6.7%)
		***Bubo bubo sibiricus* Gloger 1833**	5/0	
		Bubo scandiacus (Linnaeus 1758)	2/0	
		Strix nebulosa Forster 1772	1/0	
Casuariformes	Casuariidae	*Casuarius casuarius* (Linnaeus 1758)	12/0	
Struthioniformes	Dromaiidae	***Dromaius novaehollandiae* (Latham 1790)**	20/0	
Rheiformes	Rheidae	***Rhea americana* (Linnaeus 1758)**	7/2	*Entamoeba* spp. (4-nucleated) (28.6%)
Struthioniformes	Struthionidae	*Struthio camelus* Linnaeus 1758	12/5	*Entamoeba polecki*-like (33.3%), *Balantioides coli* (8.3%)

Table 5. List of parasites found in the mammal hosts at Faunia Park. Species in bold are also housed at ZooAquarium.

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
Afrosoricida	Tenrecidae	*Echinops telfairi* Martin 1838	2/0	
Artiodactyla	Bovidae	***Capra hircus***	40/22	*Entamoeba bovis*-like (55.0%), *Eimeria* spp. (2.5%)
		Madoqua kirkii (Günther 1880)	43/15	*Entamoeba bovis*-like (11.6%), *Entamoeba* spp. (8-nucleated) (27.9%), *Trichuris* spp. (2.3%)
		Ovis aries	26/10	*Entamoeba bovis*-like (34.6%), *Eimeria* spp. (3.9%)
	Cervidae	*Subulo gouazoubira* (Fischer 1814)	12/11	*Entamoeba bovis*-like (91.7%), *Trichuris* spp. (8.3%)
		Muntiacus muntjack Zimmermann 1780	47/18	*Entamoeba bovis*-like (36.2%), *Entamoeba* spp. (8-nucleated) (2.1%), *Giardia* spp. (2.1%), *Trichuris* spp. (4.3%)
	Suidae	***Sus scrofa***	39/22	*Entamoeba polecki*-like (46.2%), *Chilomastix* spp. (7.7%), *Balantioides coli* (28.2%)
	Tayassuidae	***Dicotyles tajacu***	16/1	*Balantioides coli* (6.3%)

Table 5. *Cont.*

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
Carnivora	Ailuridae	***Ailurus fulgens***	36/0	
	Canidae	*Vulpes zerda* (Zimmermann 1780)	52/2	*Trichuris* spp. (1.9%), unidentified ascarid (1.9%)
	Felidae	*Leopardus pardalis* (Linnaeus 1758)	48/0	
	Herpestidae	*Helogale parvula* (Sundevall 1847)	32/0	
		Suricata suricatta	15/0	
	Mephitidae	*Mephitis mephitis* (Schreber 1776)	50/3	*Baylisascaris* spp. (6.0%)
	Mustelidae	***Mustela lutreola***	19/0	
		Mustela putorius furo Linnaeus 1758	5/0	
	Procyonidae	***Nasua nasua***	45/0	
		Potos flavus (Schreber 1774)	49/0	
		Procyon lotor	30/0	
	Viverridae	***Arctictis binturong***	25/0	
		Genetta genetta (Linnaeus 1758)	45/0	
Chiroptera	Phyllostomidae	*Carollina perspicillata* (Linnaeus 1758)	13/0	
	Pteropodidae	*Rousettus aegyptiacus* (Saint-Hilaire 1810)	26/0	
Cingulata	Chlamyphoridae	*Euphractus sexcinctus* (Linnaeus 1758)	16/0	
		Chaetophractus villosus (Desmarest 1804)	16/0	
		Tolypeutes tricinctus (Linnaeus 1758)	2/0	
Dasyurimorpha	Dasyuridae	*Dasyurus viverrinus* (Shaw 1800)	24/0	
Diprotodontia	Macropodidae	***Notamacropus rufogriseus***	46/4	*Entamoeba* spp. (one nucleated) (6.5%), *Entamoeba* spp. (8-nucleated) (2.2%)
Eulipotyphla	Erinaceidae	*Osphranter rufus* (Desmarest 1822)	42/0	
		Atelerix albiventris (Wagner 1841)	35/0	
Lagomorpha	Leporidae	***Oryctolagus cuniculus***	1/0	
Perissodactyla	Equidae	*Equus africanus* (Heuglin and Fitzinger 1866)	27/9	Endosymbiotic ciliates (33.3%)
		Equus caballus	35/8	Endosymbiotic ciliates (22.9%)
Pilosa	Choloepodidae	*Choloepus didactilus* (Linnaeus 1758)	46/13	*Entamoeba* spp. (8-nucleated) (28.3%)
	Myrmecophagidae	*Tamandua tetradactyla* (Linnaeus 1758)	36/0	
Primates	Aotidae	*Aotus nancymaae* Hershkovitz 1983	7/1	*Entamoeba coli*-like (14.3%)
		Aotus trivirgatus (Humboldt 1812)	9/0	
	Callitrichidae	*Callimico goeldii* (Thomas 1904)	47/0	

Table 5. *Cont.*

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
		Callithrix jacchus (Linnaeus 1758)	38/0	
		Cebuella pygmaea (Spix 1823)	11/0	
		Leontopithecus rosalia (Linnaeus 1766)	31/0	
		Saguinus geoffroyi (Pucheran 1845)	30/0	
		Saguinus imperator (Goeldi 1907)	34/0	
		Saguinus oedipus (Linnaeus 1758)	26/0	
	Cebidae	***Sapajus apella***	30/0	
		Saimiri sciureus (Linnaeus 1758)	53/0	
	Galagidae	*Galago moholi* Smith 1836	19/0	
	Lemuridae	*Eulemur albifrons* (Geoffroy 1796)	17/0	
		Lemur catta	26/0	
		Varecia variegata	3/0	
		Varecia rubra (Geoffroy 1812)	12/1	*Capillaria* spp. (8.3%)
	Lorisidae	*Xanthonycticebus pygmaeus* (Bonhote 1907)	5/0	
		Perodicticus potto (Müller 1766)	26/0	
	Pitheciidae	*Pithecia pithecia* (Linnaeus 1766)	33/2	*Entamoeba coli*-like (6.1%)
Rodentia	Heterocephalidae	*Heterocephalus glaber* Rüppell 1842	29/0	
	Caviidae	***Cavia porcellus***	54/2	*Balantioides coli* (3.7%)
		Dolichotis patagonum	24/7	*Giardia* spp. (8.3%), *Trichuris* spp. (20.8%)
		Hydrochoerus hydrochaeris	1/0	
	Dasyproctidae	*Dasyprocta azarae* Lichtenstein 1823	1/0	
		Dasyprocta fuliginosa Wagler 1832	23/3	*Trichuris* spp. (13.0%)
		Dasyprocta punctata Gray 1842	8/0	
	Dipodidae	*Jaculus orientalis* Erxleben 1777	12/3	*Entamoeba muris* (25.0%), *Chilomastix* spp. (16.7%)
	Echimyidae	*Capromys pilorides* (Say 1822)	94/15	*Trichuris* spp. (16.0%)
	Erethizontidae	*Coendou prehensilis* (Linnaeus 1758)	43/5	*Chilomastix* spp. (9.3%), *Trichuris* spp. (2.3%)
	Hystricidae	*Hystrix cristata* Linnaeus 1758	47/5	*Entamoeba* spp. (8-nucleated) (2.1%), *Giardia* spp. (8.5%)
	Pedetidae	*Pedetes capensis* (Forster 1778)	31/0	
	Sciuridae	*Cynomys ludovicianus* (Ord 1815)	4/1	*Chilomastix* spp. (25.0%)
Tubulidentata	Orycteropodidae	*Orycteropus afer* (Pallas 1766)	7/1	*Giardia* spp. (14.3%)

Table 6. List of parasites found in the avian hosts at Faunia Park. Species in bold are also housed at ZooAquarium.

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
Accipitriformes	Accipitridae	***Necrosyrtes monachus***	8/0	
		Aquila nipalensis Hodgson 1833	5/0	
		Buteo jamaicensis (Gmelin 1788)	6/0	
		Buteo regalis (Gray 1844)	5/0	
		Geranoaetus melanoleucus	13/0	
		Gyps fulvus	7/0	
		Parabuteo unicinctus	14/0	
Anseriformes	Anatidae	***Cygnus atratus***	5/0	
	Anhimidae	*Chauna torquata* (Oken 1816)	11/0	
Charadriiformes	Recurvirostridae	*Recurvirostra avosetta* Linnaeus 1758	1/0	
Falconiformes	Falconidae	*Phalcoboenus australis* (Gmelin 1788)	2/0	
Galliformes	Numididae	***Numida meleagris***	7/3	*Entamoeba gallinarum* (14.3%), capillariids (14.3%), *Ascaridia* spp./*Heterakis* spp. (28.6%), *Raillietina*-like eggs (14.3%)
	Phasianidae	***Gallus gallus***	14/1	*Ascaridia* spp./*Heterakis* spp. (7.1%)
		Meleagris gallopavo Linnaeus 1758	39/0	
Gruiformes	Gruidae	*Grus grus* (Linnaeus 1758)	6/1	Capillariids (16.7%)
		Grus virgo (Linnaeus 1758)	8/0	
Musophagiformes	Musophagidae	***Menelikornis leucotis***	11/0	
Passeriformes	Corvidae	*Calocitta formosa* (Swainson 1827)	3/0	
	Cotingidae	*Rupicola peruvianus* (Latham 1790)	22/0	
	Sturnidae	*Lamprotornis purpureus* (Müller 1776)	2/0	
Pelecaniformes	Ardeidae	*Bubulcus ibis* (Linnaeus 1758)	1/0	
	Pelecanidae	*Pelecanus onocrotalus* Linnaeus 1758	2/0	
Phoenicopteriformes	Phoenicopteridae	***Phoenicopterus ruber***	5/0	
Piciformes	Ramphastidae	*Ramphastos swainsonii* Gould, 1833	21/17	*Capillaria* spp. (81.0%)
		Ramphastos toco	16/7	*Capillaria* spp. (43.8%)
Psittaciformes	Cacatuidae	*Eolophus roseicapilla* (Vieillot 1817)	5/0	
	Psittacidae	***Amazona aestiva***	27/0	
		Ara ararauna	17/0	
		Ara chloropterus	6/0	
		Ara macao (Linnaeus 1758)	3/0	
		Ara militaris (Linnaeus 1766)	1/0	
		Ara rubrogenys	2/0	

Table 6. *Cont.*

Order	Family	Species	Samples (Total/Positives)	Parasites Found (% of Total Samples)
Strigiformes	Psittaculidae	***Aratinga solstitialis***	9/0	
		Eclectus rotarus	8/0	
		Trichoglossus haematodus (Linnaeus 1771)	3/0	
	Strigidae	***Bubo bubo hispanus***	9/0	
		Bubo bubo sibiricus	6/0	
	Tytonidae	*Tyto alba* Scopoli 1769	11/0	
Casuariiformes	Casuariidae	***Dromaius novaehollandiae***	48/0	
Rheiformes	Rheidae	***Rhea americana***	23/0	

The protists were the most frequently identified parasite group and the only one found in 34 mammal and 3 avian species in ZooAquarium and in 15 mammal species in Faunia (Tables 2–6). Helminth-only infections were found in six mammal and four avian species in ZooAquarium and in five mammal and four avian species in Faunia. Finally, both protists and helminths were recorded in 20 mammal species in ZooAquarium and 5 mammal and 1 avian species in Faunia. The host species with the higher parasite biodiversity were the dama gazelle (*Nanger dama*) in mammals and the helmeted guineafowl (*Numida meleagris*) in birds, which were infected (not simultaneously) by up to 8 and 4 different parasite species, respectively. Single parasitisms were found in most positive samples; in polyparasitisms, the maximum number of parasite species causing a simultaneous infection was 4 (in the dama gazelle). The most common parasitic genera found were *Entamoeba* (in 44 host species in ZooAquarium and 13 in Faunia), *Balantioides* (in 14 host species in ZooAquarium and 3 species in Faunia), and *Trichuris* (in 14 and 9 host species in ZooAquarium and Faunia, respectively).

3.1.1. Avian Hosts

The differences in the number of species analysed correspond to the collection design by the management of the zoological institutions. By feeding type, the number of samples analysed at ZooAquarium is proportional to the number of bird species; in the case of Faunia, the number of samples from carnivorous species is proportionally much lower (Table 1) because omnivorous and herbivorous species are kept in groups, making it easier to find valid samples for analysis than in the case of carnivorous species, which must be housed individually in most cases.

Only nematodes, including capillariid and ascarid eggs, were identified in carnivorous species (in birds of prey at ZooAquarium and Faunia, as well as in gruiformes specifically, the common crane, *Grus grus*, at Faunia) (Tables 4 and 6). In omnivorous species, only protists (*Entamoeba* spp. and *B. coli*) were detected at ZooAquarium (Table 4), while nematodes (capillariids and ascarids) and cestodes (with one observation of *Raillietina*-like eggs in the helmeted guineafowl) were exclusively found at Faunia; additionally, *E. gallinarum* was detected in the helmeted guineafowl at Faunia (Table 6).

3.1.2. Mammalian Hosts

Almost all herbivorous species were infected but generally exhibited low parasite biodiversity. The most prevalent parasites in herbivorous mammals were amoebae (*Entamoeba*) (Table 2), which were found in nearly all hoofed animals (except equids), suids, and macropodids (the yellow-footed rock-wallaby, *Petrogale xanthopus*, and Bennett's wallaby, *Notamacropus rufogriseus*) (Tables 3 and 5). The *Entamoeba* cysts found in these hosts were uninucleated in all cases, except in two samples from the dama Gazelle and one from Bennett's wallaby, where eight unidentified cysts were present. The one-nucleated *Entamoeba*

cysts were of two types; those from hoofed animals were small (4–10 μm in diameter) and were identified as *Entamoeba bovis*-like, while those from suids and the tapir were larger (15–20 μm in diameter) and were identified as *Entamoeba polecki*-like. The species from macropodids were not identified.

Giardia infection in herbivores was rare; cysts were detected on a few occasions in the dama gazelle, the red river hog (*Potamochoerus porcus*), and the Patagonian mara (*Dolichotis patagonum*) in ZooAquarium (Table 3), and in the Southern red muntjac (*Muntiacus muntjak*), the Patagonian mara, the Brazilian porcupine (*Coendou prehensilis*), and the aardvarks (*Orycteropus afer*) in Faunia (Table 5). Among the ciliates from herbivorous hosts, *B. coli*-like cysts were found in suids, the sitatunga (*Tragelaphus spekii*) and the South American tapir (*Tapirus terrestris*); the identifications were based on the cyst size (about 40 μm in diameter). In camels (*Camelus bactrianus* and *Camelus dromedarius*), the cysts were of greater diameter (around 80 μm) and were identified as belonging to *Buxtonella cameli*. Entodiniomorphid ciliates were frequently found in equids, elephants, and rhinoceronts.

Helminth infections in herbivorous species were mainly caused by trichostrongylids and trichurids in the dama gazelle and in the dorcas gazelle (*Gazella dorcas*), and by trichurids/capillariids in some hoofed animals and in rodents (the Patagonian mara). The identification of eggs belonging to genera *Trichuris* or *Capillaria* was based on the appearance of the eggshell (thick and smooth in *Trichuris*, striated in *Capillaria*). Cestode eggs (Figure 1) were found only on isolated occasions in the reed deer (*Cervus elaphus*), the hippoptamous (*Hippopotamus amphibious*), the South American tapir, and the Patagonian mara, all of them in ZooAquarium.

Among the omnivorous species, primates were the group with the greatest number of species infected, mostly by protists (Table 2); the mandrill was the species harbouring the widest range of parasites (*Entamoeba* spp., *Chilomastix* spp., *B. coli*, *Trichuris* spp., and an unidentified cestode), and *Giardia* cysts were found only in lemurs (ring-tailed lemur, *Lemur catta*, and Mayotte lemur, *Eulemur fulvus*) (Tables 3 and 5). Two different types of *Entamoeba* cysts were found in primates: one nucleated cyst identified as *E. polecki*-like and eight nucleated cysts identified as *E. coli*-like. The *E. polecki*-like cysts found in this study were clearly larger (10–16 μm in diameter) compared to those found in hoofed animals and similar to those identified as *E. polecki*-like in suids and tapirs.

In Ursidae, nematodes (*Baylisascaris*) were detected in the brown bear (*Ursus arctos*); trematode eggs (Figure 1) were found in one sample from the sun bear (*Helarctos malayanus*).

Very few carnivore species were found infected, typically by nematodes (Table 2); only the giant anteater (*Myrmecophaga trydactila*) was found infected by nematodes and protozoa (capillariid eggs, four-nucleated *Entamoeba* cysts, *B. coli*-like cysts, and trichomonad flagellates), while the bushdog (*Spheotos venaticus*) only by coccidia. Cestode and trematode eggs were found once in several species at ZooAquarium (Table 3).

3.2. Biodiversity and Prevalence in Relation to Feeding Habits and Housing Conditions

Before examining the obtained results, it is necessary to consider that the unequal number of samples analysed within some of the considered categories (Table 7) can introduce biases in the estimation of regression coefficients, wider confidence intervals, and the statistical significance of the coefficients. In the latter case, the significance would probably not be affected when "clear" significant or non-significant statistical values were obtained (i.e., $p > 0.100$ or $p < 0.001$), but in those cases where we have found p-values in the range 0.010–0.050, the interpretation of the associations should be taken with care and generalising results to broader populations of zoo animals may be challenging.

The distribution of host species according to the type of zoological institution, vertebrate class, housing conditions (isolation level and soil type), and feeding habits is shown in Table 7. None of the avian species analysed were housed in isolated spaces in either of the zoos.

Table 7. Number of positive and total (in parenthesis) mammalian and avian species analysed according to their housing conditions and feeding habits.

			Zoological Park											
			ZooAquarium Feeding Habits						Faunia Feeding Habits					
Host Class	**Isolation**	**Soil**	**Carnivorous**		**Omnivorous**		**Herbivorous**		**Carnivorous**		**Omnivorous**		**Herbivorous**	
Mammal	accessible	Natural	2	(10)	8	(13)	48	(53)	0	(1)	2	(3)	12	(19)
		Artificial	0	(0)	0	(0)	1	(1)	0	(0)	0	(0)	0	(0)
		Mixed	1	(1)	1	(2)	0	(1)	0	(0)	0	(0)	0	(0)
	isolated	Natural	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
		Artificial	0	(0)	1	(2)	0	(0)	1	(14)	5	(20)	5	(10)
		Mixed	0	(0)	0	(0)	0	(0)	0	(1)	0	(0)	0	(0)
Bird	accesible	Natural	0	(8)	3	(12)	0	(2)	0	(3)	3	(10)	0	(3)
		Artificial	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(1)
		Mixed	4	(24)	0	(3)	0	(15)	0	(11)	2	(2)	0	(10)
	isolated	Natural	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
		Artificial	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
		Mixed	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)

The initial analysis involved five independent variables (zoological institution, host class, soil, isolation, and feeding habits) (Table 8). The Hosmer and Lemeshow X^2 test (HLt; $p = 0.003$) was significant, indicating that the regression model did not fit the observed data well. The model explained 47.4% of the variation (Nagelkerke $R^2 = 0.474$) and 82.0% of the samples would be correctly classified. When soil type and host class (which were highly and statistically correlated with the other variables) were removed from the analysis, the HLt was non significant ($p = 0.807$). However, both the percentage of data variation explained by the model and the percentage of samples correctly classified decreased ($R^2 = 0.275$; 71.8% correct sample classification). Under these circumstances, we chose to use the model with all the independent variables to analyse the importance and influence of each one.

Table 8. Values and statistical significance of the regression coefficients obtained after including 5 independent variables in the binary logistic regression conducted with the results of the parasitological survey of the mammals and birds at two zoological institutions (ZooAquarium and Faunia) in Madrid, Spain. The dependent variable is "at least once infected"/"never infected".

		Function Coefficients		Wald's X^2 Test		
Parameter		**B**	**Standard Error**	**Score**	**Degrees of Freedom**	**Significance**
Feeding type				14.733	2	<0.001
	Omnivorous vs. carnivorous	1.581	0.532	8.824	1	0.003
	Herbivorous vs. carnivorous	1.911	0.501	14.555	1	<0.001
Soil type				2.401	2	0.301
	artificial vs. natural	0.063	1.710	0.001	1	0.971
	mixed vs. natural	−0.878	0.572	2.352	1	0.125
Host Class (bird vs. mammal)		−2.103	0.480	19.150	1	<0.001
Zoological institution (Faunia vs. ZooAquarium)		−0.649	0.405	2.567	1	0.109
Isolation type (isolated vs. accessible)		−1.326	1.777	0.577	1	0.456
Constant		−1.639	0.389	17.703	1	<0.001

The variables that exhibited higher importance for interpreting the data were host class (Wald test, $p < 0.001$) and feeding type ($p < 0.001$) (Table 8). There was no statistically significant increase ($p = 0.109$) in the probability of finding infected hosts in either zoological institution, although this probability was slightly lower in Faunia than in ZooAquarium (B coefficient = −0.649). The number of host species found infected was nearly identical when

the animals were kept in "natural" and "artificial" types of soil and lower in mixed soil; however, these differences were not statistically supported ($p = 0.301$). The probability of animals in enclosed spaces being infected was 1.326 times lower than those kept in open areas, although these differences were not statistically supported ($p = 0.456$). Regarding feeding type, the probability of positive samples in omnivorous and herbivorous species was similar between them and markedly higher (1.4581 and 1.911 times, respectively) and statistically significant ($p = 0.003$ and $p < 0.001$, respectively) than in carnivorous species.

In relation to host class, the probability of positive samples was 2.1 times lower in avian species than in mammalian ones. As the conditions in which mammals and birds are housed and fed differ, we conducted separate analyses for each group (Tables 9 and 10). In both cases, the regression models fit the observed data well (HLt = 0.944 and 0.860 for the mammalian and avian data, respectively); the important increase in the standard error of the constant in the equation is a consequence of the small number of data available in some categories (Table 7). The percentages of data variation explained by the models were similar (46.1% for mammalian data) or lower (27.8% for avian data) compared to the combined analysis, and the percentages of samples correctly classified (77.5% in mammals, 90.4% in birds) were similar. In the analysis of the avian samples, none of the variables had a statistically significant effect at the $p < 0.05$ level. However, feeding habits approached this limit ($p = 0.060$) due to the greater number of omnivorous species found infected compared to carnivorous ones, though not at the $p < 0.01$ level ($p = 0.018$). In mammals, a similar situation was observed regarding the number of positive species in each zoological garden (1.245 lower in Faunia than in ZooAquarium; $p = 0.019$); the only variable that was clearly significant was feeding habits, with carnivorous species being the less infected group.

Table 9. Values and statistical significance of the regression coefficients obtained after including 4 independent variables in the binary logistic regression conducted with the results of the parasitological survey in mammals at two zoological institutions (ZooAquarium and Faunia) in Madrid, Spain. The dependent variable is "at least once infected"/"never infected".

Parameter		Function Coefficients		Wald's X^2 Test		
		B	Standard Error	Score	Degrees of Freedom	Significance
Feeding type				21.061	2	<0.001
	Omnivorous vs. carnivorous	2.057	0.780	7.571	1	0.006
	Herbivorous vs. carnivorous	3.187	0.716	19.799	1	<0.001
Soil type				2.453	2	0.293
	artificial vs. natural	18.336	25,170.708	0.000	1	0.999
	mixed vs. natural	−2.016	1.287	2.453	1	0.117
Zoological institution (Faunia vs. ZooAquarium)		−1.245	0.531	5.496	1	0.019
Isolation type (isolated vs. accessible)		−18.995	25,170.708	0.000	1	0.999
Constant		−4.146	4195.118	0.000	1	0.999

Table 10. Values and statistical significance of the regression coefficients obtained after including 3 independent variables in the binary logistic regression conducted with the results of the parasitological survey of birds at two zoological institutions (ZooAquarium and Faunia) in Madrid, Spain. The dependent variable is "at least once infected"/"never infected".

Parameter		Function Coefficients		Wald's X^2 Test		
		B	Standard Error	Score	Degrees of Freedom	Significance
Feeding type				5.613	2	0.060
	Omnivorous vs. carnivorous	2.107	0.889	5.613	1	0.018
	Herbivorous vs. carnivorous	−18.913	7283.654	0.000	1	0.998

Table 10. *Cont.*

Parameter	Function Coefficients		Wald's X^2 Test		
	B	Standard Error	Score	Degrees of Freedom	Significance
Soil type			1.403	2	0.496
artificial vs. natural	0.833	40,847.685	0.000	1	1.000
mixed vs. natural	1.030	0.870	1.403	1	0.236
Zoological institution (Faunia vs. ZooAquarium)	0.121	0.684	0.031	1	0.860
Constant	−8.164	13,397.685	0.000	1	1.000

4. Discussion

In this study, we present the results obtained from the parasitological analysis of birds and mammals from two zoological facilities with different topologies. While other published studies on zoo animal parasites focused on hosts belonging to a small group of taxonomically related species [22–24] or based on their feeding habits [25–27], the present work stands out for the number and zoological diversity of the host species analyzed, including 15 orders of mammals and 23 of birds. The objective of this study is to assess the importance of various factors in the transmission and dissemination of parasites among zoo animals, as well as in relation to humans (zoo personnel and visitors).

With a few exceptions noted below, all the intestinal parasites identified in this study have been previously described in captive animals housed in zoological gardens [9,26,28–32]. We should note that this study had some limitations: no specific stainings were performed to detect the presence of *Cryptosporidium* oocysts or microsporidia spores, and the presence of *Blastocystis* was not routinely investigated. These parasites are mostly considered in specific studies, but only *Cryptosporidium* is sometimes investigated in broad host-range studies in zoos [6,7,32–34]. Another limitation is that no genetic studies have been conducted except in specific cases, thus preventing the precise identification of the species found.

4.1. On the Parasite Epidemiology, Biodiversity, and Species Identification

4.1.1. Avian Hosts

In our study, the number of infected host species was low and similar in both zoos (10.9–12.5%), although the number of positive samples was clearly higher in ZooAquarium (53.1%) than in Faunia (7.0%). In a general comparison, these values are within the range of prevalences reported in other studies, with nematodes being the most frequently mentioned group [35].

The hosts we found parasitised are mainly Accipitriformes and Galliformes; also, Gruiformes, Picirformes, Strigiformes, and ratites (Struthioniformes and Rheiformes). Galliformes are the group of zoo birds most commonly reported to be parasitized [9,29,31,36–39]. There are few studies on birds of prey (Accipitriformes, Falconiformes, and Strigiformes) in which capillariids and coccidia are the parasites most frequently found [39,40]. In our study, we did not find parasites in Psittaciformes or Passeriformes, groups that other authors found nematodes (mainly ascarids and trichurids) and coccidia with prevalences of up to 40% in some cases [9,28–30,32,36,39,41,42].

In Struthioniformes and Rheiformes, although parasitic biodiversity can be high in captive birds, especially the ostrich (*Struthio camelus*) [43], our findings were scarce and limited to protists in the ostrich and the rhea (*Rhea Americana*), similar to other results in Spain [37] and Brazil [7]; however, also in Brazil, nematodes (ascarids) were reported in the rhea and the cassowary (*Casuariius casuarius*) [18]. Coccidia and *Capillaria* were reported in the cassowary [39]. In Serbia, a low number of positive samples were reported in the emu (*Dromaius novaehollandiae*), the ostrich, and the rhea (29% in total), although the parasitic biodiversity was much higher, including unidentified ciliates (probably *B. coli*), unidentified ascarids, and strongyles (probably *Lybiostrongylus* Lane 1923, misidentified as *Strongyloides*

Grassi 1879) in the ostrich, and *Capillaria* in the rhea [44]; similar results were obtained in the ostriches in zoos in Nigeria [38].

The vast majority of parasites reported in previous studies are species with a direct life cycle; however, unidentified trematodes were also occasionally reported [9,36,39]. The cestode eggs found in our study in the helmeted guineafowl may correspond to *Raillietina*, although since the morphology of the eggs is similar to that of *Hymenolepis* Weinland 1858 eggs, the identification is tentative. Overall, depending on the cestode species and the intensity of the infection, this can be asymptomatic or lead to diarrhoea and intestinal lesions [45,46].

In our study, the most common group of nematodes was the capillariids, found in 7 species of birds (Tables 4 and 6). This generic term includes the genera *Baruscapillaria* Moravec 1982, *Capillaria*, *Echinocoleus* López-Neyra 1947, *Eucoleus* Dujardin 1845, *Ornithocapillaria* Barus and Sergeeva 1990, *Pterothominx* Freitas 1959, and *Tridentocapillaria* Barus and Sergeeva 1990 [47]. In cases where species from more than one genus were described in the corresponding host, we used the term "capillariid" (e.g., in the Steller's sea eagle, *Haliaeetus pelagicus*, where *Eucoleus dispar* Dujardin 1845 and *Capillaria tenuissima* (Rudolphi 1809) were described). We identified the eggs found in the Harris's hawk (*Parabuteo unicinctus*) as *Trichuris* based on their morphology; however, we have not found any species of *Trichuris* or *Capillaria* described in this bird, so the possibility of spurious parasitism cannot be ruled out. Pérez-Cordón et al. [33] identified *Trichuris* in some of their samples but without specifying the host species. The clinical significance of these parasites depends on their location in the host and the intensity of infection; in fatal cases, there may be no clinical signs, or they may be nonspecific (e.g., diarrhoea, anorexia, weakness) [47].

The other group of nematodes found in birds includes the ascarids, found in Galliformes (*Ascaridia*/*Heterakis*) and Accipitriformes (*Porrocaecum*). The genus *Porrocaecum* includes two cosmopolitan species that affect birds of prey, *Porrocaecum angusticolle* (Molin 1860) and *Porrocaecum depressum* (Frölich 1802); in both cases, infections typically do not produce clinical signs or severe disease in birds, although they may occasionally lead to death [48]. On the other hand, several species of *Ascaridia* and *Heterakis* have been reported in the helmeted guineafowl and the red junglefowl/chicken (*Gallus gallus*) [49], with the eggs of species from both genera being very similar; for this reason, we considered it preferable to identify them as *Ascaridia*/*Heterakis*. The symptoms produced by these nematodes are nonspecific and can result in the death of the affected animal. In wild populations, infections by these parasites can have a negative impact on development, lower host body condition, and worse rates of survival and reproduction [49].

In the present study, we did not find coccidia in the bird samples, although this is generally the most frequently encountered group of protists, with a range of 9–100% of positive samples when present [9,18,31,39]. The most common genera of coccidia in birds are *Eimeria* and *Isospora* Schneider 1881 [50,51], although *Caryospora* Leger 1904, *Tyzzeria* Allen 1936, and *Wenyonella* Hoare 1933 have also been reported in birds [52]. In general, the symptoms caused consist of diarrhoea, enlarged abdomen, loss of appetite and weight, and even death.

The only protists identified in birds in this study were ciliates (*B. coli*) and amoebae (*Entamoeba* spp.). *Balantioides coli* were found only in the ostriches, as reported in other studies [7,38,44]. Additionally, three species of *Entamoeba* were identified, forming uninucleated cysts (in the ostriches), tetranucleated cysts (in the rheas), and octonucleated cysts (in the chickens and the guineafowl). Uninucleated cysts are commonly found in ratites, especially the ostriches [43], where the species *Entamoeba struthionis* Ponce-Gordo et al. 2004 was described [53,54]. However, in other studies, *Entamoeba* was not reported, but other protists (ciliates) were recorded [38,44]. In the rhea, *E. polecki* and *Entamoeba suis* Hartmann 1913 were identified through genetic analysis [55,56], as well as an unidentified species forming octonucleated cysts [43]. The tetranucleated cysts found here were genetically analysed and correspond to *Entamoeba dispar* Brumpt 1925 [57]. In captive emus in Brazil, uninucleated cysts identified as *Entamoeba* spp. were also found [58]. Regarding *Entamoeba*

gallinarum Tyzzer 1920, a species forming octonucleated cysts described in galliforms, there are few studies reporting its presence, and always with a low prevalence [9,59].

4.1.2. Mammalian Hosts

The number of species found infected varied according to the zoological institution, being overall and by animal group (according to their diet) higher in ZooAquarium than in Faunia. However, the values found in ZooAquarium are within the range of results published by other authors [25,31,32,60], and compared to other European zoos, the values are also similar or even lower [26,34,44,61]. These overall data, however, require a more detailed analysis as there are significant differences depending on the mammalian group considered.

Among carnivorous animals, we only found positive samples in some species of the order Carnivora and in the giant anteater (order Pilosa) (Tables 3 and 5). In species of the order Carnivora, the vast majority of findings occurred only once or twice over the 10 years of sampling, and except for four positive samples for *Capillaria*, the parasites now found do not correspond to those generally detected in other studies, which report ascarids, whipworms, and strongyles [13,25,31,34,44,60,62].

The highest number of infected species, and positive samples, was observed in herbivores and omnivores. Almost all species of Artiodactyla tested positive for amoebas (*Entamoeba*), and those of Perissodactyla, for ciliates. In some hoofed animals, cestode eggs were found (the species could not be determined), and some species also showed persistent nematode infestation by *Trichuris*, *Capillaria*, and/or trichostrongylids; however, the overall helminth prevalence relative to protists was lower. In other studies, helminth infections in hoofed animals were predominant [6,37].

Non-human primates (NHP) are one of the mammal groups commonly studied in zoo animal research [35]. The parasites typically reported in these animals include *Entamoeba*, *Giardia*, and *Trichuris* [3,7,31]; other protists (*Giardia*, coccidians, *Cryptosporidium*, ciliates) and helminths (such as strongylates, ascarids, oxyurids, and spirurids) were also occasionally documented [7,23,30,34,44,61,63–65]. Similar to hoofed animals, helminths were typically reported in NHPs with a higher prevalence than protists [7,31,32,34]; however, in some studies, protists (mainly *Entamoeba*, *Giardia*, and ciliates) were more common [22].

By parasite group, *Entamoeba* spp. were the most frequently encountered protozoa in herbivorous and omnivorous mammals. In carnivores, *Entamoeba* cysts were found in the giant anteater, and they were genetically identified as *E. dispar* [57]. The remaining species found belong to either the *E. polecki* group (forming uninucleated cysts) or the *E. coli* group (forming octonucleated cysts). The species *Entamoeba ovis* Swellengrebel 1914 and *E. bovis* (forming uninucleated cysts) were described in various ruminant species, but due to the difficulty in morphological differentiation (the size ranges overlap), we have preferred to identify them as *E. bovis*-like [66]. The uninucleated cysts observed in suids are larger, but there are several morphologically indistinguishable species that can infect them (*E. polecki*, *E. struthionis*, and *E. suis*). In NHPs, the one-nucleated cysts are commonly identified as *Entamoeba chattoni* Swellengrebel 1914 [67–71] or as *E. polecki* [72–74], both morphologically indistinguishable. Therefore, in suids and NHPs, we identified the uninucleated cysts as *E. polecki*-like [66]. The eight-nucleated cysts found in NHPs would correspond to *Entamoeba coli*, but since it is actually considered a species complex [75], it would be best to identify the findings as *E. coli*-like. In general, the *E. bovis*-like, *E. polecki*-like, and *E. coli*-like species are considered non-pathogenic, although [76] reported a case of symptomatic infection in humans by *E. polecki*, [77] suggested an association between the presence of *E. bovis* and diarrhoea in cattle, and Coke et al. [78] reported a fatal case in which unidentified *Entamoeba* and *Acanthamoeba* Volkonsky 1931 were found in gastric ulcers in an 11-month-old female giant anteater. Except for the *E. bovis*-like species, all other *Entamoeba* spp. from zoo mammals can infect humans.

Ciliates are the second most commonly encountered group of protists in our study in terms of findings and infected hosts; however, they are usually not reported except in

specific studies. There is a great diversity of ciliates described in Artiodactyla, Perissodactyla, and Proboscidea, mostly corresponding to species of the orders Entodiniomorphida and Vestibuliferida [79–84]. Since the identification of different genera and species requires specific staining methods, and most of these species are considered commensal/endosymbionts, we made a generic identification of the findings in the hippopotamus, equids, rhinoceroses, and elephants as "endosymbiotic ciliates". From a human and animal health viewpoint, the ciliate species with greater relevance are vestibuliferid ciliates (*Balantioides* and *Buxtonella*) from some hoofed animals (camels, suids, and tapirs) and from NHPs. They can be transmitted to humans (at least *B. coli*) and have been considered by several authors as potentially pathogenic for their hosts [85–88]. Based on cyst size, our findings in some hoofed animals (the sitatunga, *Tragelaphus spekii*, the moose, *Alces alces*, the collared peccary, *Dicotyles tajacu*, the red river hog, and the pigs) and in NHPs would correspond to *B. coli*, while in large bovids (the European bison, *Bison bonasus*, the yak, *Bos grunniens*, and the African buffalo, *Syncerus caffer*), the ciliate was identified as *Buxtonella sulcata*. The species infecting camels, usually reported as *B. coli*, is *Buxtonella cameli* [88]. The identification of *B. coli* of the NHP cysts should be considered tentative, as an unnamed *Buxtonella* sp. whose cysts are similar to those of *B. coli* could also infect NHPs [87]. When these protists are reported in studies on zoo animals, their prevalence is highly variable, ranging from 10–22% in hoofed animals to 9.5–80% in NHPs (the macaques, the chimpanzees, and the orangutans) [6,7,32]. Also, in NHPs, in the present study, we found some gorilla samples positive for *Troglodytella*, a rare finding in zoo populations. Our findings occurred after a new gorilla from an England zoo was introduced to the group. While this ciliate is common in wild African great apes (and *B. coli* is rare), the different diet in captivity leads to the opposite situation in zoo animals and even to the disappearance of *Troglodytella* [89,90].

Giardia cysts were observed in several host species. *Giardia duodenalis* is considered a species complex, with its genetic variants typically regarded as assemblages [91]. A recent proposal for taxonomic revision [92] has been made to assign these assemblages to defined species. The new findings in NHPs (in the common brown lemur, *Eulemur fulvus*, and the ring-tailed lemur, *Lemur catta*) would correspond to the *G. duodenalis* Stiles 1902 assemblage B/*Giardia enterica* Grassi 1881, according to previous records [93]. Maesano et al. [61] also found *Giardia* in the ruffled lemur (*Varecia variegata*), the gorilla (*Gorilla gorilla*), and the capuchin monkey (*Sapajus apella*), although they did not specify the species. According to the recent taxonomic proposal [92], the findings we made in other mammals (hoofed animals, the crested porcupine, *Hystrix cristata*, and the red river hog) may correspond to *G. duodenalis*, *Giardia intestinalis* (Lambl 1859), or *G. enterica*; the findings in the aardvark cannot be presumptively assigned to any of the newly (re)described species.

The finding of tapeworm eggs in species housed in zoos is rarely reported [6,64]. In our study, the findings were occasional, and the morphology of the eggs did not correspond to that of the tapeworm species cited in the corresponding hosts, suggesting that they could be spurious parasitoses. The presence of eggs resembling *Hymenolepis* in Madagascar lemurs was mentioned [94], but no species were described.

In nematodes, the most frequent findings corresponded to *Trichuris* and *Capillaria* eggs. We found capillariid eggs in different anteater individuals at the ZooAquarium, but it is not possible to identify the genus because there are no previous descriptions in this host species; Diniz et al. [95] indicated that 28% of the samples they analysed were positive for *Trichuris*, although they did not provide specific details or indicate the possible species.

Several species of *Trichuris* could infect hoofed animals (*Trichuris ovis* (Abidgaard 1795), *Trichuris discolor* (von Linstow 1906), and *Trichuris skrjabini* Baskakov 1924), so it is not possible to make a specific identification with the available data. The *Capillaria* eggs in the fallow deer (*Dama dama*) could correspond to *Capillaria bovis* (Schnyder 1906) [96]. In NHPs, spurious parasitosis would explain occasional findings in the mandrill (*Mandrillus sphinx*), the Müller's gibbon (*Hylobates muelleri*), and the lemurs; however, the repeated findings in the colobus (*Colobus guereza*) and the baboons (*Papio* spp.) would indicate true infections.

The species in NHPs are typically identified as *Trichuris trichiura* (Linnaeus 1771) [97], but *Trichuris colobae* Cutillas et al. 2014 was also described in the colobus [98], *Trichuris ursinus* Callejón et al. 2017 in the baboon [99], and *Trichuris lemuris* Rudolphi 1819 in the lemurs [100]. In other studies in zoo NHPs, *Trichuris* spp. were found with prevalences between 20 and 100% [34,101]. At least *T. trichiura* can be transmitted to humans. Mild infections are usually asymptomatic, but fatal cases have been described in NHPs [102].

Regarding the trichostrongylids, the only findings occurred in hoofed animals; NHP samples were always negative. The morphological and size similarity of the eggs found in hoofed animals makes it difficult, if not impossible, to differentiate the eggs of different genera, so identifications are usually conducted generically as "strongyle type" or "trichostrongylids" [32,103–105]; if anything, *Nematodirus*, due to its size, can be identified separately [61,104]. Depending on the helminth species and the intensity of the infection, animals may be asymptomatic or suffer from gastrointestinal symptoms (especially in trichostrongylid infections); *Nematodirus* can be highly pathogenic and cause death within a few days after the onset of symptoms [106].

The ascarid eggs found in carnivores belong to *Baylisascaris*. In the Brown bear, the species could correspond to *Baylisascaris transfuga* (Rudolphi 1819), which was identified in wild host species in Europe and Asia [105,107]. In the case of the striped skunk (*Mephitis mephitis*) samples, it could correspond to *Baylisascaris columnaris* Leidy 1856, which was detected in other European zoos [108]. We did not find *Toxocara*/*Toxascaris* infections despite their prevalence potentially being high in zoo animals [109]. In equids, *Parascaris equorum* can be recorded in zoo animals with a low prevalence, usually below 15% [32,33,105,110].

4.2. Effect of Housing Conditions

Considered collectively, the results obtained in both zoological parks show parasitic prevalences (Table 1) lower than those observed in many other studies [6,24,33,37,44,111,112]. The differences between the results from different zoos can be attributed to a multitude of factors such as animal density, their immune status, the design of the facilities, perimeter barriers, or preventive medicine programmes (staff control, biosecurity measures, cleanliness, routine monitoring of the animals) [35]. In the present case, the animal density and the preventive medicine programmes were the same, as both institutions belong to the same leisure park operator and have the same protocols; the only differences between the two centres are the location and design of the facilities and the animal collection housed. In other comparative studies between zoos [34,104], differences among centres were attributed to the type of facility, the possibility of herbivore grazing, and the frequency of faeces collection and cleaning. Other important factors include possible water or food contamination (animal carcasses for carnivores, grass and herbaceous material for herbivores) [37,113,114]. In the present study, control over water and food provided to the animals is similar in both zoos, so the differences in the observed results between them must have another origin.

We have observed that some parasites appeared more frequently (those with high detection percentages in Tables 3–6), but there was not an apparent direct relationship with population size. For example, in ZooAquarium, there were numerous groups of dama gazelles and a small group of fallow deer; both species commonly had *Entamoeba bovis*-like infections (70–80% of positive samples), but nematode infections were occasional (e.g., *Trichuris* spp. was found in 15–16% of gazelle samples and *Capillaria* in 5% of fallow deer samples).

Although the results obtained in our study concerning accessibility by uncontrolled fauna are not statistically significant, the overall presence of parasites was 1.3 times higher in species in accessible environments compared to those in controlled environments. In this regard, there are no major differences in the general typology of bird facilities between ZooAquarium and Faunia. All birds are in accessible environments, and the incidence of parasitised species is similar in both centres; the slightly higher (statistically non-significant) incidence of parasitism in Faunia may be due to the greater presence of multi-species installations and aviaries, which would facilitate transmission among birds. However,

in the case of mammals, the likelihood of a species harbouring parasites in accessible environments was more than 19 times higher than in isolated ones, but no statistically significant differences were found between ZooAquarium and Faunia (where there are a greater number of species in controlled environments) most likely due to mathematical issues (highly unbalanced number of cases in each factor combination; see Table 7). It has been proposed that the possible existence of microclimates within the parks may provide the necessary humidity and temperature for the survival of some pathogens [115]. However, this circumstance would not explain the differences between ZooAquarium and Faunia; while ZooAquarium has a greater number of interior concrete sleeping quarters, where eggs/cysts/oocysts can be maintained in more humid environments and protected from solar radiation, Faunia has a greater number of controlled, enclosed installations without direct sunlight.

One of the likely most important factors to consider is the entry of parasites transported by carriers (i.e., insects) or transmitted by local wildlife that enter the zoo in search of food [14,16,18]. This would allow for similar prevalences of direct-cycle parasites in animals from outside and inside the park [9,10,17]. In the case of parasites with an indirect life cycle, the uncontrolled entries of infected intermediate hosts can lead to the occurrence of infections by adult cestodes in some cases, while the entrance of infected adult hosts (i.e., mesocarnivores) into reserved areas could result in the emergence of larval cestodiasis, which can be lethal for zoo animals [16]. ZooAquarium and Faunia are located close to each other in the same city (about 15 km apart in a straight line), so, a priori, there is not a significant difference in the potential wild animals that may introduce parasites into both centres. The climatic conditions are also similar, and these do not seem to differentiate the results between different zoos [115]. However, ZooAquarium is situated within the largest urban park in Madrid, where sheep and goats graze during certain times of the year, and there is a greater presence of wildlife in the surrounding environment compared to Faunia, which is situated in a more urban setting. In the case of cestode eggs found in some mammals at the zoo, regardless of whether they are genuine or spurious parasitosis, their origin should be linked to infected animals from the outside (intermediate hosts with larvae, adult hosts excreting eggs in faeces) that entered into the zoo facilities. In addition to local wildlife, the public can also introduce parasites (e.g., eggs, cysts, or oocysts on footwear) from the outside to the inside of the facilities. An effective way to prevent contagion would be to limit public and wildlife access to animal facilities or keep the hosted animals in isolated environments, both circumstances being more prevalent in Faunia than in ZooAquarium installations.

The sanitary control of food is another important factor. In carnivorous species, food is often frozen for a few days before use, which helps kill tissue forms of protists and helminths. However, this pretreatment is not usually carried out with vegetables to maintain their appearance and palatability, and if they are not processed with extensive washing, there is a high probability of transmission of cysts/oocysts/eggs. Several studies have shown the contamination of fruits and vegetables sold to consumers with parasitic structures (cysts, oocysts, and eggs) in countries across all continents [116–119]. In Europe, Federer et al. [120] studied the presence of taeniid eggs in the vegetables and fruits fed to gorillas in Basel Zoo (Switzerland). Despite the vegetables being of high quality, processed at high hygienic standards, and pre-washed by the farmer, the authors later identified the DNA of several taeniid species (*Taenia crassiceps* (Zeder 1800), *Taenia hydatigena* Pallas 1766, *Taenia multiceps* Goeze 1782/*Taenia serialis* (Gervais 1847), *Taenia saginata* Goeze 1782, and *Hydatigera taeniaeformis* (Batsch 1786)) in wastewater obtained after the routine processing of the food in the zoo food preparation station. The risk exists, but the problem is that there are not always well-established, standardised, or validated methods for detecting parasites in food [121].

Another possible cause of the greater impact generally experienced by herbivorous and omnivorous species compared to carnivores may be their feeding behaviour and the substrate on which they feed. The herbivorous/omnivorous species directly ingest food

from the ground or, in the case of primates, using their hands, which are also used for locomotion; thus, contact with parasite transmission forms present in the soil is easier. The type of soil is important due to the varying difficulty in cleaning it [122], which may allow for the persistence of transmission forms. As mentioned earlier, this circumstance should be especially considered in sleeping quarters or in isolated themed environments, where microclimates that serve as foci for parasitic infection can develop (of particular relevance in direct life cycle parasites). Among carnivores, the giant anteater is a special case; being in an outdoor installation with natural soil, it can also easily become infected by arthropods or annelids that it preys upon in the enclosure.

4.3. Transmission Risks between Animals and Humans

Almost all parasite species identified in this study followed a direct life cycle. The trematode eggs discovered in two samples from bears at ZooAquarium were not identified, thus hindering the evaluation of transmission risk to other animals. The bears were housed in enclosures with minimal vegetation, limiting the presence of snails that could serve as intermediate hosts; however, their enclosure features a safety moat where plants grow, and snails might live there and could potentially continue the parasite cycle. The fact that the eggs were detected only once suggests that the infection was likely due to metacercariae in the supplied food rather than an active ongoing cycle, but a natural infection cannot be ruled out.

The adult stage of cestodes exhibits some host specificity, while the larval stages could frequently affect a wide range of intermediate hosts. The guineafowl releasing *Raillietina*-like eggs would probably become infected after ingesting parasitised insects that freely accessed the bird facilities. This was an exceptional situation, as the finding occurred only once in the last 10 years. In mammals, the cestode eggs found were also detected only once in each positive host species; the fact that the morphology of the eggs does not correspond to any species previously described in the hosts suggests that some or all of them may be spurious parasitoses. Despite the greater abundance of wildlife around ZooAquarium, and until it can be confirmed that they are real parasitoses, there is no evidence for a higher rate of transmission of indirect life cycle parasites in one or the other zoo; however, the possibility of their occurrence exists. In none of the positive cases, the eggs found corresponded to taeniids, which could be the most dangerous cestodes for humans and other mammals as cysticerci could develop in their internal organs and may cause death. Cysticerci were recovered three times in the surgeries or necropsies of some animals at the ZooAquarium in the last 20 years (*T. crassiceps* cysticerci found in 2007 in a ring-tailed lemur [123]; unidentified cysticerci found in 2009 in a dorcas gazelle, and *T. crassiceps* cysticerci found in 2017 in a ring-tailed lemur [124]). The origin of these infections was not established, but at least for the 2007 lemur cysticercosis, it was suspected to have occurred before the animal arrived at the ZooAquarium [123]. However, in the park of origin, the infection would have occurred through any of the routes already mentioned in Section 4.2.

The nematodes found in birds are not infective species for mammals, so they do not pose a risk to zoo staff or visitors, although they can be transmitted to wild birds that enter the facilities seeking food. In the zoos considered in this study, this transmission seems unlikely, as most of the nematodes were detected in birds of prey, and wild fauna would avoid entering the areas within their range. At Faunia, findings were occasional in Galliformes and Gruiformes; the exception being toucans, where *Capillaria* infection recurs over time; however, in this case, the birds are in a closed installation inaccessible to wild avifauna from the area.

Transmission of parasites between zoos is possible due to the exchange of animals that may be parasitised [44]. Pre-transportation analyses of animals or quarantine periods at the receiving zoo are essential to prevent parasite dissemination. Likewise, there is a risk of parasite transmission from zoo animals to wildlife when zoos participate in breeding and species repopulation/reintroduction programs in their original habitats. It has been proposed that pre-exposure to some pathogens (i.e., parasites) can increase host survival rates

once released into the wild [24,125,126]. However, zoos can serve as hotspots for gastrointestinal parasites [115,127], and hidden host–parasite co-reintroductions could occur [128]. This can affect both the reintroduced animals and/or the target population [127], leading to difficulties or failure in some reintroduction programs [24]. Husbandry practices are of special relevance to avoid reintroduction of apparently healthy but parasitised animals into wild populations [24,115]. The gastrointestinal parasites that can be involved in the success or failure of reintroduction programs should be considered on a case-by-case basis; in general, coccidia and nematodes would be the most important ones [115,129,130].

In our study, we identified certain parasites that are potentially transmissible between animals and humans (*Entamoeba* spp., *Giardia* spp., *B. coli*, *Trichuris* spp.). In a previous molecular-based investigation [15], there was no evidence of transmission between these parasites and the personnel at ZooAquarium and Faunia; however, zoonotic transmission was detected for *Cryptosporidium hominis* Morgan-Ryan et al. 2002 and *Blastocystis* (Alexeieff 1911) spp. In some studies conducted in other zoos, potential transmission of parasites to zoo personnel was also suggested [11–14,131,132]. The risk of transmission to visitors is low, as contact with animals is generally limited or nonexistent; however, zookeepers and veterinarians are exposed during handling and cleaning operations. Regular analyses of the animals and a personnel health program incorporating proper training, periodic testing, and health monitoring would minimise transmission risks between animals and caretakers [122].

5. Conclusions

The findings of this study largely align with those reported by other authors, indicating that parasites with direct life cycle, including protists and helminths, are predominant in captive animals. Most of the parasite species identified exhibit low or no pathogenicity; the predominance of this type of parasite could be attributed to a combination of factors: (1) Non-pathogen species are typically not investigated or treated in animals, thus facilitating their spread. Conversely, potentially pathogenic species are detected and treated in animals. (2) Animals are fed a controlled diet, which helps prevent or at least limit infections.

Cleaning and disinfecting soil is relatively easy to achieve in artificial (usually concrete) substrates. However, in natural soils, this is often not feasible, and parasites with direct life cycles are difficult to eliminate from the facilities, leading to periodic reinfections. In these circumstances, animals that feed on the ground (e.g., herbivores) have an increased likelihood of becoming infected. This also applies to non-human primates, as they commonly use their hands for locomotion and food manipulation. Regular analyses and preventive/therapeutic antiparasitic treatments would be the optimal approach to maintaining a low intensity of parasite infections and to reduce the risk of zoonotic transmission.

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High Prevalence of Bovine Cardiac Cysticercosis in Upper Egypt: An Epidemiological and Histopathological Study

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Simple Summary: Bovine cysticercosis is considered one of the major parasitic zoonoses that carry an important food safety concern. The disease can lead to a range of symptoms and health problems based on the location and number of cysts. Moreover, bovine cysticercosis can cause considerable economic losses in livestock production. Clearly, there is an urgent need for a practical and sensitive method for the detection of infected carcasses. Little information is known about the prevalence of cardiac cysticercosis in bovines from Upper Egypt and the potential use of histopathological and immunohistochemical methods in the localization of the pathogen. The present study used a set of morphological, morphometric, histopathological and immunohistochemical techniques to investigate the distribution of the cardiac cysticercosis in cattle from Upper Egypt. Furthermore, the measurement of various biochemical markers was performed to identify their possible association with the infection.

Abstract: Bovine cysticercosis is categorized as a serious parasitic zoonotic infestation. The infection is mainly caused by the tapeworm *Taenia saginata*, which infects cattle and humans. The larval stage, *Cysticercus bovis (C. bovis)*, is found in the skeletal and cardiac muscles of infected cattle. Despite its potential public health concern, few studies have been conducted on cardiac cysticercosis in Upper Egypt. This study investigates the prevalence, epidemiology, and impact of cardiac cysticercosis in Upper Egypt, emphasizing how histopathological changes in cardiac muscle and physiological

parameters might be associated with the infection. From December 2022 to October 2023, a total of 941 animals from Assiut province, Upper Egypt, were slaughtered and their cardiac muscles were examined for the presence of *C. bovis*. Cysts were classified as viable or degenerated through macroscopic inspection. The overall prevalence of *C. bovis* infected hearts made up 10.8% of the total examined. The highest prevalence rate was in the summer season followed by spring; winter had the lowest infections. The histopathological examination of infected tissues revealed immune cell infiltration around Cysticercus-infected areas. Additionally, Bax immunostaining demonstrated the apoptotic effect of cysticercosis. Regarding the measured physiological parameters, there were non-significant changes in plasma levels of total protein and albumin in cattle infected with cysticercosis compared with control animals. Moreover, there was a significant decrease in total antioxidant capacity (TAC) combined with a significant increase in lipid peroxide (Malondialdehyde) (MDA), troponin T, and lactate dehydrogenase (LDH) activity in infected animals. The present work documented a set of epidemiological and pathological findings, revealing that *C. bovis* is a potentially harmful parasite and can cause significant health problems in both cattle and humans.

Keywords: *Cysticercus bovis*; cattle; prevalence; physiological parameters; meat inspection; histopathological

1. Introduction

Bovine cysticercosis is a parasitic disease caused by the larval stage (*Cysticercus bovis*) of the cestode *Taenia saginata*. Cattle serve as the intermediate host in the two-host life cycle, while humans represent the definitive hosts. Globally, around 50 million human cases were reported resulting in ~50 thousand fatalities annually [1,2]. Generally, humans get infected by consuming raw or undercooked meat containing infective *cysticerci* that develop into full grown tapeworms three months later. Taken into account, human taeniasis is asymptomatic or presented through mild gastrointestinal symptoms, nausea, vomiting, abdominal discomfort, flatulence, and diarrhea. However, other complications such as weight loss and vitamin deficiency, excessive loss of appetite, weakness, and intestinal blockage might take place. Moreover, psychological stress caused by the active migration of proglottids from the anus and the length of the tapeworm has been reported [3]. Importantly, infected people can shed millions of eggs daily into the environment; these eggs can survive for up to seven months and could be transmitted to the intermediate host [4]. Commonly, cattle become infected through the ingestion of grass, grains, or water contaminated with eggs [4,5]. Then, the hatching of ingested eggs occur within the small intestine to produce oncospheres that penetrate the intestinal wall and migrate through the bloodstream to the muscles where they may grow into infectious cysts. Humans become infected by ingesting raw or inadequately cooked meat containing *C. bovis*. Typically, the masseter muscles, heart, tongue, diaphragm, and other skeletal muscles contain live cysticerci, while there are rarely found in fat or visceral organs. Cysticerci undergo degeneration after a period of time that can range from weeks to years followed by calcification [6]. In cattle, natural infections are normally asymptomatic. However, heavy infestation by larvae may cause myocarditis or heart failure [7].

Although the epidemiological distribution of bovine cysticercosis is worldwide, the incidence of the disease in developing countries is higher than developed countries. Prevalence rates of 30–80% have been reported in some East African countries [7,8]. In the meat industry, economic losses are significantly associated with infection by these parasites. Carcasses must be completely condemned they have heavy infestations. However, light infection or localized cysticercosis leads to the condemnation of the infected parts. Additionally, the carcass must be kept in freezing storage fir up to 3 weeks to inactivate the parasites [9]. In this regard, the projected cost of the handling, transporting, and refrigerating of infected carcasses in the United Kingdom was estimated to exceed GBP 4.0 million

yearly [10]. Similarly, bovine cysticercosis causes significant losses in Africa that have been estimated to be approximately USD 1.8 billion annually [9].

Regarding the diagnosis of the infection, a visual examination of the cysticerci during the post-mortem examination of carcasses is typically employed for the detection of the parasites. Nevertheless, traditional methods have major drawbacks and limited diagnostic capability [1,11]. Moreover, standard inspection techniques are ineffective for detecting bovine cysticercosis at low concentrations. In Egypt, the diagnosis of detecting of bovine cysticercosis is totally based on the postmortem examination of the carcass. In the same context, lesions in the heart or masseter muscles that include entirely transparent, cheesy, or calcified cysts are thought to be *Cysticercus* cysts [5,6]. Taken into account, the presence of abscesses, granulomas, or parasites such as *Sarcocystis* spp. associated with eosinophilic myositis may develop similar macroscopic lesions [8]. The developed immunohistochemical test provides an inexpensive, reliable means of diagnosis in tissue sections and differentiating them from normal tissues and other pathogenic lesions [10,12]. Despite its zoonotic potential, few studies have been conducted to investigate the current status of bovine cyctsercosis in Upper Egypt. Furthermore, limited information is reported about the morphology and morphometric measurements of cysticerci in this area of Egypt. This study aimed to determine the prevalence of *C. bovis* in cattle slaughtered in Assiut province in Upper Egypt using a set of different diagnostic serological and immunohistochemical techniques. The work also included a descriptive histopathological study of viable and calcified cysts by using light and scanning electron microscopes. Furthermore, biochemical tests and immunohistochemistry (IHC) were employed for confirmation, which might represent a step towards establishing a more dependable method for identifying the effects of *C. bovis* on bovine tissue.

2. Materials and Methods

2.1. Study Area, Period and Design

This study included a cross-sectional study conducted from December 2022 to October 2022 to determine the prevalence rate of *C. bovis* in Assiut province located in Upper Egypt.

2.2. Animals

A total of 941 animals admitted to various slaughterhouses in Assiut province, including (*n* = 852) native bred cattle and (*n* = 89) cases from other breeds (Holstein and Brazilian breeds), were included in the present study. Antemortem examinations of the animals were performed for the evaluation of their general conditions. All data, including sex, breed, and season of sample collection, were recorded routinely. Carcasses were subjected to complete postmortem examination via visual inspection, palpation, and multiple incisions of the organs. The present study was focused on the examination of hearts found to be positive with *Cysticercus bovis*. The samples used for this study were selected from the condemned infected heart specimen after routine examinations in Assiut slaughterhouse. The ethics committee of the Faculty of Veterinary Medicine, Assiut University, Egypt, approved this study (no. 06/2022/0014). Hearts were also collected to perform the morphological and ultrastructural identification of the cyst.

2.3. Blood Collection

Blood samples were collected from all live cases studies through the study period and then were placed in tubes containing EDTA. The positive and control samples were effectively recognizable after slaughter, with five samples from infected animals and five from healthy ones. Then, samples were centrifuged at 3000 rpm for 10 min and plasma was obtained and stored at −20 °C.

2.4. Biochemical Determinations

Plasma of negative and positive *C. bovis* cattle (*n* = 5 each) was used for the estimation of LDH activity spectrophotometrically using reagent kits purchased from Human Gesell

Schaft fur Biochemical und Diagnostic mbH, Germany, while plasma total protein and albumin levels were determined using reagent kits purchased from DiaSys Diagnostic System GmbH, Germany. Plasma levels of TAC and MDA were estimated using reagent kits obtained from Biodiagnostic, Giza, Egypt.

Plasma cardiac troponin T (cTnT) (a protein integral to the contraction of heart muscles) levels were measured using the ELISA technique (Dynatech microplate reader model MR 5000, Chantilly, VA, USA) and reagent kits (No. SG-10127) purchased from SinoGeneClon Biotech Co., Ltd., No. 9 BoYuan Road, Yu Hang District, Hangzhou, China.

2.5. Cyst Collection and Preservation

Cysticerci were collected and classified into degenerated and viable cysts after macroscopic examination. Cysts that were filled with translucent fluid were considered viable, while cysts that were empty or those with solid contents were considered degenerating or non-viable [13]. Cysts were then washed using PBS to remove debris and prepared specimens were carefully subjected to horizontal incision and preserved in 3% glutaraldehyde for further examination using a scanning electron microscope (SEM). For histopathological examination, cysts were preserved in a neutral buffer formalin, 10%. All samples were transported in sterile containers directly to Assiut University, Faculty of Veterinary Medicine, Parasitology Department.

2.6. Scanning Electron Microscopical Examination of the Cyst

The incised specimens were dehydrated through graded series of ethanol, critical-point-dried, coated with gold, and examined at the Electron Microscopy Unit at Assiut University.

2.7. Histopathological Examination of the Cyst and the Surrounding Heart Tissue

The cysts and surrounding heart tissue from five infected animals were fixed in neutral-buffered formalin, processed in serial concentrations of ethyl alcohol, cleared in xylene, and then paraffin-embedded. The histological sections were stained using hematoxylin and eosin (H & E) and photographed using a Canon digital camera (Canon Powershot A95) mounted on a Leitz Dialux 20 microscope (Wetzlar, Germany).

2.8. Semi-Thin Sections and Transmission Electron Microscopical Examination of the Cyst and the Surrounding Heart Muscle

Small specimens from the normal and cyst-infected hearts were preserved through immersion in a mixture of 3% PFA–glutaraldehyde in 0.1 mol/L Na-cacodylate buffer at a pH of 7.2 for 48 h at 4 °C. The samples were washed with the same buffer and then postfixed in 1% osmic acid in 0.1 M Na-cacodylate buffer for 2 h at room temperature (RT). The specimens were dehydrated in an ascending series of ethanol and embedded in an Araldite–Epon mixture. The sections were cut at a thickness of 1 µm and stained with 1% toluidine blue in line with our previous study [14]. The stained sections were examined using the Leitz Dialux 20 microscope and the photos of these sections were captured using a Canon digital camera (Canon Power shot A 95). Ultrathin sections were cut using an Ultrotom VRV (LKB Bromma, Oberkochen, Germany). Subsequently, the sections (70 nm) were stained with uranyl acetate and lead citrate and then examined using the JEOL 100CX II transmission electron microscope (TEM) (JEOL, Tokyo, Japan) at the Electron Microscopy Unit at Assiut University.

2.9. Immunohistochemical Analysis

Immunohistochemistry was performed as described previously [15–18]. The paraffin sections were deparaffinized and hydrated and then washed with 1 mML of PBS (three times, 5 min each). To unmask the antigen epitopes, antigen retrieval was carried out using 1 mML of sodium citrate buffer solution (pH 6). The sections were washed with PBS (pH 7.4) for 15 min. After blocking the endogenous peroxidase activity with 3% H_2O_2,

sections were washed with PBS (3 × 5 min) and then were incubated overnight at 4 °C with Bax (Rabbit Polyclonal-Polyscience Lab, Seoul, Korea).

2.10. Statistical Analysis

Prevalence data were verified, coded, and analyzed using IBM-SPSS 24.0 (IBM-SPSS Inc., Chicago, IL, USA). Regarding the descriptive statistics, frequency and percentages were calculated and the Chi-square test/Fisher's exact test were used to compare the differences in frequency between groups as appropriate. Measurements of biochemical data were represented as mean ± standard error of the mean (SEM). The results were then analyzed utilizing Student's *t*-test analysis using Prism software (version 8.0.1; GraphPad Software, Inc., San Diego, CA, USA). A *p*-value of <0.05 was considered significant.

3. Results

3.1. Prevalence of Cardiac Cysticercosis

The prevalence rate of *C. bovis* among examined hearts was 10.8% from the total examined (102 out of 941). The collected (n = 102) cysts were of different sizes and ages (including viable and degenerated ones). In relation to breeds, bovine cysticercosis was reported among the native cattle breeds and not recorded in the imported cattle breeds (Holstein and Brazilian breeds). All the infections were only recorded among male animals aged from 1.5 to 2 years old (Table 1). Concerning season, the prevalence rate was highly significant with regard to the summer season (79 out of the 102 cases) followed by spring, while the lowest infection rate was in winter.

Table 1. Prevalence rate of *C. bovis* in examined slaughtered cattle in relation to breed, sex, and season.

N = 941	**Positive *C. bovis* (n = 102)**	***p* Value**
Breed		
Native breed	102 (100%)	<0.001 *
Others	0 (0%)	
Sex		
Female	0 (0%)	=0.006 *
Male	102 (100%)	
Season		
Fall	4 (3.9%)	
Winter	7 (6.9%)	<0.001 **
Spring	12 (11.8%)	
Summer	79 (77.5%)	

(*) Fisher's exact test was used to compare frequency between groups and (**) the Chi-square test was used to compare frequency between groups.

3.2. Gross and SEM Studies

The collected positive hearts were carefully examined and a total of 102 cysticerci were dissected and their size ranged from 0.5 to 1.4 cm. As depicted in Figure 1A, the macroscopic examination of cysticerci revealed the presence of two viable cysticerci and their wall was relatively thinner; the cyst was containing translucent to faint white material and a visible invaginated scolex. The two viable cysticerci were found in the summer season from two different cattle that were apparently healthy. The calcified ones (comprising the other 100 samples) were in different stages of degeneration and their walls were hard, containing solid materials and a salty exudate (Figure 1B). The SEM of the incised susceptible, viable or recently degenerated cysts showed the presence of a chamber containing the invaginated scolex with tegumental fold at the bladder opening (Figure 1C). The cyst was surrounded by a relatively thin fibrous layer separating it from the surrounding cardiac muscle (Figure 1D). The invaginated scolex was visible from the neck and septa and the site of the beginning of proglottide generation (Figure 1E). On the other hand, the degenerating cysticerci showed the presence of several calcified fragments and parts of the degenerated neck within the

cavity of the cyst. The fibrous layer was relatively thick, while the tegumental fold was not noticed, nor was the bladder opening (Figure 1F). The site of attachment between the bladder and the muscle tissue showed the strong attachment between microtriches and muscle fibers (Figure 1F). The fibrous layer was relatively thicker than that of viable cysts (Figure 1D), while the tegumental fold was not noticed, nor were the bladder opening and site of proglottide generation (Figure 1F).

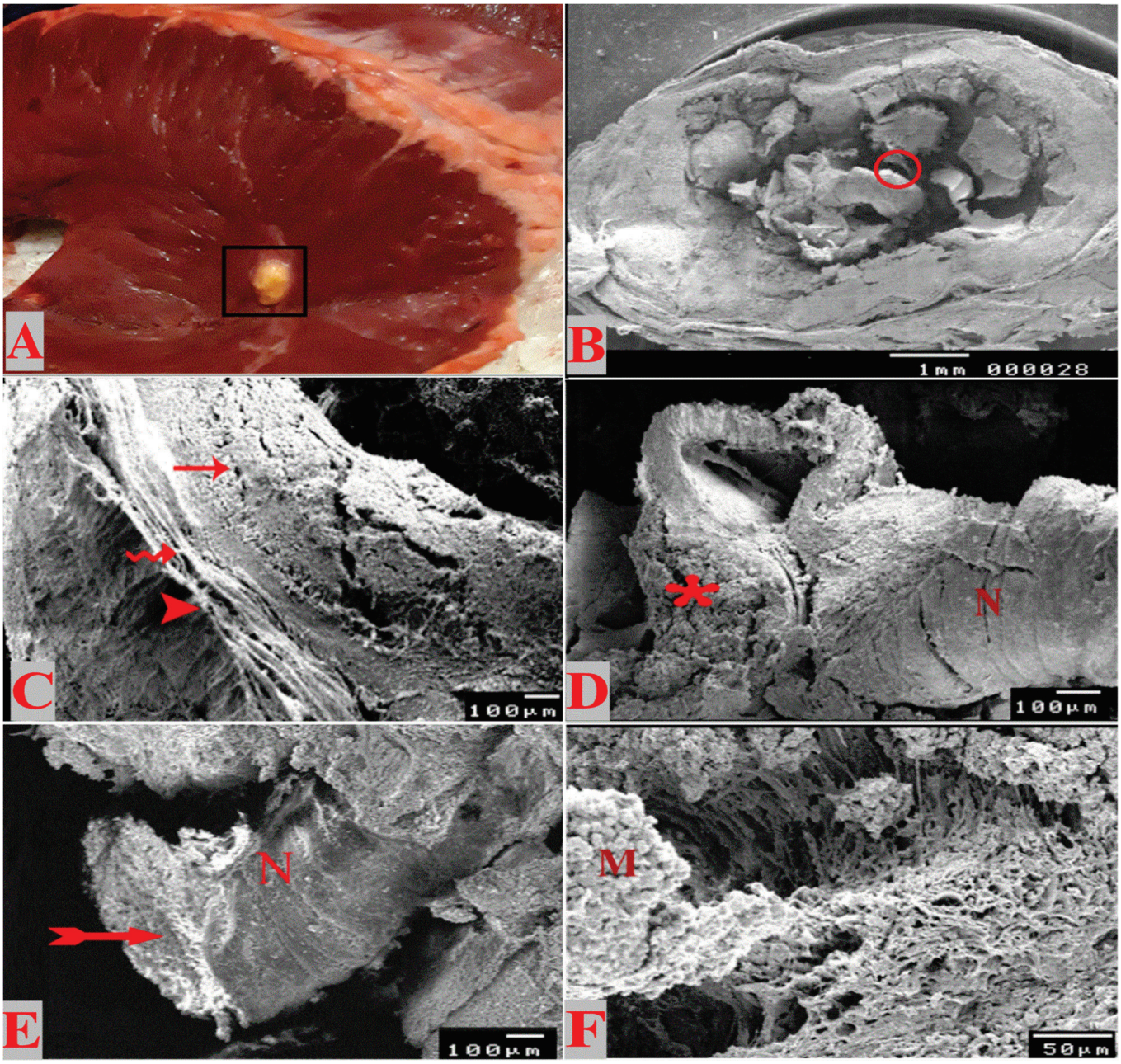

Figure 1. Gross and SEM photographs of *Cysticercus bovis* affecting cattle heart. (**A**) Calcified non-viable cyst (square), (**B**) SEM photograph of the invaginated scolex (circle), (**C**) calcified cyst at a higher magnification of the bladder showing heart (head arrow), attachment of microtriches with heart muscle (wavy arrow), and thick fibrous layer (thin arrow), (**D**) higher magnification of the neck (N) and evagination site (asterisk), (**E**) calcified cyst showing calcified parts of the neck (N) and calcified parts (arrow), and (**F**) the attachment of the cyst with the heart muscle through microtriches (M).

3.3. Histopathological and Semi-Thin Sections of Cysticerci

Histological sections of the viable *Cysticercus* showed that the whole cyst was surrounded by a thin fibrous layer that lies between the cyst and the cardiac muscles (Figure 2E). There was a cavity around the cyst; the bladder wall was thin surrounding the invaginated scolex (Figure 3). The cyst had a bladder opening from which the parasite invaginated. The invagination of the scolex and the neck formed a spiral canal leading to the deepest part of the cyst where the scolex invaginated inside (Figure 2A). This canal was markedly corrugated and lined with the tegumental layers that were analogues of the future adult cestode; the suckers were located toward the blind side of the cyst (Figure 2B). The folded membrane of the spiral canal was lined with a highly eosinophilic homogenous cuticle that was markedly branched. It was lined with the three layers of the cuticular layer, followed by the middle cellular layer, and then by the reticular layer; the parenchyma was thicker around the scolex and suckers and the surface of the parenchymatous portion facing the bladder cavity were lined with a fibrous tissue (receptaculum) (Figure 2A,B).

The fibrous layer surrounding the cyst was thin followed by a cellular layer (where intense inflammatory reaction occurs) between the cardiac muscle and the thin fibrous layer of the cyst. This cellular layer, together with the fibrous layer (host originated), represented the immune response of the host where many inflammatory cells were accumulated, including ellipsoidal cells and multi-nucleated giant cells (Figure 2D). This formal layer was then followed by the cardiac muscles.

On other hand, the histological examination of the degenerated cysticerci showed the impossibility of noticing the scolex or any parts of the parasite tegumental layers. The cyst had a central necrotic area with caseation, extensive mineralization (calcification), and foci were found inside the cyst (Figure 2B). There was a layer of giant cells between the mineralized area and the surrounding cellular layer (Figure 2E) that comprised an extensive pyogranulomatous reaction with mixed inflammatory cells. Another section of degenerated cyst showed an aggregation of diagnostic calcareous corpuscles in the necrotic core (Figure 2F). Semi-thin sections of the susceptible, viable, or recently degenerated cysticerci showed the parasite cuticle had three distinct layers. The first layer was the microtriches that were carried by dense apical plasma membrane and appeared to be different in length with regard to the facing side; they appeared longer in the host side than the bladder side (Figure 2G,H). The second layer, following microtriches, was the cuticular-(vesicular layer) dense cytoplasmic layer that contained electron-dense granules and many translucent vesicles (Figure 2G,H). The third layer was the sub-tegumental layer containing distinct tegumental cells that appeared spindle-shaped with large nuclei containing dense chromatin bodies and perinuclear cytoplasms; it was connected to the distal cytoplasm through cytoplasmic extensions (Figure 2G,H). In addition, calcareous corpuscles were noticed in this layer. The following layer was the muscular layer that contained circular and longitudinal muscle bundles and numerous muscle fibers (Figure 2H).

3.4. Histopathological, Transmission Electron Microscopic and Immunohistochemical Analysis of the Cardiac Muscle

Normally, the cardiac muscle is striated, branched, and arranged in densely packed myofibrils as well as separated by a narrow interstitial space that is occupied by rare connective tissue (Figures 3A and 4A). The infected heart muscle showed degenerative changes including a loss of striations, the fragmentation of myofibrils, an increase of the perinuclear spaces, and disorganization of the muscle fibers, especially around the cyst (Figure 3). Furthermore, the infected cardiac muscle showed intense collagen fiber disposition between the cardiac muscle fibers that contained dilated and congested blood capillaries and fibroblast infiltrations (Figure 4B). There was a storm of immune cell infiltration, including the lymphocytes, plasma, macrophages, and mast cells between the muscle fibers and within the cyst (Figure 4D). Additionally, much fibroblast infiltration led to intense deposition of the collagen fibers in the interstitium and around the cyst (Figure 4C). At the ultrastructural level, the degenerative cardiac muscle fibers showed

unclear banding of the sarcomeres and disorganization of the mitochondria. The wall of the cyst consisted of mast cells, macrophages, and many fibroblast cells (Figure 5). The Bax immunohistochemical staining showed a negative reaction in the cardiac muscle of the control group but strong positive staining within the cardiac muscle that surrounded the cyst as well as in the inflammatory cells infiltrating the cyst (Figure 6).

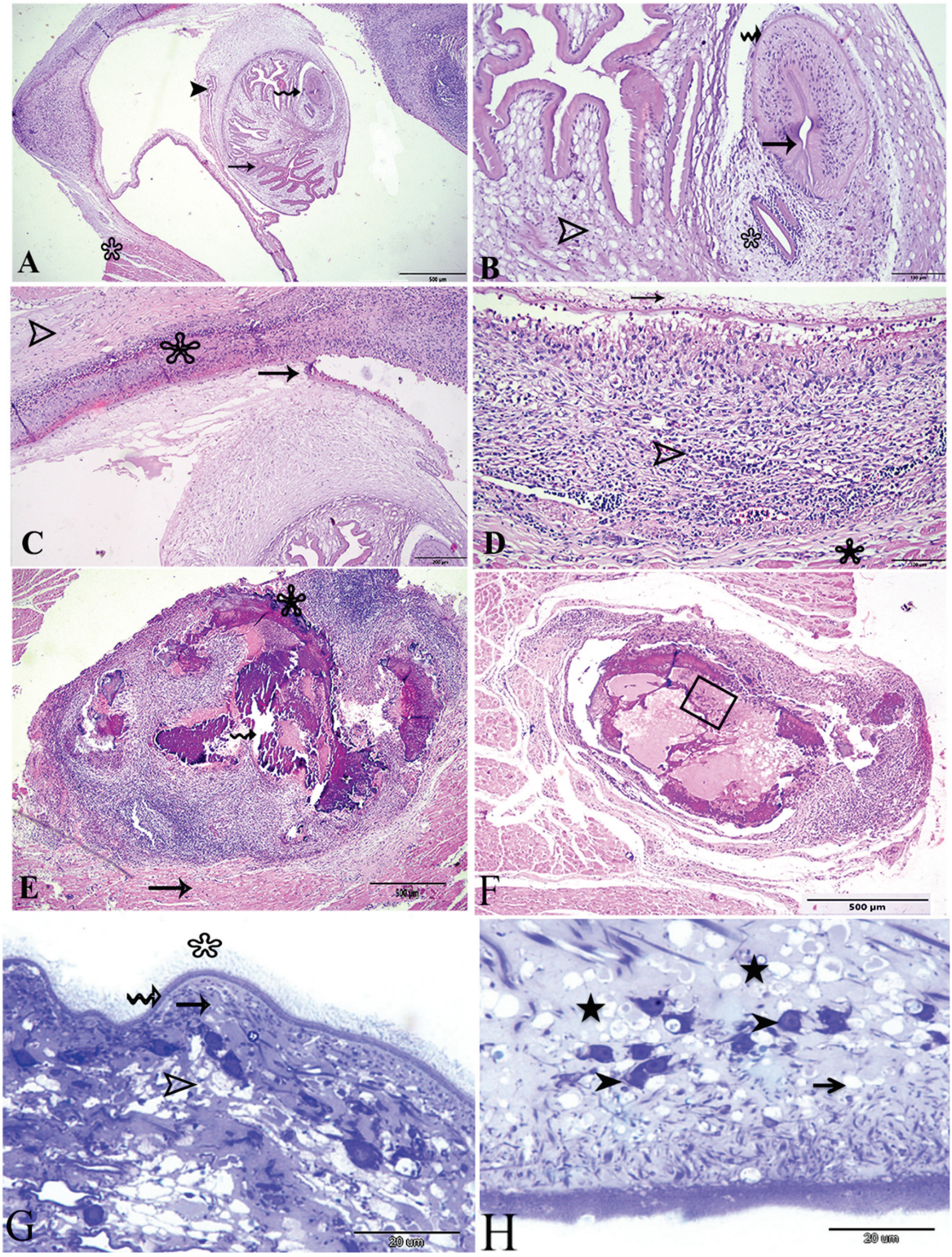

Figure 2. Photomicrograph sections of *Cysticercus bovis* affecting cattle heart stained with H&E showing (**A**) viable or young Cysticercus, convoluted spiral canal (thin arrow), invaginated scolex (wavy arrow),

vestibule (head arrow), and thin wall of the cyst attached to heart muscles (asterisk); (**B**) higher magnification of the cyst showing the reticular parenchymatous layer (head arrow), sucker (asterisk), the scolex (wavy arrow), and sucker (thin arrow); (**C**) inflammatory reaction (asterisk) separating the cyst wall (thin arrow) from the heart muscle (arrow head); (**D**) mixed inflammatory cells (minor) (head arrow) between the heart muscle (star) and cyst fibrous layer (thin arrow); (**E**) calcified cyst formed through a central cavity necrotic core (wavy arrow) and an intense pyogranulomatous reaction (major) (asterisk) within the heart muscle (thin arrow); (**F**) calcified cyst with aggregation of calcareous corpuscles (square); (**G**) photomicrograph of a semi-thin section of *C. bovis* stained with toluidine blue showing the *C. bovis* layers and the three distinctive layers of the Cysticercus, with the outer tegumental layer (wavy arrow) having straight filamentous microtriches (asterisk), the middle cellular layer (thin arrow) containing tegumental cells (spindle shape), and the innermost reticular layer (head arrow); and (**H**) translucent vesicles (thin arrow), tegumental cells (head arrows), and calcareous corpuscles (star).

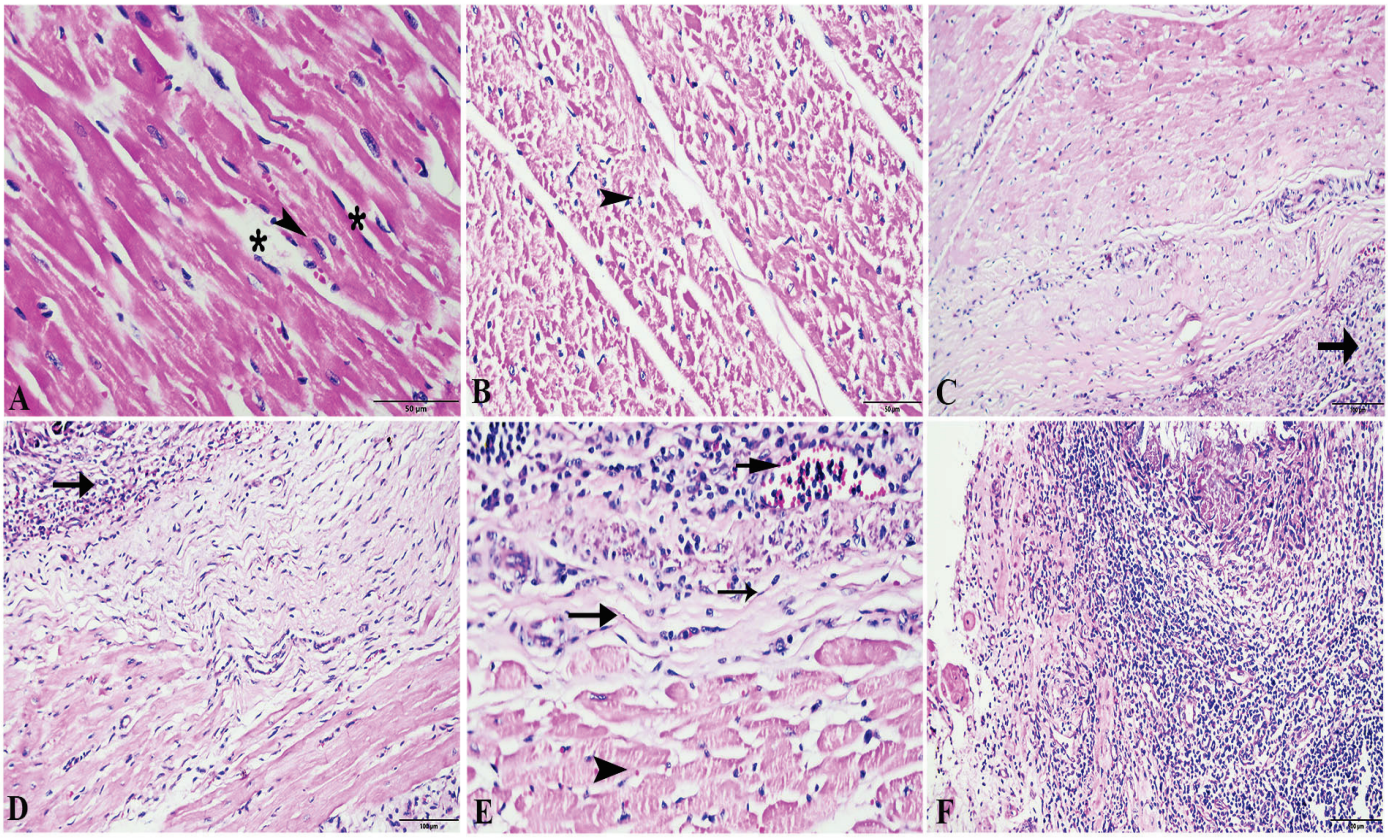

Figure 3. Photomicrographs of the bovine cardiac muscle stained with H & E showing (**A**) the normal appearance of the cardiac muscle fibers with normal striated and branching cardiac cells with the nucleus (arrowhead) as well as perinuclear spaces (asterisk) and (**B**–**F**) abnormal degenerated cardiac muscle fibers (arrow heads) with heavy inflammatory cells and fibroblast infiltrations (arrows).

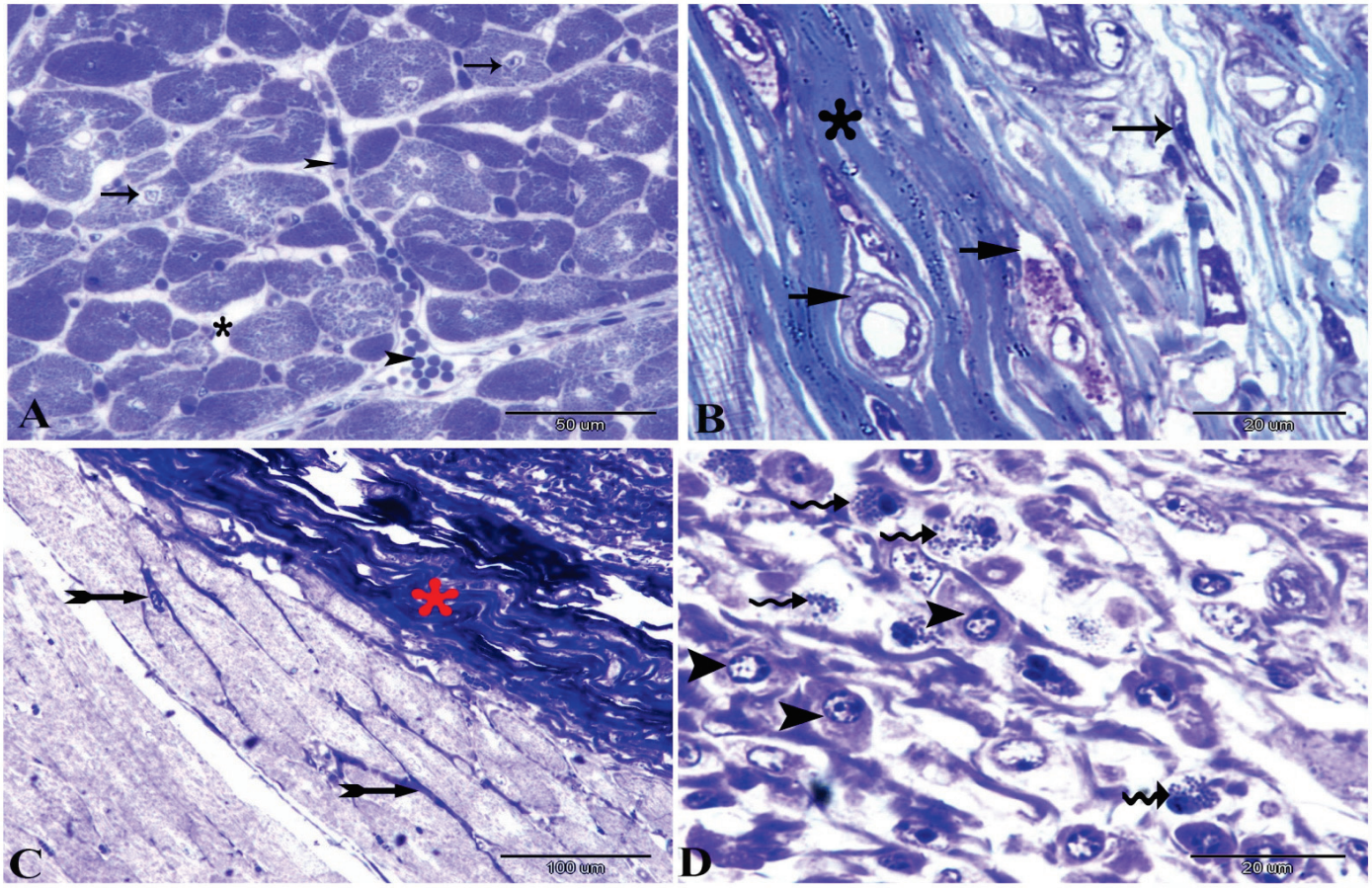

Figure 4. Photomicrographs of a semi-thin section of the cardiac muscle stained with toluidine blue showing (**A**) cross-section of the normal cardiac muscle fibers with central nuclei (arrows) and scanty connective tissue (asterisk) between the cardiac muscle fibers containing secretory granules (arrow heads); (**B**) intense collagen fiber (asterisk) disposition between the cardiac muscle fibers containing dilated and congested blood capillaries (short arrows) and fibroblast (arrow) infiltrations; (**C**) increase in the interstitial connective tissue (arrows), especially near the cyst (note the dense collagen fibers (asterisk)); (**D**) localization of mast (wavy arrow) and plasma cells (arrow heads) within the infiltration that surround the parasite.

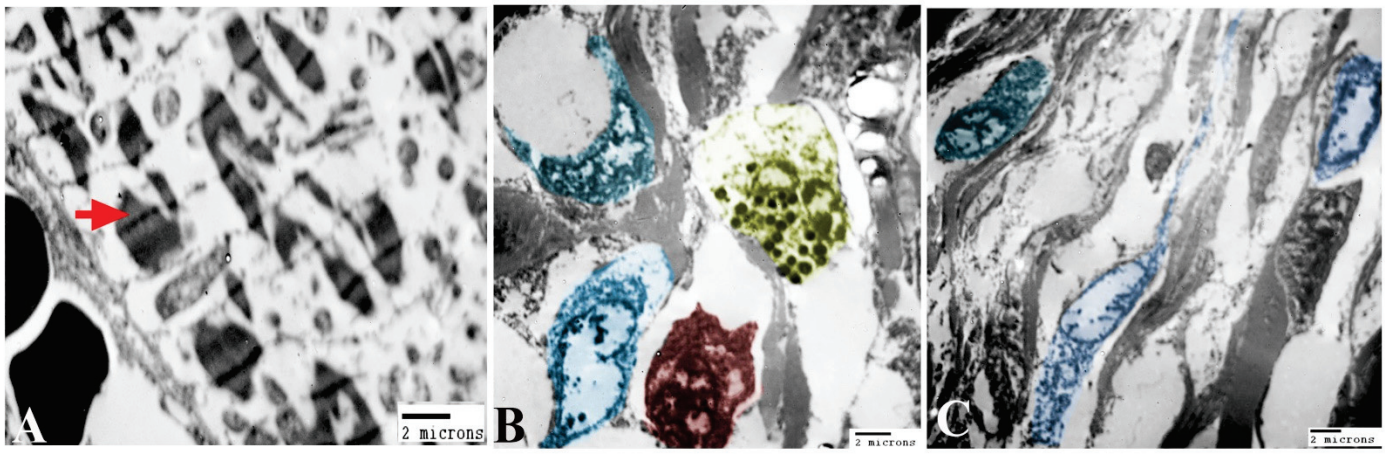

Figure 5. Photomicrographs of a TEM showing (**A**) degeneration of cardiac muscle fibers and unclear banding of sarcomeres (arrow) and disorganization of mitochondria (arrows) and (**B**,**C**) the wall of the cyst consists of mast (yellow), macrophage (red), and many fibroblast (blue) cells.

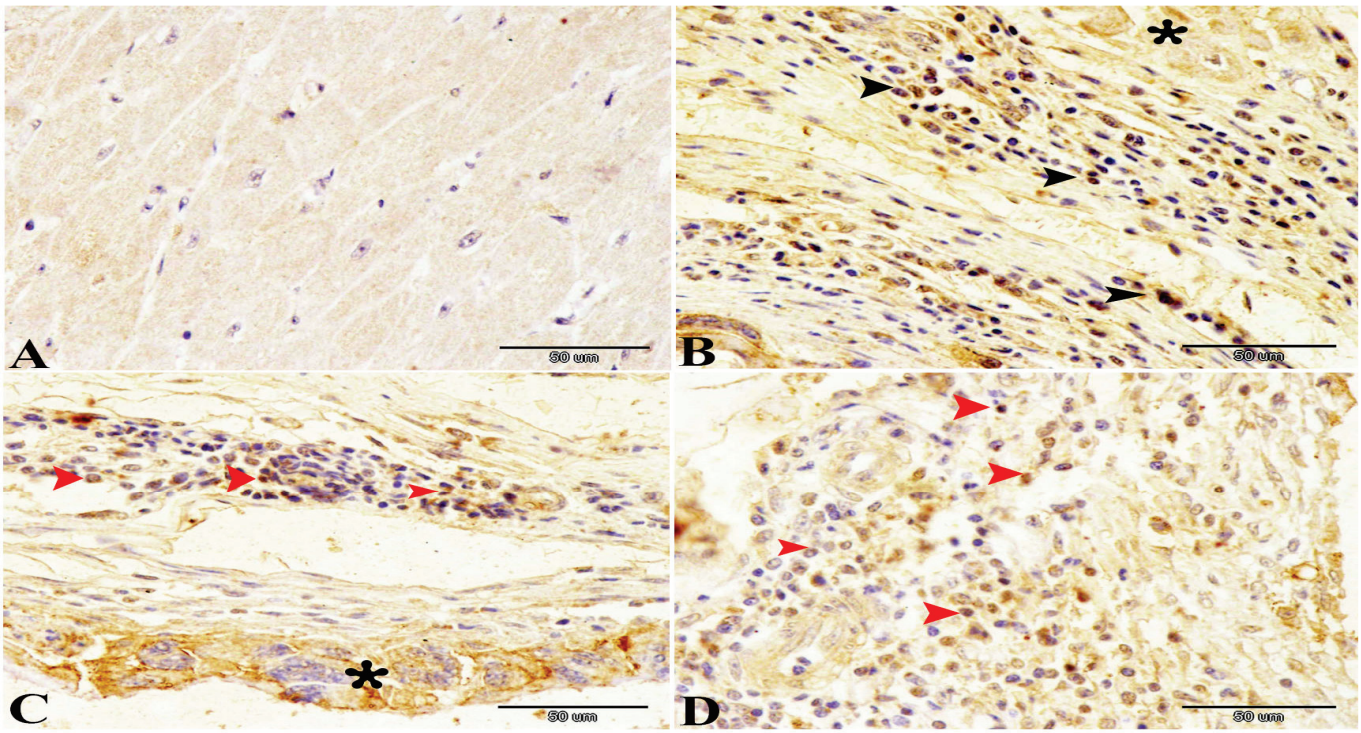

Figure 6. Photomicrographs of Bax immunohistochemical staining showing (**A**) negative reaction in the cardiac muscle of the control group and (**B**–**D**) strong positive staining within the cardiac muscle (asterisk) that surrounds the cyst and in the inflammatory cells (arrow heads) infiltrating the cyst.

3.5. Biochemical Parameters of Positive and Negative Cases

Effects of *C. bovis* infection on the levels of plasma total proteins, albumin, MDA, TAC, troponin T, and LDH are presented in Table 2. There were non-significant changes in the levels of total proteins and albumin between the control and cattle infected with cysticercosis ($p > 0.05$). However, there was a significant loss of levels of TAC in cattle infected with cysticercosis compared with the control ($p < 0.05$). While there was a significant increase in levels of MDA, troponin T, and LDH in cattle infected with cysticercosis compared with the control ($p < 0.05$).

Table 2. The effect of *Cysticercus bovis* on plasma total proteins, albumin, MDA, TAC, troponin T, and LDH ($n = 5$ in each group).

Groups/ Parameters	Negative *Cysticercus bovis*	Positive *Cysticercus bovis*	*p* Value
Total protein (g/dL)	6.653 ± 0.0437	6.733 ± 0.1257	0.5803
Albumin (g/dL)	3.190 ± 0.0819	2.940 ± 0.2100	0.3295
MDA (nmol/mL)	1.943 ± 0.5261 [a]	3.551 ± 0.1802 [b]	0.0445
TAC (mM/L)	1.096 ± 0.0305 [a]	0.953 ± 0.0195 [b]	0.0168
Troponin T (pg/mL)	6.590 ± 0.1418 [a]	8.267 ± 0.4153 [b]	0.0188
LDH (U/L)	2247 ± 169.2 [a]	2805 ± 102.3 [b]	0.0479

Means ± SE, [a, b] the least square mean with an arrow is lacking a common superscript differ ($p < 0.05$).

4. Discussion

Despite causing a considerable economic loss in the meat industry due to the condemnation of meat or carcass downgrading in slaughterhouses, limited data are available about the prevalence and risk factors associated with bovine cysticercosis in developing coun-

tries. Clearly, providing daily disease records of all slaughtered animals is mandatory [19]. Our study reports bovine cardiac cysticercosis with a prevalence rate of 10.8% among the examined animals. The prevalence of cysticercosis varies between the different regions of Egypt and some areas still have a higher prevalence [13]. Previous works [13,20] report a prevalence rate of 4.2% and 3.63% in cattle in Egypt, which are less than the current results. However, another previous study [21] agrees with the present results and reports a prevalence rate of 7.5% in Aswan city (Upper Egypt). *C. bovis* is considered endemic in many parts of Africa, particularly in areas with poor sanitation and inadequate meat inspection practices. The prevalence in cattle has been reported to be as high as 50% and 46.7% in Africa and Asia, respectively [22,23]. Nevertheless, *C. bovis* may be underestimated in many regions due to inadequate meat inspection practices, a lack of awareness among consumers and producers, and limited access to diagnostic tools [24]. The prevalence of *C. bovis* in slaughtered cattle has been reported to be 21.8%, 22.6%, 27.3%, and 26.2% in Nigeria, Uganda, Ethiopia, and Tanzania, respectively [2,25–27]. However, it should be underlined that the prevalence of *C. bovis* might vary in different regions of the world depending on various factors such as geographic location, cultural practices, and animal husbandry practices. Importantly, the prevalence of *C. bovis* in African countries might be higher than reported as many slaughterhouses lack proper meat inspection and hygiene practices. Moreover, studies on the prevalence of *C. bovis* often use different diagnostic methods and sampling techniques, making comparisons between studies difficult [26,27]. The studied area is considered one of Upper Egypt's governorates that have many rural areas where native breeds of cattle are reared. Before entering the slaughterhouses, most animals have been grazing in surrounding pastures, while imported breeds are reared through intensive farming breeding systems. It is important to note that the prevalence of cysticercosis may be influenced by various factors, including climate and animal husbandry practices [28]. Regarding seasonal variation, there is limited information available on the seasonal prevalence of *C. bovis* in Egypt. This study reveals a higher prevalence of *C. bovis* during hot weather (June–August) that might be attributed to the length of the grazing season and the proportion of grazed grass in the diet [28]. Taken into account, the onset of cardiac lesions after the ingestion of eggs on the pasture can be linked with the latency time. Furthermore, it is important to note that the exact reasons for the higher prevalence of bovine cardiac cysticercosis in the summer season may vary and could be influenced by factors such as temperature, farm management practices, and sampling methods [29]. There are many contradictions in seasonal variation between the previous studies [19]. The highest prevalence rate has been detected during summer and declines in spring and autumn, while the lowest prevalence rate is in winter and this result is consistent with our results [19]. It is important to note that the exact reasons for the higher prevalence of bovine cardiac cysticercosis in the summer season may vary and could be influenced by factors such as temperature, farm management practices, and sampling methods [29]. Other studies in Egypt report that winter season has the highest infection rate [30]. The variations in infection rate might be due to differences in temperature degree and humidity. Moreover, the decline in prevalence in urban cities can be attributed to the implementation of control measures such as the prohibition of feeding raw meat to animals.

Meat inspection plays a crucial role in preventing the transmission of *C. bovis* to humans through contaminated meat. In Egypt, the government has implemented strict regulations regarding meat inspection at different levels, including at the abattoirs and at the retail markets [13]. For the precise diagnosis of *C. bovis* through meat inspection, the viable cysticerci are more easily identified and confirmed by their morphological characteristics such as the slightly transparent smooth wall, invaginated scolex, and the translucent fluid filling it. These characteristics can be confirmed using SEM, TEM, and histopathology, while the judgment on the calcified (mineralized) cysts in the heart that are to be calcified as cases of *C. bovis* through macroscopic examination may overestimate the results of the prevalence rate of bovine cysticercosis due to confusion with other lesions. Moreover, the specific diagnosis in slaughterhouses during postmortem inspection is

very difficult and meat inspection has a low sensitivity [1]. Degenerating cysts are more frequently found during postmortem inspection than the viable cysts [12]. In the present study, degenerating cysts are the most prevalent (100 out of 102) among the encountered cysts. Taken into account, the demonstration of a scolex in a degenerate lesion is diagnostic of tapeworm infections; however, this is not always possible. In this respect, SEM could help in the visualization of some remnants of the parasite.

Bovine cysticercosis could be misdiagnosed with other macroscopic lesions, such as actinobacillosis [12] and sarcocystosis, which can be found in cattle carcasses [31]. Molecular diagnosis can be performed to discriminate between these diseases, but the expensive cost and limited financial resources remain a major barrier to performing these techniques at a large scale, particularly in developing countries. Clearly, confirmatory tests should be performed for certain diagnoses using histopathology and TEM which are helpful in the identification of the causative agent through the scolex, sucker, and tegumental layers. In the case of mineralized cysts, where there is no way to visualize the scolex or any tegumental parts, histopathological sections can aid in the diagnosis through the presence of granulomatous reactions containing multinucleated giant cells that indicate inflammation. Different pathogens contribute to the granuloma formation that is characterized by focal necrosis and calcification as well as mixed inflammatory cells [32,33]. However, in the current study, all inflammatory and the multinucleated giant cells are detected, suggesting cystic lesion and cysticercosis rather than other pathogens. The histopathological detection of bovine cysticercosis in cases of calcified cysts is undertaken through the detection of some microscopic, characteristic structures such as the calcareous corpuscles that are represented by small elliptical blank spaces [1]. Semi-thin sections of the viable cysts help in the identification of the parasitic tegumental layers that represent the basic components of other larval cestodes and the future adult cestode. The basic layers' components are microtriches, the vesicular (cellular) layer, and the reticular layer. As observed in the current study, the first layer is the microtrichia [34] and their function includes absorption, transportation, protection, and anchorage [35]. The second layer is the vesicular layer, which has the common characteristics of the distal cytoplasm layer of the cestode in addition to many dense inclusion bodies and lucent vacuoles, suggesting their secretory, excretory, or pinocytotic functions [34]. The tegumental layer contains many spindle-shaped tegument cells, suggesting their neurosecretory nature [36] and, possibly, that they absorb nutrients from the host in the described cysticerci [34]. The parenchyma, with its numerous inclusions, has a storage function and muscular contractions are probably responsible for the movement of fluids within the interstices between the parenchymal tissues. All the previous data help in the confirmation of the diagnosis and providing more descriptive information of *C. bovis.*

The present study demonstrates the localized response of the cardiac muscle around the parasite and the infiltration of the immune cells like macrophages, plasma, mast cells and lymphocytes in the areas around the *Cysticercus*. The severity of the histological lesions depends on the intensity of the inflammatory response [37]. The previous studies classify the lesions according to the severity of the immune response into different stages [38,39]. These stages start when the viable parasite is surrounded by a thin layer of the collagen fibers, then by the invasion of mononuclear inflammatory cells, and ending with the formation of granulomatous tissue with centers containing amorphous materials and the destructed parasite in a state of chronic infection. This occurs in agreement with the present results that show how some lesions that contain a layer of collagen fiber are infiltrated by the immune cells which are surrounded by the parasite and how other lesions had large number of the fibroblasts and dense layers of connective tissue. Furthermore, the current study uses immunohistochemical staining of Bax to demonstrate the apoptotic effect of the cysticercosis infection. The Bax protein is considered an apoptotic inducer, present in many tissues like hepatocyte, lymphocyte, and myocyte [40]. Apoptosis is considered the main defense action against intracellular infections [41]. The present study shows strong positive

staining within the cardiac muscle that surrounds the cyst and in the inflammatory cells infiltrating the cyst, which is consistent with several previous studies [40,41].

Additionally, cysticercosis has various effects on the physiological condition of the heart depending on the severity and location of the infection. One of the most common effects of cysticercosis on the heart is inflammation and damage to the heart muscle, which can lead to myocarditis and cardiomyopathy [42]. The effect of cysticercosis on the physiological condition of the heart can be significant and potentially life-threatening [43]. To ascertain the impact of cysticercosis on the physiological state of sthe cardiac muscle, we have investigated various physiological parameters, such as the measurement of albumin, total proteins, troponin T, TAC, MDA, and LDH plasma levels. The present study shows insignificant changes in total protein and albumin levels that agrees with the previous study [44] reporting no changes in total proteins in naturally and experimentally infected cattle with *C. bovis*. Another study [45] finds significantly lowered serum total protein ($p < 0.05$) in infected animals (buffalo/cattle) that is attributed to decreased levels of albumin in comparison with a non-infected group. Others reports reveal a significant decrease in total serum proteins ($p < 0.05$) while there are high levels of albumin and globulin in infected cattle compared to the non-infected group [46]. These differences in the magnitude of the antibody response could be correlated with the number of cysts found at slaughter and the intensity of the infection (light, moderate, or severe) [44].

Cardiac troponin is a sensitive biomarker for the determination of minimal myocardial damage due to various conditions including trauma, inflammation, exposure to necrosis, and toxins [47,48]. Myocardial dysfunction could be detected through the blood levels of cardiac biomarkers such as troponin T, creatine kinase, LDH, and myoglobin. The present work reveals that there is an increase in the LDH and troponin T plasma levels of infected cattle as a marker of the muscle, cell, and tissue damage associated with many diseases and conditions [49]. The increasing level of serum creatine kinase and LDH during bovine cysticercosis could be due to the necrosis of muscular tissue that is enhanced by muscle fiber integration due to inflammatory cell infiltration because of degenerated or active metacestodes [44].

In addition, we found that the infected animals with *C. bovis* experience an increase in MDA levels and a decrease in TAC levels in plasma, reflecting the oxidative stress. The antioxidant enzymes, including TAC, superoxide dismutase, glutathione peroxidase, and catalase, play indispensable and fundamental roles in the protective capacity of biological systems against free radicals. Our results agree with a previous study [50] and reveal a statistically significant increase in MDA levels as a biomarker of lipid peroxidation, while there is a significant decrease in superoxide dismutase, glutathione peroxidase, and catalase in cattle tissues infected with cysticercosis compared with control samples. Moreover, *C. fasciolaris* infection leads to oxidation and apoptosis in rat livers as detected through antioxidant enzymes that show marked decline in the level of glutathione-S-transferase and glutathione activity, leading to the generation of reactive oxygen species [51]. Our results point out the pathological damage of *C. bovis* which is consistent with the idea that cysticercosis causes an oxidative stress due to the imbalance between free radical induction and the ability of the body to counteract or detoxify their harmful effects via neutralization by antioxidants, resulting in inflammation by cytokine infiltrations with damages to the host cells and tissue.

5. Conclusions

The current study reveals a set of epidemiological, morphological, and histopathological findings about the occurrence of bovine cardiac cysticercosis in Upper Egypt. It is important to note that bovine cysticercosis remains a public health concern in Egypt and further efforts are needed to sustain the decline in prevalence and prevent its spread. However, more research is needed to determine the epidemiological trend of *C. bovis* prevalence in Egypt over the years and the factors that influence its prevalence combined with the biomarkers associated with the infection.

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Prevalence and Genetic Characterization of *Blastocystis* in Sheep and Pigs in Shanxi Province, North China: From a Public Health Perspective

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Simple Summary: *Blastocystis* is one of the most prevalent parasites, which can infect humans and many animal species worldwide, and the infection can result in public health problems and economic losses. Sheep and pigs are economically important animals in Shanxi province, north China; however, it is yet to be determined whether they are infected with *Blastocystis*. Thus, the present investigation was conducted to reveal the prevalence of *Blastocystis* in sheep and pigs in three representative counties in Shanxi province by examining 492 sheep feces and 362 pig feces using a molecular approach. The overall prevalence of *Blastocystis* in sheep and pigs were 16.26% and 14.09%, respectively. Five subtypes were found in sheep and pigs via DNA sequence analysis, of which ST5 was the dominant subtype in the three study counties. This study is the first to report the prevalence and subtypes of *Blastocystis* in sheep and pigs in Shanxi province. The findings not only extend the geographical distribution of *Blastocystis* but also provide baseline data for the prevention and control of *Blastocystis* infection in humans and animals in Shanxi province.

Abstract: *Blastocystis* is a common zoonotic intestinal protozoan and causes a series of gastrointestinal symptoms in humans and animals via the fecal–oral route, causing economic losses and posing public health problems. At present, the prevalence and genetic structure of *Blastocystis* in sheep and pigs in Shanxi province remains unknown. Thus, the present study collected 492 sheep fecal samples and 362 pig fecal samples from three representative counties in northern, central and southern Shanxi province for the detection of *Blastocystis* based on its SSU rRNA gene. The results showed that the overall prevalence of *Blastocystis* in the examined sheep and pigs were 16.26% and 14.09%, respectively. Sequences analyses showed that four known subtypes (ST5, ST10, ST14 and ST30) in sheep and two subtypes (ST1 and ST5) in pigs were detected in this study, with ST5 being the predominate subtype among the study areas. Phylogenetic analysis showed that the same subtypes were clustered into the same branch. This study reveals that sheep and pigs in Shanxi province are hosts for multiple *Blastocystis* subtypes, including the zoonotic subtypes (ST1 and ST5), posing a risk to public health. Baseline epidemiological data are provided that help in improving our understanding of the role of zoonotic subtypes in *Blastocystis* transmission.

Keywords: *Blastocystis*; subtype; prevalence; sheep; pig; Shanxi province

1. Introduction

Blastocystis is a common intestinal eukaryotic parasite that is frequently detected in feces in a variety of hosts, including mammals, reptiles and birds [1–4]. Up to now, one to two billion people worldwide have been infected with *Blastocystis* [5]. The prevalence of *Blastocystis* in most developing nations is higher than that in industrialized countries, which is highly related to socio-economic levels, sanitation infrastructures and geographical areas [6]. The infected hosts show different symptoms according to the host's susceptibility [7]. After infection, asymptomatic or very mild symptomatic infections occur in most individuals, whereas immunocompromised individuals commonly exhibit gastrointestinal symptoms, such as abdominal pain, vomiting, flatulence and urticaria [8,9]. In addition, some reports have shown that *Blastocystis* was a possible risk factor for anemia in pregnant women [10], and the key nutrients needed for pregnancy, such as iron, glucose, lipids, proteins and so on, produced competitive effects. This competition may have a devastating effect on the growth and development of the fetus, resulting in bleeding during pregnancy [11]. At present, the pathogenicity of *Blastocystis* is controversial, and increasing evidence demonstrates that this depends on the interaction with intestinal microbiota, infection subtypes and the host immune response [7]. Previous reports indicated that infectious cysts of *Blastocystis* could persist in the environment (water and soil) for a long period of time when they were shed with the feces, until infecting the next individual via the fecal–oral route [12–14]. The World Health Organization's drinking water quality publication mentions that *Blastocystis* is a water-related pathogen [15], indicating that *Blastocystis* has a significant impact on public health.

Different diagnostic methods for *Blastocystis* show different levels of sensitivity. Traditionally, detection of *Blastocystis* depends on light microscopy examination of fecal smears; however, various morphological forms of *Blastocystis* make diagnosis difficult [9,16]. Polymerase chain reaction (PCR) has been applied to detect the presence and subtype of *Blastocystis* based on the small subunit ribosomal RNA (SSU rRNA) gene, because a PCR-based method is more sensitive and specific than microscopic and immunological methods [17–19]. Previously, numerous epidemiologic studies on *Blastocystis* collectively reported that 30 valid subtypes (ST1–ST17, ST21, ST23–ST34) had been detected from humans and animals, of which ST1–4 contributed to over 95% of *Blastocystis* infections in humans [20–22]. ST5 was a frequently identified subtype in hoofed animals worldwide, including pigs and sheep, and considered as a potential zoonotic subtype because it was occasionally detected from farmers that had close contact with animals [23]. ST10 was commonly detected in livestock around the world and, recently, it has been detected in Senegalese school children and Thai adults [24,25]. In addition, enzootic subtypes (ST12, ST14, ST26, etc.) were commonly reported, and their zoonotic potential serves to be evaluated in the future when more data are available.

To date, *Blastocystis* has been detected in many animals worldwide [19,26]. Sheep and pigs are economically important animals in Shanxi province, north China, and they provide valuable meat and furs to the market. Infection of sheep and pigs with *Blastocystis* may reduce livestock performance and pose a zoonotic risk [27]. Thus, understanding the transmission characteristics of *Blastocystis* is of significance to local animal husbandry development and public health. At present, there are no data on *Blastocystis* infection in sheep and pigs in Shanxi province, except a report on alpacas [28]. This study reports the prevalence and subtypes of *Blastocystis* in sheep and pigs in Shanxi province for the first time, which contributes to understanding the prevalence, subtype distribution and public health implications of *Blastocystis* in China.

2. Materials and Methods

2.1. Sampling Sites

According to the China Statistical Yearbook—2020 (http://www.stats.gov.cn/sj/ndsj/2020/indexeh.htm, accessed on 25 September 2020) and the Shanxi Statistical Yearbook—2019 (http://tjj.shanxi.gov.cn/tjsj/tjnj/nj2019/zk/indexeh.htm, accessed on 25 September 2020),

fecal samples were randomly collected from sheep and pigs in three representative counties in Shanxi province, north China. In November 2020, a total of 854 fresh fecal samples (492 sheep feces and 362 pig feces) were collected from central, southern and northern Shanxi province, north China (Figure 1). All samples were directly collected from the rectum with a sterile swab to ensure no cross-contamination and placed in a tube labeled with information (area and age), then kept in a box at a low temperature. Next, all samples were transported to the Laboratory of Parasitic Diseases, College of Veterinary Medicine, Shanxi Agricultural University, and kept in a −20 °C freezer. Before DNA extraction, each sample was thawed at 4 °C.

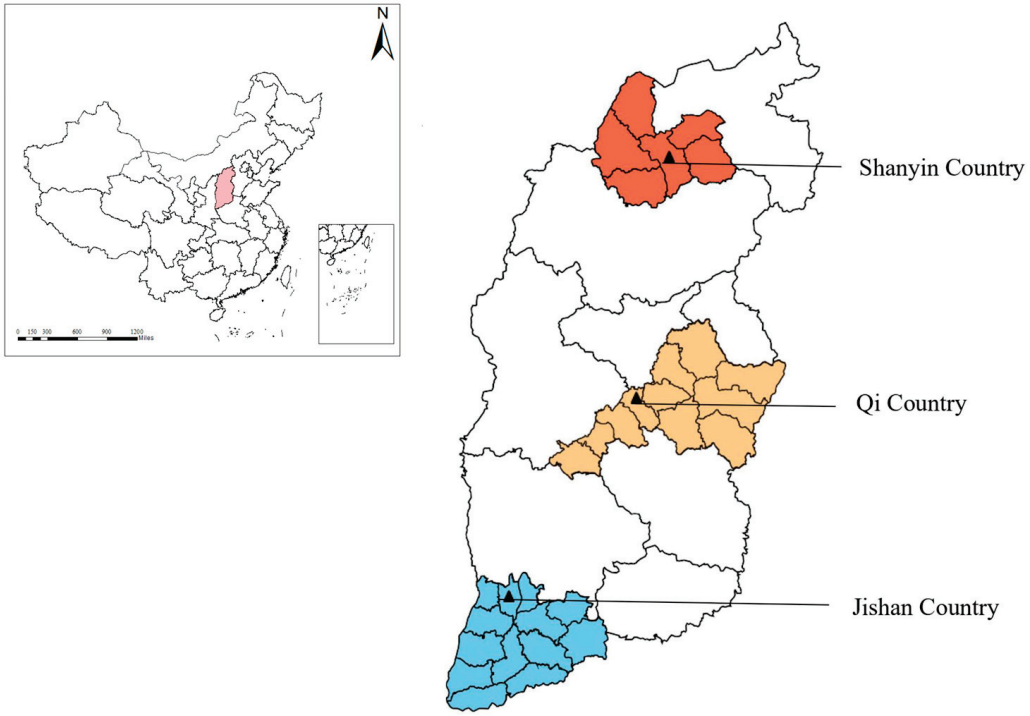

Figure 1. Sampling sites of sheep and pig feces in Shanxi province, north China. The map is based on the standard map service system of the Ministry of Natural Resources of China, drawing review number GS (2019) 1822.

2.2. DNA Extraction and PCR Amplification

The genomic DNA of each fecal sample was extracted using an E.Z.N.A.® Stool DNA Kit (Omega, Bio-Tek Inc., Winooski, VT, USA) according to the manufacturer's instructions, and stored at −20 °C until the PCR amplification. The *Blastocystis*-positive fecal samples of sheep and pigs were determined by PCR amplification of an ~600 bp fragment of the SSU rRNA gene. The primers BhrDR (5′-GAGCTTTTTAACTGCAACAACG-3′) and RD5 (5′-ATCTGGTTGATCCTGCCAGT-3′) were used in this study, as advised in a previous report [29]. Each 25 μL PCR mixture contained 14.75 μL of ddH_2O, 2.0 μL of dNTPs, 2.0 μL of $MgCl_2$, 2.5 μL of 10 × PCR buffer (Mg^{2+}free), 0.5 μL of each primer, 0.25 μL of *Ex*-Taq DNA polymerase (5 U/μL) and 2.5 μL of DNA template. The PCR conditions were as follows: an initial denaturation at 94 °C for 5 min; followed by 30 cycles denaturing at 94 °C for 1 min, annealing at 65 °C for 45 s and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. All PCR products were analyzed by electrophoresis in 1.5% agarose gels with ethidium bromide (EB), and the positive products were sent to Sangon Biotech Co., Ltd. (Shanghai, China) for bidirectional sequencing.

2.3. Sequencing and Phylogenetic Analysis

The obtained nucleotide sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi, ac-

cessed on 10 December 2022). The representative sequences of each subtype of *Blastocystis* detected in sheep and pigs in this study were deposited in GenBank under the accession numbers ON062964–ON062987 (sheep) and OM859017–OM859041 (pig), respectively. The phylogenetic analysis was conducted using the neighbor-joining (NJ) method, and Kimura two-parameter (K2P) genetic distances were also calculated using the software MEGA v7.0.26. A bootstrap with 1000 replicates was used to determine the support for the clades generated [28,30]. The outgroup was set to *Proteromonas lacertae* (U37108).

2.4. Statistical Analysis

In this study, a chi-squared ($\chi 2$) test was used to calculate the statistical difference between the prevalence of *Blastocystis* and risk factors (region and age) using the software SPSS 26.0 (IBM, Chicago, IL, USA). In addition, the odds ratio (OR) and 95% confidence interval (95% CI) were calculated to evaluate the correlation strength between prevalence and test conditions.

3. Results

3.1. Prevalence of Blastocystis in Sheep and Pigs

In this study, 80 out of 492 sheep fecal samples were detected as positive for *Blastocystis*, with an overall prevalence of 16.26% (80/492) (Table 1). The highest prevalence of *Blastocystis* in sheep was detected in Qi County (32.99%, 32/97), followed by Shanyin County (22.96%, 31/135) and Jishan County (6.54%, 17/260). Statistically significant difference in prevalence were found among the three areas ($\chi 2 = 42.439$, $p < 0.001$). However, the prevalence of *Blastocystis* detected in lamb aged less than 6 months (16.11%, 34/211) was only slightly less than that in older sheep aged more than 6 months (16.37%, 46/281); thus, no statistically significant difference was found between the two age groups ($\chi 2 = 0.003$, $p = 0.939$).

Table 1. Factors associated with prevalence of *Blastocystis* in sheep in Shanxi province.

Factor	Category	No. Tested	No. Positive	Prevalence% (95% CI)	OR (95% CI)	*p*-Value
Region	Qi County	97	32	32.99 (23.63–42.35)	7.04 (3.68–13.46)	$p < 0.001$
	Shanyin County	135	31	22.96 (15.87–30.06)	4.26 (2.26–8.04)	
	Jishan County	260	17	6.54 (3.53–9.54)	1	
Age	≤6 M	211	34	16.11 (11.15–21.07)	1	$p = 0.939$
	>6 M	281	46	16.37 (12.04–20.70)	1.02 (0.63–1.65)	
Total		492	80	16.26 (13.00–19.52)		

M: month.

Meanwhile, 51 out of 362 pig feces samples were successfully amplified and identified as *Blastocystis*-positive in the present study, and the overall *Blastocystis* prevalence in pigs was 14.09% (51/362) (Table 2). In the three study areas, the prevalence of *Blastocystis* in Jishan County was 19.67% (36/183), higher than that in Qi County (17.65%, 12/68) and Shanyin County (2.70%, 3/111). Among age groups, the prevalence of *Blastocystis* in pigs aged less than 6 months (18.57%, 44/237) was significantly higher than that in older pigs aged more than 6 months (5.60%, 7/125). Statistical analysis showed that the prevalence of *Blastocystis* was significantly different among regions ($\chi 2 = 17.31$, $p < 0.001$) and ages ($\chi 2 = 11.37$, $p < 0.001$).

Table 2. Factors associated with prevalence of *Blastocystis* in pigs in Shanxi province.

Factor	Category	No. Tested	No. Positive	Prevalence% (95% CI)	OR (95% CI)	*p*-Value
Region	Qi County	68	12	17.65 (8.59–26.71)	7.71 (2.09–28.47)	$p < 0.001$
	Shanyin County	111	3	2.70 (0–5.72)	1	
	Jishan County	183	36	19.67 (13.91–25.43)	8.82 (2.65–29.38)	
Age	≤6 M	237	44	18.57 (13.62–23.52)	3.84 (1.68–8.81)	$p < 0.001$
	>6 M	125	7	5.60 (1.57–9.63)	1	
Total		362	51	14.09 (10.50–17.67)		

M: month.

3.2. Subtype Distribution of Blastocystis in Sheep and Pigs

In order to better understand the correlation between prevalence and subtypes of *Blastocystis* in sheep and pigs in study areas, 80 *Blastocystis*-positive sheep samples and 51 *Blastocystis*-positive pig samples were further sequenced and analyzed. Among the 80 sheep-derived samples, four known subtypes (ST5, ST10, ST14 and ST30) of *Blastocystis* were identified (Table 3), with the most prevalent subtype being ST5 (n = 40). Notably, ST5 and ST10 subtypes were found in all of the three sampled areas, whereas ST14 was detected in Shanyin County and Jishan County, and ST30 was only detected in Shanyin County. Among the 51 pig-derived samples, two known subtypes (ST1 and ST5) were identified in all of the three study regions (Table 3), and ST5 (n = 47) was the predominant subtype.

Table 3. Prevalence and subtypes of *Blastocystis* in sheep and pigs in Shanxi province.

Host	Factor	Category	No. Positive/Tested	Prevalence (%)	Subtype (*n*)
Sheep	Region	Qi County	32/97	32.99	ST5 (31), ST10 (1)
		Shanyin County	31/135	22.96	ST5 (8), ST10 (11), ST14 (11), ST30 (1)
		Jishan County	17/260	6.54	ST5 (1), ST10 (13), ST14 (3)
		Subtotal	80/492	16.26	ST5 (40), ST10 (25), ST14 (14), ST30 (1)
Pig	Region	Qi	12/68	17.65	ST5 (11), ST1 (1)
		Shanyin	3/111	2.70	ST5 (2), ST1 (1)
		Jishan	36/183	19.67	ST5 (34), ST1 (2)
		Subtotal	51/362	14.09	ST5(47), ST1(4)
		Total	131/854	15.34	ST5 (87), ST10 (25), ST14 (14), ST1(4), ST30 (1)

ST: subtype.

3.3. Phylogenetic Analysis

The *Blastocystis* sequences obtained from sheep and pigs in this study corresponded to the sequences ST1, ST5, ST10, ST14 and ST30 obtained from the GenBank database, while the outgroup was in a single branch (Figure 2). We found that the corresponding ST5 sequence from pigs in the present study was highly similar to that of sheep isolates and human isolates. For example, 100% sequence homology was observed for *Blastocystis* ST5 between a pig isolate (OM859032) and sheep isolate (ON062966). In addition, the obtained sequence of the ST1 subtype (OM859039) from pigs was highly similar to that isolated from humans (AY618266).

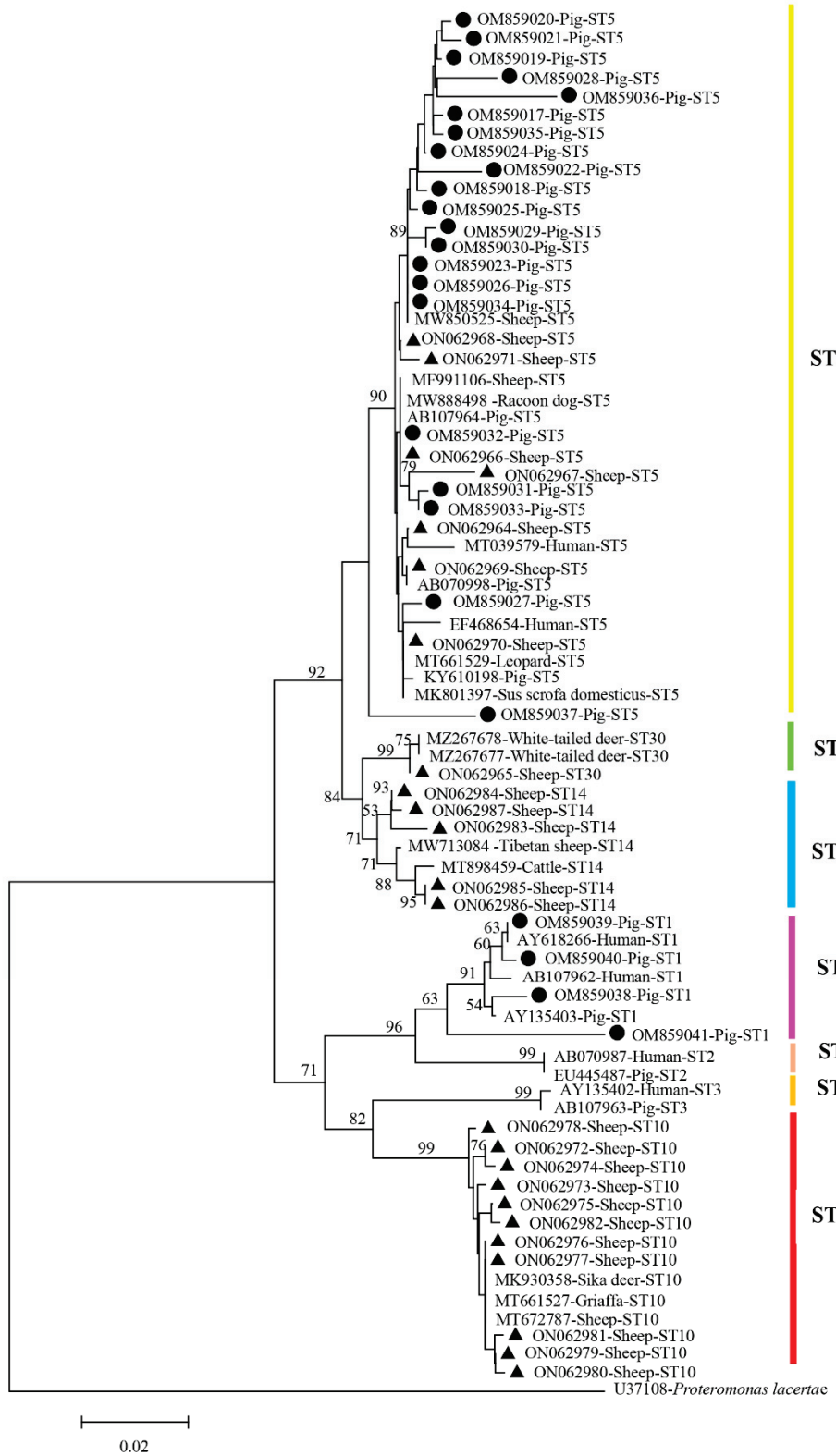

Figure 2. Phylogenetic relationships of *Blastocystis* subtypes based on SSU rRNA sequences. Obtained *Blastocystis* sequences from sheep and pigs in the present study are marked with black triangle (▲) and black circle (●), respectively. The bootstrap value < 50% is hidden.

4. Discussion

Blastocystis is an important microorganism, which has been frequently detected in fecal samples from humans and animals worldwide, and the shared *Blastocystis* STs between humans and animals indicate a potential risk of zoonotic transmission to humans [2]. In the present study, the *Blastocystis* prevalence in sheep and pigs in Shanxi province were 16.26% (80/492) and 14.09% (51/362), respectively. The *Blastocystis* prevalence in sheep in Shanxi province was lower than that in Turkey (38.2%, 84/220) [31], the United Arab Emirates (63.6%, 7/11) [32], Italy (81.8%, 9/11) [33], Brazil (33.3%, 1/3) [34], Iran (20.9%, 14/67) [35], and some provinces of China, such as Jiangsu province (24.00%, 18/75) and Shandong province (16.67%, 10/60) [36]. However, the 16.26% *Blastocystis* prevalence in sheep in Shanxi province was higher than that in Anhui province (6.4%, 22/697) and the Qinghai-Tibetan Plateau (8.55%, 53/620) [36,37]. For pigs, *Blastocystis* was highly prevalent worldwide, with a range of prevalence: 12.00% in Slovakia [38], 45.20% in Cambodia [39], 57.89% in Brazil [40], 76.40% in Thailand [41], 76.70% in Australia [39] and 22.89% to 74.80% in many provinces of China [42–44]. The differences in the prevalence of *Blastocystis* between different studies may be due to sample size, animal immune status and geographical environment [22,45].

In this study, there were significant differences in *Blastocystis* prevalence in both sheep and pigs among the sampled regions ($p < 0.001$). With regards to three regions, the highest *Blastocystis* prevalence in sheep was detected in Qi County (32.99%, 32/97), followed by Shanyin County (22.96%, 31/135) and Jishan County (6.54%, 17/260). The possible factor for high *Blastocystis* prevalence in Qi County may be its unique geographic location, because it is close to Taiyuan city, the capital of Shanxi province, which has a dense population and the largest transportation network, and high accessibility can easily lead to the spread of pathogens. In addition, different climates may also contribute to the different prevalence of *Blastocystis* in the three study areas. The *Blastocystis* prevalence in pigs in Jishan County was 19.67% (36/183), higher than that in Qi County (17.65%, 12/68) and Shanyin County (2.70%, 3/111), and the prevalence of *Blastocystis* in pigs gradually decreased with increasing latitude (Figure 1). We speculated that the mild and warm climate in the lower latitudes of Jishan County may contribute to the different prevalence of *Blastocystis* compared to the other study areas. The difference in *Blastocystis* prevalence between sheep and pigs in the same area may be due to differences in sampling size, managing differences and different husbandry patterns, as well as the different degrees of susceptibility of sheep and pigs to *Blastocystis* infection.

With regards to the age groups, there was no statistically significant difference in *Blastocystis* prevalence in sheep ($p = 0.939$); however, a statistically significant difference was found in pigs ($p < 0.001$). Our results were consistent with those previously reported in sheep and in pigs [36,46]. The age factor is considered an important factor influencing *Blastocystis* transmission among animals, but this point is still controversial [47]. A study reported that the higher *Blastocystis* prevalence in young pigs might be due to their imperfect immune system [46]. However, a high prevalence of *Blastocystis* was also found in older pigs in different studies [44,48]. Furthermore, host age had no significant effect on *Blastocystis* infection in a previous study [36]. Therefore, it is difficult to explain the discrepancies in *Blastocystis* prevalence between different studies.

The phylogenetic tree revealed that all five subtypes were clustered into their corresponding branches of subtypes. Among the four subtypes (ST5, ST10, ST14, ST30) and two subtypes (ST1, ST5) detected in sheep and pigs in this study, respectively, ST5 was the most prevalent subtype in both sheep (50.0%, 40/80) and pigs (92.2%, 47/51). ST5, a dominant subtype generally identified in hoofed animals [44], has also been detected in humans with close animal contact, highlighting its potential for zoonotic transmission to humans [23,49]. Previous reports indicated that ST10 and ST14 were only detected in ruminants [45,50,51]; however, both ST10 and ST14 were recently detected in Senegalese school children and Thai adults [24,25], suggesting that its transmission dynamics warrant further study. At present, the prevalence and subtype characterization of *Blastocystis* ST30

is still limited, which was only identified in sheep and camel in China since it was detected in deer in the USA for the first time [20,52,53]. The four zoonotic STs (ST1, ST5, ST10, ST14) obtained in this study highlight that both sheep and pigs may play a very prominent role in the transmission cycle of *Blastocystis* zoonotic subtypes.

Parasitic diseases increase the burden of infectious diseases all over the world to a great extent [54]. The transmission of parasites between humans and animals is an issue of public health and veterinary significance. Animals play a critical role in the One-Health Strategy with regards to the prevention and control of zoonotic diseases [55]. *Blastocystis*, as a ubiquitous zoonotic parasite with a worldwide distribution [5], will not only cause irritable bowel syndrome (IBS) but also compete with pregnant women for nutrient elements, resulting in bleeding during pregnancy [11]. Although it is still controversial as to the pathogenicity of *Blastocystis*, the clinical symptoms caused by *Blastocystis* infection still need to be treated. At present, metronidazole (MTZ) is the drug of choice for *Blastocystis* treatment, but it does not have a good power to improve gastrointestinal symptoms [56]. Therefore, prevention remains the top priority to control *Blastocystis* infection. Firstly, other studies have shown that intestinal protozoans can cause zoonotic diseases related to livestock and domestic pets (transmitted from animals to humans) [57], so people in close contact with animals need to take some necessary safety measures, such as hand hygiene and keeping distance with animals [25,39]. Secondly, it is necessary to optimize the breeding environment, especially in terms of fecal cleaning and drinking water safety. Since the fecal–oral route is the principal means of transmission for *Blastocystis* infection, improving the management of the feeding environment can reduce the risk of infection in the external environment. Finally, steps should be taken to increase the molecular epidemiological surveillance of *Blastocystis* in humans and animals with low immune function [58]. The above measures can effectively prevent and control the spread of *Blastocystis*, and then reduce the adverse effects of *Blastocystis* on public health.

5. Conclusions

This study reported the prevalence and subtypes of *Blastocystis* in sheep and pigs in Shanxi province for the first time. The prevalence of *Blastocystis* in sheep and pigs were 16.26% and 14.09%, respectively. Four zoonotic subtypes (ST1, ST5, ST10 and ST14) and one species-specificity subtype (ST30) were identified according to *Blastocystis* SSU rRNA sequences, indicating a potential zoonotic transmission risk to humans and other animals. The results of the present study indicate that measures should be taken to reduce the risk of *Blastocystis* infection in humans and animals.

Author Contributions: S.-C.X., X.-Q.Z. and W.-W.G. conceived and designed the study. C.-N.W., R.-L.Q. and Z.-H.Z. performed the experiments. C.-N.W. analyzed the data and wrote the manuscript. W.-B.Z., Q.L. and W.-W.G. participated in the collection of fecal samples and implementation of the study. S.-C.X. participated in data analysis. S.-C.X. and X.-Q.Z. critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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animals

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Article

Molecular Detection and Epidemiology of Potentially Zoonotic *Cryptosporidium* spp. and *Giardia duodenalis* in Wild Boar (*Sus scrofa*) from Eastern Spain

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Simple Summary: *Cryptosporidium* spp. and *Giardia duodenalis* are widely distributed pathogens in vertebrates. Both protozoa are among the major causes of diarrhoea in humans. Wild boars are known hosts of both parasites and are able to harbour zoonotic species. The main goal of this study was to molecularly evaluate the presence of *Cryptosporidium* spp. and *Giardia duodenalis* in faecal samples taken from hunted wild boar in eastern Spain. This area is experiencing a rapid increase in the wild boar population, which is colonising all habitats, including urban and peri-urban areas, thereby increasing interactions with humans. Both parasites were found in our study, evidencing a high prevalence, mainly of *Cryptosporidium scrofarum* and *Cryptosporidium suis*, which have been previously reported to affect humans. These results point out the potential for wild boar-human transmission because of close contact interactions, such as space sharing or dressing for meat consumption.

Abstract: The protozoans *Giardia duodenalis* and *Cryptosporidium* spp. are common causes of gastrointestinal disease in humans and animals. While both are commonly documented in domestic animals, few studies have analysed their presence in wildlife. To assess the prevalence of both parasites in wild boar (*Sus scrofa*) in the Valencian Community (eastern Spain), 498 wild boar faecal samples were collected from 2018 to 2022. *Cryptosporidium* spp. was detected by performing a nested PCR targeting a 578 bp sequence of the small subunit ribosomal RNA gene (SSU rRNA), followed by sequencing and phylogenetic analysis. For *G. duodenalis*, a qPCR amplifying a fragment of 62 bp from the SSU rRNA was employed. Positive samples were genotyped for glutamate dehydrogenase and β-giardin genes. Different epidemiological factors were considered potential modulating variables in the transmission of both parasites. *G. duodenalis* prevalence was 1.20%, while *Cryptosporidium* spp. prevalence reached 21.7%. Coinfection was observed in 0.2%. Genotyping of *G. duodenalis* isolates only detected genotype E. Two species of *Cryptosporidium* spp. were identified: *Cryptosporidium scrofarum* and *Cryptosporidium suis*. The results of this study demonstrate that the exposure to *Cryptosporidium* spp. in wild boars is high, particularly among young individuals belonging to the Typical Mediterranean climate. Moreover, the probability of infection is dependent on both the season and the density of wild boars. On the other side, exposure to G. duodenalis seems scarce and is influenced, in turn, by the climate. Both *Cryptosporidium* species detected in the present study have been reported in humans. Due to wild boar increasing in number and their colonisation of urban and peri-urban areas, this could represent an inherent health risk for the human population.

Keywords: *Cryptosporidium scrofarum*; *Cryptosporidium suis*; epidemiology; *Giardia duodenalis*; molecular characterisation; Spain; *Sus scrofa*; wild boar; zoonoses

1. Introduction

European wild boar (*Sus scrofa*) populations have experienced a continuous rise in the last 40 years throughout Europe [1]. The Valencian Community (eastern Spain) is not an exception, with a 159.7% hunting bag increase in the last 10 years, becoming the most popular big game species in the territory [2]. This situation has additional implications, as wild boar are known to carry a plethora of zoonotic pathogens [3–5] that can be transmitted to humans when close-contact situations arise, including protozoa. The increase in human–wildlife contacts due to the overlapping of wildlife habitats and human settlements [6,7], handling carcasses while meat dressing [8], and foodborne infections [9,10] are some of the most common scenarios where zoonotic transmission can occur.

Giardia and *Cryptosporidium* have been singled out as some of the most important zoonotic protozoa [6]. *G. duodenalis* is one of the most common enteric parasites in humans and domestic animals [7,8]. It is responsible annually for around 280 million cases of human diarrhoea worldwide [9,10], especially affecting children [11,12], and it infects more than 40 animal species [13], particularly in neonates [14]. *Cryptosporidium* spp. is the fifth most important foodborne pathogen, with more than 8 million cases being notified every year [15]. In human medicine, immune-compromised patients can develop a chronic disease, leading to a severe, sometimes fatal, outcome [15,16].

G. duodenalis and *Cryptosporidium* have other characteristics that make these parasites some of the most common causes of parasitic diarrhoea in humans [14,15]. The ability of *Cryptosporidium* spp. oocysts and *Giardia duodenalis* to resist conventional water treatments [12,13] and their very low infection dose [16] enhance their risk of transmission [16], as both are mainly foodborne and waterborne parasites. Only ten oocysts of *Cryptosporidium hominis* [17] or *Cryptosporidium parvum* [18,19] or ten cysts of *G. duodenalis* [20] are enough to promote symptomatic disease in healthy human adults. Additionally, there is a lack of effective treatment against *Cryptosporidium* spp. [15,21–23], and *Cryptosporidium*'s high environmental resistance is considered a key factor in its transmission [24].

From the eight different assemblages found for *G. duodenalis* (A to H), only A and B have been identified as zoonotic, and both are found in wild boars [25,26]. Among the different species of *Cryptosporidium* described to date, *C. suis* and *C. scrofarum* (formerly known as Crypto pig genotype II [27]) are the most commonly reported from wild boars [28–30]. Both species have been detected in human beings, indicating their zoonotic potential [31–34]. Moreover, swine could act as the origin of contamination of human water and food supplies, as both cryptosporidia species have been found in untreated water [35].

Therefore, given the importance these two protozoa have within the field of Public Health and that both parasites can be found in the ever-increasing population of wild boar, the main aim of this study was to determine the prevalence of both parasites in the wild boar population of the Valencian Community (eastern Spain). Additionally, this study aimed to evaluate the potential effects of climatic, human-related, and host-related factors in their epidemiology.

2. Materials and Methods

2.1. Study Area and Wild Boar Sampling

The Valencian Community is an autonomous region in eastern Spain. The territory is divided into 31 counties, all of which have a confirmed wild boar presence [2]. Sampling was conducted in areas where hunting activity is allowed (on both private and public land), comprising 1,916,454.75 ha (82.4% of the total territory) [36].

Sampling size (*N*) for each county was calculated based on the expected prevalence of wild boar [37–39] over the declared hunting bags [2] using *WinEpi 2.0 Software* [40]

(Table 1). Three counties were discarded because the number of wild boars to sample was <1 specimen. Sampling periods involved four game seasons (from October to February) during the years 2018/19 to 2021/22. The total number of collected samples was 498, most of them from hunting events (N = 494), while road kills (N = 2) and found dead individuals (N = 2) were anecdotal. Additional information about the sampled wild boars (location, sex, age, weight, and pregnancy in females) was recorded. Sex was established by observing the genitalia, and age was calculated by dental eruption patterns [41,42].

Table 1. Description of the sampling area and achieved samples. Wild boar population determined the minimum sampling size. Dimensions, climatic characteristics, and human influence (human density, use of the land, and fertilisation with slurry) in the given counties are shown.

County	Climate	Human Density	Land Use	Slurry	Hunting Area (ha)	Wild Boar Hunting Bag	Wild Boar Density	Minimum Sample Size	Achieved Sample Size
Alto Mijares	CM	R	F	1	53,261.19	857	H	6.7	12
BaixMaestrat	TM	R	F-I	1	104,635.04	1480	H	11.6	15
Els Ports	CM	R	F	1	79,599.80	731	S	5.8	33
L'Alcalatén	CM	R	F-I	0	62,539.34	712	H	5.6	7
Alt Maestrat	CM	R	F	1	64,532.02	518	S	4.1	16
Plana Alta	TM	I	F-I	1	74,611.73	1283	H	10.1	38
Plana Baixa	TM	I	I	0	47,337.87	544	H	4.3	9
Alto Palancia	CM	R	F-I	0	87,353.56	1292	H	10.2	13
TOTAL CASTELLÓN					**573,870.59**	**7416**		**58.4**	**143**
Hoya de Buñol	TM	R	I	0	74,865.52	913	H	7.2	10
Ribera Alta	TM	I	I	1	80,757.26	1021	H	8.0	7
Camp de Morvedre	TM	I	I	0	19,424.64	317	H	2.5	4
Camp de Turia	TM	I	F-I	1	64,412.67	518	S	4.1	19
Rincón de Ademuz	CM	R	F	0	36,663.14	490	H	3.9	2
Valle de Cofrentes-Ayora	TM	R	F	1	123,981.80	2765	H	21.8	70
L'Horta	TM	U	I	0	15,956.24	95	S	0.7	1
Canal de Navarrés	TM	R	F	0	55,325.44	577	H	4.5	9
La Costera	TM	I	F-R	0	45,618.45	1216	E	9.6	13
La Plana de Utiel-Requena	TM	R	R	1	162,835.77	2799	H	22	19
Ribera Baixa	TM	I	I	-	14,854.79	87	S	0.7	-
La Safor	TM	I	I	0	30,855.04	2399	E	11	27
La Valld'Albaida	TM	I	F-R	1	68,677.48	916	H	7.2	12
Los Serranos	CM	R	F	1	144,613.83	1393	H	11	10
TOTAL VALENCIA					**938,842.14**	**14,505**		**114.1**	**203**
Baix Segura	DM	I	R	-	40,695.07	149	S	1.2	-
BaixVinalopó	DM	U	R	-	18,503.75	62	S	0.5	-
El Comtat	TM	R	F	0	35,521.49	878	E	6.9	50
VinalopóMitjà	DM	I	R	0	58,736.34	894	H	7	9
L'Alacantí	DM	U	R	0	39,683.17	349	S	2.7	6
L'Alcoià	TM	I	F-R	1	46,424.67	1099	H	8.6	14
L'AltVinalopó	DM	R	R	1	56,473.58	739	H	5.8	31
Marina Alta	TM	I	F-I	0	56,811.46	2034	E	16	22
Marina Baixa	TM	I	F	0	43,580.76	975	H	7.7	20
TOTAL ALICANTE					**396,430.34**	**7179**		**56.5**	**152**
TOTAL VC					**1,909,143.08**	**29,100**		**229**	**498**

Abbreviation key: **Climate:** CM: Continental Mediterranean; DM: Dry Mediterranean; TM: Typical Mediterranean. **Human Density:** R: Rural; I: Intermediate; U: Urban. **Land use:** F: Forested; I: Irrigated; R: Rainfed. **Slurry**: 1: yes; 0: no. **Wild boar density:** S: Sustainable; H: High; E: Extreme. **VC**: The Valencian Community.

Refrigerated faecal samples were transported to the Veterinary Faculty CEU-UCH, Alfara del Patriarca (Valencia), where they were kept at 4 °C and processed within 24 h post-collection [37]. After DNA extraction, samples were labelled and stored at −18 °C until further molecular analysis.

2.2. Environmental and Population Data Collection

2.2.1. Climate, Rainfall Regime, and Seasonality

According to Köppen–Geiger's classification [43], the territory can be subdivided into three variants of the Mediterranean climate (Table 1). The Typical Mediterranean (also known as Csa) has moderate winters with rare snowfalls, while summers are dry and hot, with temperatures above 30 °C. The Continental Mediterranean climate (Csa-Bsk) has frequent snowfalls during the winter but experiences long, dry summers, even reaching 40 °C. The Dry Mediterranean (Bsh-Bsk) is located in the southernmost area of the territory, where winters are mild (around 10 °C) and the maximum temperature in summer is above 30 °C.

The rainfall regime is concentrated in spring and autumn, usually in a few days with heavy rains [44,45], while drought is common during the summer. The maximum annual rainfall is 800 mm, commonly seen in some coastal mountain ranges and in the northwestern extremity of the territory. On the southern end, where the climate is dry, the annual rainfall value is around 300 mm, which is the minimum annual rainfall value of the Valencian Community [45]. To analyse the effect of the climate on parasite presence, the sampled counties were clustered according to the bioclimatic area they mainly belong to and the average rainfall of the sampled municipalities [46].

Sampling dates were registered in order to classify the samples according to the astronomical-meteorological seasons in the Northern Hemisphere (spring includes samples taken from mid-March to mid-June, summer from mid-June to mid-September, autumn from mid-September to mid-December and winter from mid-December to mid-March) [47].

2.2.2. Land Use

Land use allows us to classify the territory into urbanised, irrigated cropland (mainly citrus production and growing vegetables), rain-fed crops (olive, carob, and almond trees), and forest land (almost 60% of the total) [36]. The origin of the animals was registered at the municipal level to know the possible effect of land use on parasite prevalence.

Crop fertilisation is a common way to dispose of the slurry from pig farms, which can carry *Giardia* sp. and *Cryptosporidium* sp. infective cysts. But transport costs limit the use to a close buffer from the origin [48]. Counties were classified according to the presence or absence of this practice.

2.2.3. Wild Boar Population

The geographic information about the origin of the samples allowed us to compare the effect of wild boar population density in the above-mentioned parasites. The territory was classified into three categories based on hunting bags: sustainable density with 0.3–1 wild boar/km^2; high density from 1.1 to 3 wild boar/km^2; and extreme density, 3.1–6.7 wild boar/km^2, in agreement with the classification made by local authorities [2]. Age groups were established in piglets ($\leq$6 months old (m.o.), striped coat), juveniles (7 to 12 m.o., reddish colour), sub-adults (12 to 18 m.o.), and adults ($\geq$18 m.o.) by dental eruption and coat colour patterns [49,50].

2.2.4. Human Population

To evaluate the potential relationship between wild boar positivity and a possible anthropogenic disturbance, the territory was classified according to human density. Counties were categorised as rural (<100 inhabitants/km^2), intermediate (100–499 inh/km^2), and urban (>500 inh/km^2) [51] (Table 1).

2.3. DNA Extraction

DNA extraction was performed within 24 h after sampling using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

2.4. Molecular Detection and Characterisation of Giardia duodenalis and Cryptosporidium spp.

Samples were tested for *G. duodenalis* presence with a real-time PCR (qPCR) protocol, amplifying a 62 bp segment of the small subunit ribosomal RNA (SSU rRNA) gene of the parasite [52]. Briefly, 3 μL of template DNA were used in a total volume reaction of 25 μL [12.5 pmol of each primer (Gd-80F/Gd-1278R), 10 pmol of the probe, and 12.5 μL of NZY Supreme qPCR Probe Master Mix (Nzytech genes and enzymes, Lisbon, Portugal)]. The detection of parasitic DNA was performed on an AriaMx real-time PCR (qPCR) instrument (Agilent Technologies, Santa Clara, CA, USA) following the already described amplification protocol [52,53]. A negative control without a template DNA and a positive control (*G. duodenalis* genotype C isolated from an infected dog) were used in each reaction.

After qPCR, positive samples were further subjected to semi-nested PCRs for glutamate dehydrogenase (*gdh*) [54] and β-giardin (*bg*) [55] specific parasite genes.

The presence of *Cryptosporidium* spp. was assessed by means of a nested PCR amplifying a 578 bp fragment from the SSU rRNA gene [56]. A total of 3 μL of DNA samples were used in a 25 μL amplification reaction, containing 12.5 pmol of each primer pair (18SicF2/18SicR2 and 18SicF1/18SicR1). Both PCR amplification reactions were carried out in a thermal cycler GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) [54]. All the PCR conducted included negative and positive controls, the latter being DNA from *Cryptosporidium ubiquitum*-positive farmed lamb. Products of positive samples with a band of the expected size were visualised on 1.5% agarose gel pre-stained with RedSafe TM Nucleic Acid Staining Solution (iNtRON Biotechnology, Seongnam, Republic of Korea).

2.5. Sequencing and Phylogenetic Analysis

Positive samples were sequenced by an external sequencing service (Genomics Department at Principe Felipe Research Centre, Valencia, Spain). The quality control and assembly of chromatograms were conducted using Chromas version 2.6.6 (Technelsyum DNA Sequencing Software, South Brisbane, QLD, Australia). The resulting sequences were blasted against *Cryptosporidium* spp. and *G. duodenalis* sequences available in the NCBI GenBank database using the online BLAST tool (http://blast.ncbi.nlm.nih.gov/blast, accessed on 15 January 2023). A phylogenetic analysis was applied to the *Cryptosporidium* spp. SSU rRNA partial sequences were obtained, which exhibited <100% identity with the closest reference sequence, using the MEGA X 10.1 [57]. The partial sequences were aligned with selected *Cryptosporidium* species sequences retrieved from Genbank, distance matrices were calculated, and the phylogenetic tree was inferred by the Maximum Likelihood (ML) method (bootstrap test on 1000 replicates). *Cryptosporidium* spp. partial sequences obtained were deposited in GenBank under the accession numbers OR030357–OR030362 and OR030363–OR030373.

2.6. Data Analysis

Statistical analysis was performed using the R programme and RStudio Version 4.1.0 [58]. From the original database containing 498 individuals, 24 wild boars were excluded because of incomplete data. Therefore, the final analysis was carried out using a total of 474 individuals with complete datasets. To assess the correlation between explanatory variables, different types of statistical tests were used depending on the nature of the data. Briefly, Cramer's V index was used when both explanatory variables were categorical; Pearson's product–moment correlation (Pearson's correlation coefficient) was applied when both explanatory variables were numerical; and the Kruskall–Wallis rank sum test was used to assess the correlation between numerical and categorical variables.

Data regarding the positive status of *Giardia duodenalis* or *Cryptosporidium* spp. were analysed separately and by applying different statistical models due to the difference in the number of positive animals for *Giardia duodenalis* (5/474) and *Cryptosporidium* spp. (109/474).

2.6.1. *Giardia duodenalis*

Binary logistic regression was used to examine differences in the presence or absence of *Giardia duodenalis*. A backward stepwise model selection process was applied to select the most parsimonious model, using Akaike's criteria (AIC) as the election factor. The variables included in the saturated models were sex, the interaction between age category and wild boar density, the type of human population, climate, slurry, and the sampling season. The backward stepwise selection was performed using the "step AIC" function that can be found within the "MASS" R 7.3-60 package [59]. McFadden R2 was calculated using the "pscl" package from R [60] for each final chosen model to assess model fitting. The importance of each predictor variable in the final model was assessed using the varImp function from the "caret" package [61]. Finally, the Variance Inflation Factor (VIF) was calculated for each final model to test for the presence of multi-collinearity, also using the "caret" package [61].

2.6.2. *Cryptosporidium* spp.

A Classification and Regression Tree (CART) model was applied to determine the contribution of the following explanatory variables in testing positive for *Cryptosporidium* sp.: sex, age category, wild boar density, climate, use of nitrogen as fertiliser, and the sampling season. As the variable response is binomial (positive/negative), a classification tree was built to fit the data. Two of the main problems faced by this type of model are finding good data splits and data overfitting [62]. In our analysis, the information gain criteria were applied to determine the best split, and the complexity parameter was used to prune the tree and thus avoid data overfitting. Assessment of model reliability was performed by calculating the prediction error rate (accuracy test). The "rpart" library [63] was used to fit the classification tree, and its graphical representation was conducted using the "rpart.plot" library [64].

3. Results

The total number of wild boars sampled was 498. The prevalence found for *G. duodenalis* and *Cryptosporidium* spp. was diverse depending on the different study variables, which were considered possible modulators for the transmission of both parasites (Table 2).

Table 2. Prevalence of infection by *Giardia duodenalis*, *Cryptosporidium suis*, and *Cryptosporidium scrofarum* in wild boars (*Sus scrofa*) in relation to the studied variables.

		Giardia duodenalis	***Cryptosporidium suis***	***Cryptosporidium scrofarum***
Climate	CM	4.3% (4/93)	1.1% (1/93)	23.7% (22/93)
	TM	0.6% (2/359)	3.3% (12/359)	19.5% (70/359)
	DM	0% (0/46)	0% (0/46)	8.7% (4/46)
Season	Summer	3.22% (1/31)	0% (0/31)	6.5% (2/31)
	Autumn	0.5% (1/209)	1% (2/209)	22.9% (48/209)
	Winter	0.5% (1/195)	3.6% (7/195)	18.5% (36/195)
	Spring	0% (0/63)	4.8% (3/63)	12.7% (8/63)
Land use	Forested land	1.3% (4/307)	2.3% (7/307)	20.1% (64/307)
	Irrigated land	0.9% (1/108)	4.6% (5/108)	18.5% (20/108)
	Rainfed land	1.2% (1/83)	1.2% (1/83)	14.5% (12/83)
Human density	Rural	1.7% (5/297)	8.4% (7/83)	65.1% (54/83)
	Intermediate	0.5% (1/194)	3.1% (6/194)	21.6% (42/194)
	Urban	0% (0/7)	0% (0/7)	0% (0/7)
Wild boar density	Sustainable	2.7% (2/75)	0% (0/75)	18.7% (14/75)
	High	0.9% (3/311)	3.9% (12/311)	21.5% (67/311)
	Extreme	0.9% (1/112)	0.9% (1/112)	13.4% (15/112)

Table 2. *Cont.*

		Giardia duodenalis	*Cryptosporidium suis*	*Cryptosporidium scrofarum*
Age group	Piglet	0% (0/30)	6.7% (2/30)	30% (9/30)
	Juvenile	0% (0/83)	2.4% (2/83)	31.3% (26/83)
	Sub-adult	2.7% (1/37)	0% (0/37)	13.5% (5/37)
	Adult	1.2% (4/324)	3.1% (10/324)	15.1% (49/324)
	Unknown	4.2% (1/24)	0% (0/24)	20.8% (5/24)
Sex	Male	1.3% (3/244)	3.6% (8/244)	19.6% (44/244)
	Female	0.8% (2/237)	2.5% (6/237)	19.8% (47/237)
	Unknown	5.9% (1/17)	0% (0/17)	17.6% (3/17)

3.1. Giardia duodenalis

The general prevalence was 1.2% (6/498; CI 95% 0.3–2.2%). The median values of the generated cycle threshold (Ct) were 34.7 (range: 25.9−39.8). Genotyping was successful in only one sample by using the gdh gene, resulting in the assemblage E, typical of the artiodactyl order [65].

After assessing the correlation between explanatory variables, those selected for the model were climate (in order to consider vegetation cover and environmental humidity) as a factor directly related to the cyst's survival [66]; fertilisation with slurry, an indicative value of potential cross-contamination from pig farms [67]; and, finally, the season in relation to temporary environmental conditions.

The simplest model able to explain the maximum variability of the data, according to backward selection and AIC, included only the variable climate (Table 3).

Table 3. Model results for *G. duodenalis*. Reference category: Continental Mediterranean.

Factor	Estimate	S.E.	*p*-Value
Dry Mediterranean	−17.48	2672.95	0.99
Typical Mediterranean	−2.74	1.12	0.015

Observed prevalences for the Continental Mediterranean, Typical Mediterranean, and Dry Mediterranean were 4.3% (4/93; CI 95% 0.2–8.4%), 0.6% (2/359; CI 95% 0.0–1.3%), and 0% (0/46; CI 95% 0.0–6.3%), respectively. There are significant differences (p-value = 0.015) between Continental and Typical Mediterranean climates; no other significant differences among climates were detected (Table 3 and Figure 1).

3.2. Cryptosporidium spp.

The general prevalence of *Cryptosporidium* was 21.7% (108/498; CI 95% 18.1–25.3%). BLAST results revealed two *Cryptosporidium* species present, namely *C. suis* (2.8%; 14/498; CI 95% 1.4–4.3%) and *C. scrofarum* (18.9%; 94/498; CI 95% 15.5–22.3%) (Table 4). The phylogenetic reconstruction supports BLAST results. The 11 identified *C. scrofarum* isolates clustered with the reference sequence MT071828, while the six *C. suis* isolates formed another cluster with the reference sequences MT071826 and KX668209. Despite the relatively small size of the sequenced regions, the overall topology of the tree is consistent with the known topology for the *Cryptosporidium* genus (Figure 2).

Coinfections between both species could not be assessed with the deployed methods. One mixed infection among *G. duodenalis* and *C. scrofarum* was detected (0.2%, CI 95% 0.00–0.591%).

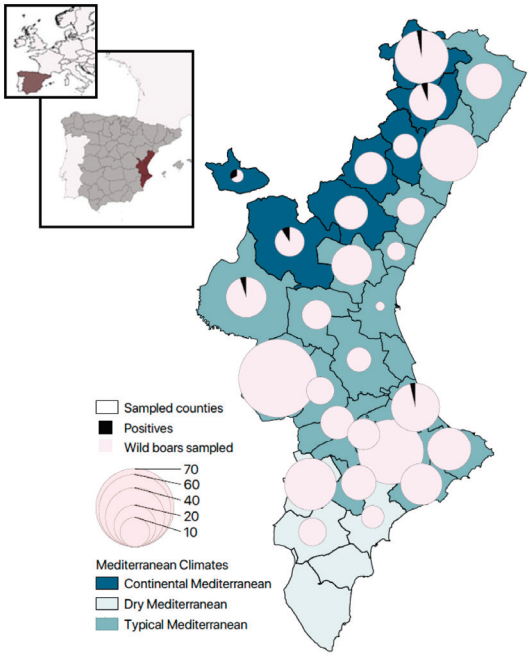

Figure 1. Insert: Location of the Valencian Community and Spain in relation to the European continent. Main map: Relationship of positives to *G. duodenalis* over the total sampled animals per county, classified according to the climate. Number of positive animals is shown in the map as the total number of wild boars (*n*) that were positive for *G. duodenalis*/total number of wild boars sampled in the county.

Table 4. *Cryptosporidium* spp. isolates are classified per species according to the closest reference sequence.

Species	N	Reference Sequence	% ID	SNV	Accession Number
C. suis	6	MT071826	100	None	OR030357–OR030362
	3	MT071826	99.52–99.62	InsT; T → A	
	3	KX668209	99.78–99.81	DelA	
	1	MT561508	100	None	
C. scrofarum	82	MT071828	100	None	OR030363–OR030373
	5	MT071828	99.78–99.8	T → Y	
	4	MT071828	99.8–99.81	T → C	
	1	MT071828	99.8	A → R	
	1	MT071828	99.56	C → T; T → C	

ID: identity; SNV: single nucleotide variant; Ins: insertion; Del: deletion.

The most parsimonious tree model to predict the probability of testing positive for *Cryptosporidium* spp. ($p = 0.22$) was fitted using four variables (i.e., age, climate, season, and wild boar density) (Figure 3). Independently of climatic conditions, seasonality, and wild boar density, piglets and juveniles have higher probabilities ($p = 0.35$) of testing positive than adults and sub-adults ($p = 0.18$). Within piglets and juveniles, those living in areas with a Typical Mediterranean climate (TM) have a higher probability of being positive ($p = 0.41$) than those living in Continental and Dry Mediterranean counties ($p = 0.15$). In turn, in areas with TM, the infection probability for piglets and sub-adults depends on the season, being higher in winter ($p = 0.50$). During the other seasons, infection probability is dependent on wild boar density (i.e., higher probability in counties with extreme density) (Figure 4).

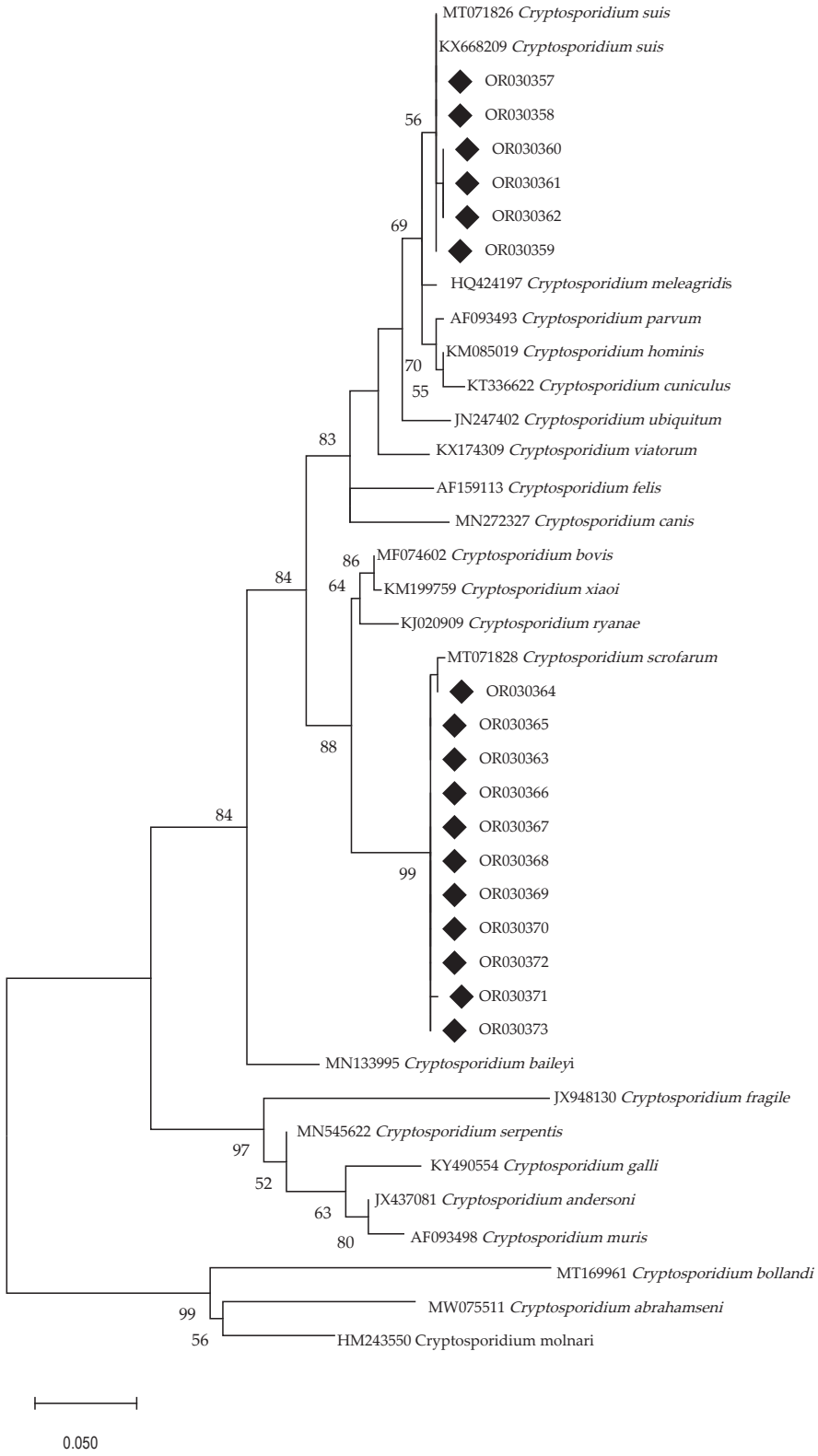

Figure 2. Phylogenetic relationships for *Cryptosporidium* SSU rRNA partial sequences from the present study (◆). The model was inferred by the Maximum Likelihood (ML) method based on the T92+G substitution model. The tree with the highest likelihood (−1859.60) is shown. The percentage support (>50%) for each cluster is indicated at the left of the supported node. The tree is at scale, with the scale bars referring to the phylogenetic distance expressed in nucleotide substitutions per site.

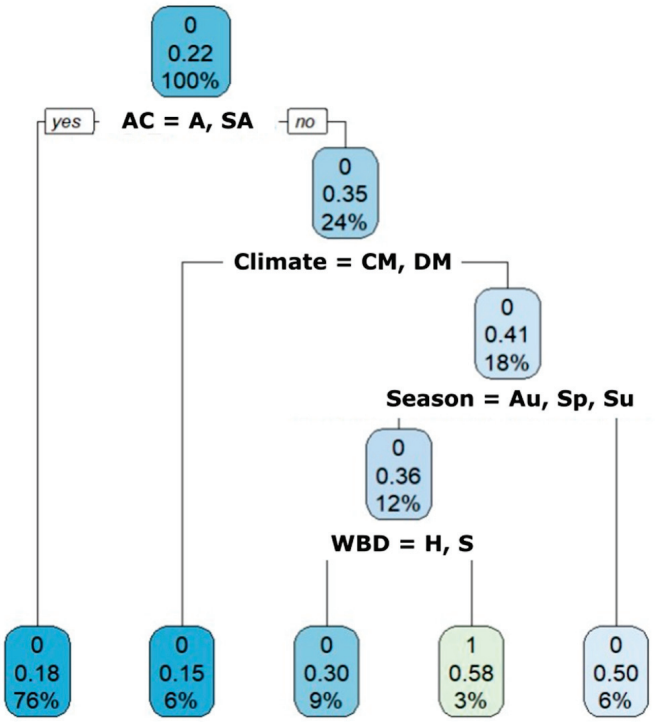

Figure 3. Tree-based classification modelling evaluates the relevance of different risk factors related to *Cryptosporidium* spp. infection. Abbreviation key: **AC**: Age Category. A: Adult (>24 m); SA (12–24 m): Sub-adult; *p* (0–6 m): Piglet; J (6–12 m): Juvenile. **Climates**: Continental Mediterranean (CM), Dry Mediterranean (DM), Typical Mediterranean (TM). **Seasons**: Au: Autumn; Sp: Spring; Su: Summer; Wi: Winter. **WBD**: Wild Boar Density. H: High; S: sustainable; E: extreme.

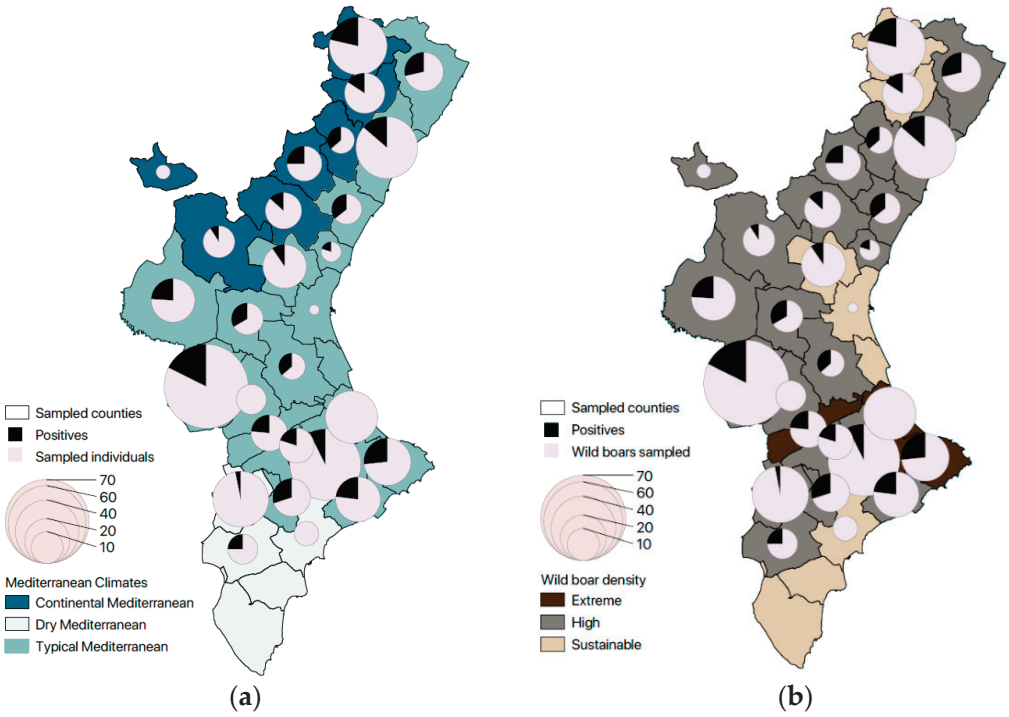

Figure 4. Relation of positives to *Cryptosporidium* spp. over the total sampled animals per county, (**a**) classified according to the climate and (**b**) according to wild boar density.

4. Discussion

To the authors' knowledge, this is the first time that the presence of *Giardia duodenalis* and *Cryptosporidium* spp. in wild boar has been comprehensively studied in the Valencian Community (eastern Spain). We detected a high prevalence of *Cryptosporidium* spp. and a low prevalence of *G. duodenalis*. Moreover, the role of different epidemiological variables has been assessed, identifying potential risk factors in *Cryptosporidium* spp. transmission.

There are great differences in the prevalence of *Giardia duodenalis* (1.2%; 4/498) and *Cryptosporidium* spp. (21.7%; 108/498). Both parasites have simple life cycles and low infective doses (only 10 (oo)cysts [19,20]). Thus, it is crucial to study host- or environmental-related factors that can explain the differences in the observed prevalence. Among them, climate seems to be the most plausible, comparing the relative resilience of *Cryptosporidium* spp. oocysts and the vulnerability of *Giardia duodenalis* cysts to some environmental stressors, essentially dryness and UV radiation exposure [66,68,69].

The overall observed prevalence for *G. duodenalis* is similar to other results reached by previous studies conducted in the Iberian Peninsula (1.3% in Galicia or 0% in Portugal) [37,70]. A slightly higher prevalence (i.e., 5.6%) has been detected in a recent study that includes samples from the different bioregions of Spain, probably because this study encompasses regions with high climatic diversity [71]. The highest prevalence in the Iberian Peninsula was detected in Cordoba (southern Spain), where the positives reached 22.5% [39]. In the European context, previous studies have found a similar prevalence (1–4%) to our results [72,73]. Generally, the differences in *G. duodenalis* prevalence between studies could mainly be related to climatic conditions as well as sampling data (due to variations in the temperature and rainfall regime). In accordance with this argument, in our dataset, the climate is the most relevant variable related to *Giardia* prevalence (Table 3), with significant differences observed between Continental Mediterranean and Typical Mediterranean climates. Higher prevalence was found in counties with CM climates, probably related to the pluviometric regime (as high as 800 mm) that enables the growth of denser vegetation cover. Under these conditions, *G. duodenalis* cysts, which are highly susceptible to drying, extreme temperatures, and UV radiation [73], are more likely to survive. It is known that *G. duodenalis* cysts remain infective for several months in humid and fresh areas, enabling rapid accumulation in the environment [74]. Another key factor in *G. duodenalis* infectiveness, closely influenced by the pluviometric regime, is the presence of water points such as ponds and dams. In these water sources, cysts survive up to 56 days from 0 °C to 7 °C or up to 28 days at 17–20 °C. The survival period is even more prolonged in rivers (84 days at 0–4 °C and 28 days between 20 and 28 °C) [74]. It is worth noting that rivers can constitute a potential point of pathogen transmission, as both wildlife and humans make use of them.

However, due to the very low prevalence of *G. duodenalis* found in the sampled populations of wild boar, statistical results must be interpreted with caution.

Only one of the positive samples (1/6) was successfully genotyped with glutamate dehydrogenase (*gdh*) and was found to be assemblage E. None of the positive samples could be genotyped with beta-giardin (*bg*). Similar amplification rates have been observed in wild mammals in previous studies [39,71,75–77], in contrast to other groups, like humans or birds [78,79], with higher rates of success. Artiodactyls are a host type for assemblage E [65]. However, this assemblage has been occasionally detected in humans from Europe [80] and developing countries [81]. Therefore, some authors consider it zoonotic [80].

The general prevalence of *Cryptosporidium* spp. (21.7%) is higher in comparison to previous studies carried out in northwestern and southern Spain (6–8%) or in Portugal (1.4%) [30,37,39,70,75]. Local climatic conditions in eastern Spain (mild temperatures and the regulating effect of the Mediterranean Sea) lengthen the survival of the oocysts [74], thus enhancing the risk of transmission. Wild boar populations have been surging in numbers and expanding their range during the last few years. Population growth favours disease transmission among suids, and close interactions with human beings (hunting, butchering, and dressing [82]) enhance the risk of disease transmission.

Age was the most relevant factor related to prevalence. Piglets and juveniles were the most susceptible groups to infection; this result is consistent with previous studies

showing decreasing *Cryptosporidium* spp. prevalences with age. Furthermore, susceptibility to different *Cryptosporidium* species appears to differ between groups based on age [83–85]. Additionally, *Cryptosporidium* spp. infections seem to be modulated by climate and season. The Mild Typical Mediterranean climate shows the highest prevalence, probably related to favourable environmental conditions for oocyst survival, in comparison to the more challenging Continental and Dry Mediterranean climates [86]. Winter is the season with the highest probability of *Cryptosporidium* spp. infection, presumably related to changes in the behaviour that favour transmission (aggregation, group mixing, increased travelling, and physical contact) due to mating season [87].

In coincidence with previous studies, wild boar density was also an element involved in the observed results [88]. In our study area, extreme densities (established at >3.1 wild boars/km^2 and even reaching 6.7 wild boars/km^2 in some counties) [2] increase the prevalence of the parasite over the expected prevalence (enhancing contact among individuals). This constitutes a risk factor for environmental and public health.

The two detected species, namely *C. suis* and *C. scrofarum*, are the main *Cryptosporidium* species found in wild boars, with similar proportions registered in Central European countries like Austria, the Czech Republic, Poland, and Slovakia [28,29]. Both have been reported in human beings [31–34]; therefore, wild boars could potentially act as an important source of infection for people. *C. scrofarum* is the predominant one in this species, as shown by prior studies [29,30,39,71], infecting all wild boar age groups [30].

Previous studies have found (oo)cysts of *Cryptosporidium* spp. and *G. duodenalis* in leachate from croplands related to the farm industry [89–92]; hence, we considered the fertilisation with slurry from pig farms and main land use from the sampled areas as potential risk factors worth investigating. Although our results showed these variables were not statistically significant, this must be cautiously interpreted. In this study, information concerning the presence/absence of pig farms surrounding the sampling areas was not accessible. Therefore, we relied on data indicating the use of slurry as a natural fertiliser, although quantities, previous treatments, and its origin were not available. Further investigation is needed to discern the role that malpractices related to manure management may play in determining the transmission risk of *G. duodenalis* and *Cryptosporidium* spp.

5. Conclusions

The results of this study emphasised the importance of expanding *Cryptosporidium* spp. and *G. duodenalis* monitoring beyond domestic species, including wildlife, in disease surveillance programs. Further studies might be necessary to evaluate the potential effect of fertilisation with slurry on the microbiological contamination of croplands and its transference to humans, domestic animals, and wild animals. It is necessary to further investigate the connections between pathogens, environmental factors, human activities, and wildlife as a way to prevent future outbreaks from a health perspective.

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Occurrence of *Giardia duodenalis* in Cats from Queretaro and the Risk to Public Health

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Simple Summary: *Giardia duodenalis* is a flagellated protozoan that has been reported worldwide. Its resistance to adverse climates and its wide host range makes this parasite a serious problem specifically in developing countries where personal hygiene practices are inadequate. The magnitude of the risk of transmission from cats to humans is not well known; to date, in Mexico, there has not been a single study of the presence of *G. duodenalis* in cats. Therefore, the objective of this work is to determine the frequency and importance of the cat as a potential zoonotic reservoir of *Giardia*. Sampling of feces was performed in private clinics and an animal control unit. A direct microscopy diagnosis was carried out, and once positive results were obtained, PCR and RFLP were performed in order to obtain assemblages. The results obtained in the present study, in terms of the genetic characterization, showed only assemblage A, which indicates that the cat is an important source of transmission to humans, with consequences for public health.

Abstract: *Giardia* is a protozoan that affects humans as well as a wide range of domestic species. It is distributed worldwide, and the highest frequency is seen in developing countries. Due to the potential for domestic cats to be carriers of this parasite and subsequently transmit the infection to humans, it is important to know the risk of transmission. For this reason, the objective of this study was to determine the frequency of this parasite in the cat population of the city of Santiago de Queretaro, Mexico, and identify the assemblages present to determine the role this host plays in public health, this being the first study of its type to be performed in the country. This was a cross-sectional study during which 200 fecal samples were collected from cats of both sexes and varying ages and strata of origin. The samples were analyzed by microscopy following the flotation technique, having obtained a general frequency of 25%. *Giardia* cysts were found at higher frequency in pasty stools. The assemblages found were zoonotic, specifically assemblage A, which suggests that the cat poses an important risk for the dissemination of the parasite to humans, making it an important public health problem.

Keywords: *Giardia*; zoonoses; assemblages; cats; risk; Mexico

1. Introduction

Giardia duodenalis is one of the most common gastrointestinal parasites that affects humans. Its resistance to adverse climates and its wide host range make it a serious problem, more so in places that lack good personal hygiene practices [1,2]. Because giardiosis can occur following the ingestion of only 10 cysts, this protozoan is considered a risk to public health [3]. Despite having been discovered 400 years ago, the disease is considered a re-emerging disease [4]. Worldwide, about 200 million people are reported to be infected with the parasite, mainly in Asia, Africa, and Latin America, and approximately 500,000 new cases are reported annually [5].

Giardia is transmitted through the fecal–oral route, frequently due to the ingestion of food and water contaminated with the parasite's cysts, or by host-to-host contact [6], The most susceptible section of a population includes infants in daycare, daycare workers, travelers to regions where the protozoan is endemic, people with immunodeficiencies and cystic fibrosis, and those who participate in oral and anal sexual practices [7].

Giardia duodenalis has a direct life cycle that is composed of two morphologically different stages. On the one hand, the infective stage, or cyst, is responsible for causing the infection. The cyst can survive in the environment for months under optimal conditions (4 °C) [8]. On the other hand, the invasive stage, or trophozoite, is responsible for causing the disease due to its colonization in the upper parts of the small intestine. Previously, the cyst was considered to be a cryptobiotic form; however, it was found that its oxygen consumption is 15% of that of trophozoites and it is capable of passing through the stomach and excysting in the duodenum [9].

Giardiosis can manifest symptomatically and asymptomatically; the fact that this parasite is found to a greater extent in asymptomatic patients, including cats, increases the risk of transmission to humans [10]. Although it is said that the presence of clinical signs could be linked to host factors, such as immune response, it may also be due to virulence factors of the strain of the infecting parasite [11]. However, the risk factors that may trigger the clinical disease are not yet known with certainty [12]. The most common signs of giardiosis include diarrhea, steatorrhea, abdominal pain, and weight loss [11]. Clinical manifestation of giardiosis may also be related to the assemblages present, with assemblage A being associated with acute disease and the presence of explosive diarrhea, and assemblage B being associated with chronic disease [4,13,14].

Studies indicate that cysts are passed through the stool intermittently, making diagnosis difficult and mistaking it with other gastrointestinal diseases. The low sensitivity of coproparasitoscopic tests makes detection of the disease even more difficult, for which a minimum of three serial tests are needed to observe the morphological forms [15].

The presence of *G. duodenalis* depends on variables such as geographic area, sanitary conditions, and number of animals sharing the same space. Epidemiological studies allow us to understand the importance of giardiosis in different regions of the world, characterizing the presence of the zoonotic and non-zoonotic assemblages and the risk of people living with companion animals, especially cats [16,17].

The reported prevalence of *Giardia* in humans in Mexico is variable, ranging from 2 to 50%, depending on the region, with preschool-age children being the most susceptible [18,19]. With regard to cats, studies have reported prevalences ranging from 1 to 43% (Table 1).

Table 1. Occurrence, origin, age, and assemblages of *Giardia duodenalis* in cats.

Location	Age and Origin	Total	Occurrence (%)	Assemblage	Reference
Germany	Owned, stray, Shelter (Adults and kittens)	145	17.9%	D	2018 [20]
Italy	Owned, Stray (Kittens, Adults)	181	6.1%	A, F	2011 [21]
Romania	Owned (Kittens, Adults)	181	28%	Not reported	2011 [22]
Poland	Owned (No age)	160	3.75%	A, B, D	2011 [23]
USA	Owned (No age)	250	13%	AI, AII, F	2007 [24]
USA	Owned (Kittens, Adults)	211,105	0.58%	Not reported	2006 [25]
Japan	Kennels, Owned (Kittens, Adults)	600	40%	Not reported	2005 [26]
Colombia	Stray (Kittens, Adults)	46	6.5%	F	2006 [27]
Colombia	Owned (Kittens, Adults)	203	20%	Not reported	2019 [28]
Brazil	Owned, shelters (No age)	19	Not reported	AI, F	2007 [29]
Chile	Owned (No age)	230	19%	Not reported	2006 [30]
Costa Rica	Owned (No age)	9	57.1%	Not reported	2011 [31]

Giardia affects a wide range of species, and the fact that it can infect humans and animals raises concern regarding the risk to public health posed by companion animals [32]. The level of risk depends on the prevalence and assemblages present, and since cats are reservoirs of this parasite, this causes public health problems, affecting the health of humans and animals [33].

To date, the magnitude of the risk of transmission from cats to humans is not well known. Studies on the genetic variability of *G. duodenalis* have identified four main genetic groups. Groups A and B generally affect humans, while groups C and D affect dogs, and group F is more specific to cats. However, there are reports of groups A and B infecting cats and dogs. Group A includes four subgroups: AI to AIV. Subgroups AI and AII can be found in both humans and animals, but AIII and AIV are exclusive to animals [34,35]. Currently, mixed infections with assemblages belonging to humans and cats have been reported; therefore, from an epidemiological point of view, this suggests that there is a potential environmental reservoir for giardiosis in urban areas [36]. In Mexico, studies have been performed regarding the prevalence and presence of zoonotic assemblages in dogs, sheep, and cattle [19,37,38]; however, there are no reports of the occurrence of *G. duodenalis* or its assemblages in cats. For this reason, the present study aims to determine the occurrence of assemblage A and its sub-assemblages (AI, AII) in cats that affect humans as well as the role that cats have as potential transmitters of this protozoan to humans.

2. Materials and Methods

2.1. Study Setting and Sampling

The study was performed in the city of Santiago de Queretaro, located in the center of Mexico. This city has a territorial extension pf 263 km^2, a population of 1,049,777, and a temperate, semi-arid climate (20 °C annual average temperature, 720 mm^2 per year, 38.2% humidity). For the study, 200 fecal samples were obtained from cats from two different strata of origin: (1) without an owner or "unowned" (stray and feral cats), from the municipal animal control unit (UCAM), and (2) with an owner (pet cats), from private veterinary clinics. The samples were labeled and data regarding age and sex of the cats and consistency of the feces was recorded. The samples were then transported to the parasitology lab of the Autonomous University of Queretaro (UAQ), where they were stored at 4 °C until further processing.

A total of 200 samples were distributed, as follows: by strata of origin, 103 were from cats with owners and 97 from cats without owners; by age, 72 were from cats under

6 months and 128 were from cats over 6 months; by gender, 109 were from females and 91 were from males; and by stool consistency, 139 stools were firm and 61 were pasty.

2.2. Coproparasitoscopic Analysis

Two grams of each fecal sample were processed using the flotation technique with zinc sulfate, as described by Dryden et al. [39]. A drop of fecal suspension was transferred to a microscope slide with a cover slip and examined at 40X magnification for identification of cysts. A sample was considered positive if cysts were observed.

2.3. Sample Pooling

After identifying the positive samples, seven pools were made. It was not possible to make more pools because of the low volume of the samples obtained from the cats. All of the positive samples with a sufficient quantity of feces were pooled. Each pool consisted of four positive samples (totaling 28 samples) in order to obtain a minimum of 5 g of feces, which is required for cyst concentration with the sucrose gradient method [40]. This technique was required due to the necessity of obtaining a sufficient number of cysts to perform an optimal DNA extraction [41].

2.4. DNA Extraction

DNA extraction was performed following the technique described by Babaei et al. [42], with some modifications to obtain more DNA from each sample. A 200 µL volume of the concentrated cyst suspension was transferred into a 2 mL Eppendorf tube with 200 µL of glass beads (0.1 mm) and 500 µL of a buffer lysis solution (100 mM NaCl, 50 mM Tris HCl, 100 mM EDTA, 1% SDS, pH 7.4) and homogenized in a Powerlyzer 24 (MO BIO, Carlsbad, CA, USA) with five 2 min cycles in liquid nitrogen and boiling water. A 40 µL volume of proteinase K and 10 µL of SDS (1 M) were added, and the reaction was incubated at 55 °C for 4 h. Subsequently, DNA was extracted using the CTAB method, as described by De Almeida et al. [43].

2.5. PCR, RFLP, and Sequencing

Fragments of the *β-giardin* gene were amplified in two phases using the following primers: G7 5′-AAGCCCGACGACCTCACCCGCAGTGC-3′ and G759 5′-GAGGCCGCCC-TGGATCTTCGAGACGAC-3′ for an initial reaction, and G376 5′-CATAACGAC-GCCAT-CGCGGCTCTCAGGAA-3′ and G759 for a second reaction. The cycling conditions were those described by Caccio et al. [44]. The reaction mixture consisted of 6.25 µL of GoTaq Green Master Mix 2X (Promega, Madison, WI, USA), 0.4 µL of each primer (10 µM), 1 µL of BSA, 2.45 µL of nuclease-free water, and 2 µL of DNA (100 ng/µL). PCR products were analyzed by 2% agarose gel electrophoresis (Thermo Scientific Waltham, MA, USA), submerged in TAE 1X buffer (55 min, 75 volts), including a molecular weight marker of 100 pb (Promega, Madison, WI, USA), and blue/orange 6X loading buffer (Promega, Madison, WI, USA).

For genetic characterization, restriction fragment length polymorphism (RFLP) analysis was performed using 10 µL of the second PCR product (380 bp) and 0.25 µL of restriction enzyme HhaI (Promega, Madison, WI, USA), for a total reaction volume of 10.25 µL, and then incubated in a water bath at 37 °C for 2 h [44]. Banding patterns were analyzed by 3% agarose gel electrophoresis (Thermo Scientific Waltham, MA, USA), submerged in TAE 1X buffer (90 min, 70 volts), including a molecular weight marker of 100 pb (Promega, Madison, WI, USA), and blue/orange 6X loading buffer (Promega, Madison, WI, USA).

To validate the resulting amplicon as *Giardia duodenalis*, the second PCR product was sequenced, also using the G759 and G376 primers mentioned above. Sanger sequencing was performed by the National Laboratory of Genomics for Biodiversity (LANGEBIO-CINVESTAB, Irapuato, Guanajuato, Mexico) using a [PacBio/Illumina/Ion Torrent/etc instrument]. For validation, the sequences were compared against *G. duodenalis* reference

sequences found in NCBI using the BLAST (blastn) web tool, which is available at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Accesed on 5 May 2022.

2.6. Statistical Analysis

A χ^2 analysis was used to determine associations between the presence of the parasite and any of the variables (age, sex, strata of origin, and stool consistency) using SPSS Statistics software, version 25.0 (IBM Corp, Armonk, NY, USA). Associations were considered statistically significant if $p < 0.05$.

3. Results

3.1. Coproparasitoscopic Analysis

Based on the zinc sulfate flotation technique, cysts were detected by microscopy in 50 out of the 200 samples analyzed, obtaining an overall occurrence of 25%. Significant statistical associations for the presence of the parasite were determined for age and stool consistency (Table 2). Detection of *G. duodenalis* was similar in cats with and without an owner ($p > 0.05$). Regarding age, the occurrence was higher in young cats (33%) than in adults (20%) ($p < 0.05$). For stool consistency, the occurrence was higher in cats with pasty stool (44%) as opposed to those with firm stool (16%) ($p < 0.05$). Finally, sex didn't show significant association, as the occurrence was similar in males (24%) and females (25%) ($p > 0.05$).

Table 2. Distribution of samples and statistical association analysis by stratum, age, stool consistency, and gender.

	Positive	Negative	Occurrence (%)	*p* Value	Odds Ratio	95% CI
Total	50	150	25%			
Strata of origin						
Owned	29	74	28	0.28827	1.41	0.731–2.7
Unowned	21	76	21			
Age						
<6 Months	24	48	33%	0.041227	1.96	1.02–3.76
>6 Months	26	102	20%			
Stool consistency						
Pasty	27	34	44%	0.000031	4.00	2.03–7.8
Firm	23	116	16%			
Sex						
Male	22	69	24%	0.805723	0.92	0.48–1.75
Female	28	81	25%			

3.2. PCR, RFLP, and Sequencing

Seven groups of pooled samples were obtained based on age, sex, and strata of origin. These pools showed a good number of cysts (at least 10 cysts per field with a 40x objective). A fragment specific to the *β-giardin* gene was amplified from all of them, obtaining a 753 bp product for the first PCR reaction and a 384 bp product for the second (Figure 1). Original figures were shown in Figure S1.

RFLP on the fragments corresponding to the second PCR product (384 bp) resulted in the detection of assemblage A in all the pools. Three were further determined as the AI assemblage, with a fragment pattern of 70, 100, and 190 bp, and four were assemblage AII, consistent with a fragment pattern of 70 and 210 bp (Figure 2).

Two sequences were recovered from the sanger sequencing results, one from the G759 primer and one from G376 (Appendix A). BLAST results showed 99% identity similarity to the *β-giardin* gene sequences reported in GenBank. These sequences were submitted to the GenBank: PRJNA945414.

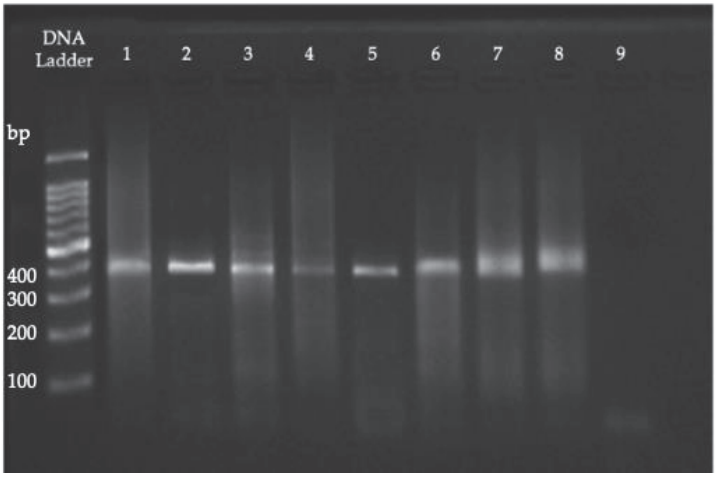

Figure 1. Amplicons of 384 bp in size from the second PCR reaction (*G. duodenalis*). Lane 1 corresponds to control; lanes 2–8 correspond to the pools analyzed; lane 9 corresponds to the negative control.

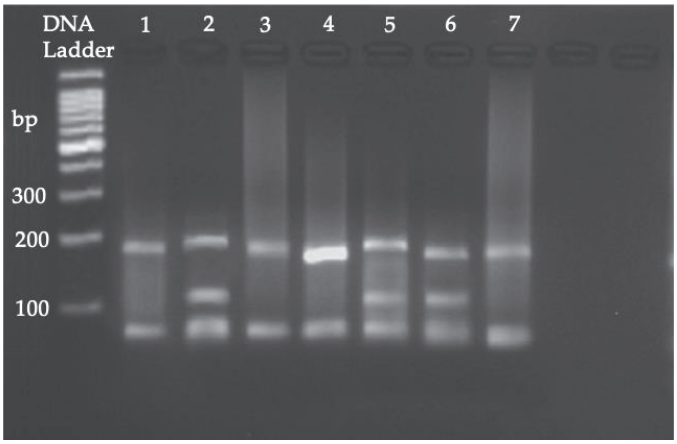

Figure 2. RFLP analysis of a 384 bp fragment of the *β-giardin* gene (*G. duodenalis*). Lanes 2, 5, and 6 correspond to the AI assemblage (190, 100 and 70 bp) and lanes 1, 3, 4, and 7 correspond to the AII assemblage (210 and 70 bp).

4. Discussion

The estimated occurrence of *G. duodenalis* obtained in this study and the assemblages detected indicate that cats represent an important risk for the transmission of this protozoan to humans in Santiago de Queretaro, this being the first study of its type carried out in Mexico. The overall occurrence was 25%, similar to that obtained by studies on cats from Serbia and Colombia [28,45]; however, higher than that reported worldwide (12%) [46] and lower than that reported in Brazil (32%) [47]. The diagnostic method used in the present study was zinc sulfate with centrifugation, which has proven to have a sensitivity of over 72% if a single sample is analyzed [48,49]. This technique has been considered the gold standard for the diagnosis of *Giardia* in dogs and cats [39]. However, due to the intermittent excretion of cysts and because only one sample was collected rather than the three recommended [49] the occurrence obtained in this study could be underestimated.

Differences in occurrence were seen in each of the categories defined, such as strata of origin (owned vs. unowned), age, sex, and stool consistency. Regarding their origin, the occurrence was slightly higher for cats with owners (28%) than for cats without owners (21%); in Mexico, it is more common for household cats to have access to the exterior and roam the streets, which inadvertently puts their owners at higher risk for infection [50].

A significant association with age was identified in this study, where young cats (<6 months) had a higher occurrence (30%) than adults (21%) ($p < 0.05$). Similar findings were reported by Nikolic et al. [45], in which *G. duodenalis* was detected in young cats at

a frequency of 30.4% but only in 19% of adults. Therefore, the age of the animals could potentially be a risk factor for becoming infected with this parasite, most likely due to their adaptive immunity not being fully developed [45]. Consequently, these young cats can excrete high amounts of cysts per gram of feces; therefore, they represent a significant source of infection [45,51,52]. In contrast, sex was not a statistically significant factor ($p > 0.05$), obtaining a frequency of 24% for males and 25% for females, coinciding with previously published studies [22,53].

As our results show, stool consistency was an important factor for detecting giardiosis in cats, as diarrhea or pasty stool with steatorrhea are among the main clinical signs observed in cats with this disease [2]. Therefore, in the present study, cats with pasty stools (44%) were significantly ($p < 0.05$) more likely to be infected than those that had firm stools (16%) (Table 2). These findings agree with those of other studies in which *G. duodenalis* was detected more frequently from animals with diarrhea [53–55].

For the molecular analysis, we pooled the positive samples to ensure a high yield of DNA, and this strategy has proven to be cost-effective and efficient for obtaining precise results [56]. Pooling of samples has also been commonly used in veterinary medicine as a fast and safe method for estimating prevalene based on microscopy results [57,58]. Additionally, molecular studies have used this pooling method to increase sensitivity at the group level, which has shown to be a reliable method for the detection of several microorganisms [46,59,60]. Here, PCR was performed on each of the seven pools targeting a fragment of the *β-giardin* gene due to its specificity for *G. duodenalis* and its high sensitivity for genetic characterization, both of which were essential for the objectives of this study [38,44,61].

The results show assemblage A, which suggests that the cat could be an important transmitter of this protozoan to humans [24] In this study, assemblage B was not analyzed due to the fact that its presence in Mexico is very low and it has only been found twice in humans and never in domestic animals [19,62]. The presence of assemblage A raises the concern of zoonotic transmission due to the association with the presence of clinical signs of the disease [13]. This coincides with other reports in which the zoonotic assemblages dominated in the cat population [24,63–65]; in contrast, some studies have detected cat-specific assemblage (F) at a higher frequency [66–68]. Here, further genetic characterization showed that assemblage AII was present at a higher proportion (57%) than assemblage AI (42%), coinciding with Procesi et al. [63]. Because AII is predominantly present in humans and is associated with virulence factors that may trigger clinical manifestations, this finding raises the concern for zoonotic transmission [69,70]. Studies carried out in North America have reported three assemblages in cats: AI and AII from group A and groups B and F [71,72], coinciding also with assemblage A detected here. In Mexico, the current cat population is at approximately 16.2 million; pet cats are not regularly spayed/neutered and usually have unrestricted access to the outdoors. Also, some can become free-roaming cats, without necessary preventative care to control these diseases. Gerold and Jessup have described that free-roaming cats represent a significant source of zoonotic diseases, including rabies, toxoplasmosis, larva migrans, tularemia, and plague [33]. Consequently, the high level of interaction among cats can lead to an increased risk of cat-to-cat transmission of pathogens, with subsequent transmission to humans and other animals.

The close contact that exists between humans and their pet cats needs to be taken into consideration due to the high occurrence and assemblages observed [73]. From this perspective, it is important to emphasize the need to develop effective strategies for the control of these zoonotic diseases, as well as raise awareness among the population and encourage responsible ownership and proper management in order to reduce the risk of transmission.

5. Conclusions

The high occurrence rate of *G. duodenalis* observed in this study, as well as the presence of assemblages A only, makes evident the source of infection that cats represent for humans

and other domestic animals. With this knowledge, control measures need to be carried out in order to minimize or eliminate the risk of *Giardia* transmission to cat owners.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13061098/s1, Figure S1: Original figures.

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Appendix A

Appendix A.1. Sequencing Analysis for the forward Primer G736 > B_Giardin_G376_Partial Sequence

CGAGAAGGTCGCAGAGGGCTTCGCCCGCATCTCCGCCGCGATCGAGAAGGAGA CGATCGCCCGCGAGAGGGCCGTTAGTGCTGCCACGACAGAAGCGCTCACAAACACGAAGCTCGTCGAGAAGTGCGTCAACGAGCAGCTCGAGAACGTCGCCTCGGA GATCCGCGCTATCCAGGAGGAGATCGACCGCGAGAAGGCCGAGCGCAAGGAG GCAGAGGACAAGATCGTCAACACTCTCGAGGACGTCGTCTCGAAGATCCAGGG CGGCCTCA

Table A1. BLAST results for the sequence obtained by PCR amplification of the *G. duodenalis β-giardin gene* (primer G376).

Description	Max Score	Total Score	Query Cover	E Value	Ident.	Accession
Giardia intestinalis isolate CAT clone 2 beta-giardin gene, complete cds	496	496	99%	1×10^{-135}	99.63%	EU014386.1
Giardia intestinalis isolate B7V_oct'11 note assemblage AI beta-giardin gene, partial cds	497	496	100%	3×10^{-136}	99.63%	KF963547.1
Giardia intestinalis isolate 34H beta-giardin gene, partial cds	497	497	100%	3×10^{-136}	99.63%	DQ466765.1

Appendix A.2. Sequencing Analysis for the forward Primer G759 > B_Giardin_G759_Partial Sequence

GGATCTCCGAGGCGACGTCCTCGAGCTGCTCGTTGACGCACTTCTCGACGAGCT TCGTGTTTGTGAGCGCTTCTGTCGTGGCAGCGCTAACGGCCCTCTCGCGGGCGAT CGTCTCCTTCTCGATCGCGGCGGAGATGCGGGCGAAGCCCTCTGCGACCTTCTCG T-

TGAGCTGGTCGTACATCTTCTTCCTTTCTGCGTTCTCCGTGGCAATGCGTCTCGA GA-
TCGTTCAGGCTCTTGAGGGCCTCCTTCCTGAGAGCCGCGATGGCGTCGTTATG A

Table A2. BLAST results for the sequence obtained by PCR amplification of the *G. duodenalis β-giardin gene* (primer G759).

Description	Max Score	Total Score	Query Cover	E Value	Ident.	Accession
Giardia intestinalis isolate 9C beta-giardin gene, partial cds	508	508	100%	1×10^{-139}	99.64%	DQ466729.1
Giardia intestinalis isolate Z1 beta-giardin gene, partial cds	507	507	99%	5×10^{-139}	99.64%	MN629930.1
Giardia intestinalis strain VANC/96/UBC/126 beta-giardin (bg) gene, partial cds	507	507	99%	5×10^{-139}	99.64%	KP687765.1

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