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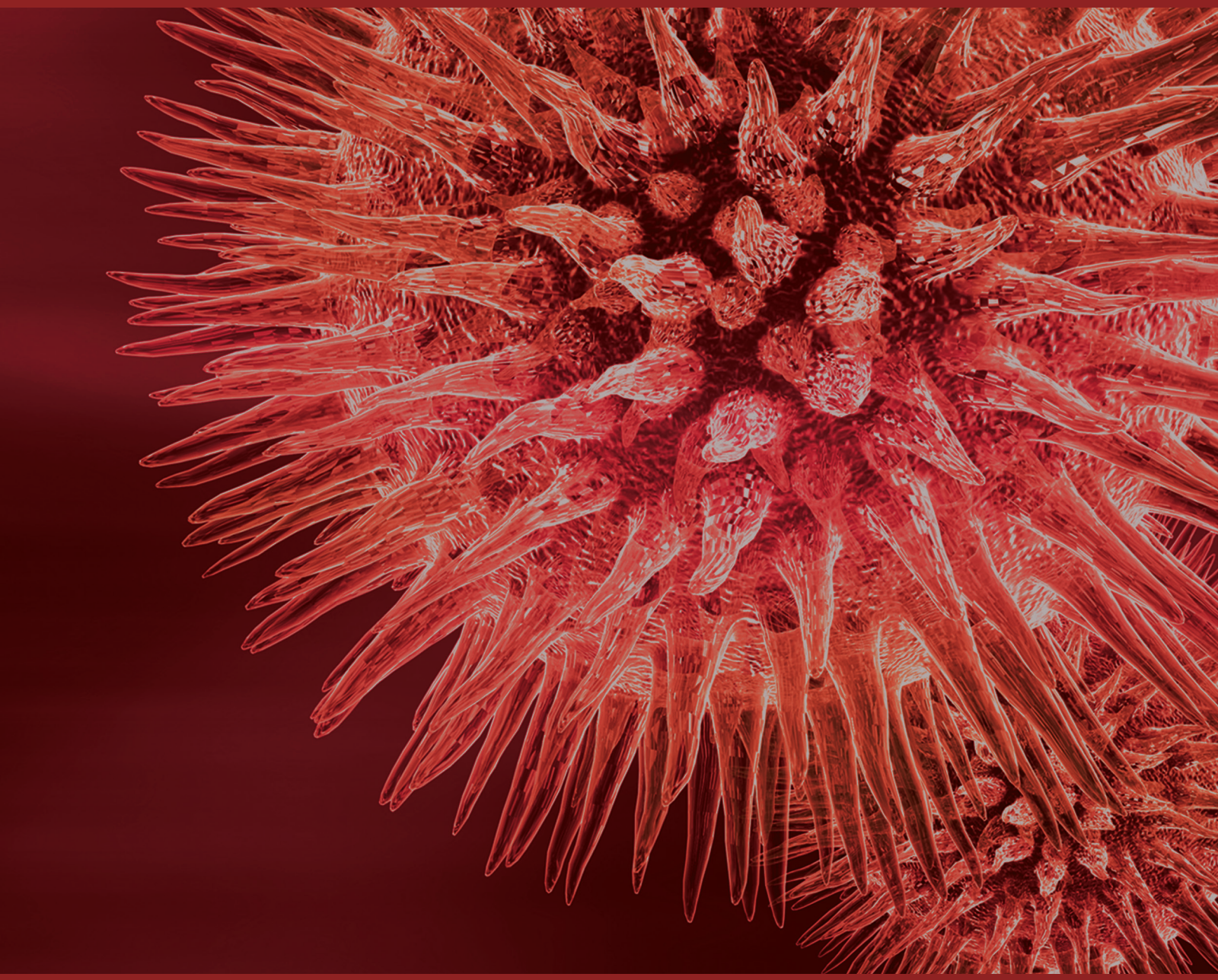
Zoonotic Neglected Tropical Diseases: New Approaches to Combat Old Enemies

Guest Editors: Fabio Ribeiro Braga, Pedro Mendoza de Gives, Adolfo Paz Silva, Filippe Elias de Freitas Soares, and Jackson Victor de AraújoJia



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Editorial

Zoonotic Neglected Tropical Diseases: New Approaches to Combat Old Enemies

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Neglected tropical diseases are a group of tropical infections which are especially endemic in low-income populations in developing regions of Africa, Asia, and the Americas. Different groups define the set of diseases. In this context, we mention roundworms (ascariasis), whipworms (trichuriasis), hookworms (necatoriasis and ancylostomiasis), trematodes (schistosomiasis), lymphatic filariosis, leishmaniasis, American trypanosomiasis, hanseniasis, African trypanosomiasis, Guinea worm (dracunculiasis), and Buruli ulcer. Due to the fact that thousands of people become affected, the social and economic impacts of these diseases are tremendous. Together, they cause an estimated 500,000 to 1 million deaths per year and cause an overall charge higher than the HIV-AIDS, possibly due to the lack of adequate tools for their diagnosis and treatment. They affect the poorest populations in the least developed countries of the world and therefore do not constitute a lucrative market for the pharmaceutical industries.

It should also be emphasized that these diseases represent an enemy that takes advantage of social and economic fragility. However, articles which also cover other diseases such as malaria, toxoplasmosis, and cryptosporidiosis were welcomed. In another context, in this special section, we call attention to research that focuses on the Trematoda *Paragonimus westermani* (paragonimiasis).

In this special edition some proposals for advances in controlling and reducing the risk of these diseases have been reported, with an approach to biotechnology and biomedicine. Moreover, the main objective was to bring to the "light of knowledge" further research to combat neglected tropical diseases.

Thus, the quick growth in the adoption of the concept of a healthcare, in particular in developed countries over the last six years, was demonstrated, where the interesting focus of this research was to observe the advantages and benefits to the best approach to combating zoonoses [1]. In another moment, the edition included an experience in the field of campylobacteriosis in chickens and, recording with that, the concern with poultry production, which in many situations may be associated with other creations of domestic animals and, with that, the close association and opportunity for movement of *Campylobacter* spp. among these host species ("*MLST genotypes and antibiotic resistance of Campylobacter spp. isolated from poultry in Grenada*").

In a study conducted in Brazil, valuable information in the field of diseases and pathogens of buffalos in the northern region has been reported and may benefit management programs and control of disease in these animals [2].

In the field of the study of protozoa, two investigations have been reported, one evaluating the direct effect of two new compounds on the viability and infectivity of tachyzoites of *Toxoplasma* [3] and the second about the cellular immunity, especially for the remission of lesions and protection against infection from cutaneous leishmaniasis [4]. Finally,

in the area of combating helminths by natural antagonists stood out the research of filamentous fungi from the genera ovicides and predators to combat liver *Fasciola hepatica,* causing fasciolosis, an important emerging zoonosis ("*Mixed production of filamentous fungal spores for preventing soil-transmitted helminth zoonoses: a preliminary analysis*").

Fabio Ribeiro Braga
Pedro Mendoza de Gives
Adolfo Paz Silva
Filippe Elias de Freitas Soares
Jackson Victor de Araújo

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Research Article

Canine Filarial Infections in a Human *Brugia malayi* Endemic Area of India

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A very high prevalence of microfilaremia of 42.68 per cent out of 164 canine blood samples examined was observed in Cherthala (of Alappuzha district of Kerala state), a known human *Brugia malayi* endemic area of south India. The species of canine microfilariae were identified as *Dirofilaria repens, Brugia malayi*, and *Acanthocheilonema reconditum*. *D. repens* was the most commonly detected species followed by *B. pahangi*. *D. immitis* was not detected in any of the samples examined. Based on molecular techniques, microfilariae with histochemical staining pattern of "local staining at anal pore and diffuse staining at central body" was identified as *D. repens* in addition to those showing acid phosphatase activity only at the anal pore. Even though *B. malayi* like acid phosphatase activity was observed in few dogs examined, they were identified as genetically closer to *B. pahangi*. Hence, the possibility of dogs acting as reservoirs of human *B. malayi* in this area was ruled out.

1. Introduction

Major filarial parasites of dogs are *Acanthocheilonema reconditum, Acanthocheilonema dracunculoides, Brugia malayi, Brugia pahangi, Brugia ceylonensis, Brugia patei, Cercopithifilaria grassii, Dirofilaria immitis*, and *Dirofilaria repens* [1–4]. Microfilariae of *Brugia* spp. are sheathed while those of others are not sheathed.

Canine filariosis was reported from various parts of India mainly from states like Kerala, Tamil Nadu, Karnataka, Orissa, West Bengal, Bihar, Uttar Pradesh, and Maharashtra. The species of microfilariae detected from these states include *D. immitis* from Kerala [5], *C. grassi* from Tamil Nadu [6], *D. immitis* from Himalayas [7], *D. immitis* and *A. reconditum* from West Bengal [8], *D. immitis* and *D. repens* from Orissa [9], *D. repens* from Kerala [10], *D. repens* and *A. reconditum* from Karnataka [11], *A. reconditum, D. immitis, and D. repens* from Maharashtra and New Delhi, *and Microfilaria auquieri* and a novel species of *Acanthocheilonema* from Ladakh, India [12]. In general, it is believed that *D. immitis* is mostly prevalent in north eastern India [13] while *D. repens* is confined to southern parts of the country [14, 15].

Currently, there is paucity of information on the prevalence of filarial worms of dogs in the genus *Brugia* from India. The disease caused in humans by subperiodic *B. malayi*, in Malaysia and Indonesia, is considered zoonotic due to

the existence of animal reservoir hosts like cats and dogs [16]. Brugian filariosis is endemic in Alappuzha district of Kerala State [17–19] and thus the presence of *B. malayi* in dogs is always suspected. Even though *B. malayi* like microfilariae [20, 21] were detected from cats and dogs from India, they were not confirmed as *B. malayi*. The present study focuses on the detection and differentiation of microfilariae of domestic canines inhabiting a *B. malayi* endemic area of southern India (Alappuzha district of Kerala state) based on histochemical staining and molecular techniques.

2. Materials and Methods

2.1. Ethics Statement. All study procedures and protocols were approved by the institutional animal ethics committee of College of Veterinary and Animal Sciences, Pookode, Kerala (which follows committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines, Ministry of Environment and Forest, Government of India). The committee did not deem it necessary for this research group to obtain formal approval to conduct this study. National Centre for Diseases Control, directly under Directorate General of Health Services, Ministry of Health and Family Welfare, Government of India, is authorized to collect microfilaria positive human blood samples. Written informed consent was obtained from the human patients and owners of dogs before collection of samples.

2.2. Preliminary Screening and Collection of Samples. Samples were collected from dogs by collaborating veterinarians at "prophylactic antirabies vaccination and blood parasite detection camps" in the south Indian panchayats of Kerala state, namely, Kuthiathode (KUT), Kadakkarappally (KAD), and Pattanakkad (PKT). Brugian filariosis is endemic in this area [17–19]. Before sample collection data regarding the age, sex, and breed of these dogs were recorded.

Blood samples were collected from the saphenous vein. A drop of blood was examined by wet film technique at the camp site itself. Thick smears were prepared, fixed with methanol, stained using Giemsa, and examined for the presence of microfilariae with or without sheath. Whole nonheparinized (1 mL) and heparinized (1 mL) blood samples were also collected from the saphenous vein of all these dogs. A total of 164 blood samples of dogs (37 from Kadakkarappally, 57 from Kuthiathode, and 70 from Pattanakkad) were collected.

Blood sample of a known *B. malayi* infected human patient from Alappuzha, the endemic district of Kerala, was also collected to serve as positive control for histochemical staining and PCR techniques.

2.3. Histochemical Differentiation. The nonheparinized blood was allowed to clot and the serum fraction was centrifuged in an Eppendorf tube for 5 minutes at 1000 rpm. After discarding the supernatant, the sediment containing the microfilariae was resuspended in the remaining serum and a drop of this fluid was used for preparation of smears. They were air dried, fixed in absolute chilled acetone for 1 minute, further air dried, and stored in −20°C until used. These smears were used for histochemical staining within two weeks after preparation.

Histochemical staining was performed [22] to study the differences in the acid phosphatase enzyme activity in microfilariae for identification of the species [22–26]. Briefly, 20 mL of solution I (Michaelis Veronal Acetate Buffer, pH 10.0) was mixed with 50 mL of distilled water and 4 mL of solution II (0.05 g Naphthol AS-TR phosphate, sodium salt (Sigma) in 5 mL of N, N-dimethyl formamide) was added to it in a beaker. In another beaker, 3.2 mL each of solutions III (1.0 g Pararosaniline hydrochloride, 5.0 mL concentrated hydrochloric acid, and 20.0 mL distilled water) and IV (4 per cent sodium nitrite) was mixed and then added to the mixture in the first beaker. The pH of the mixture was adjusted to 5.0 with 0.1 N sodium hydroxide solution. The final solution was prepared fresh every time before the staining procedure.

The air dried smears were incubated in the substrate for 1 hour at 37°C, rinsed in distilled water, counterstained in solution V (mixture of 77.1 mL of 0.2 M sodium phosphate, 122.9 mL 0.1 M nitric acid, and 2 g methyl green) for 5 minutes, and rinsed in distilled water. The slides were then dehydrated in 95 per cent and absolute ethyl alcohol, respectively, rinsed in xylene, and mounted using DPX. The smears were examined for the precipitated red azo dye indicating acid phosphatase activity.

2.4. Polymerase Chain Reaction. DNA isolation from the heparinised whole blood samples of dogs and human was based on phenol-chloroform isoamyl alcohol (PCI) method [27]. The leukocyte DNA from a three-day-old pup served as negative control. For the *Brugia* specific PCR, the DNA isolated from a known *B. malayi* infected human patient's blood served as positive control. Canine blood samples with heavy (one microfilaria in every low power field) monoinfection with *D. repens* and *A. reconditum* (confirmed by histochemical staining) served as positive controls for these species. The reactions were carried out in a thermal cycler (Eppendorf, Germany); the products were visualized (Alpha Innotech, USA) after electrophoresis on a 1.5 per cent agarose gel and documented.

2.4.1. D. repens Specific PCR. PCR assay for the amplification of 246 bp direct tandem repeats of *D. repens* using specific primers [28] 5′-CCGGTAGACCATGGCATTAT-3′ (Forward) and 5′-CGGTCTTGGACGTTTGGTTA-3′ (Reverse) custom synthesized from IDT, USA, was standardized. The PCR amplification was performed in 25 μL reaction volume containing 2.5 μL 10x PCR buffer, 1 μL (0.25 mM) dNTP, and 30 pmol of each primer, 1.5 U *Taq* polymerase, and 5 μL of template DNA. Reaction conditions were as follows: after the initial denaturation at 94°C for 5 minutes, 40 cycles each with 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 25 seconds were run. A final extension at 72°C for 5 minutes was also given. One product of *D. repens* specific PCR was directly sent to Chromos Biotech, Bangalore, for sequencing.

Table 1: Prevalence of canine microfilariae in a human *B. malayi* endemic area of Kerala, India, based on wet film examination, Giemsa staining, histochemical staining, and polymerase chain reaction.

Place	Samples examined	W. f	G	Histochemical reaction/acid phosphatase reaction					Polymerase chain reaction		
				D. repens	*A. reconditum*	*B. pahangi*	New reaction	*B. malayi* like	*D. repens*	*A. reconditum*	*Brugia* sp. (*Hha*1)
Kadakkarappally	37	11	16	11	0	3	11	3	10	0	6
Kuthiathode	57	23	29	18	2	3	28	0	28	2	7
Pattanakkad	70	17	25	16	0	12	19	3	26	0	10
Total	164	51	70	45	2	18	58	6	64	2	23

W. f: wet film examination; G: Giemsa staining.

2.4.2. A. reconditum Specific PCR. PCR assay for the amplification of 348 bp ITS2 fragment of *A. reconditum* using primers [29] 5′-CAGGTGATGGTTTGATGTGC-3′ (Forward) and 5′-CACTCGCACTGCTTCACTTC-3′ (Reverse) custom synthesized from IDT, USA, was standardized. The PCR amplification was performed in 25 μL reaction volume containing 2.5 μL 10x PCR buffer, 1 μL (0.25 mM) dNTP, and 0.3 mM of each primer, 2.5 U *Taq* polymerase, and 5 μL of template DNA. The PCR cycling consisted of a denaturation step at 94°C for 3 minutes and 30 cycles each with denaturing at 94°C for 3 minutes, annealing at 63°C for 1 minute, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. One product of *A. reconditum* specific PCR was directly sent to Chromos Biotech, Bangalore, for sequencing.

2.4.3. Brugia Specific PCR. PCR amplification of a 322 bp *Hha*1 fragment [30] using specific primers 5′-GCGCATAAATTCATCAGC-3′ (Forward) and 5′-GCGCAAAACTTAATTACAAAAGC-3′ (Reverse) constituted the genetic diagnostic criterion for *Brugia* infection in the study dogs. The PCR amplification was performed in 25 μL reaction volume containing 2.5 μL 10x PCR buffer, 1 μL (0.25 mM) dNTP, and 10 pmol of each primer, 2.5 U *Taq* polymerase, and 5 μL of template DNA. The PCR procedure consisted of an initial denaturation step at 94°C for 5 minutes and 35 cycles each of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. PCR products were cloned and sequenced.

2.4.4. Amplification of 5.8S-ITS2-28S. In order to confirm the identity of microfilariae with new acid phosphatase staining pattern, PCR assay for the amplification of 5.8S-ITS2-28S fragment of rDNA gene ofsuspected samplesusing specific primers [3], 5′-AGTGCGAATTGCAGACGCATTGAG-3′ (Forward), 5′-AGCGGGTAATCACGACTGAGTTGA-3′ (Reverse), was standardized. The PCR amplification was performed in 25 μL reaction volume containing 2.5 μL 10x PCR buffer, 1 μL (0.25 mM) dNTP, and 100 pmol of each primers, 1.5 U *Taq* polymerase, and 5 μL of template DNA. Reaction conditions were as follows: after the initial denaturation at 94°C for 2 minutes, 32 cycles each with 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds were run. A final extension at 72°C for 7 minutes was also given. PCR products were cloned and sequenced.

2.5. Cloning and Sequencing of PCR Products. PCR products were eluted from the gel using QIA quick gel extraction kit (QIAgen, Germany) based on manufacturer's protocol. The eluted product (8 μL) was used for cloning into a pTZ57R/T vector (InsTAclone PCR cloning kit, Fermentas, USA) which was used to transform the JM107 strain of *E. coli* (Fermentas, USA). The transformed cells were plated on an LB agar with ampicillin (50 μg/mL) as the selective antibiotic. After overnight incubation at 37°C, positive white colonies were selected and subcultured in LB broth overnight. The presence of the insert was confirmed by restriction digestion of the isolated plasmid from the subculture using *Eco*R1 and *Sal* I enzymes (GeneJET plasmid mini prep kit, Fermentas, USA). The stab culture of the colony containing the insert was sent for automated sequencing to Chromos Biotech, Bangalore. In order to verify mixed infection or recombination, 2 clones from each PCR products were sequenced.

2.6. Sequence Analysis. The sequence analysis was done using ClustalV method of MegAlign programme (DNASTAR, USA).

3. Results

3.1. Preliminary Screening. A total of 164 blood samples of dogs were collected from Kadakkarappally (37), Kuthiathode (57), and Pattanakkad (70). Out of 164 dogs, 114 were from nondescript animals while 50 were from exotic breeds (Spitz, German shepherd, Dachshund, and Doberman).

Wet film examination revealed microfilariae in 11 (29.72 per cent) samples from Kadakkarappally, 23 (40.35 per cent) from Kuthiathode and 17 (24.29 per cent) from Pattanakkad (Table 1). The overall prevalence of microfilaremia based on wet film examination was 31.17 per cent.

Giemsa staining technique revealed microfilariae in 16 (43.24 per cent) samples from Kadakkarappally, 29 (50.87 per cent) from Kuthiathode, and 25 (35.71 per cent) from Pattanakkad. Overall, the prevalence of microfilaremia based on Giemsa staining was 42.68 per cent. Four dogs revealed microfilariae with sheath.

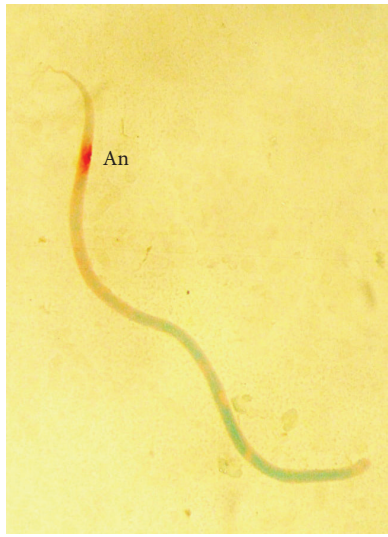

Figure 1: *D. repens* microfilaria showing acid phosphatase activity at the anal pore (An).

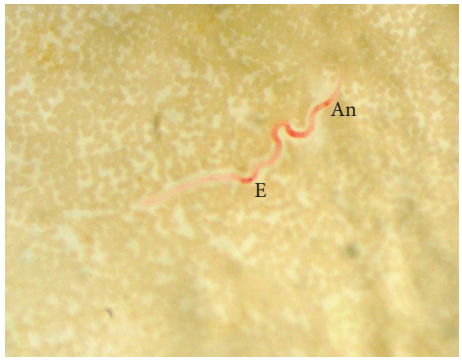

Figure 2: *A. reconditum* microfilaria showing acid phosphatase activity throughout the entire body, especially between the excretory (E) and anal pores (An).

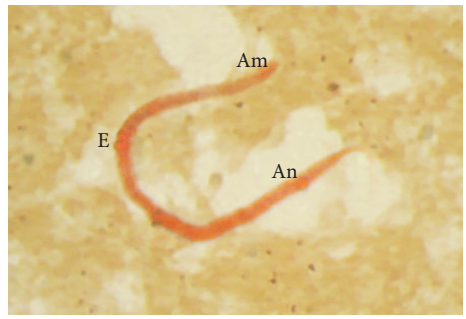

Figure 3: *B. pahangi* microfilaria showing heavy and diffuse acid phosphatase activity along the entire body length. The excretory (E) and anal pores (An) are recognizable.

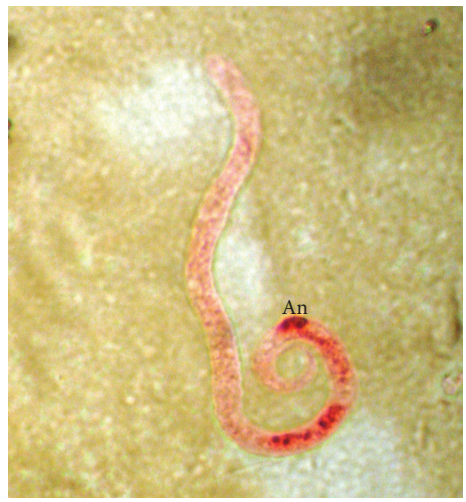

Figure 4: Microfilaria showing acid phosphatase activity with red spot at anal pore (An) region and diffuse red staining at the central body.

Fifty two per cent of exotic breeds and 38 per cent of nondescript breeds of dogs harboured microfilariae. Prevalence of microfilaraemia was more common in dogs above two years of age. Also, male dogs exhibited higher prevalence (Table 2).

3.2. Histochemical Differentiation. The *D. repens* microfilariae showed single locus of intense acid phosphatase staining in the region of the anal pore (Figure 1). This type of reaction was observed in 27.4 per cent of dogs.

The entire body, especially between the excretory and anal pores, was stained bright red in microfilariae of *A. reconditum* (Figure 2). Only two out of 164 smears examined revealed this type of reaction.

For *B. pahangi* microfilariae, the heavy and diffuse acid phosphatase activity was observed along the entire body length, although the excretory and anal pores were still recognizable (Figure 3). A total of 18 smears (11 per cent) showed this type of reaction.

The acid phosphatase staining pattern, with local staining at the anal pore and diffuse staining in the central body (Figure 4), was observed in 58 cases (35.4 per cent). Most of the samples contained a mixture of *D. repens* and this type of microfilariae.

Blood smears prepared from human patients with confirmed *B. malayi* infection contained microfilariae with loci of acid phosphatase activity in the areas of the amphids, the excretory pore, the anal pore, and the phasmids (Figure 5). Out of 164 dogs tested, 6 harboured microfilariae with histochemical reaction similar to *B. malayi* (Figure 6).

3.3. Polymerase Chain Reaction

3.3.1. D. repens. PCR detected the 246 bp *D. repens*-specific product in 64 out of 164 samples. PCR product from a sample that contained microfilariae showing a single locus of staining in the region of the anal pore was sequenced for confirmation (accession number JN830762).

Table 2: Microfilaraemia in different breeds, age groups, and sexes of dogs in a *B. malayi* endemic area of Kerala, India, based on Giemsa staining technique.

Blood smears	Area	Breed					Age group		Sex		Total
		ND	Exotic								
			Spitz	G.S.D	Dach	Dob	<2 years	>2 years	Male	Female	
Examined	Kadakkarappally	29	6	2	0	0	14	23	33	4	37
	Kuthiathode	36	10	5	4	2	13	44	39	18	57
	Pattanakkad	49	17	2	1	1	31	39	56	14	70
	Total	**114**	**33**	**9**	**5**	**3**	**58**	**106**	**128**	**36**	**164**
Positive	Kadakkarappally	11	3	2	0	0	4	12	16	0	16 (43.24%)
	Kuthiathode	16	6	2	3	2	4	25	24	5	29 (50.87%)
	Pattanakkad	17	6	1	0	1	3	22	20	5	25 (35.71%)
	Total	**44 (38.6%)**	**15 (45.46%)**	**5 (55.56%)**	**3 (60%)**	**3 (100%)**	**11 (18.97%)**	**59 (55.66%)**	**60 (46.88%)**	**10 (27.78%)**	**70 (42.68%)**

ND: Nondescript, G.S.D: German shepherd, Dach: Dachshund, Dob: Doberman.

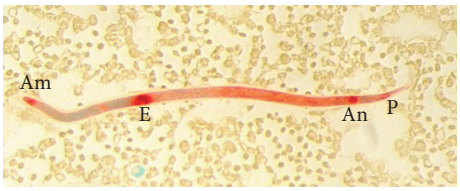

Figure 5: Human *B. malayi* microfilaria showing acid phosphatase activity in the areas of amphid (Am), excretory pore (E), anal pore (An), and phasmid (P).

3.3.2. A. reconditum. PCR with primers specific for *A. reconditum* amplified the expected 348 bp product from two samples containing microfilariae with histochemical reactions typical of *A. reconditum*. The partial sequence of the PCR product was submitted to GenBank (accession number JQ039745).

3.3.3. Brugia Species. PCR with primers specific for *Brugia* sp. amplified the expected 322 bp product from 23 (14 per cent) samples.

One *Hha*1 PCR product each from samples showing only *B. pahangi* (PKT 94) (accession number JN601135), human *B. malayi* (accession number JN413104), and *B. malayi* like parasite of dog (two clones from KAD 19) (accession numbers JN601136 and JN601137) were cloned and sequenced. The phylogenetic tree (Figure 7) and distance matrix (Figure 8) plotted based on ClustalV method of MegAlign programme (DNASTAR, USA) demonstrated that the microfilariae with histochemical reaction similar to *B. malayi* were genetically closer to *B. pahangi* of dogs.

3.3.4. Amplification of 5.8S-ITS2-28S. For confirmation of the species of microfilariae with the acid phosphatase staining reaction pattern "local staining at the anal pore and diffuse staining in the central body," sequence analysis of PCR product of 5.8S-ITS2-28S fragment of rDNA gene was resorted to. The 584 bp fragment was sent for sequencing. When submitted to NCBI-BLAST, the 492/498 bp stretches of the 584 bp product (accession numbers JQ039743 and JQ039744) shared 97 per cent identity with the previously reported *D. repens* sequence AY 693808.

4. Discussion

Filarial infections in dogs can be diagnosed through morphological observations of the circulating microfilariae, detection of circulating antigens, histochemical staining of circulating microfilariae, or through molecular approaches. Proper identification of circulating microfilariae based on morphology requires the involvement of a well-trained parasitologist. Filarial infection with multiple species and morphological alterations of microfilariae due to incorrect preventive treatment of dogs are not easily differentiated morphologically even by trained persons [31].

Histochemical staining to detect acid phosphatase activity can overcome most of these problems; however, this technique requires fresh samples to yield optimal results. Besides being time consuming and labour intensive, both staining methods require expertise to identify and confirm the species [32]. Molecular methods based on species specific PCRs are simple and easy to perform. Use of properly designed primers and control template DNAs can make PCR assays species specific. PCR assays are very sensitive due to the exponential amplification of target DNA. Additionally, it may be possible with a PCR to detect circulating DNA liberated from host destroyed microfilariae or from adult worms [30].

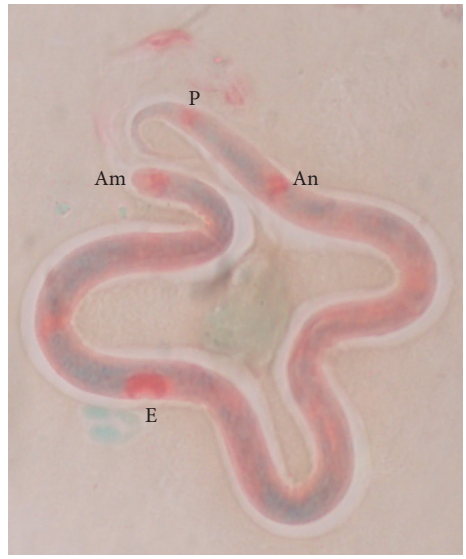

Figure 6: Canine *B. malayi* like microfilaria showing acid phosphatase activity in the areas of amphid (Am), excretory pore (E), anal pore (An), and phasmid (P).

Microscopical examination of Giemsa stained blood smears were used previously for detection of the presence of microfilaremia in dogs of Thrissur, Kerala. The prevalence varied from 7 to 26.5 per cent [10, 12, 33]. In the present study, using the same technique, the overall prevalence of microfilaremia in dogs was 42.68 per cent and the highest prevalence (50.87 per cent) was observed in Kuthiathode. Only two samples revealed sheathed microfilariae. Other techniques (histochemical staining and PCR) employed in the study detected more number of dogs positive for microfilariae especially *Brugia* species.

Even though the most pathogenic canine filaria, *D. immitis*, was reported previously from the Kerala state [5], it was not identified in any of the dogs tested in the present study. Unambiguous identification of *D. repens* and *A. reconditum* in dogs from the study population by histochemical staining and sequencing of PCR products was possible in this study.

Recently, *A. reconditum* (9.3 per cent) was identified as the most common species in North India [12] followed by *D. repens* (6.7 per cent) and *D. immitis* (1.5 per cent). In the present study, the microfilariae with a new histochemical staining pattern (local staining at the anal pore and diffuse staining at the central body) were more common. Based on

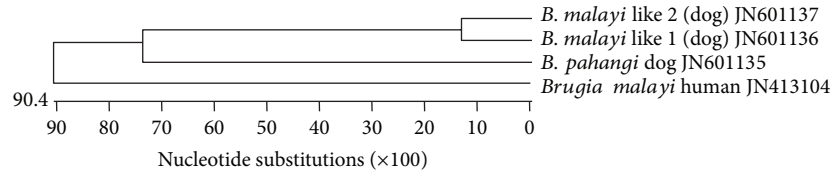

Figure 7: Phylogenetic tree constructed based on *Hha*1 sequence of different *Brugia* species.

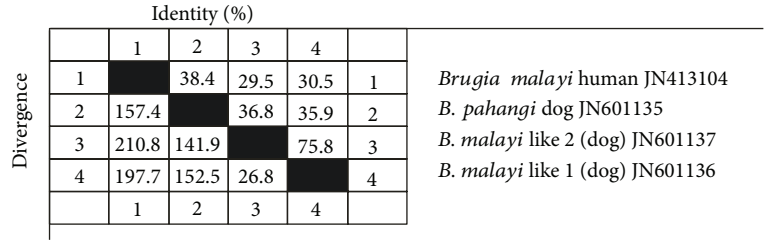

Figure 8: Distance matrix based on *Hha*1 sequence of different *Brugia* species.

97 per cent identity of 5.8S-ITS2-28S region of rDNA gene [3] of this form of microfilariae with that of *D. repens*, the species was confirmed as *D. repens*. Therefore, *D. repens* is identified as the most common species of filarial worm in the study area. However, 97 per cent identity may also indicate an interspecific difference and therefore the possibility of a new species could not be ruled out.

Another important finding of the study was the presence of *Brugia* species in the dogs of the area. The most prevalent *Brugia* species identified in the study area was *B. pahangi*. Out of 164 dogs examined, 18 (11 per cent) harboured microfilariae with acid phosphatase staining pattern specific to that parasite. Histochemical staining pattern similar to *B. malayi* was observed in 6 out of 164 dogs examined. PCR specific for *Brugia* sp. (*Hha*1) revealed the diagnostic 322 bp product in 23 samples that included *B. malayi* like microfilariae and *B. pahangi* as indicated by histochemical staining. *Hha*1 PCR products amplified in samples of dogs showing only either of these parasites based on histochemical staining were selected for sequencing. Similarly, the *Hha*1 fragment of the *B. malayi* (human) was also sequenced. Phylogenetic analysis of the sequences revealed that the suspected *B. malayi* like parasite was genetically closer to *B. pahangi* (dogs) than to *B. malayi* (human). Therefore, the results of the present study did not indicate potential of dogs acting as reservoirs of *B. malayi*.

However, a recent study conducted at Thrissur, Kerala, (100 km away from the study area) revealed that out of 100 dogs with symptoms of filariosis (fever, anorexia, conjunctivitis, oedema of limb, and scrotum) circulating microfilariae were detected in 80 cases, of which all the 16 cases with sheathed microfilariae were identified as *B. malayi* based on histochemical staining and PCR [20]. The primers specific for amplification of 294 bp trans-spliced exon 1 (SLX) region (5S r RNA) of *Brugia* species [16] followed by sequence analysis (90 bases with query coverage of 32 per cent) was used for confirming the identification of *B. malayi* in dogs. The authors concluded that the high prevalence of *B. malayi* in Thrissur emphasized the possible role of dogs in the transmission of human filariosis. The results of the present study are contrary to the above report.

Lymphatic filariosis is one of the major diseases of mankind in tropical and subtropical countries and the global burden of this type of disease is 119.1 million cases [34] of which 12.9 million are affected by Brugian filariosis. In India, *B. malayi* was reported from a few foci only, the largest single tract being Travancore-Cochin state (present study area) [17]. *B. malayi* is known to occur in two forms, periodic and subperiodic. The disease caused by subperiodic *B. malayi*, in Malaysia and Indonesia, is considered zoonotic due to the existence of animal reservoir hosts like cats and dogs [16, 35]. Previously, two out of 57 cats examined in Orissa state, India, were found infected with *B. malayi* like microfilariae [21].

Other epidemiological factors also should be considered before confirming the absence of reservoir status in dogs for *B. malayi* in Cherthala. The zoonotic subperiodic *B. malayi* is transmitted in nature by *Mansonia bonnae* while *M. dives, M. annulata*, and *M. uniformis* are efficient laboratory vectors. The Indian situation is different. Microfilariae of *B. malayi* in India are purely nocturnal in their periodicity [21]. In Cherthala "Taluk" (an administrative subdivision of a district) of Alappuzha district of Kerala, Brugian filariosis is transmitted by *M. annulifera, M. uniformis*, and *M. Indiana* [17–19]. The efficient vector (*M. bonnae*) for the transmission of zoonotic Brugian filariosis is not reported from this area. Also, a 90.7 per cent decline in the trend of disease rate for human filarial infections in Cherthala Taluk was also reported [36]. If animal reservoirs like dogs were there in existence, there would have been further increase in the number of outbreaks of the disease because of the availability of reservoirs (including stray dog population) and absence of microfilaricidal therapy in them. Also, when *B. malayi* could not be detected from Cherthala, the endemic hotspot, it is difficult to detect the parasite from a place which is 100 km away from the endemic area. So, the possibility for dogs acting as reservoirs of *B. malayi* in these areas is very remote. In addition, the clinical symptoms reported in dogs of Thrissur,

Kerala [20], were similar to that of dogs infected with *B. pahangi* [37] which include varying levels of microfilaraemia, episodic lymphadenopathy, lymphangitis, and limb oedema. For these reasons, the detection of *B. malayi* in dogs of Thrissur might be a false detection.

The microfilariae of three species, namely, *B. tupiae* (reported previously from Malaysia, Thailand, and Vietnam), *B. ceylonensis*, and *B. pahangi*, are liable to be confused with those of *B. malayi* [38]. *B. ceylonensis* was first described in lymphatics of dogs in 1962 from Sri Lanka [39]. This parasite, which is transmitted by *Aedes aegypti*, was reported from Kerala too as early in 1974, but there is no recent documentation of this parasite from the state. By contrast, *B. ceylonensis* was recently reported from the conjunctiva of a human patient in Sri Lanka [40]. Also, a Sri Lankan survey of 65 dogs revealed 7 per cent prevalence of single infection with *B. ceylonensis* [41]. The present study could not differentiate the *B. malayi* like microfilariae of dogs from *B. ceylonensis* due to the absence of reports on the typical histochemical staining reaction of *B. ceylonensis* microfilariae or gene accessions. The *B. malayi* like microfilariae observed in dogs of the study area could be a new *Brugia* species, *B. ceylonensis*, or a genetic variant of *B. pahangi*.

5. Conclusion

Microfilariae of *D. repens*, *A. reconditum*, and *B. pahangi* occur in dogs of Cherthala Taluk, Alappuzha district, Kerala, the human *B. malayi* endemic area of south India. Sequence analysis of 5.8S-ITS2-28S fragment of rDNA gene of microfilariae with the acid phosphatase staining pattern of local staining at the anal pore and diffuse staining in the central body revealed 97 per cent homology with *D. repens*. The *B. malayi* like microfilariae observed in dogs of the study area could be a new *Brugia* species, *B. ceylonensis*, or a genetic variant of *B. pahangi*.

Abbreviations

DNA: Deoxyribonucleic acid
PCR: Polymerase chain reaction
rDNA: Ribosomal DNA
DPX: Distyrene, plasticiser (dibutyl phthalate), and xylene.

Conflict of Interests

The authors declare that there is no professional or financial conflict of interests related to this paper.

Acknowledgments

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Research Article

A Retrospective Study of Ectoparasitosis in Patients Referred to Imam Reza Hospital of Mashhad, Iran

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This cross-sectional study was performed on all patients suspected to be suffering from ectoparasites who were referred to the parasitology laboratory of Imam Reza Hospital of Mashhad during 15 years (April 1995 to April 2010). All patients' data were collected from the questionnaires and then analyzed statistically. From 1814 suspected patients to be suffering from ectoparasites, 375 patients had scabies and, 99 suffer from pediculosis. The mean age of patients was 26/18 ± 17/68. The most common age of scabies was 10–19 (27/7%) and pediculosis 0–9(9/6%) (P value = 0.00). The highest incidence of pediculosis was in women (3.6%) and scabies in men (13.7%) (P value = 0.00). Pediculosis is more common in children (9/9%) and scabies in workers (32%)(P value = 0.00). Scabies and pediculosis were more prevalent in patients from Razavi Khorasan Province with 18.7% and 5%, respectively (P value = 0.08).

1. Introduction

Ectoparasitic infestation can induce persecutor diseases. Some of the common diseases of this group are pediculosis and scabies. Pediculosis and scabies are caused by ectoparasites; patients usually present with itching. Body lice are vectors of *Rickettsia prowazekii, Borrelia recurrentis*, and *Bartonella quintana,* the etiological agents of epidemic typhus, relapsing fever, and trench fever, respectively. Proper hygienic condition can prevent these diseases. Although these illnesses are not the concern of health care systems, they can cause high morbidity. Their incidence varied around the world depending on type and place of living. Ectoparasitic infestation can be as sporadic, endemic or epidemic [1].

Pediculus is a blood sucking parasite that is specific to humans. *Pediculus humanus var capitis* involved human head, *Phthirus pubis* involve genital area, and *Pediculus humanus var corporis* infest human body and use it as a warm place to live and feed. *Pediculus capitis* is the most common type in this group of ectoparasites especially in age groups of 3–11 years. Since 1970, the incidence of *Pediculus capitis* is increasing in the world [2].

Sarcoptes is an obligatory skin parasite and is important in dermatology. *Sarcoptes* usually involved hand skin including area between fingers and wrist, as well as elbow, feet, testis, and other sites of body [2].

The prevalence of *Pediculus capitis* in school children was reported by some studies as follows: Yasuj 11% [3], Babol 2.2% [4], Kerman 3.8% [5], Hamedan 1.3% [6], Turkey 6.8% [7], Korea 4.1% [8], and Egypt 12% [9]. In a study on Iranian prisoners, 9% of patients with dermatology complain hadpediculosis and 57% had scabies [10].

In poor communities the prevalence of scabies is reported more than 20% [11]. The prevalence of scabies was reported 0.4% in Turkey [7], 2.09% in Sari, and 1.3% [12] in Somehsara.

Since mentioned diseases are considered among important parasitic skin diseases and show the level of public health, by considering their high prevalence in our country and the necessity of identification of region of common infections, the dominant species in the region, and the mode of their transmission to human, we decided to report a 15-year period retrospective statistics study of patients who referred to parasitology laboratory of Imam Reza Hospital of Mashhad, Iran, one of the most important laboratories of east Iran.

Table 1: Frequency distribution of final diagnosis based on gender and place of living.

Final diagnosis	Gender		Place of living		
	Female	Male	Razavi Khorasan	North Khorasan	South Khorasan
Scabies					
Number	127	248	341	10	2
Percent	14.2	28.1	21.1	28.6	6.7
Pediculosis					
Number	65	34	92	2	3
Percent	7.3	3.8	5.7	5.7	10
Negative results*					
Number	702	602	1185	23	25
Percent	75.5	68.1	73.2	65.7	83.3

*Cases with final diagnosis except ectoparasites.

Table 2: Frequency distribution of final diagnosis based on age.

Age group	Final diagnosis						Total number	
	Scabies		Pediculosis		Negative			
	Number	Percent	Number	Percent	Number	Percent		
0–9	65	20.1	31	9.6	227	70.3	323	100
10–19	101	27.7	28	7.7	235	64.6	364	100
20–29	84	20.0	16	3.8	320	76.2	420	100
30–39	49	17.6	10	3.6	220	78.9	279	100
40–97	69	18.9	12	3.3	285	77.9	366	100
Total	368	21.0	97	55	1287	73.5	1752	100

2. Objects and Method

In this cross-sectional study which was performed in February 2012, records of 1851 patients who had been suspicious of suffering from ectoparasitosis and had been referred to parasitology laboratory of Imam Reza Hospital of Mashhad during a 15-year period (from April 1995 to April 2010) were evaluated. In this study, diagnostic method forpediculosis was inspection and microscopic examination and for diagnosis of scabies direct examination from eruption and then microscopic examination had been used.

The data were collected from their health records by a researcher made questionnaire. Patient demographic data including age, gender, occupation, place of birth and place of living, and final diagnosis were collected. Data were analyzed by SPSS v 15, using Chi square test. P value less than 0.5 was considered significant.

3. Results

In the present study, 1814 patients were assessed, 375 patients had scabies and 99 had pediculosis. The patients' minimum age was one month and maximum age was 97 years. *Sarcoptes* was more common among males with incidence of 13.7%, and pediculosis was more common among females with incidence of 7% ($P = 0.00$) (Table 1). The mean age of patients was 26.18 ± 17.68 years. The most common incidence of scabies and pediculosis was observed in age groups of 10–19 years and 0–9 years, respectively ($P = 0.00$) (Table 2).

Considering occupation, scabies was more common among workers with incidence of 32% and pediculosis was more prevalent among children with incidence of 9.9% ($P = 0.00$) (Table 3).

Regarding the place of living, the highest percentage of patients with *Sarcoptes* infestation were from Razavi Khorasan Province (18.7%), and the highest percentage of patients with pediculosis were from Razavi Khorasan Province as well (5%) ($P = 0.08$) (Table 1).

The laboratory results confirmed initial diagnosis, 23.7% of scabies and 45.1% of pediculosis ($P = 0.00$).

4. Discussion

Pediculosis is one of the common ectoparasitic infestations that are still considered as one of the health problems in the world [6, 7].

Our study revealed that ectoparasitic infestation is gender-dependent; the pediculosis is more common among females and scabies is more common among males. Many other studies have also shown that pediculosis is more common among females, which is similar to our results [8, 12]. It can be related to the women's hair length.

However, some of the researches about scabies have declared higher incidence in women rather than men [13]. Furthermore, the incidence of scabies in age groups of 31–40 and 41–50 is higher in women and in age groups 11–20 is higher in men [14]. These results are partly different from our findings. Poudat and Nasirian, in their study, reported similar

Table 3: Frequency distribution of final diagnosis based on occupation.

Occupation	Final diagnosis						Total number	
	Scabies		Pediculosis		Negative			
	Number	Percent	Number	Percent	Number	Percent		
Employee	24	16.2	6	4.1	118	79.7	148	100
Worker	16	32.0	2	4.0	32	64.0	50	100
Self employed	73	30.8	7	3.0	157	66.2	237	100
Housewife	58	11.6	22	4.4	419	84.0	499	100
Student	98	27.8	23	6.5	231	65.6	352	
Child	52	20.6	25	9.9	176	69.9	253	100
Retired/unemployed	44	22.4	11	5.6	141	71.9	196	100
Total	365	21.0	96	5.5	1274	73.4	1735	100

prevalence among two the genders [10]. It seems that more studies about scabies prevalence in Iran have to be performed.

In the present study, the highest incidence of scabies was among age groups of 10–19 years, and the highest incidence of pediculosis was among age groups of 0–9 years. In Lassa et al.'s study, England has the highest incidence of scabies which is also reported among groups of 10–19 years [15] which is similar to our results. The maximal incidence of disease was reported under 10 years old by Amro in Palestine [16] but in rural area of Brazil greater prevalence was under 4 years old [17]. These differences could be related to differences in lifestyle and hygienic conditions in different societies. Another paper from Occupied Palestine showed the highest rate of pediculosis in age groups of 4–11 years [18] which is in concordance with our results.

In the present study, the maximal rate of scabies observed among patients who were workers can be related to lower income, poor hygiene, and low education and the highest rate of pediculosis observed among children can be due to poor hygiene in childhood. Sim reported that increased economic status and increased parental concern about children might have resulted in decrease of head lice infestation in Korea [13]. A study in Iran reported that children with unemployed fathers have more incidence of head pediculosis [12]. Another Iranian study on soldiers showed greater incidence of scabies among soldiers whose parents were farmers [19]. A Study in Bushehr showed that children, whose fathers are workers or unemployed, are more likely to have scabies [20]. As it was described our results are in agreement with these findings.

The prevalence of ectoparasites varied around the world depending on hygienic condition of the communities [4–7, 9–11, 21–23]. The prevalence of head pediculosis among primary school children was reported to be 11% in Yasuj [3], 2.2% in Babol [4], 3.8% in Kerman [5], and 1.3 % Bahar Hamedan [6].

The prevalence of scabies is reported to be more than 20% in communities with low socioeconomic status. Studies that had been performed in Iran reported the incidence of scabies among primary school children, 2.09% in Sari [22], and 1.3% in Someasara [21]. According to the present study, the greater incidence of scabies and pediculosis was observed among patients from Razavi Khorasan Province, 18.7 and 5% separately.

A study in Germany showed that the relation between initial diagnosis and final diagnosis varied depending on the used diagnostic method; in diagnosing scalp pediculosis, this relation is 90.5% when wet combing method was used, and it is 28.6% when the diagnostic method is visual inspection [24]. Laboratory findings confirmed initial diagnosis of scabies and pediculosis cases 23.7% of 45.1% respectively. In this study, diagnostic method forpediculosis was inspection and microscopic examination and, for diagnosis of scabies, direct examination from eruption and then microscopic examination had been used.

5. Conclusion

Scabies is a prevalent dermatologic disease in Iran and is transmitted from person to person or from dressing or bed sheets to others. In this study, the highest incidence of scabies among different occupations was observed in workers with 32% incidence and between genders it was more common among males with incidence of 28.1%. Therefore, it seems that education about the signs and transmission method of this disease to high risk groups will help greatly to reduce the prevalence of scabies and prevent probable future epidemy. Increasing knowledge of high risk people and having good hygiene are the proper methods for controlling scabies in the community.

We found the most common incidence of pediculosis in age groups of 0–9 years; regarding gender, it was more common between females with incidence of 7.3%. Considering scalp pediculosis transmission way which is usually head to head, it will justify its higher incidence in primary school children and female gender. The risk factors for pediculosis are long hair, crowded family, age, personal hygiene, and contact with infected person as well as education levels. Therefore, to prevent pediculosis, physical contact with infected people must be restricted, and sharing dress and bed must be prevented. Training people about *Pediculus* life cycle, correct treatment method, and the importance of washing cloths and bedding with warm water or dry cleaning for eradicating insect and its eggs is very valuable. Besides periodic assessment of school children regarding pediculosis

is necessary. The authors believe that considering pediculosis high incidence in kindergarten and school children, educating parents and teachers is an important method for preventing and controlling pediculosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Putative Bronchopulmonary Flagellated Protozoa in Immunosuppressed Patients

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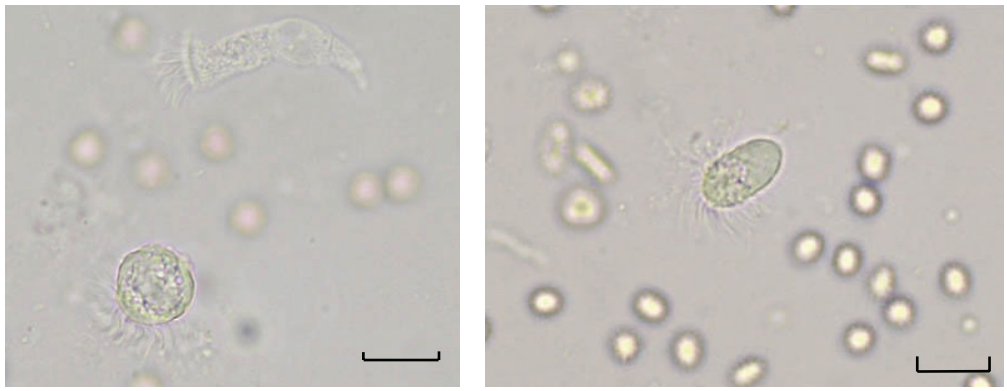
Flagellated protozoa that cause bronchopulmonary symptoms in humans are commonly neglected. These protozoal forms which were presumed to be "flagellated protozoa" have been previously identified in immunosuppressed patients in a number of studies, but have not been certainly classified so far. Since no human cases of bronchopulmonary flagellated protozoa were reported from Turkey, we aimed to investigate these putative protozoa in immunosuppressed patients who are particularly at risk of infectious diseases. Bronchoalveolar lavage fluid samples of 110 immunosuppressed adult patients who were admitted to the Department of Chest Diseases, Hafsa Sultan Hospital of Celal Bayar University, Manisa, Turkey, were examined in terms of parasites by light microscopy. Flagellated protozoal forms were detected in nine (8.2%) of 110 cases. Metronidazole (500 mg *b.i.d.* for 30 days) was given to all positive cases and a second bronchoscopy was performed at the end of the treatment, which revealed no parasites. In conclusion, immunosuppressed patients with bronchopulmonary symptoms should attentively be examined with regard to flagellated protozoa which can easily be misidentified as epithelial cells.

1. Introduction

Protozoa such as Microsporidia, *Cryptosporidium, Entamoeba histolytica,* and *Leishmania* which are single-cell eukaryotes, are amongst the microorganisms that rarely cause infections in the respiratory system. Severity of these infections can vary according to the immune status, which is altered in AIDS, organ transplantation, cancer, and corticotherapy [1]. *Lophomonas blattarum* is another example of a single-cell eukaryote that occasionally appears to infect humans. Only a few articles reported that *Lophomonas blattarum* and other flagellated protozoa caused bronchopulmonary infections in humans [2–6]. No human cases of bronchopulmonary flagellated protozoa were reported from Turkey, but only a few cases were diagnosed as *L. blattarum* by Levent Doğancı (Bayındır Hospital, Turkey, personal communication).

Ribas et al. reported that, in the case of pulmonary infections in immunosuppressed patients, examining bronchial secretions including bronchoalveolar lavage fluid (BALf) would be more useful to detect certain microorganisms [7]. It has been argued that it was difficult to distinguish protozoal forms from epithelial cells as the morphological features are quite similar. In addition to the difficulty in differentiating the emerging parasite, *L. blattarum*, and the other flagellated protozoa in bronchial secretions, bronchial epithelial cells could easily be misidentified as flagellated protozoa [8, 9]. *Lophomonas blattarum* is a flagellated protozoon found in order Hypermastigida and suborderLophomonadina. It is accepted as an endocommensal in the intestine of cockroaches such as *Periplaneta americana* and *Blattella orientalis*. *L. blattarum* is approximately 20–60 μm in length and has round to oval shape [10].

Mode of transmission of these flagellated protozoa still remains a mystery. The most frequent symptoms in humans are fever, cough, and sputum expectoration [3]. Radiology may reveal signs of pneumonia, bronchiectasis, pulmonary abscess, and pleural effusion. Successful treatment



(a) (b)

Figure 1: (a) A round shaped flagellated protozoon with granular cytoplasm and wavy, not combed flagella of different lengths without a terminal bar in BALf smear. At the top it is possible to observe a columnar ciliated epithelial cell (wet mount preparation ×400, case number 1). (b) An oval shaped flagellated protozoal form in BALf. Long flagella of variable length are inserted around the cytoplasm (wet mount preparation ×400, case number 2). Scale bar = 25 μm.

by metronidazole has been reported [1–3]. Many researchers concluded that microscopic examination of the respiratory secretions was the essential method for diagnosis of protozoal forms [3, 7, 9].

Even though bronchoscopy is an invasive method, parasitic examination of BALf was performed in immunosuppressed patients in whom fiberoptic bronchoscopy (FOB) was indicated for other pathologies. The aim of this study was to investigate bronchopulmonary flagellated protozoa in immunosuppressed patients.

2. Materials and Methods

BALf samples were obtained by FOB (Olympus, EVIS EXERA II CV-180, Tokyo, Japan) from 110 immunosuppressed adult patients who were admitted to the Department of Chest Diseases, Celal Bayar University, Manisa, Turkey, between 2011 and 2012. BALf collection was performed by wedging the tip of the bronchoscope into the nondependent lobes, especially middle lobe of the right lung and lingula of the left lung in each patient. The BALf collected lobe was determined by the images of the lesion with the greatest radiological abnormality. About 100 mL of sterile physiologic saline warmed to the body temperature was instilled in 20 mL aliquots. Gentle manual suction was applied to retrieve the saline. BALf was collected in sterilized containers and brought to the laboratory. Apart from these, additional invasive surgery was not applied to the patients.

BALf samples were examined directly and after centrifugation at 1000 ×g for 5 minutes under the light microscope (×400) within half an hour. A drop of BALf was put on the slide and covered with a coverslip for direct wet mount examination. This preparation was used primarily to observe the movements of cilia or flagella of putative protozoan and epithelial cells. Round or oval, motile trophozoites (20 to 60 μm in length) with granular cytoplasm and wavy, not combed flagella of different lengths without a terminal bar were considered to be flagellated protozoa (Figures 1(a) and 1(b)). Round or columnar shaped cells with straight, combed, and uniform length cilia with rhythmic and synchronous movements were considered to be ciliated bronchial epithelial cells (Figure 1(a)).

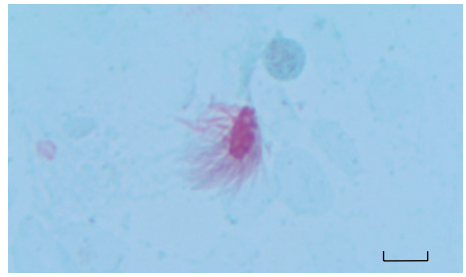
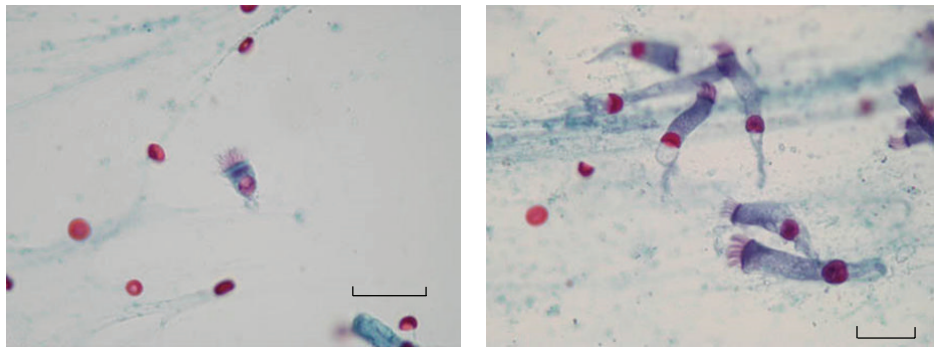


Figure 2: A putative bronchopulmonary flagellated protozoon in stained BALf smear (Wheatley's Trichrome stain ×1000). Long and irregular flagella are inserted around the cytoplasm. Scale bar = 25 μm.

BALf samples were stained using the trichrome technique of Wheatley [11]. Briefly, the BALf smeared slides were allowed to air dry for a few minutes following fixation in Schaudinn's fixative for at least 30 minutes. Then the staining process was performed as follows. First, slides were immersed in 70% alcohol for 5 minutes, followed by removal of mercuric chloride by 70% alcohol plus iodine for 1 minute. Iodine was then removed from the smear in two changes of 70% alcohol for 5 minutes of each and stained with trichrome stain for 10 minutes. For destaining, the slides were immersed in 90% alcohol plus acetic acid for 1 to 3 seconds and dipped several times in 100% alcohol as a rinsing step. Two changes of 100% alcohol for 3 minutes of each were used for dehydration followed by two changes of xylene for 5 to 10 minutes to complete the dehydration step. Finally, slides were covered with a coverslip and examined under light microscope using ×100 ocular piece. Protozoal forms and ciliated epithelial cells were distinguished based on the characteristic features as previously described by Ribas et al. [7]. A putative bronchopulmonary flagellated protozoon stained by Wheatley's trichrome is presented in Figure 2. Columnar cells



(a) (b)

Figure 3: (a) A ciliated epithelial cell in BALf smear. Straight and combed cilia of the same length, inserted into a terminal bar, can be seen along one edge. A clear nucleus is also seen at the end of the cytoplasm (Wheatley's Trichrome stain ×1000). (b) A group of columnar epithelial cells in BALf smear. (Wheatley's Trichrome stain ×1000). Scale bar = 25 μm.

having short, regular cilia and discernible terminal bar were considered to be ciliated bronchial epithelial cells (Figure 3).

Cases positive for protozoal forms were treated with metronidazole (500 mg *b.i.d.* for 30 days). Second FOB was carried out in positive cases as a follow-up control after treatment. A questionnaire was also given to positive cases.

Before the study, approval was obtained from the Ethics Committee of Faculty of Medicine, Celal Bayar University (approval number 118, dated May 18, 2011) and all patients were informed and written consents were taken.

3. Results

Flagellated protozoa were found in nine of 110 (8.2%) immunosuppressed patients. Figure 1 presents protozoal forms of case number 1 and case number 2 in the BALf samples. Of these nine positive cases, eight were male, and one was female. It was found that most of the cases were farmers or factory laborers with low socioeconomic status. Sociodemographic, clinical, and laboratory findings of these cases were given in Table 1.

No flagellated protozoa were detected in BALf samples of all positive cases after treatment. In follow-up controls, it was observed that the initial pulmonary symptoms were considerably recovered in all cases.

4. Discussion

Only a few human cases have been reported in the world on bronchopulmonary infection caused by flagellated protozoa [7, 8]. However, presence of *L. blattarum,* which has similar morphology like other protozoal forms, has been reported in some studies [2–4, 12]. A detailed review based on extensive search of PubMed and Google Scholar about *L. blattarum* and bronchopulmonary protozoal infections has recently been published [13].

Ribas et al. [8] and Martínez-Girón et al. [9] have submitted "Letters to the Editor" claiming that some figures presented in the related manuscripts did not actually depict flagellated protozoa/*L. blattarum*, arguing that the bronchial epithelial cells could easily be misidentified as flagellated protozoa, which is in agreement with our experience in our series of 110 patients. A detailed table was reported which can be very useful to distinguish protozoal forms from ciliated epithelial cells [7]. Based especially on this table and the other literature, we were able to detect the protozoal forms and the bronchial epithelial cells in BALf samples though we could not differentiate *L. blattarum* from other protozoal forms. Thus, we defined all the parasitic forms we detected as flagellated protozoa. We observed nearly 1 or 2 bronchopulmonary flagellated protozoan cells per 10 microscopic fields (×400) while numerous epithelial cells were seen in each field in direct examination of BALf samples. Moreover, epithelial cells formed clusters in most of microscopic fields, so the protozoan cells could easily be overlooked. This paper highlights the need for molecular techniques to detect their presence and to differentiate the protozoal forms in respiratory secretions. Apart from these, culture studies we performed with Trypticase-Yeast-Maltose, Cystein-Peptone-Liver-Maltose, and Novy-Nicolle-McNeal media had unfortunately failed. We think successful cultivation of these parasites will enable us to describe detailed morphological features, to improve specific molecular techniques, and to develop novel treatment choices.

A cytological study [7] in which sputum smears of 106 immunocompromised patients (83 AIDS, 23 non-AIDS patients) were evaluated in terms of protozoal forms and compared with nonimmunocompromised group (control group, $n = 85$) with different respiratory disorders showed a greater number of protozoal forms in the sputa of patients with AIDS (86.7%) in comparison to the other two groups. Protozoal forms were found in 34.8% and 18.8% in non-AIDS immunocompromised patients and in control group, respectively, in that study. In a Letter to the Editor which reported an AIDS case [8], the authors stated that a multi-flagellated protozoan cell was found in the aspiration fluid. Because FOB is an invasive method, healthy control group was not included in the present study, so it is unclear whether the protozoa observed in immunosuppressed cases are more common than healthy controls.

Table 1: Sociodemographic, clinical, and laboratory findings of nine cases detected with flagellated protozoal forms.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9
Gender	Male	Male	Male	Male	Female	Male	Male	Male	Male
Age	48	60	56	76	71	49	74	78	67
Occupation	Worker	Farmer	Worker	Farmer	Housewife	Worker	Farmer	Farmer	Retired
Socioeconomic status	Low	Low	Low	Low	Low	Low	Low	Low	Low
Presence of cockroaches in the house	Yes	No	Yes	No	Yes	Yes	No	No	Yes
Symptom	Weakness	Weight loss	Dyspnea	Weight loss	Cough	Dyspnea	Dyspnea	Dyspnea	Weakness
Comorbidity	Diabetes mellitus	Psoriasis	Lung cancer	Kaposi sarcoma	COPD	COPD	COPD, hypertension	Alzheimer, nasopharyngeal carcinoma	Psoriasis
Radiological findings	Reticular infiltration	Reticular infiltration and bronchiectasis	Alveolar opacities in right upper lung	Reticular infiltration	Reticular infiltration	Lung abscess and pyothorax	Lobar infiltration	Multilobar infiltration and atelectasis	Reticular infiltration
White blood cell ($\times 10^9$)	7.7	10.8	9.6	6.5	14.2	14.7	11.6	27.0	13.7
Neutrophil (%)	65.4	58.5	63.7	45.7	69.3	64.1	52.4	71.4	56.9
Lymphocyte (%)	25.1	30.2	22.5	38.6	20.1	22.6	21.6	22.6	27.2
Eosinophil (%)	2.4	4.7	4.1	7.1	3.3	1.4	11.3	2.8	7.4
Erythrocyte sedimentation rate	65	88	69	81	46	94	53	104	59
Presence of malignancy	No	No	Yes	Yes	No	Yes	Yes	Yes	No
Usage of corticosteroid	Yes	No	No	Yes	No	Yes	No	Yes	No
Usage of anti-TNF-α drugs	No	Yes	No	No	No	No	No	No	Yes
Duration of metronidazole treatment (days)	30	30	30	30	30	30	30	30	30
Flagellated protozoa after treatment	Eradicated	Eradicated	Eradicated	Eradicated	Eradicated	Eradicated	Eradicated	Eradicated	Eradicated

COPD: Chronic obstructive pulmonary disease.

In a study in which the relationship between protozoa and asthma was evaluated [6], data supported the hypothesis that protozoa were more prevalent in patients diagnosed with asthma than in comparable controls. That study also investigated the relationship between presence of protozoa in sputum and dampness in the house and the data did not support the hypothesis that an increased prevalence of protozoa in sputum was associated with living in damp houses. However, as all of our positive cases were from low socioeconomic background, it was considered that the home in which the patients lived and hygienic conditions might contribute to the occurrence of the parasites. Furthermore, it was thought that the presence of cockroaches in abundance in such houses could play a crucial role for infection [1], but in our cases such a risk was not encountered.

In our study, the cases infected with flagellated protozoa were treated with metronidazole and after the treatment a second FOB and chest radiography were performed for follow-up control. One of the common points in the literature was to use metronidazole in the treatment [4, 8]. However, the dosage and the length of the treatment period were different from one case to another. We considered that the considerable recovery of symptoms and improvements in radiological findings of our cases in the follow-up controls supported the accuracy of our diagnosis and the treatment choice as well.

We conclude that immunosuppressed patients with bronchopulmonary symptoms should attentively be examined with regard to flagellated protozoa which can easily be misidentified as epithelial cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Individual Subject Meta-Analysis of Parameters for *Giardia duodenalis* Shedding in Animal Experimental Models

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Giardia duodenalis is a zoonotic protozoan parasite with public health importance worldwide. While articles about animal model infectivity have been published for *G. duodenalis*, the studies have used diverse protocols and parameters to evaluate the infectivity of this protozoan parasite. Hence, the objectives of this study were to (1) conduct a meta-analysis of published literature for cyst shedding and diarrhea outcomes in animal models and (2) develop recommendations to help standardize experimental dose response studies. Results showed that, for the outcome of cyst shedding in faeces, the covariates of infective stage (cyst versus trophozoite), *Giardia* dose, and the interactions between doses and infective stage, as well as dose and species of experimental host, were all significant (P value ≤ 0.05). This study suggests inoculation of the experimental host with cysts rather than trophozoites and administration of higher doses of *Giardia* will most likely result in cyst shedding. Based on the results of this meta-analysis, the infective stage (cyst versus trophozoite), parasite dose, and the interactions between dose and infective stage, as well as dose and species of experimental host, should be considered when designing experimental dose response studies that will assist in the study of zoonotic neglected tropical diseases globally.

1. Introduction

Giardia duodenalis is a zoonotic waterborne pathogen known to be released into the environment through human and animal faeces [1]. Infection and gastrointestinal disease in humans are caused by ingestion of small doses (>10 cysts) of *Giardia* cysts [2] directly from faeces or indirectly from contaminated water and food products [1, 3]. *Giardia* is considered the most common cause of protozoan diarrhea in developing countries and worldwide [1]. It has been estimated that in Africa, Asia, and Latin America about 200 million people have symptomatic giardiasis with some 500000 new cases being reported each year [4]. The number of reported cases in the US was 18,913 in 2008 [5]. In 2004, *Giardia* was included in the World Health Organization's Neglected Disease Initiative for its link with poverty [6]. *G. duodenalis,* also called *G. intestinalis* and *G. lamblia,* is the only species found in humans [1, 3] and will be referred to as *G. duodenalis* throughout this paper.

Animal and human dose-response models as well as in vitro cell culture have been widely used to evaluate *Giardia* spp. infectivity. At present, human infectivity studies are not

considered to be practical because of the ethical concerns involved. Therefore, very few experiments have involved the attempted infection of humans with *Giardia* isolates [2, 7]. Instead, experimental animal models have been frequently used to estimate whether *G. duodenalis* is strictly host specific or zoonotic [1] and to provide information used for human health risk assessments. In vitro cell culture has been proposed as a potential alternative to animal assays because cultivation has the advantage of being cheaper and less time consuming and does not raise the ethical concerns associated with using animal models [8]. However, experimental animal model studies are often preferred when performing risk assessment, as they provide the most realistic scenario of what is happening with the pathogen once it enters the host. For this reason, this meta-analysis focuses on the diverse range of experimental animal model studies available in the peer-reviewed literature.

Numerous cross-transmission experiments involving the infection with isolates of *Giardia* in a variety of animal species have been published. Each of the experimental studies used different parameters such as animal species, age, cysts or trophozoites, dose, and detection methods to quantify cysts in faeces and evaluate the infectivity of *G. duodenalis* as well as outcomes ranging from laboratory detection of cyst shedding to clinical symptoms. Using diverse variables and protocols can be problematic in experimental studies because the variations in protocols between studies can affect the outcome of the experiments and thus their comparability [1, 9]. Consequently, a standardization of the experimental dose-response studies is required.

Hence the main objective of the present study was to develop recommendations for the standardization of animal dose-response experiments by conducting a meta-analysis of individual subjects and exploring study design characteristics that cause heterogeneity between included studies. The hypothesis tested in this meta-analysis was that selected experimental factors of interest are associated with increased *G. duodenalis* cyst shedding and diarrhoea. The results of this meta-analysis will be useful for more standardized and comparable studies of *Giardia* infectivity, so that in the future there may be fewer animal species and resources used to provide dose-response information more quickly and efficiently.

2. Methods

2.1. Literature Search Strategy and Selection of Studies. The search of published animal and/or human dose-response studies in all languages was performed using the electronic databases PubMed and Web of Science from March to December, 2010. The process is described in Figure 1, with search criteria consisting of the following algorithms: (i) protozoan infectious doses for humans, (ii) *Giardia* and dose response, (iii) *Giardia* and infectivity, (iv) *Giardia* and meta-analysis, (v) giardiasis and experimental and model, and (vi) experimental and infection and *Giardia*. Unpublished studies (grey literature) were not included in this meta-analysis.

The articles identified by means of these search criteria were subject to a further selection process consisting of the removal of the complete study or individuals within the articles that met any of the exclusion criteria (Table 1). One reviewer examined the titles and abstracts of all the articles found using the search criteria mentioned above. The full article for each of the relevant studies was then assessed by the same reviewer and a second reviewer was consulted as needed. Only those articles that met at least one of the search criteria and none of the exclusion criteria were included in the data analysis. Furthermore, the reference lists of all included articles were searched for further possible papers, but no additions were identified. Ethical approval was not required for this meta-analysis.

2.2. Data Extraction. Data regarding the following variables for each individual animal were extracted and recorded from each article: (i) *Giardia species* (*Giardia*), (ii) isolate analyzed, such as HP-10 (Isolate), (iii) assemblage of the *Giardia sp.* such as A (Assemblage), (iv) original source of isolate (Isolate Source), (v) whether cysts or trophozoites were subject to passage before challenge (Passage), (vi) storage time of cysts or trophozoites (in weeks) prior to inoculation of experimental host (Storage Time), (vii) method used to confirm cyst or trophozoite viability prior to inoculation (Viability Method), (viii) animal species used as experimental host (Experimental Species), (ix) number of subjects per group inoculated (Number), (x) age of the experimental host (Age), (xi) whether the experimental host was subject to immunosuppression (Immunosuppression), (xii) method of immunosuppression of the experimental host (Immunosuppression Cause), (xiii) whether cysts or trophozoites were administered to the experimental host (Infective Stage), (xiv) cyst or trophozoite dose administered to the experimental host (*Giardia* Dose), (xv) administration route used to inoculate the cysts or trophozoite dose to the experimental host (Administration Route), (xvi) cyst detection method in faeces of experimental hosts after inoculation (Detection Method), and (xvii) the number of animals that shed cysts and/or presented with diarrhoea after inoculation.

The unit of analysis was the individual animal; thus, the value for each variable was collected for each animal and included in the analysis. If a variable was not reported at the animal level, the variable was left blank. Table 2 provides information regarding the number of subjects and missing values by variable and study. The primary outcomes of interest were the presence of cyst shedding and diarrhoea. An animal was considered to have shedding or diarrhoea when the condition of the animal was described with the words diarrhoea or cyst shedding in the study from which the information was extracted. All of the studies included in this analysis evaluated shedding over prolonged periods so even if shedding was intermittent, any report of cysts observed in the feces was considered as positive for shedding. For diarrhoea and cyst shedding, the classification of Yes/No was used. Initially, attempts were made to contact authors for clarification, but, due to lack of responses, this approach was not systematically implemented throughout the entire study.

2.3. Classification of Variables. The extracted variables were classified according to the information provided by the

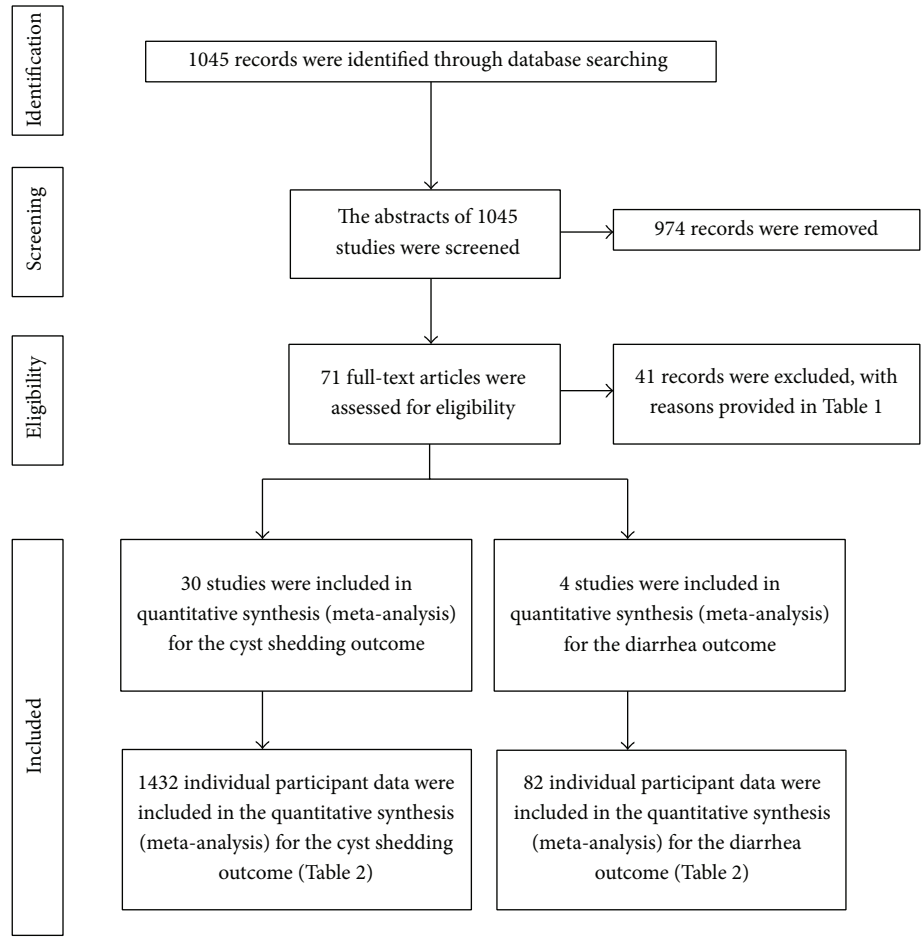

Figure 1: Flow of information through the different phases of a systematic literature review for the *Giardia duodenalis* meta-analysis.

Table 1: Exclusion criteria for studies and number of articles excluded from the *Giardia duodenalis* meta-analysis.

Exclusion criteria	Articles excluded
(1) *Giardia* different than *G. duodenalis*, *G. intestinalis*,or *G. lamblia* were used	19
(2) Infection was assessed only by histology on tissue sections	6
(3) Number of animals with diarrhoea or shedding was not reported	4
(4) Animal study groups were subject to treatments other than the single inoculation/single outcome design used in this meta-analysis	3
(5) Infection was assessed by cyst shedding and histology together and it was not possible to distinguish how many animals were shedding	4
(6) Experimental dose response was measured in humans only	2
(7) Study was not an experimental dose response experiment	1
(8) Cyst dose administered to the experimental host was not provided or a range was provided	1
(9) Number of animals used was not provided	1

selected studies for each outcome (Table 3). In the case of the variable "Isolate Source," the classification "Other than Humans" was composed of ruminants (cattle and lambs), primates, rodents, and drinking water. For the variable "Passages," the classification "Yes" was composed of in vivo (animal passage), in vitro (cultures), and both in vivo and in vitro passages, while the classification "No" corresponded to cysts that were not subject to any passage. For the "Storage Time" variable, the class "<1 week" included animals that were inoculated with cysts or trophozoites that were stored for less than one week; "≥1 week" included all those animals that were inoculated with cysts or trophozoites that were stored for one week or more. Treating the variable "Storage Time" as a continuous variable, with or without log transformation, did not

improve the model fit. Regarding the variable "Experimental Species," the category "Other Animals" included dogs, cats, and cattle, while the class "Other Rodents" comprised rats and hamsters. The variable "Age" was categorized following published criteria (Table 4), which were consistent with the criteria used in the studies included in this meta-analysis. The classification of age was dependent on the animal model as animal species mature at different rates. For instance, 2-month-old kittens are in a different maturity stage than 2-month-old gerbils and thus might have different susceptibilities to protozoan infection. When ranges of age were reported, the mean of the range was used in the meta-analysis. The classification "Young" in the "Age" variable for the shedding outcome consisted of newborn and weanling animals, while the classification "Adult" included only adult animals. For the diarrhoea outcome, no adult animals with diarrhoea were reported. Therefore, the "Age" variable for the diarrhoea outcome was composed of "Newborn" and "Weanling." For the variable "Administration Route," "Gastric Intubation" included the terms gastric intubation, intragastric route, and stomach tube, while "Other Than Gastric Intubation" included the terms orally, intraesophageal route, and nasogastric. The variable "Detection Method" was only included in the cyst shedding outcome and included three classes: "Hemocytometer," "Microscopy," and "Flotation Techniques." "Microscopy" included indirect fluorescent antibody (IFA), fluorescent microscopy, and Nomarski interference contrast (NIC) microscopy. "Flotation Technique" included parasite concentration methods such as zinc sulfate flotation and sucrose gradient flotation. The variable "*Giardia* Dose" was a continuous variable. As the values in the "*Giardia* Dose" variable ranged from 4 to 10^7 cysts or trophozoites, the data for this variable were log transformed to make the distribution of the data more normal. The mean of the whole dose series (mean log scale = 4.12) was subtracted from every dose value to center the data. This enabled interpretation of the main effect in the presence of an interaction term at a meaningful value (the mean) [10]. For instance, in the multivariable model, the main effect of "Infective Stage" in the model that also includes the interaction "*Giardia* Dose by Infective Stage" corresponded to a difference between cysts and trophozoites at a mean dose. If the mean of the whole dose series was not subtracted, this comparison would have been the difference between cysts and trophozoites at a log dose of zero (dose of 1).

2.4. Statistical Analyses. In this meta-analysis, the statistical analysis and publication bias assessment were done as described in Adell et al. [11]. Final models were selected based on inclusion of the four key variables "Experimental Species," "Age," "*Giardia* Dose," and "Infective Stage," allowing for as many interaction terms as possible and having a combination of variables that generated narrower confidence intervals or more precision. The fit of the model was assessed by the ratio of the generalized chi-square statistic and its degrees of freedom (generalized chi-sq/df), which could not exceed a value of 1, and residual plots. The statistical software JMP 9 (SAS 2010) and SAS 9.3 (SAS 2011) were used to perform all the analyses.

3. Results

3.1. Search Strategy and Selection of Studies. As shown in Figure 1, the initial search identified 1045 potentially relevant studies. Subsequent to reviewing the abstracts, 71 studies were considered for further screening, of which 41 were not eligible since at least one of the exclusion criteria (Table 1) was met. After examination of the full text, 30 studies were included for the cyst shedding outcome while 4 were incorporated for the diarrhoea outcome. The studies included in this meta-analysis were published between 1978 and 2010 and are shown in Table 2. The total number of individuals included in this meta-analysis was 1432 individuals for the shedding outcome and 82 individuals for the diarrhoea outcome.

3.2. Analysis for the Cyst Shedding Outcome. The bivariate analysis showed that the covariates "Passage," "Experimental Species," "Age," "Infective Stage," and "*Giardia* Dose" were associated with cyst shedding in faeces (P value $\leq$ 0.2) (Table 5). These variables were then incorporated in the multivariable analysis which provided one model that best fulfilled the selection criteria. The final multivariable model (Table 6) shows that the variables "Infective Stage" and "*Giardia* Dose" and the interactions "*Giardia* Dose by Experimental Species" and "*Giardia* Dose by Infective Stage" have at least one category with a statistically significant difference (P value $\leq$ 0.05) from the reference category (Table 6). The variables "Experimental Species," "Age," and "Administration Route" were not significant, but they were incorporated into the model to control for confounding and effect modification. "Administration route" and "Age" were identified as a potential confounder (an epidemiologic term specifically describing a variable associated with a change in the coefficient estimate of at least one variable when placed in the model) of the relationship between "*Giardia* dose" and cyst shedding and "Experimental Species" and cysts shedding, respectively. "Experimental species" was part of the interactions "*Giardia* Dose by Experimental Species."

The multivariable model indicated that inoculating cysts into the experimental host had 5.02 times higher odds of cyst shedding than inoculating trophozoites at the mean log dose of 0.0538 (P value $\leq$ 0.001; CI: 2.63, 9.56). For each 1 unit of change in the log of trophozoite dose administered to the reference experimental host "Gerbil," the odds of cyst shedding increased 2.67 times (P value $<$ 0.0001; CI: 1.81, 3.94), whilst the odds ratio corresponding to an increase of one in log dose in cysts was 0.57 times that in trophozoites (P value = 0.002; CI: 0.41, 0.81).

The model also indicated that, for each unit of increase in the log dose administered to "Mice," the odds of cyst shedding increased 4.36 times compared to "Gerbils" (P value: 0.004; CI: 1.59, 11.93). Whereas, for each unit increase in the log dose administered to "Other Rodents," the odds of having cyst shedding were reduced 0.09 times compared to "Gerbils" (P value: 0.02; CI: 0.01, 0.72). Figure 2(a) shows the range of doses compared to the odds of detecting shedding by experimental species, illustrating how the choice of the experimental animal species does not have an effect until beyond a log scale 6, with mice only having an increase in

Table 2: Studies included for *Giardia duodenalis* meta-analysis of diarrhoea (D) and shedding (S) outcomes (chronological order).

Reference	Year	Outcome	Sample size (number of animals)								
			IS[1]	P[2]	ST[3]	ES[4]	A[5]	IS[6]	GD[7]	AR[8]	DM[9]
[23]	1978	S[10]	20	20	20	20	20	20	20	20	20
[24]	1979	S[10]	47	47	NR[11]	47	47	47	47	47	NR[11]
[17]	1982	S[10]	11	11	11	11	11	11	11	11	NR[11]
[19]	1982	S[10]	150	150	150	150	150	150	150	150	150
[25]	1984	S[10]	49	49	NR[11]	49	49	49	49	49	NR[11]
[26]	1985	S[10]	25	25	14	25	NR[11]	25	25	25	25
[18]	1986	S[10]	60	60	NR[11]	60	49	60	60	60	60
[27]	1988	S[10]	204	204	107	204	204	204	204	204	204
[28]	1989	S[10]	10	10	NR[11]	10	NR[11]	10	10	10	10
[29]	1990	S[10]	75	75	NR[11]	75	75	75	75	75	75
[30]	1991	S[10]	NR[11]	NR[11]	NR[11]	10	10	10	10	10	10
[31]	1991	S[10]	109	109	109	109	109	109	109	109	109
[32]	1992	S[10]	NR[11]	14	NR[11]	14	14	14	14	14	14
[33]	1993	S[10]	62	62	NR[11]	62	62	62	62	62	62
[34]	1994	S[10]	4	16	NR[11]	16	16	16	16	NR[11]	16
[35]	1996	S[10]	10	10	10	10	10	10	10	10	10
[36]	1997	S[10]	NR[11]	10	NR[11]	10	10	10	10	10	10
[37]	2002	S[10]	NR[11]	94	NR[11]	94	94	94	94	94	94
[38]	2005	S[10]	56	56	NR[11]	56	56	56	56	56	56
[39]	2006	S[10]	NR[11]	6	NR[11]	6	6	6	6	6	6
[40]	2007	S[10]	97	97	97	97	97	97	97	97	97
[41]	2007	S[10]	6	6	NR[11]	6	6	6	6	6	6
[42]	2008	S[10]	NR[11]	12	NR[11]	12	12	12	12	12	12
[43]	2008	S[10]	NR[11]	60	NR[11]	60	60	60	60	60	60
[44]	2010	S[10]	40	40	NR[11]	40	40	40	40	40	40
[45]	2010	S[10]	33	33	33	33	33	33	33	33	33
[46]	1995	S and D[12]	70[13], 28[14]	70[13], 28[14]	30[13], 8[14]	70[13], 28[14]	70[13], 28[14]	70[13], 28[14]	70[13], 28[14]	40[13], 20[14]	70[13], NA[15]
[47]	1995	S and D[12]	52[13], 30[14]	52[13], 30[14]	22[13], NR[11]	52[13], 30[14]	52[13], 30[14]	52[13], 30[14]	52[13], 30[14]	52[13], 30[14]	52[13], NA[15]
[48]	1997	S and D[12]	10[16]	10[16]	NR[11]	10[16]	10[16]	10[16]	10[16]	10[16]	NA[15]
[49]	2010	S and D[12]	14[16]	14[16]	NR[11]	14[16]	14[16]	14[16]	14[16]	14[16]	NA[15]

[1]Isolate Source, [2]Passage, [3]Storage Time (weeks), [4]Experimental Species, [5]Age, [6]Infective Stage, [7]*Giardia* Dose, [8]Administration Route, [9]Detection Method, [10]Shedding outcome, [11]Information was not reported in study; thus no individual data were included, [12]Shedding and Diarrhoea outcome, [13]Number of individuals for shedding outcome, [14]Number of individuals for diarrhoea outcome, [15]Does not apply, [16]Number of individuals for both outcomes.

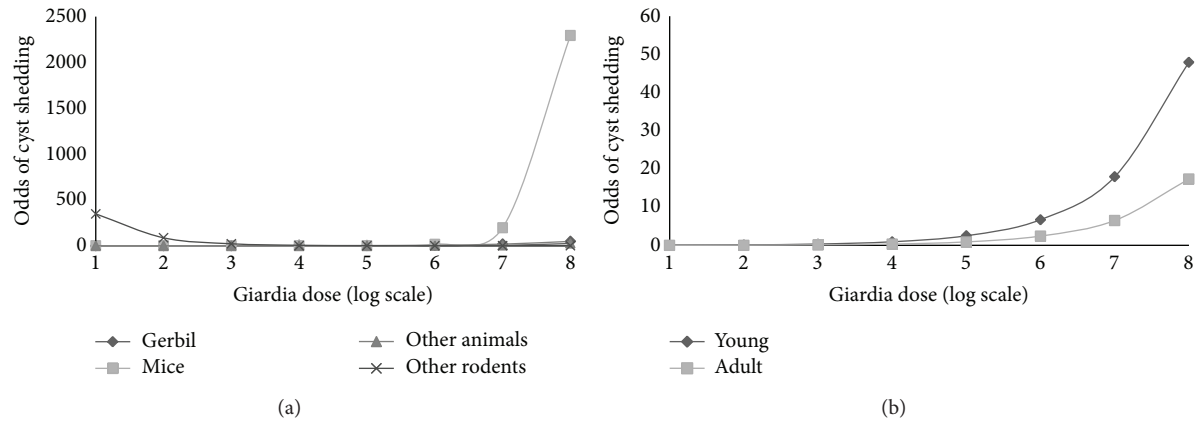

Figure 2: Range of *Giardia* dosages captured in the articles included in this meta-analysis by the odds of the cyst shedding outcome for the categories "Age" and "Experimental Species." (a) Range of *Giardia* dosages (log scale) by odds of cyst shedding by Experimental Species. (b) Range of *Giardia* dosages (log scale) by odds of cyst shedding by Age.

Table 3: Classification of categorical variables for both *Giardia duodenalis* cyst shedding and diarrhoea outcomes.

Variable	Shedding outcome			Diarrhoea outcome		
	Classification of variables[1]	Number of studies	Number of individuals	Classification of variables[1]	Number of Studies	Number of individuals
Assemblage	A	3	148	A and E	1	14
	B	1	49			
	E	1	6			
	A and E	1	14			
Isolate Source	Humans	19	1128	Humans	1	28
	Other Than Humans	4	86	Other Than Humans	3	54
Passage	No	17	755	No	2	24
	Yes	16	667	Yes	2	58
Storage Time	<1 week	10	420	<1 week	1	8
	≥1 week	3	183			
Experimental Species	Mice	7	180	Other Animals	2	24
	Other Animals	8	87	Gerbils	2	58
	Other Rodents	4	202			
	Gerbils	15	963			
Age	Adult	12	458	Newborn	2	24
	Young	18	828	Weanling	2	58
Infective Stage	Cysts	20	1021	Cysts	3	64
	Trophozoites	18	411	Trophozoites	2	18
Administration Route	Gastric Intubation	6	233	Gastric Intubation	2	50
	Other Than Gastric Intubation	23	1153	Other Than Gastric Intubation	2	24
Detection Method	Hemocytometer	15	749	Not analyzed		
	Microscopy	3	53			
	Flotation Technique	9	523			

[1] Categories not listed indicate that there were no animals from those categories in the studies.

Table 4: Criteria used to determine age categories for experimental species (alphabetical order).

Animal species	Criteria used		
	Newborn	Weanling[1]	Adult
Cattle	<21 weeks	21–64 weeks [50]	≥65 weeks [51]
Cats	<8 weeks	8–25 weeks [52]	≥26 weeks [51]
Dogs	<8 weeks	8–29 weeks [53]	≥30 weeks [51]
Gerbils	<4 weeks	4–7 weeks [54]	≥8 weeks [55, 56]
Hamsters	<3 weeks	3 weeks [57]	>3 weeks
Mice	<3 weeks	3 weeks [58]	>3 weeks [51]
Rabbits	<4 weeks	4 weeks–17 weeks [59, 60]	≥18 weeks [51]
Rat	3 weeks	3–7 weeks [61]	≥8 weeks[51]

[1] Weanling was merged with the newborn category to create the classification "Young" in the "Age" variable for the cyst shedding outcome.

odds. When "Mice" was removed from the analysis, the odds of shedding cysts were similar for all animals until log 7 after which the odds of shedding were greater in "Gerbils" as opposed to "Other animals." Figure 2(b) shows the ranges of *Giardia* doses compared to the odds of detecting shedding and how choosing young experimental animals had a higher impact on shedding in response to different doses being investigated compared to adult experimental animal.

The fit of the model was assessed by the ratio value for generalized chi-sq/df value and residual plots. The generalized chi-sq/df for the multivariable model was 0.66, indicating that the model had good fit. The residual plots (not shown), in which the residual values were plotted against the linear predictor, showed that two observations in each model had large residuals (≥15) and were possible outliers from the cluster of observations. Removing the individuals that had large residuals did not change the significance of the other variables of the model; thus, they were kept in the model.

In this meta-analysis, we found evidence of possible publication bias for the cyst shedding outcome. The funnel plot showed a higher number of studies over the mean value (Figure 3). The Egger regression test suggested a significant

Table 5: Bivariate analysis results for risk factors associated with *Giardia* cyst shedding outcomes.

Variable[1]	*P*-value	Statistical association between variable and cyst shedding (Yes/No)[2]
Passage	<0.0001[3]	Yes
Storage Time	0.9269[3]	No
Experimental Species	<0.0001[3]	Yes
Age	0.0023[3]	Yes
Infective Stage	<0.0001[3]	Yes
Giardia Dose	0.0061[4]	Yes

[1]Data sparseness prevented analytic calculations for Isolate Source, Administration Route and Detection Method.
[2]Significant level ≤0.2.
[3]Mantel-Haenzel bivariate analyses analysis taking into account the correlation between studies.
[4]GLIMMIX bivariate analyses.

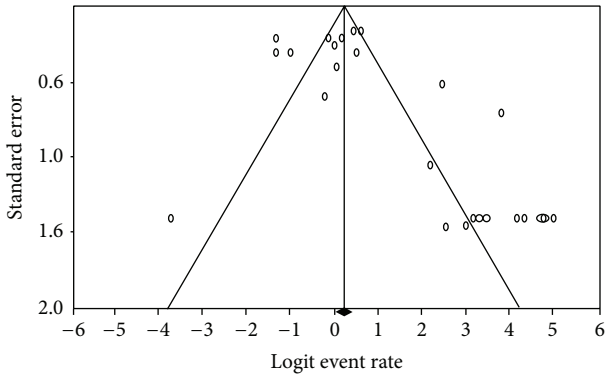

Figure 3: Funnel plot of standard error by logit event rate for cyst shedding outcome after *Giardia* infection showing possible publication bias as evidenced by a higher concentration of studies to the right of the mean.

association between study size and study effect (*P* value of 0.002). The Duval and Tweedie trim and fill method suggested adding 10 studies to the left side of the funnel plot, which under the random effects model would shift the point estimate of the prevalence of cyst shedding of all the studies included in this meta-analysis from 0.77 (CI: 0.68, 0.85) to 0.61 (CI: 0.49, 0.72), improving the estimate. The results of Cochran's *Q* test indicated significant heterogeneous results (*P* value < 0.001) among different studies and the I^2 statistic determined that 88% of variation across studies was due to significant heterogeneity rather than random chance.

3.3. Analysis for the Diarrhoea Outcome. As the data for the diarrhoea outcome were sparse, the analysis done for the shedding outcome could not be performed for the diarrhoea outcome. Therefore, the bivariate analysis was done considering the data as a pool across studies to estimate associations rather than accounting for the correlation between studies as done for the shedding outcome. The bivariate analysis showed that, for the outcome of diarrhoea in exposed animals, the covariates "Isolate Source" and "Experimental Species" were significant (*P* value ≤ 0.1) (Table 7). Due to the smaller number of studies reporting on the outcome of diarrhoea and variability in the reporting of these potential covariates across studies, it was not possible to create a multivariable model for this outcome. The publication bias for diarrhoea outcome was not assessed due to the low number of studies (4 studies) included in this meta-analysis.

4. Discussion

This meta-analysis evaluated the effects of multiple experimental covariates on cyst shedding or diarrhoea as indicators of *G. duodenalis* infection. The results obtained in this meta-analysis identified covariates that potentially cause heterogeneity between the outcomes of the dose results experiments and suggest that administering cysts or trophozoites to experimental hosts and the dose administered can all significantly impact the incidence of cyst shedding.

Based on the results of this meta-analysis, it would be appropriate to make the following recommendations for future dose-response experiments on *G. duodenalis* when assessing infection by means of the presence of cyst shedding in experimentally infected animals to make the studies more comparable and increase likelihood of cyst shedding: (i) use cysts to infect the experimental animals rather than trophozoites, (ii) consider the infective stage used (cysts versus trophozoites) and the *Giardia* dose administered together, (iii) consider the animal species used as an experimental host and dose together, and (iv) taking this into account, consider using mice as experimental hosts rather than gerbils, rats, hamsters, dogs, cats, and cattle. These parameters should be considered when designing experimental dose-response studies, as once the designs of the dose-response studies are more standardized, they will provide better information and more comparable results for more accurate risk assessments that consider infection as the outcome.

In the case of assessing infection by means of the presence of diarrhoea in experimentally infected animals, more experimental studies in animal models should be conducted, as not enough studies have been reported to obtain estimates of the effect of different experimental parameters on diarrhoea in individual animals. It should be noted that while the diarrhoea outcome is of clinical relevance, the presence of asymptomatic infected individuals is a limitation of using diarrhoea to represent infectious status. Based on the bivariate analyses, it would be appropriate to consider and report the following in future dose-response experiments: (i) the assemblage being inoculated into the experimental host, (ii) original source of the cysts being inoculated, (iii) whether cysts or trophozoites were subject to any passage before inoculation into the experimental host or not, (iv) animal species used as experimental hosts, (v) age of the experimental host, and (vi) the administration route used to inoculate the cysts or trophozoites into the experimental host. These studies will provide better information and more comparable results for risk assessments that consider illness as an outcome.

Table 6: Multivariable generalized linear mixed model showing risk factor associations for *Giardia duodenalis* cyst shedding outcomes[1].

Variable	Categories	Odds ratio estimate	95% Wald confidence limits	*P* value
Experimental Species	Gerbils[2]			
	Mice	0.16[3]	(0.003, 8.87)	0.37
	Other Animals	0.03[4]	(<0.001, 5.05)	0.18
	Other Rodents	4.30[5]	(0.10, 183.05)	0.45
Age	Young[2]			
	Adult	0.36	(0.05, 2.43)	0.30
Infective Stage	Trophozoites[2]			
	Cysts	5.02[6]	(2.63, 9.56)	<0.001[12]
Giardia Dose	Not applied (continuous variable)	2.67[7]	(1.81, 3.94)	<0.001[12]
Administration Route	Gastric Intubation[2]			
	Other Than Gastric Intubation	10.39	(0.19, 573.61)	0.25
Giardia Dose by Experimental Species	Mice	4.36[8]	(1.59, 11.93)	0.004[12]
	Other Animals	2.12[9]	(0.12, 37.45)	0.61
	Other Rodents	0.09[10]	(0.01, 0.72)	0.02[12]
Giardia Dose by Infective Stage	Cysts	0.57[11]	(0.41, 0.81)	0.002[12]

[1]Pseudo-AIC = 7013.49.
[1]Generalized chi-square statistic and its degrees of freedom (gener. Chi-sq/df) = 0.66.
[2]Corresponds to the reference category.
[3]The odds ratio estimate corresponds to Mice versus Gerbils at the mean log dose.
[4]The odds ratio estimate corresponds to Other Animals versus Gerbils at the mean log dose.
[5]The odds ratio estimate corresponds to Other Rodents versus Gerbils at the mean log dose.
[6]The odds ratio estimate corresponds to Cysts versus Trophozoites at the mean log dose.
[7]The odds ratio estimate corresponds to an increase of one in log dose in Gerbils with Trophozoites.
[8]The odds ratio corresponding to an increase of one in log dose in Mice is 4.36 times that in Gerbils.
[9]The odds ratio corresponding to an increase of one in log dose in Other Animals is 2.12 times that in Gerbils.
[10]The odds ratio corresponding to an increase of one in log dose in Other Rodents is 0.09 times that in Gerbils.
[11]The odds ratio corresponding to an increase of one in log dose in Cysts is 0.57 times that in Trophozoites.
[12]Risk factors with statistically significant results.

Table 7: Bivariate analysis results for risk factors associated with diarrhoea outcome after *Giardia* infection.

Variable[1]	*P* value	Statistical association between variable and presence of diarrhoea (Yes/No)[2]
Isolate Source	<0.0001[3]	Yes
Passage	0.35[3]	No
Experimental Species	0.07[3]	Yes
Age	0.35[3]	No
Infective Stage	0.25[3]	No
Giardia Dose	0.25[4]	No
Administration Route	0.32[3]	No

[1]Data sparseness prevented analytic calculations for Storage Time.
[2]Significant level ≤0.2.
[3]Mantel-Haenzel bivariate analyses not taking into account the correlation between studies.
[4]GLIMMIX bivariate analyses.

Interestingly, the significant "*Giardia* Dose by Experimental Species" interaction suggests that an increase of one in log of dose administered has a larger impact on cyst shedding in "Mice" than "Gerbils" as experimental hosts, indicating a larger difference between the experimental host-groups as the administered dose increases. But, an increase of one in log of dose administered had a protective impact on cyst shedding in "Other Rodents" compared to "Gerbils" as experimental hosts. Nevertheless, differences in cyst shedding among experimental animal hosts may depend on the dose of *Giardia* administered, and it is advisable to consider these two variables together when designing experimental studies. In addition, it is important to take into consideration that some species might vary in their susceptibilities to different *G. duodenalis* assemblages. For instance, dogs are the only species that have been reported to be susceptible to *G. duodenalis* assemblage C and D, cats to assemblage F, hoofed livestock to assemblage E, and rats to assemblage G [1, 3, 12], while a wide variety of animals, such as cattle, dogs, cats, rodents, and other wild animals and humans are susceptible to *G. duodenalis* assemblage A [1, 3, 12]. Thus, choosing to use mice, other rodents, or other animals as experimental hosts would be appropriate only for some study objectives, such as evaluating the infectivity of the different *G. duodenalis* assemblagesby means of cyst shedding.

Pathogen shedding patterns for newborns, weanlings, and adults can be quite different across host species. For instance, it has been reported that weanling calves generally lack a strong specific humoral immune response to *G. duodenalis* infection, while newborns may be protected by the anti-*Giardia* activity of colostrums [13], indicating that weanlings may be more susceptible to *Giardia* infection than newborns or adults. Unfortunately, in this meta-analysis there were not enough studies to analyse newborn and weanling animals separately, thus both categories had to be merged and analyzed as "Young." Based on the meta-analysis results, young animals were not statistically different from adult animals

with regard to cyst shedding. This finding is in contrast to numerous studies that reported the prevalence and cyst excretion peaks in young animals [14–16]. However, our results from this meta-analysis are in concordance with the findings reported by Hewlett et al. [17], where young mongrel dogs were not found to be more susceptible than adults, and Woo and Paterson [18] where adult and young dogs and cats did not present with infection after being challenged with 300000 cysts. The data available for this meta-analysis were unfortunately inadequate to provide estimates to determine how the dose and the choice of the age of the experimental species impact the odds of cyst shedding.

The multivariable model suggested that inoculating the experimental host with cysts would increase the likelihood of cyst shedding compared to inoculation with trophozoites. However, the model also indicated that an increase of one log in dose had less of an impact on cysts than it did on trophozoites. Therefore, differences by using cysts versus trophozoites for host infection depend on the Giardia dose used, and it is advisable to consider these two variables together when designing experimental studies. Results of the multivariable model also suggest that when a higher dose of trophozoites is administered to gerbils, the odds of cyst shedding increase. Numerous studies in which different doses of Giardia cysts or trophozoites have been administered to animal models have been published. However, whether the infectious dose may contribute to symptom variability is still unclear [1]. Studies have shown that the infectivity ratio is directly related with the number of cysts inoculated to the experimental host [19, 20]. Nevertheless, all of these reports were individual studies; therefore, there is the possibility that those results were influenced by the experimental design or other factors. Among the strengths of meta-analyses is that it provides an overall estimate of an association or effect based on a number of independent scientific studies and explores the variation in the observed effect across studies, thus obtaining a gain in statistical power to detect effects [21]. The result obtained in the multivariable model indicated that when a higher dose is administered, the odds of having cyst shedding increases.

Information for the variables "Assemblage," "Isolate Source," and "Storage Time" was scarce and thus the association between these variables and the shedding outcome could not be assessed. Passage of cysts or trophozoites used to infect experimental hosts was identified as a risk factor for cyst shedding in the bivariate analysis. However, information on this variable was not reported for all studies, which made it challenging to consider it jointly in a multivariable model. Once more experimental studies providing information regarding these variables are published, it would be beneficial to evaluate the association with the shedding outcome and incorporate them in the multivariable model "Experimental Species," "Age," "Infective Stage," and "*Giardia* Dose" to ascertain the independent effect of these possible covariates on the presence of cyst shedding.

This meta-analysis showed evidence of possible publication bias for the cyst shedding outcome. This finding can be explained by the fact that smaller studies are more likely to be published if they have larger than average effects, which makes them more likely to meet the criterion for statistical significance [22]. The addition of studies by the trim and fill procedure improved the point estimate of the prevalence of cyst shedding of all the studies included in this meta-analysis. However, the adjusted estimate is similar to the original effect and thus indicates that the reported trends may be valid. Caution is advised when interpreting these results because there is evidence of high heterogeneity among the studies, thus precluding a full evaluation of publication. These findings suggest that it is important to publish those studies that have negative or nonsignificant results as well those that have significant or positive results in order to reduce or avoid the bias.

Based on the results of this meta-analysis, it is crucial that more experimental studies in animal models are conducted to assess infectivity of *Giardia* by means of cysts shedding and diarrhoea. These studies would provide useful data for risk assessments that consider either infection or illness as an outcome.

5. Conclusions

When assessing *G. duodenalis* infection using a cyst shedding outcome measure, this study suggests that differences among the animal species used as experimental hosts depend on the dose of *Giardia* administered. It is therefore advisable to consider these two variables (host and dose) together when designing experimental studies. Taking this into account, mice appear to be the most appropriate animal model in which to assess infection when using a cyst shedding outcome, as they were more likely to shed cysts than other animal species.

Young and adult animals were similarly likely to shed cysts. Nevertheless, additional studies are needed for increased statistical power to ascertain effects of the log dose increment for different age groups on the presence of cyst shedding.

For considering whether cysts or trophozoites are used to challenge the experimental hosts to assess infection by means of cyst shedding, the multivariable analysis results suggest that it would be more appropriate to use cysts. However, it also indicated that an increase of one log dose has less of an impact for cysts than for trophozoites; thus, differences between inoculating cysts or trophozoites in the cyst shedding depend on the dose administrated. As expected, administering higher doses of cysts or trophozoites increases the odds of cyst shedding.

When using a diarrhoea outcome measure in experimental studies, the source of the isolate and species of experimental animal host should be considered when designing experimental studies. As additional studies are published, greater power will be possible to distinguish individual and joint effects of the identified covariates.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Analysis of Structures, Functions, and Epitopes of Cysteine Protease from *Spirometra erinaceieuropaei* Spargana

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Spirometra erinaceieuropaei cysteine protease (SeCP) in sparganum ES proteins recognized by early infection sera was identified by MALDI-TOF/TOF-MS. The aim of this study was to predict the structures and functions of SeCP protein by using the full length cDNA sequence of SeCP gene with online sites and software programs. The SeCP gene sequence was of 1 053 bp length with a 1011 bp biggest ORF encoding 336-amino acid protein with a complete cathepsin propeptide inhibitor domain and a peptidase C1A conserved domain. The predicted molecular weight and isoelectric point of SeCP were 37.87 kDa and 6.47, respectively. The SeCP has a signal peptide site and no transmembrane domain, located outside the membrane. The secondary structure of SeCP contained 8 α-helixes, 7 β-strands, and 20 coils. The SeCP had 15 potential antigenic epitopes and 19 HLA-I restricted epitopes. Based on the phylogenetic analysis of SeCP, *S. erinaceieuropaei* has the closest evolutionary status with *S. mansonoides*. SeCP was a kind of proteolytic enzyme with a variety of biological functions and its antigenic epitopes could provide important insights on the diagnostic antigens and target molecular of antisparganum drugs.

1. Introduction

Sparganosis is a serious parasitic zoonosis caused by infection with spargana, the plerocercoid larvae of some *Diphyllobothrium* tapeworms that belong to the genus *Spirometra* [1]. The most important species of the genus *Spirometra* tapeworms with plerocercoids that can produce sparganosis in human include *Spirometra erinaceieuropaei* (syn. *Spirometra erinacei* or *Spirometra mansoni*) which is the most common in Asia, and *Spirometra mansonoides* which is mainly distributed in North America [2]. The adults are intestinal parasites of some species of Canidae and Felidae; the first intermediate hosts are freshwater copepods (cyclops), whereas the second intermediate or paratenic hosts belong to different species of vertebrates (frogs, snakes, pigs, etc.) [3, 4]. Human is an accidental host. Human infection results mainly from drinking raw water contaminated with cyclops harboring procercoid, ingesting raw fleshes of frogs and snakes infected with plerocercoids, or placing frog or snake flesh on open wound for treatment of skin ulcers or eye inflammations [5, 6].

Human sparganosis is reported in many countries of the world but is most common in Eastern Asia and the Far East [7]. Sparganosis poses a serious threat to human health; the plerocercoids usually lodge in the subcutaneous tissues and muscles but sometimes invade the abdominal cavity, eye, and central nervous system causing blindness, seizures, headache, epilepsy, paralysis, and even death [8]. Ocular sparganosis is especially prevalent in China and Vietnam [9]. The clinical diagnosis of sparganosis is rather difficult and often misdiagnosed because the larvae have no predilection site in humans and the specific signs or symptoms are lacking. A definite diagnosis of subcutaneous sparganosis can be achieved by detection of the larvae in a biopsy specimen from the lesion, but the confirmative diagnosis is very difficult for visceral and cerebral sparganosis since the larva is found only by surgical removal [10]. The ELISA using the crude or excretory-secretory (ES) antiagens of plerocercoids has high sensitivity for the detection of sparganum infection in humans, but the main disadvantage is the false negative results during the early stage of infection and the cross-reactions with serum samples from patients with other parasitic diseases (cysticercosis, paragonimiasis, clonorchiasis, etc.) [11, 12].

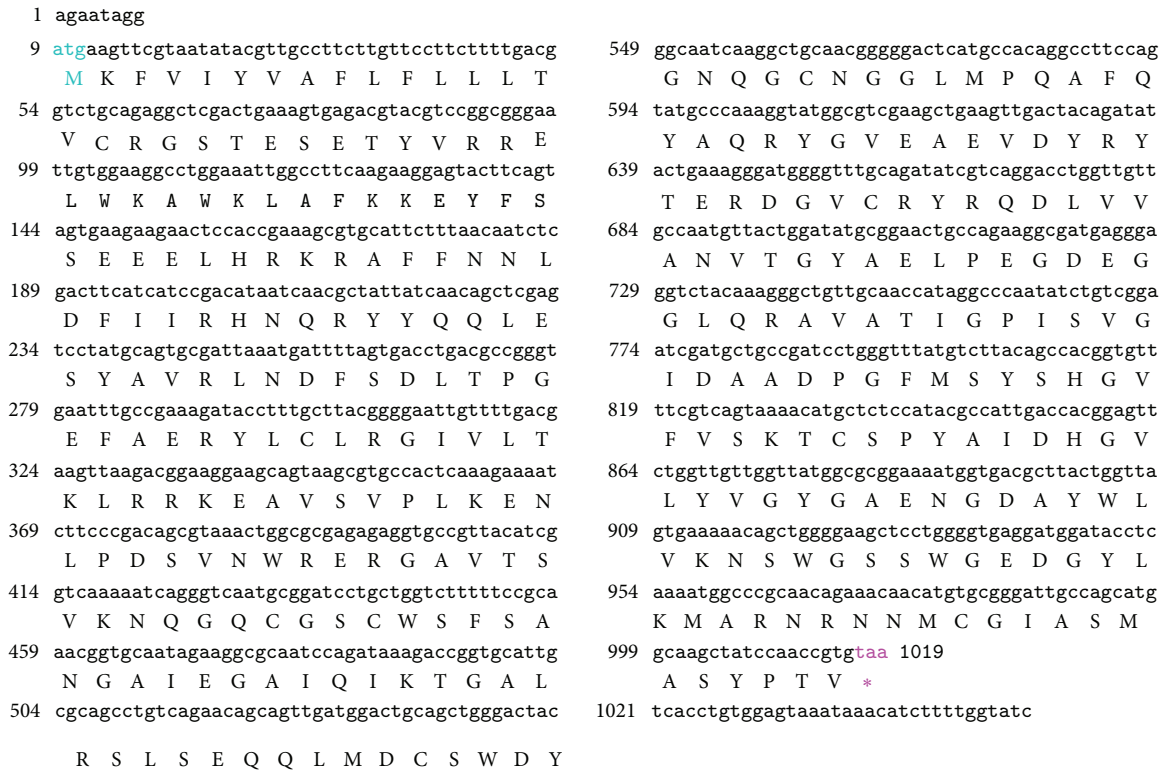

Figure 1: Sequences and amino acid residues of SeCP. The SeCP sequence was of 1 053 bp length with a 1011 bp biggest OFR from 9 bp (ATG) to 1019 bp (TAA), which encoded 336-amino acid protein with 3′UTR locating at the positions 1020–1053 bp.

In order to separate the early specific diagnostic antiagens, the ES proteins of *S. erinaceieuropaei* sparganum were analyzed by two-dimensional electrophoresis (2DE) and Western blot probed with early sera from infected mice at 14 days after infection. Three immunoreactive protein spots were successfully identified by MALDI-TOF/TOF-MS and characterized as the *S. erinaceieuropaei* cysteine protease (SeCP) [13]. In this paper, the full-length cDNA sequence of SeCP (GenBank accession no. 1834307) was analyzed; its structure and function were predicted by using bioinformatics techniques.

2. Materials and Methods

The full-length cDNA sequence of SeCP (GenBank accession no. 1834307) was used in this study. The structure domain and function domain were predicted by online analysis http://smart.embl-heidelberg.de/. The amino acid sequence was submitted to http://www.expasy.org/tools/protparam.html and its physical and chemical properties were predicted. Signal peptide was predicted by a web-based tool (http://www.cbs.dtu.dk/services/SignalP/), and subcellular localization was predicted using http://psort.nibb.ac.jp/form2.html. Hydrophilic prediction was predicted at http://www.expasy.org/cgi-bin/protscale.pl. Transmembrane domain was predicted through http://www.cbs.dtu.dk/services/TMHMM-2.0/. The secondary structures were constructed using the software PSIPRED v3.0 http://bioinf.cs.ucl.ac.uk/psipred/ [14, 15]. The 3D models of proteins were constructed by I-TASSER, a protein structure server on the website http://zhanglab.ccmb.med.umich.edu/I-TASSER/, which is considered to predict protein 3D structures that have more than 100 amino acids [16–18]. Visual molecular dynamics (VMD) was used to read standard Protein Data Bank (PDB) files and display the contained structure [19–21]. VMD is a molecular visualization software for displaying, animating, and analyzing large biomolecular systems using 3D graphics and built-in scripts http://www.ks.uiuc.edu/Research/vmd/. Amino acid sequence was submitted to http://www.cbs.dtu.dk/services/BepiPred/ in order to predict its antigen epitopes. Conserved HLA-restricted CD8+ T cells epitopes were also predicted using the software from IEDB http://www.immuneepitope.org/ which could identify novel HLA-class I restricted $CD8^+$T cell epitopes. Other cysteine protease amino sequences of model organisms of other parasites used in this study were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and listed as follows: *Clonorchis sinensis* (AAD29130.1), *Homo sapiens* (CAB42883.1), *Spirometra mansonoides* (AAB17051.1), *Taenia solium* (BAH03395.1), *Paragonimus westermani* (AAF21457.2), *Schistosoma japonicum* (CAX71578.10), *Schistosoma mansoni* (P25792.1), *Taenia pisiformis* (AEE69034.1), *Haemonchus contortus* (ACS36090.1), *Entamoeba histolytica* (CAA62836.1), *Trichinella spiralis* (XP_003377240.1), *Plasmodium vivax* (AAA60368.1), *Brugia malayi* (XP_001896823.1), *Echinococcus multilocularis* (BAF02516.1), *Arabidopsis thaliana* (AAB67626.1), *Mus musculus* (AAA37445.1), *Drosophila melanogaster* (AAB18345.1), *Caenorhabditis elegans* (AAA98785.1), *Haemaphysalis longicornis* (BAH86062.1), and *Aedes aegypti* (ABE72970.1). The multiple

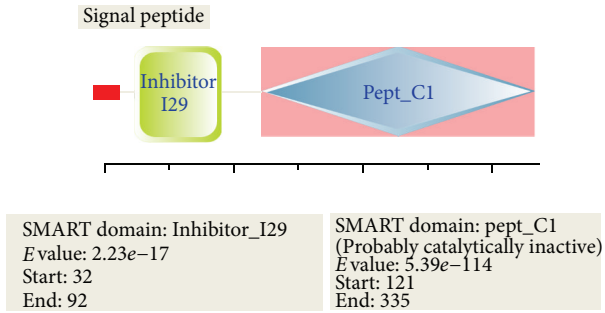

Figure 2: Prediction of structure domains of SeCP by SMART servers. The confidently predicted SeCP structure domains contained a complete cathepsin propeptide inhibitor domain (I29) located at 32aa–92aa and a peptidase_C1A with an active site located at 39aa–303aa and a S2 subsite 189aa–330aa, which has the function of cysteine-type peptidase activity.

sequence alignment of SeCP and the above-mentioned sequences were carried out by Clustal X; then, molecular evolutionary tree was constructed by MEGA4.1 [22]. Phylogenies were estimated under the neighbor-joining (N-J) method [23].

3. Results

3.1. The Basic Properties of SeCP Sequence. The SeCP sequence was of 1 053 bp length with a 1011 bp biggest OFR from 9 bp (ATG) to 1019 bp (TAA), which encoded 336-amino acid protein with 3′UTR locating at the positions 1020–1053 bp. Nucleotide sequence and deduced amino acid sequence were shown in Figure 1.

3.2. Physical and Chemical Properties of SeCP. The SeCP had the molecular weight of 37.87 kDa and theoretical isoelectric point (pI) of 6.47. Extinction coefficients are 74300 M^{-1} cm^{-1}, at 280 nm measured in water, assuming all pairs of Cys residues form cysteines. The half-life was 30 h, >20 h, and >10 h in mammalian reticulocytes (*in vitro*), yeast (*in vivo*), and *Escherichia coli* (*in vivo*), respectively. The instability index (II) was computed to be 32.11. This classifies the protein as stable. Aliphatic index is 75.74. Grand average of hydropathicity (GRAVY) is −0.321.

3.3. Structural Domain, Hydrophobicity, Signal Peptide, Subcellular Localization, and Transmembrane Domain. The confidently predicted SeCP structure domains contained a complete cathepsin propeptide inhibitor domain (I29) located at 32aa–92aa and a peptidase_C1A with an active site located at 39aa–303aa and a S2 subsite 189aa–330aa, which has the function of cysteine-type peptidase activity (Figure 2). Using the scale Hphob./Kyte and Doolittle, the SeCP protein has an obvious hydrophobic regions at 5′ (Figure 3).

The prediction results of SeCP signal by Signal P-4.1 showed that there was a peak fraction at 19aa residue position (Figure 4). The score was 3.87 which was high enough with split site. So, the SeCP protein had a cleavable signal peptide (from 1 to 19) and with possible cleavage site between 19aa and 20aa.

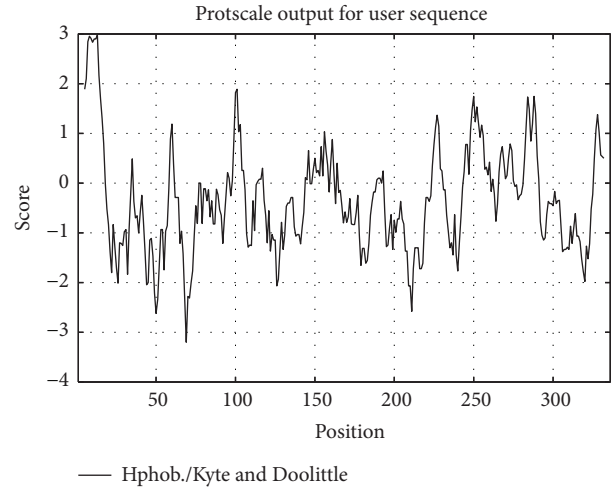

Figure 3: Hydrophobicity of SeCP. The SeCP protein has an obvious hydrophobic regions at 5′ predicted by using the scale Hphob./Kyte and Doolittle.

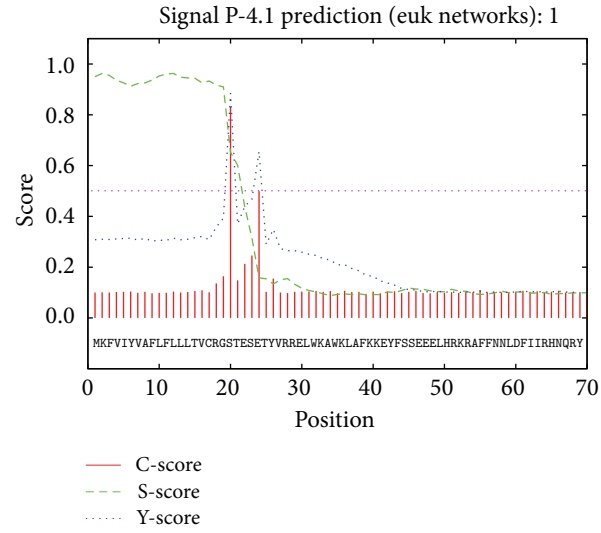

Figure 4: Prediction of SeCP signal peptide. There was a peak fraction at 19aa residue position and the score was 3.87 which was high enough with split site. The SeCP protein had a cleavable signal peptide (1 to 19) with possible cleavage site between 19aa and 20aa.

Results of the k-NN prediction of SeCP suggested that the peptide chain was located in the extracellular (including cell wall), vacuolar, mitochondrial, and endoplasmic reticulum, with the possibility of 55.6%, 22.2%, 11.1%, and 11.1%, respectively. The maximum possible location was in the extracellular ($k = 23$).

Prediction of transmembrane domain of SeCP with TMHMM Server v. 2.0 suggested that the SeCP had no transmembrane domain, located outside the membrane.

3.4. 2D Structure Alignment for SeCP. PSIPRED v. 3.3 was used to predict the secondary structures of SeCP which had 8 α-helixes, 7 β-strands, and 20 coils (Figure 5).

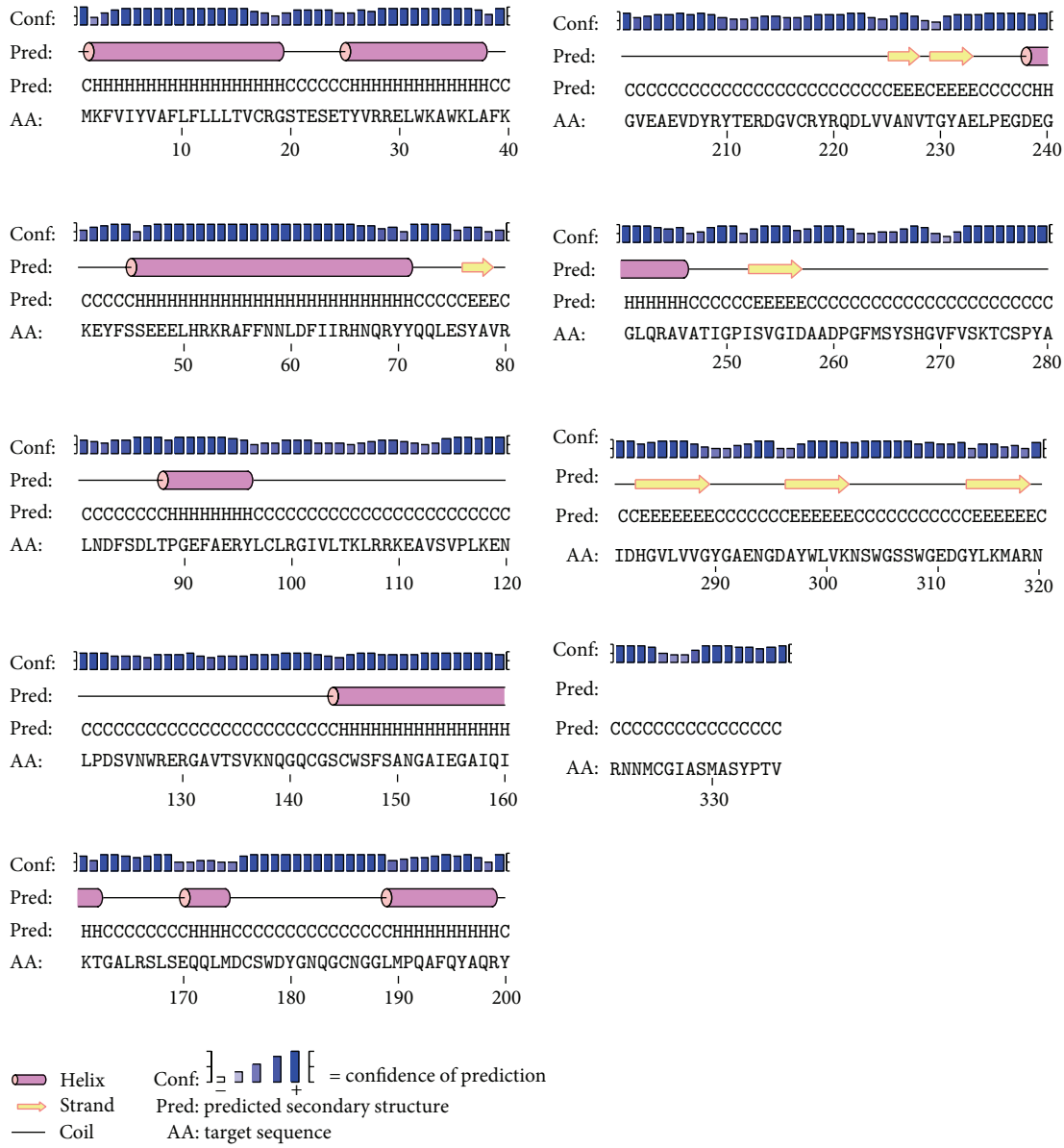

Figure 5: The predicted secondary structure of SeCP by using PSIPRED. There were 8 α-helixes, 7 β-strands, and 20 coils of the predicted secondary structure of SeCP.

3.5. Construction of 3D Model and Enzyme Activity Predicting. Five models were set up for each protein by Dr. Zhang's lab [16]. We selected the model with the highest confidence C-score (Figure 6), which estimates the quality of predicted models by I-TASSER. It was calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of $[-5, 2]$, and a model with a C-score above 2 suggested a high confidence. Enzyme homologs in PDB predicted by I-TASSER showed that the most reliable enzyme classification (EC) number prediction was 3.4.22.43, with the highest $\text{Cscore}^{\text{EC}}$ (0.664). $\text{Cscore}^{\text{EC}}$ is the confidence score for the EC number prediction. $\text{Cscore}^{\text{EC}}$ values range in between [0-1], where a higher score indicates a more reliable EC number prediction. The recombinant enzyme hydrolyzes proteins (serum albumin, collagen) and CA synthetic substrates (Z-Phe-Arg-NHMec > Z-Leu-Arg-NHMec > Z-Val-Arg-NHMec). The maximum activity of the enzyme was at pH 5.7 and was unstable at neutral pH. Compound E-64, leupeptin, and chicken cystatin are inhibitors of cysteine protease which belongs to peptidase family C1 (http://enzyme.expasy.org/EC/3.4.22.43).

3.6. Antigenic Epitope of SeCP. The sequence of SeCP was compared with the host's homologous sequences corresponding regional sequence by BepiPred 1.0b Server. The SeCP had 15 potential antigen epitopes (19aa–26aa, 44aa–50aa, 86aa–92aa, 118aa–128aa, 130aa–143aa, 151aa–155aa, 179aa–189aa, 199aa–205aa, 207aa–214aa, 231aa–243aa, 254aa–264aa, 276aa–280aa, 291aa–296aa, 305aa–312aa, and 332aa–336aa).

Table 1: The predicted HLA restricted $CD8^+$ T cell epitopes for SeCP.

Allele	Start	End	Peptide	Method	Percentile_rank
HLA-A*02:01	3	16	FVIYVAFLFLLLTV	Ann	0.3
HLA-A*02:01	263	272	FMSYSHGVFV	Consensus (ann/smm)	0.4
HLA-A*02:01	194	206	FQYAQRYGVEAEV	Ann	0.4
HLA-A*02:01	194	202	FQYAQRYGV	Consensus (ann/smm/comblib_sidney 2008)	0.5
HLA-A*02:01	188	197	GLMPQAFQYA	Consensus (ann/smm)	0.55
HLA-A*02:01	6	16	YVAFLFLLLTV	Consensus (ann/smm)	0.65
HLA-A*02:01	3	14	FVIYVAFLFLLL	Ann	0.8
HLA-A*02:01	9	16	FLFLLLTV	Consensus (ann/smm)	0.9
HLA-A*11:01	124	137	SVNWRERGAVTSVK	Ann	0.3
HLA-A*11:01	33	40	KAWKLAFK	Consensus (ann/smm)	0.45
HLA-A*11:01	307	316	SSWGEDGYLK	Consensus (ann/smm)	0.5
HLA-A*11:01	262	274	GFMSYSHGVFVSK	Ann	0.6
HLA-A*11:01	33	41	KAWKLAFKK	Consensus (ann/smm)	0.6
HLA-A*11:01	266	274	YSHGVFVSK	Consensus (ann/smm)	0.6
HLA-A*11:01	264	274	MSYSHGVFVSK	Consensus (ann/smm)	0.7
HLA-B*07:02	277	286	SPYAIDHGVL	Consensus (ann/smm)	0.35
HLA-B*07:02	317	330	MARNRNNMCGIASM	Ann	0.5
HLA-B*07:02	277	288	SPYAIDHGVLVV	Ann	0.6
HLA-B*07:02	106	117	KLRRKEAVSVPL	Ann	0.7

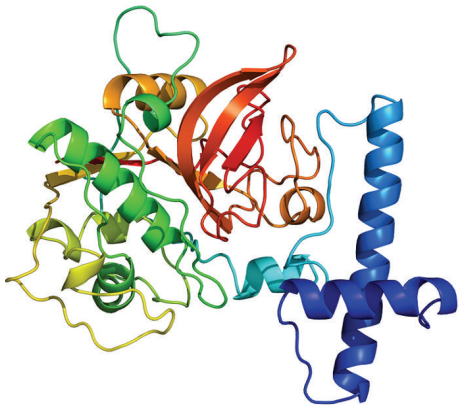

Figure 6: The 3D model of SeCP with highest confidence C-score, which estimates the quality of predicted models by I-TASSER.

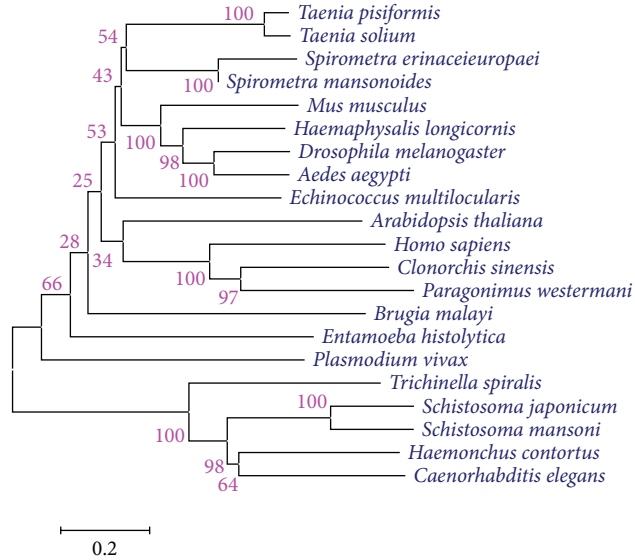

Figure 7: Neighbor-joining phylogenetic tree referred from cysteine protease amino acid sequence of *Spirometra erinacei*. Bootstrap values are indicated on branches.

Epitope prediction algorithm consensus was used to predict peptides that could stimulate human to induce effective and protective immune response against *S. erinaceieuropaei*, when the conserved HLA-restricted CD8+ T cells, epitopes of SeCP were predicted. The SeCP had 19 conserved peptides based on a high HLA allele binding score (percentile rank < 1) (Table 1).

3.7. Multiple Sequence Alignment and Molecular Evolution of SeCP. Multiple sequence alignment and phylogenetic analysis of SeCP with the cysteine protease of other species were displayed in Figure 7. Based on the phylogenetic analysis of SeCP, *Spirometra erinaceieuropaei* has the closest evolutionary status with *Spirometra mansonoides*.

4. Discussion

Cysteine protease is a kind of proteolytic enzyme, which contains cysteine residues in the center of enzyme activity. It has been shown that the cysteine protease of many parasites acts extracellularly to help invade tissues and cells, to uptake nutrient, to hatch, or to evade the host immune system [24–26]. Cysteine protease is the key factor in the parasitic pathogenicity, either by inducing tissue damage and

facilitating invasion or by empowering the parasites to salvage metabolites from host proteins [27, 28]. Cysteine protease has been detected in *S. erinacei* [29, 30]. The plerocercoids of *S. erinacei* is also known to secrete a large amount of cysteine proteases [31]. The cysteine protease from *S. erinacei* can hydrolyze collagen, hemoglobin, and immunoglobulin G (IgG) *in vitro* and may be concerned with digestion of host tissue in pathogenesis [32, 33]. Our previous study on 2DE analysis showed that the ES proteins of *S. erinacei* plerocercoids had a total of approximately 149 proteins spots with molecular weight varying from 20 to 115 kDa and isoelectric point (pI) from 3 to 7.5. When probed with sera from infected mice at 14 days after infection, seven protein spots with molecular weight of 23–31 kDa were recognized and analyzed by MALDI-TOF/TOF-MS. Three of seven spots were successfully identified and characterized as the same protein SeCP [13]. The SeCP might come from the excretory and secretory products and the cuticles (membrane proteins) and are directly exposed to the host's immune system and are the main target antiagens which induce the immune responses.

Based on the construction of full-length cDNA library of SeCP, the sequence of SeCP gene was of 1 053 bp length with a 1011 bp biggest ORF encoding 336-amino acid protein with a complete cathepsin propeptide inhibitor domain and a peptidase_C1A conserved domain. The predicted molecular weight and isoelectric point of the deduced SeCP protein were 37.87 Da and 6.47, respectively. Based on the phylogenetic analysis of SeCP, *Spirometra erinaceieuropaei* has the closest evolutionary status with *Spirometra mansonoides*. The secondary structure of SeCP contained has 8 α-helixes, 7 β-strands, and 20 coils. The SeCP had 15 potential antigenic epitopes and 19 HLA-I restricted epitopes. These predicted antigenic epitopes could provide important insights on the diagnostic antiagens and target molecular of antiparasitic drugs for sparganosis.

Acknowledgments

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