PCR-positivity of gerbils and their ectoparasites for *Leishmania* spp. in a hyperendemic focus of zoonotic cutaneous leishmaniasis in central Iran

Sahar Azarmi
Tehran University of Medical Sciences
Alireza Zahraei-Ramazani (alirezazahraei@yahoo.com)
Tehran University of Medical Sciences
Mehdi Mohebali
Tehran University of Medical Sciences
Yavar Rassi
Tehran University of Medical Sciences
Amir Ahmad Akhavan
Tehran University of Medical Sciences
Amrollah Azarm
Iran University of Medical Sciences School of Behavioral Sciences and Mental Health
Samira Elikaee
Tehran University of Medical Sciences
Sina Mohtasebi
Tehran University of Medical Sciences
Rahimeh Abdoli
Tehran University of Medical Sciences
Mohsen Mahmoudi
Tehran University of Medical Sciences
Sayed Hussain Mosawi
Ghalib University

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Abstract

Background: Sand flies are the only known vectors of Leishmania parasites. Various arthropods, including ticks have been suggested as secondary vectors of Leishmania spp. many years ago. This study was conducted to determine PCR-positivity of zoonotic cutaneous leishmaniasis reservoir hosts and their ectoparasites for Leishmania spp. in central Iran.

Methods: Microscopic examination and nested polymerase chain reaction (Nested-PCR) were used to detect and identify species of Leishmania and the results were confirmed by two methods, PCR-restriction fragment length polymorphism (PCR-RFLP) and sequencing.

Results: Totally, 93 rodents (Rhomombis opimus (n=92) and Nesokia indica (n=1)) were captured during different seasons and 9 different species of ectoparasites were collected from them. Out of 92 collected R. opimus, 14 were positive for Leishmania spp. by microscopic examination while one N. indica and 77 R. opimus were positive by nested-PCR. The infection rate of rodents with Leishmania major and Leishmania turanica was 39.79% (37) and 15.05% (14), respectively. Mixed natural infections with L. major and L. turanica were seen in 15.05% of the rodents (14), in 7.53% of the rodents (7) with L. major and Leishmania gerbilli, and in 6.45% of the rodents (6) with the three species. The leishmanial infection rate of the rodents was the highest (94.29%) in summer and lowest (45.45%) in the winter. Moreover, 39 out of 54 fleas (72.22%), 5 out of 8 mites (75%), and 1 tick nymph (100%) were PCR-positive for Leishmania parasites.

Conclusions: The highest rate of infection with L. major and L. turanica in R. opimus populations was observed in the summer and spring respectively, and the highest percentage of L. major and L. turanica coinfection was seen in the winter. It is suggested that the role of L. turanica and the probable role of ectoparasites in the transmission of epidemiology should be investigated carefully. Xenodiagnostic testing is recommended for future testing.

Background

Zoonotic Cutaneous Leishmaniasis (ZCL) caused by *Leishmania major* is a public health problem in several countries affecting a large number of people [1]. The disease is endemic in many rural areas of Iran and in 17 out of 31 provinces. Gerbils are the main reservoir hosts of ZCL in Iran. *Rhombomys opimus* acts as a reservoir host of ZCL in Esfahan Province, central Iran [2]. The main and biological vector of *L. major* is *Phlebotomus papatasi* [3]. However, secondary forms of leishmaniasis transmission have been reported in the literature, but their role is still unclear in the epidemiology of leishmaniasis [4-7]. Some arthropods, such as *Rhipicephalus sanguineus*, have been introduced as probable mechanical vectors for some *Leishmania* parasites. Ticks and fleas may play a role in the transmission of *Leishmania infantum* between dogs [8]. Two recent studies found that the *Leishmania* parasite could be transmitted by tick and flea bites [6, 7].

Ticks that have a high reproductive rate and a long lifespan, are widely distributed and abundant, and can maintain high population densities. They are potential vectors of several vertebrate pathogens [9]. According to reports of human parasitism [10] and transmission of *Leishmania* parasites by *R. sanguineus* [5, 6], ticks may have a role in the survival of *Leishmania* parasites between dogs and sporadic transmission of this parasite from dogs to humans [11].

Similar to ticks, fleas, which are widely distributed due to their high reproductive rate, can attain a high population density. In the case of regular blood feeding, fleas are able to live for 200 days. Their blood feeding habit and high longevity may turn fleas into a potential vector of leishmaniasis in endemic areas over time [12]. A recent study found that the *Ctenocephalides felis felis* fleas collected from dogs showed the presence of promastigotes in smears stained by Giemsa. Moreover, it was also shown that fleas could transmit *Leishmania chagasi* from the infected dogs to hamsters in laboratory conditions. This investigation was confirmed by polymerase chain reaction (PCR) and the indirect fluorescent antibody test (IFAT) assays [13].

This study was conducted to determine PCR-positivity of ZCL reservoir hosts and their ectoparasites for *Leishmania* spp. in a hyperendemic area of Esfahan Province, Iran.

Methods

Study area

This study was done in Segzi (32° 41' N, 52° 7' E), 35 km east of the city of Esfahan, Esfahan Province over a period of 12 months from October 2016 to October 2017. Segzi is located at an average height of 1545 m above sea level and has hot summers and cold winters. Most of the vegetation in the desert area of Segzi is *Salsola rigida* and Haloxylon *ammodendron* (black saxaul).

Collection and identification of rodents and their ectoparasites

According to leishmanial infection of the ZCL reservoirs in the hyperendemic areas in different parts of the country (on average 40%) [1], the following formula was used to estimate the number of rodents.

[Please see the supplementary files section to view the equation.]

Rodents were captured using 30 Sherman traps (18×18×30 cm) at intervals of 45 days, 2 times in each season from different residential, agricultural and desert areas in Segzi. The traps were placed in front of the holes of active colonies before sunset and sunrise. In the laboratory of the Esfahan Health Research Station, captured rodents were anesthetized using chloroform-impregnated cotton. Ectoparasites were collected from each rodent by brushing the hairs of the hosts on a water surface and stored in 96% alcohol separately. To identify ectoparasites, microscopic slides were prepared. Then, ectoparasites were identified morphologically [14-18]. The rodents were identified through their morphological and morphometrical characteristics using identification keys [19].

Microscopic examination

While the rodents were anesthetized, two different microscopic slides (smear) were prepared from each ear lobe and cutaneous lesions for the examination of the presence of amastigotes. After Giemsa staining, the slides were evaluated using a light microscope (×1000). Before and after smear preparation, the ears of the rodents were sterilized with 70% alcohol [1]. All rodents were transferred to the Esfahan Health Research Station animal house for breeding. Animal experiments were admired by the Ethics Committee of the Tehran University of Medical Sciences (the ethical code: IR.TUMS.SPH.REC.1396.2804) and performed in accordance with the Animals (Scientific Procedures) Act, 1986 (UK). A completed ARRIVE guidelines checklist was prepared (Additional file 1).

Nested PCR assay

The genomic DNA of ectoparasites collected from infected rodents with *Leishmania* spp. and the smears of the rodents' ear lobes and cutaneous lesions were extracted and purified according to the GeneAll® kit instruction. The extracted DNA samples were kept in 40 µl of elution buffer at -20 °C until use. The sequence of the internal transcribed spacer (ITS2) in the ribosomal RNA gene (rDNA) was used for detection and identification of different species of *Leishmania* parasites. The sequences of the primers were as follows: external forward primer (5'-AAA CTC CTC TCT GGT GCT TGC-3'), external reverse primer (5'-AAA CAA AGG TTG TCG GGG G-3'), internal forward primer (5'- AAT TCA ACT TCG CGT TGG CC-3') and internal reverse primer (5'-CCT CTC TTT TTT CTC TGT GC-3') [20].

Three microliters of template DNA, 1.5 µl of each external primer, 12.5 µl of Ampliqon (Taq DNA Polymerase 2x Master Mix Red-MgCl2 1.5 mM/ 2mM and made in Denmark), and 6.5 µl of sterile distilled water were used in the initial PCR. Thermal cycle steps of the initial PCR were as follows: at 95°C for 5 minutes (initial denaturation), 30 cycles at 94°C for 30 seconds (denaturation), 60°C for 45 seconds (annealing), and 72°C for 1 minute (extension) with a final extension step at 72°C for 5 min.

The second-round (nested) PCR included 3 µl of the diluted product of the first-round PCR (1:10 dilution in distilled water), 1.5 µl of each internal primer, 10 µl of Ampliqon, and 6 µl of sterile distilled water. The thermal cycle steps of the second-round PCR were as follows: 95°C for 2 minutes (initial denaturation), 30 cycles at 94°C for 15 seconds (denaturation), 62 °C for 30 s (annealing) and 72°C for 45 seconds (extension) with a final extension step at 72°C for 5 min.

The PCR products were analyzed by 1.5% agarose gel electrophoresis. Bands created in the gel were obtained using UV ray and gel stain and photographed. The standard strain *L. major* (MRHO/IR/75/ER) was used as positive control and distilled water was used as negative control. The predicted size of ITS2 products in the second-round PCR was 247 bp for *L. major* (GenBank accession numbers: FJ753394), 206 bp for *L. gerbilli* (GenBank accession numbers: AJ300486), and 141 bp for *L. turanica* (GenBank accession numbers: AJ272382).

Confirmation of identified species by nested PCR

The results of the second-round PCR products were confirmed by two methods, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using restriction digestion with *MnlI* and sequencing. PCR-RFLP was done by mixing 1 µl of the *MnlI* enzyme and 1.5 µl of G buffer with 12.5 µl of the positive nested PCR product in a final volume of 15 µl [21]. In this technique, 120, 73, 43 and 11 (bp) band lengths were expected for *L. major* (GenBank accession number: FJ753394), 158, 37 and 11 (bp) band lengths for *L. gerbilli* (GenBank accession number: AJ300486), 131 and 10 (bp) band lengths for *L. turanica* (GenBank accession number: AJ272382).

Moreover, 30 µl of 10 positive nested PCR products were sent to Macrogen, South Korea for sequencing after purification using the Bioneer kit. The fragments were sequenced using internal forward and reverse primers. Nucleotide sequences were edited and aligned using the Chromas Pro v2.1.3 and were compared with the sequences in the GenBank using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST). Then, the phylogenetic tree was constructed by the Mega 7 software and Maximum Likelihood Tree method with a bootstrap value of 1000 for sequences. All the sequences were recorded in the GenBank.

Statistical analysis

Fisher's exact test was used to evaluate the difference in the infected rodents between different seasons by SPSS 22.00 software and the p-values less than 0.05 were considered significant. In order to estimate evolutionary relationships between two nucleotide sequences, the correlation coefficient was calculated using Mega 7 software and the pairwise distance matrices method using the *P* distance model. So, the correlation coefficient between two distance matrices was used to estimate the similarity of two sequences and infer the exact genetic relationship between species of the *Leishmania*.

Results

Totally, 92 *R. opimus* (52 females and 40 male) and 1 *Nesoki indica* (1 male) were captured. Nineteen rodents were trapped in the fall, 11 in the winter, 28 in the spring, and 35 in the summer and 9 different species of ectoparasites were collected from them. The identified ectoparasites included one tick species (1 nymph of *R. sanguineus*), one sucking lice species (1 *Polyplax* spp.), five flea species of (399 *Xenopsylla nuttalli*, 46 *Echidnophaga oschanini*, 5 *Nosopsyllus ziarus*, 5 *Coptopsylla mesghalii* and 1 *Nosopsyllus turkmenicus turkmenicus*) and two mite species (41 *Dermanyssus sanguineus* and 28 *Hirstionyssus* sp.) (Additional file 2).

The amastigote form of *Leishmania* parasites was seen in 14 out of 92 collected *R. opimus* (15.2%) using microscopic examination. While 77 of *R. opimus* and 1 *N. indica* (83.87%)

were positive using nested PCR. Table 1 shows the infection of rodents with different species of *Leishmania* in different seasons using nested PCR. All positive smears were positive by nested PCR. The percentage of rodents infected with *L. major* and *L. turanica* was 39.79% (37) and 15.05% (14), respectively. Mixed natural infections were seen in 15.05% (14) of rodents with *L. major* and *L. turanica*, 7.53% (7) of the rodents with *L. major* and *L. gerbilli*, and 6.45% (6) of the rodents with the three species. *N. indica* was infected with *L. major* (Additional file 3). Fisher's exact test showed a significant difference in the leishmanial infection rate between different seasons ($P = 0.004$). The *Leishmania* spp. infection rate of the rodents was the highest (94.29%) in the summer and lowest (45.45%) in the winter. Mixed leishmanial infection of *L. major* and *L. turanica* was observed in all of the seasons (Table 1).

Table 1 Detection of *Leishmania* DNA from rodents. Detection of the ITS2 gene of *Leishmania* parasites from rodents in different seasons using nested-PCR assay, Segzi area, Esfahan Province, October 2016-October 2017

Different seasons	All captured rodents	Infected rodents with *Leishmania* spp.										All infected rodents	
		L. major		*L. turanica*		mixed infection of *L. major* & *L. turanica*		mixed infection of *L. major* & *L. gerbilli*		mixed infection of *L. major*, *L. turanica* & *L. gerbilli*			
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Fall	19	11	57.9	1	5.26	1	5.26	3	15.79	0	0	**16**	**84.21**
Winter	11	1	9.09	0	0	3	27.28	0	0	1	9.09	**5**	**45.45**
Spring	28	9	32.14	7	25	3	10.72	2	7.14	3	10.72	**24**	**85.71**
Summer	35	16	45.73	6	17.14	7	20	2	5.71	2	5.71	**33**	**94.29**
Total	93	37	39.79	14	15.05	14	15.05	7	7.53	6	6.45	**78**	**83.87**

Moreover, 39 out of 54 fleas (72.22%), 5 out of 8 mites (75%), and 1 tick nymph (100%) were found PCR-positive for *Leishmania* DNA using nested PCR. *Leishmania* DNA was detected from 67.5% (27/40) of *X. nuttalli*, 100% (3/3) of *N. ziarus*, 100% (3/3) of *C.*

mesghalii, 75% (6/8) of *E. oschanini*, 80% (4/5) of *D. sanguineus*, 66.66% (2/3) of *Hirstionyssus* sp., and 100% (1/1) of the nymphs of *R. sanguineus* (Additional file 3). Of five PCR-positive specimens of *X. nuttalli* fleas in which the blood fed was digested, 1 (female) was PCR-positive for *L. major*, 2 (males) were positive for both of *L. major* and *L. turanica,* and 2 (females) for both of *L. major* and *L. gerbilli*. Moreover, one of 2 PCR-positive *Hirstionyssus* sp. mites that had *L. major* DNA was blood-digested and the rest of the PCR-positive ectoparasites were blood-fed. DNA of the three species of *Leishmania* parasites and *L. turanica* was found in 2 *E. oschanini* (male) and 1 *N. ziarus* (male) fleas, respectively (Table 2).

Table 2 Detection of *Leishmania* DNA from the ectoparasites. Detection of the ITS2 gene of *Leishmania* parasites from the ectoparasites using nested-PCR assay, Segzi area, Esfahan Province, October 2016-October 2017

Species of ectoparasites	No. of examined samples	PCR- positivity of the ectoparasites with *Leishmania* spp.							
		No. of positive samples	Blood-Fed	Blood-digested	*L. major*	*L. turanica*	*L. major* & *L. turanica*	*L. major* & *L. gerbilli*	*L. major*, *L. turanica* & *L. gerbilli*
X. nuttalli	40	27	22	**5**	23	0	2	2	0
E. oschanini	8	6	6	0	4	0	0	0	2
N. ziarus	3	3	3	0	2	1	0	0	0
C. mesghalii	3	3	3	0	3	0	0	0	0
D. sanguineus	5	4	4	0	4	0	0	0	0
***Hirstionyssus* sp.**	3	2	1	**1**	2	0	0	0	0
R. sanguineus	1	1	1	0	1	0	0	0	0
Total	63	46	40	6	39	1	2	2	2

Out of 10 positive nested PCR products, 9 were sequenced well. All these sequences were compared with the sequences of ITS2 in the GenBank. The GenBank accession numbers for the sequenced ITS2 of the *Leishmania* parasites included MK372246 (*L. turanica*; host: *R. opimus*), MK372247 (*L. infantum*; host: *R. opimus*), MK372248 (*L. major*; host:

Hirstionyssus sp.), MK372249 (*L. major*; host: *X. nuttalli*), MK372250 (*L. major*; host: *R. opimus*), MK372251 (*L. major*; host: *N. ziarus*), MK372252 (*L. major*; host: *R. sanguineus*), MK372253 (*L. major*; host: *C. mesghalii*) and MK372254 (*L. major*; host: *D. sanguineus* (synonym: *Liponyssoides sanguineus*)). One of the sequenced samples was similar to *L. infantum* with accession number MG831328.1 (Query cover= 98%, *E value*= 1e-72 and Ident= 96%).

The constructed phylogenetic tree showed that *L. tropica* (out group) was in a separate branch and seven and one of the sequenced samples were located in the same clades with recorded sequences of *L. major* (the GenBank accession number: AJ786164.1) and *L. turanica* (the GenBank accession number: HF545838.1.1), respectively (Figure 1). The genetic distance coefficient showed no difference between the sequence of *L. major* in this study and the sequence recorded in the GenBankl, and the difference of evolutionary correlations coefficient between the *L. tropica* recorded sequence in the GenBank and the sequence of *L. major* and *L. turanica* was 0.516 and 0.550, respectively. In addition, the difference of evolutionary correlations between the *L. turanica* sequence recorded in the GenBank and the sequence of *L. turanica* in this study was 0.008 (Table 3).

Table 3 Evolutionary correlations coefficient. Evolutionary correlations coefficient between isolated ITS2 gene sequences from the *Leishmania* parasites from ectoparasites and smears of ear lobes of rodents

	1	2	3	4	5	6	7	8	9	10	11	12
1. *L. turanica*: MK372246												
2. *L. turanica*: HF545838.1	0.008											
3. *L. gerbilli*: HF545839.1	0.042	0.042										
4. *L. major*: MK372248	0.203	0.211	0.203									
5. *L. major*: MK372249	0.203	0.211	0.203	0.000								
6. *L. major*: MK372250	0.203	0.211	0.203	0.000	0.000							
7. *L. major*: MK372251	0.203	0.211	0.203	0.000	0.000	0.000						
8. *L. major*: MK372252	0.203	0.211	0.203	0.000	0.000	0.000	0.000					
9. *L. major*: MK372253	0.203	0.211	0.203	0.000	0.000	0.000	0.000	0.000				
10. *L. major*: MK372254	0.203	0.211	0.203	0.000	0.000	0.000	0.000	0.000	0.000			
11. *L. major*: AJ786164.1	0.203	0.211	0.203	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
12. *L. tropica*: KR706374.1	0.550	0.559	0.542	0.516	0.516	0.516	0.516	0.516	0.516	0.516	0.516	

Note. These sequences were compared with recorded ITS2 gene sequences in the GenBank (AJ786164.1, HF545839.1, HF545838.1 and KR706374.1). The correlation coefficient was calculated using Mega 7 software and pairwise distance matrices method using *P* distance model.

Discussion

Esfahan is known as one of the important foci for ZCL in Iran. Segzi has a population of about 6500. According to the reports of Esfahan Health Centers, cutaneous leishmaniasis is reported on average 10 cases per 1000 people annually. *R. opimus* is the main reservoir host for ZCL in the northeast parts of the central plateau of Iran, and *N. indica* has been identified as the secondary reservoir in Esfahan [2]. The current study showed that *R. opimus* rodents were found in large numbers in Segzi. The distribution of *R. opimus* has a strong correlation with the climatic conditions and topography of the area, such as the seasonal rainfall and average annual temperature [22]. In recent years, because of the population growth, making factories and residential houses near the colonies of the reservoir hosts, and expansion of the city, leishmanial infections in rodent populations and humans have increased. Also, with the emergence of drought in the Segzi plain, rodents have invaded the outskirts of the city. So, contact of rodents with humans has increased and subsequently, the leishmanial infection rates have increased. In a study on ZCL by Akhavan et al in three rural districts (Borkhar, Segzi and Badrood) of Esfahan Province, 21 out of 95 *R. opimus* were positive by microscopic examination and 48 of them by nested PCR [20]. The results of our and other recent studies [20, 21] indicate that *L. major*, *L. gerbilli*, and *L. turanica*, and mixed natural infections exist in *R. opimus* populations (Table 1). In this study, the highest infection rates with *L. major* and *L. turanica* in *R. opimus* populations were observed in the summer and spring respectively, and the highest percentage of *L. major* and *L. turanica* coinfections was seen in the winter. The least infection rate of *L. major* was in the winter (Table 1). Mixed natural infections with *L. major* and *L. turanica* in *R. opimus* populations are typical in central Asia [23]. *L. turanica* raises the persistence of *L. major* infection

in *R. opimus* [24]. Therefore, mixed natural infections with *L. major* and *L. turanica* help to preserve leishmaniasis in rodent populations. According to the results of this study, it can be said that young *R. opimus* gerbils mature after the winter, with more than 40% of them (Table 1) being infected at the beginning of the active season of *Ph. papatasi* and probable vectors. Because of rodents breeding in early spring, rodent populations increase and the transmission of ZCL begins in the Segzi area when the sand flies emerge in late May. From early spring to summer, the increasing trend in the numbers of *R. opimus* declines [25] and the proportion of infected animals rises. So, the highest leishmanial infection rate of *R. opimus* is in late summer and the chances of parasite ingestion by *Ph. papatasi* and ectoparasites that bite infected rodents are then greater. In our study, phylogenetic analysis results showed no significant difference between the *L. major* sequences and they were monophyletic (Figure 1 and Table 3).

Zoonotic cutaneous leishmaniosis is increasing Esfahan Province, central Iran [26], while the leishmanial infection rates of the main vector are low in the endemic areas [27]. Therefore, it could be suggested that other vectors could have a role in disease transmission. In the Aran o Bidgol city in Esfahan province, Doroodgar et al reported that 17.8% of *R. opimus*, 71.4% of human isolates and 1.9% of *P. papatasi* infected with *L. major* [27]. The possibility of the transmission of *Leishmania* parasites by ectoparasites has long been discussed [6, 11, 28] and has been already proved in laboratory conditions [5, 13, 29]. The ectopasites collected from infected rodents were selected for genomic DNA detection of *Leishmania* parasites using nested PCR. The present study showed that ectoparasites of the rodents can easily ingest *Leishmania* parasites during blood-feeding. However, just only this evidence cannot prove that these ectoparasites are vctors of ZCL.

In our study, PCR analysis of ectoparasites specimens to detect the DNA of *Leishmania* spp. showed 73.02% (46/63 ectoparasites) positivity and the DNA of *L. major* was detected in 7 of ectoparasite species, including *R. sanguineus*, *X. nuttalli*, *E. oschanini*, *N. ziarus*, *C. mesghalii*, *Hirstionyssus* sp. and *D. sanguineus* by nested PCR (Table 2). The high positivity rate in the ectoparasites is related to their life habits and the long duration of blood feeding. Around 52.6% (41/78) of the rodents infected with *Leishmania* spp. had been infested with the ectoparasites. Two studies by K. K. McKenzie and Ferreira et al. showed transmission of *Leishmania* parasites by *R. sanguineus* and *C. felis* in laboratory conditions [6, 7].

In the mid-1980s, McKenzie, KK demonstrated that the collected *R. sanguineus* from naturally infected dogs can inject *Leishmania* parasites into the healthy dogs during blood-feeding and infect them [6]. Ferreira, MGPA et al demonstrated that the collected *C. felis* fleas from infected dogs can transmit *Leishmania* spp. to the uninfected hamsters and it was observed that 18.1% of the hamsters were positive by both methods of PCR and enzyme-linked immunosorbent assay (ELISA), 45% of the hamsters were positive only by PCR method, and 9% of the hamsters were only by ELISA [7]. Coutinho et al, found that 6 *R. sanguineus* ticks (15.4%) were positive for *L. chagasi* using the PCR technique. They showed that *R. sanguineus* could transfer *L. chagasi* from infected dogs to hamsters in laboratory conditions [5]. In another study, the promastigotes of *L. chagasi* were observed in 4 stained smears out of 207 (1.9%)

C. felis felis specimens collected from dogs, whereas *Leishmania* spp. infection was reported in 43 out of 144 (29.9%) fleas by PCR [13]. In fact, K. K. McKenzie, Ferreira, MGPA et al, Coutinho et al, and Coutinho and Linardi [5, 6, 7, 13]demonstrated that *Leishmania* parasites can be viable in the blood-sucking arthropods (such as tick and fleas) in laboratory conditions and infected their vertebrate hosts. But, they did not prove that these arthropods could act as vectors of the *Leishmania* parasite in nature.

In the current study, five specimens of *X. nuttalli* fleas and one *Hirstionyssus* sp. mite, which their blood fed was digested, were found PCR-positive for *Leishmania* DNA (Table 2). In *Xenopsylla* spp. fleas, blood digestion lasts 2-9 days depending on the temperature, relative humidity, and the host species. In fleas, the duration of digestion is shorter at low relative humidity than high relative humidity [30]. In a study by Colombo et al., *L. infantum* was detected in 23% of the fleas and 50% of the ticks collected from the infected dogs by RT-PCR, real time PCR, and ELISA. In addition, RNA analysis of the tick specimens collected from infected dogs after 10 to 7 days showed that the parasites were alive. Moreover, the alive parasites were isolated from adult ticks that had molted recently [31]. In a study conducted in Brazil, the results of immunohistochemistry (IHC) and real-time PCR (RT-PCR) showed *Leishmania* spp. promastigotes in the intestine, ovaries, and salivary glands of the *R. sanguineus* ticks collected from infected dogs [32]. Probably *Leishmania* spp. may remain in these ectoparasites such as *X. nuttalli* and *Hirstionyssus* sp., at least until the blood fed is digested. However, the detection of *Leishmania* DNA is not sufficient evidence of parasite survival in *X. nuttalli* and *Hirstionyssus* sp., and this required more careful study.

In another study in Turkey, to investigate the presence of *L. major*, the pools of *R. sanguineus* ticks on *Meriones unguiculatus* were examined by RT-PCR. The results showed that none of the pools was infected with *L. major* [33]. The study conducted in Turkey could yield more precise results if it used the main reservoirs related to study potency of ticks in the transmission of parasites. A study by Rakhshampour et al, in Iran showed that approximately 67% of the *R. sanguineus* ticks collected from dogs were infected with *L. infantum* using the semi-nested PCR. However, none of the parasitological (Giemsa staining and cultivation of parasite) and molecular (nested-PCR) tests results were positive when the transmission of *L. infantum* by stained *R. sanguineus* between dogs was studied in laboratory conditions [34].

In fact, blood-feeding arthropods are susceptible to infection with different types of pathogenic microorganisms, but it does not mean that they can transmit all of them [35]. Although there is no strong evidence indicating that ectoparasites act as a vector of the *Leishmania* parasites, recent studies suggest that this theory is important. Evolutionarily, it is unclear how long it would take for the *Leishmania* parasites to adapt to other blood-feeding arthropods. This is a fact that the *Leishmania* parasites have a long evolutionary history with the main ectoparasites of *Leishmanial* spp. reservoirs, such as *L. infantum* with *R. sanguineus* and *C. felis* (dog ectoparasites) [5, 7] and *L. major* with *X. nuttalli* (ectoparasite of *R. opimus*) [36, 37] and they have been in contact during this time. Therefore, it is possible that the ectoparasites will retain *Leishmania* parasites over time and act between reservoirs as potential vectors.

Conclusion

In conclusion, this study found that *L. major*, *L. gerbilli*, and *L. turanica* exist in *R. opimus* populations in Segzi area. The highest infection rates of *R. opimus* populations with *L. major* and *L. turanica* were observed in the summer and spring respectively, and the highest percentage of *L. major* and *L. turanica* coinfection was seen in the winter. It is suggested that ZCL should be controlled continuously and the role of *L. turanica* and *L. gerbilli* in the transmission of the disease should be indicated. According to the results of this study, it is possible that *L. major* remains in the ectoparasites such as *X. nuttalli* and *Hirstionyssus* sp., at least until the blood fed is digested. Therefore, the presence of *L. major* in the ectoparasites indicates the probable importance of rodents' ectoparasites in ZCL dispersion. Thus, to study the probable role of ectoparasites in the transmission of *L. major* in rural areas between reservoirs and humans, it should be investigated whether the parasites remain viable inside the ectoparasites and performed Xenodiagnosis test on the main reservoirs for experimental infections.

Abbreviations

ZCL: Zoonotic Cutaneous Leishmaniasis; PCR: Polymerase Chain Reaction; IFAT: Indirect Fluorescent Antibody Test; ITS2: Internal Transcribed Spacer; BLAST: Basic Local Alignment Search Tool; NCBI: National Center for Biotechnology Information; ELISA: Enzymelinked Immunosorbent Assay; IHC: Immunohistochemistry; RT-PCR: Real Time Polymerase Chain Reaction; Amplified Polymorphic DNA-Polymerase Chain Reaction: RAPD-PCR; PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism.

Declarations

Ethics approval and consent to participate

Animal experiments were admired by the Ethics Committee of the Tehran University of Medical Sciences, Tehran, Iran (the ethical code: IR.TUMS.SPH.REC.1396.2804) and performed in accordance with the Animals (Scientific Procedures) Act, 1986 (UK).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request and all authors wish to share their data.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SA performed data collection and analysis, organized the database and interpretation of results. AZR contributed to the study conception and design, and experimental studies. MM and SE participated in the statistical analysis and construed data. YR and AA helped in data collection. AAA made substantial contributions to perform nested PCR. SM was evaluated all microscopic slides of smears. RM and SHM contributed in professional translation of articles and molecular study. MoMa collaborated on the definition of intellectual content and literature search. All authors have read and approved the manuscript.

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Figures

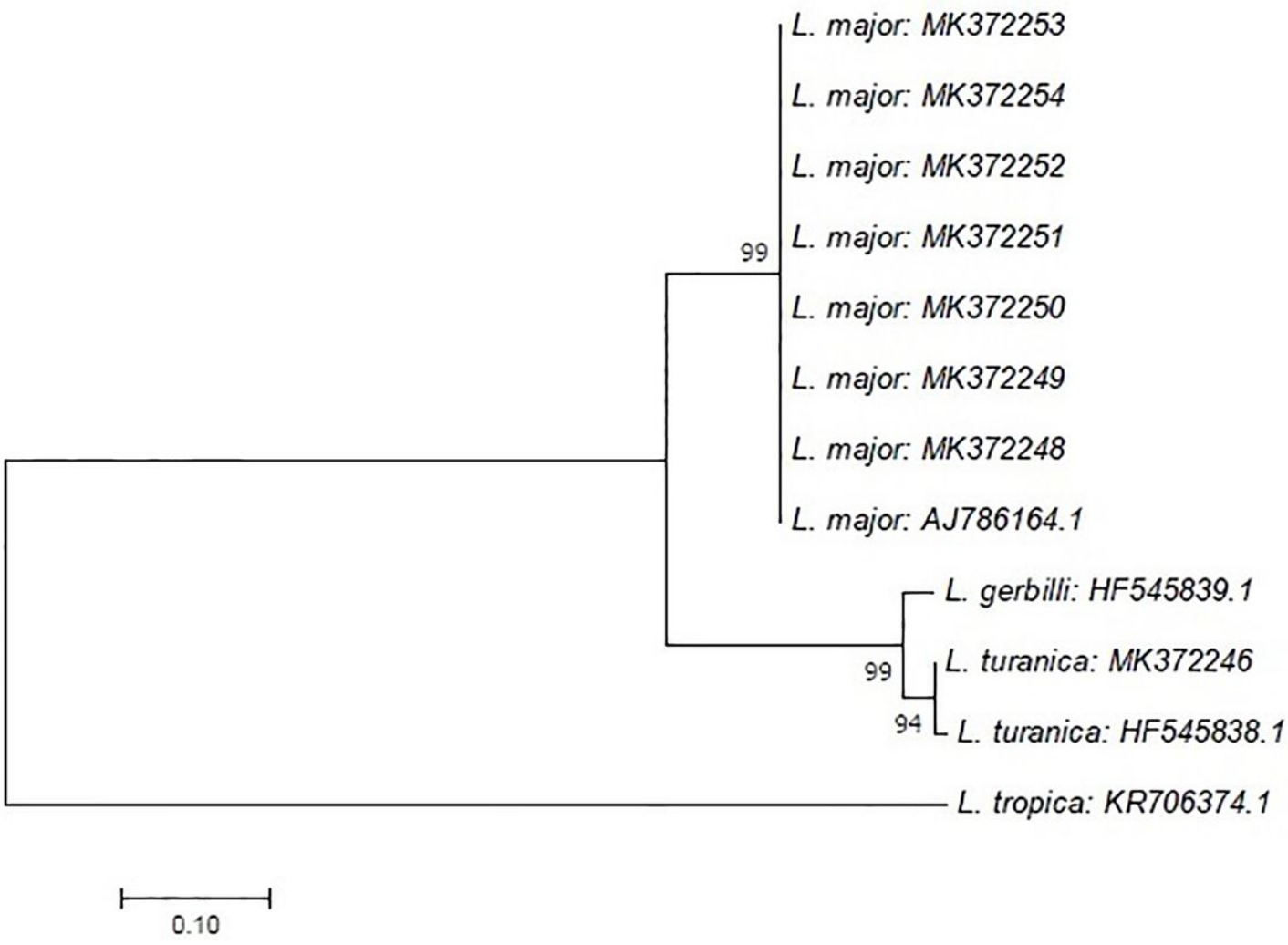

Figure 1

Phylogenetic analysis. Phylogenetic analysis of isolated ITS2 gene sequences of the Leishmania parasites from the ectoparasites and smears of ear lobes of rodents. These sequences were compared with recorded ITS2 gene sequences in the GenBank (AJ786164.1, HF545839.1, HF545838.1 and

KR706374.1). The phylogenetic tree was manufactured using Mega 7 software and maximum likelihood method with bootstrap value 1000.

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