Original Article

PCR Positivity of Gerbils and Their Ectoparasites for *Leishmania* Spp. in a Hyperendemic Focus of Zoonotic Cutaneous Leishmaniasis in Central Iran

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Abstract

Background: Various arthropods, including *Rhipicephalus sanguineus* and *Ctenocephalides felis felis* have been suggested as secondary vectors of *Leishmania* spp. many years ago. This study was conducted to determine zoonotic cutaneous leishmaniasis (ZCL) PCR positivity of reservoir hosts and their ectoparasites for *Leishmania* spp. in Segzi plain in Esfahan Province from October 2016 to October 2017.

Methods: Microscopic examination and nested PCR were used to detect and identify *Leishmania* spp. isolated from rodents' ears and ectoparasites, and then, the results were confirmed by two methods, PCR-restriction fragment length polymorphism and sequencing.

Results: Totally, 93 rodents (92 *Rhombomys opimus* and one *Nesokia indica*) and nine different species of ectoparasites (n=527) including fleas, mites, and ticks were collected during different seasons in the study area. Fourteen *R. opimus* 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% (n=37) and 15.05% (n=14), respectively. Mixed natural infections with *L. major* and *L. turanica* were seen in rodents. Moreover, 72.22% of fleas (39/54), 75.0% of mites (5/8), and 100% of tick nymph (1/1) 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 summer and spring, respectively. It is suggested that the role of *L. turanica* and the probable role of ectoparasites in the epidemiology of disease should be investigated. A Xenodiagnostic test is recommended for future study.

Keywords: Ectoparasite; *Leishmania gerbilli*; *Leishmania major*; *Leishmania turanica*; *Rhombomys opimus*

Introduction

Zoonotic Cutaneous Leishmaniasis (ZCL) caused by *Leishmania major* Yakimoff and Schokhor, 1914 is a public health problem in several countries affecting a large number of people (1). The disease is endemic in in 18 out of 31 provinces of Iran (2). The great gerbils (*Rhombomys opimus* Lichtenstein, 1823) is the main reservoir hosts of ZCL in Iran and this rodent acts as a reservoir host of ZCL in Esfahan Province, central Iran (1). The main and biological vector of *L. major* is *Phlebotomus papatasi* Scopoli, 1786 in Iran (3). However, secondary forms of *Leishmania* transmission by other arthropods have been reported in the literature, but their role is still unclear in the epidemiology of leishmaniasis (4–7). Some arthropods, such as the brown dog tick (*Rhipicephalus sanguineus* Latreille, 1806), have been introduced as probable mechanical vectors for some *Leishmania* parasites (4–8). Ticks and fleas may play a role in the transmission of *Leishmania infantum* Nicolle, 1908 between

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dogs (8). Two recent studies have suggested that the *Leishmania* parasite could be transmitted by tick and flea bites (6, 7).

Ticks that have high reproductive rates and 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 by *R. sanguineus* (10) and transmission of *Leishmania* parasites by the brown dog tick (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 (6, 11).

Like ticks, fleas, which are widely distributed due to their high reproductive rate, can attain a high population density. If a source of blood is available, fleas can 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* Bouche, 1835 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* Cunha and amp, Chagas, 1937 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 the PCR positivity of ZCL reservoir hosts and their ectoparasites for *Leishmania* spp. in a hyperendemic area of Esfahan Province, Iran.

Materials and Methods

Study area

This study was done in Segzi plain (32.694866° N, 52.120171° E), 35km 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 altitude of 1545 meters 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

Rodents were captured using 30 Sherman live traps (18×18×30cm) 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 case of trapping before sunset, the traps were collected the next morning. Also, the traps placed before sunrise were removed until noon of the same day. The captured rodents were transferred to the laboratory of the Esfahan Health Research Station and anesthetized (14). Ectoparasites were collected from each rodent by brushing the hairs of the hosts on a water surface and stored in 96% ethyl alcohol separately. The ectoparasites were mounted on microscopic slides and then identified morphologically (15–18). The rodents were identified based on their morphological and morphometrical characteristics using identification keys (19).

Microscopic examination

While the rodents were anesthetized, two different microscopic slides (thin smear) were prepared from each ear lobe and cutaneous lesions for the examination of the presence of *Leishmania* 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). The rodents were transferred to the Esfahan Health Research Station animal house and bred for other educational and research purposes.

Nested PCR assay

The prepared smears from ear lobes and cutaneous lesions in the rodents' ears were used for DNA extraction and investigation of *Leishmania* infection in rodents. The genomic DNA

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was extracted and purified according to the GeneAll® kit instruction. The extracted DNA samples were kept in 40µl of elution buffer at -20 °C. The partial sequence of the internal transcribed spacer (ITS2) of 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, 1.5µl of each external primers, 12.5µl of Ampliqon (Taq DNA Polymerase 2x Master Mix Red-MgCl2 1.5mM/2mM), and 6.5µl 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 5min.

The second-round (nested) PCR included 3µl of the diluted product of the first-round PCR (1:10 dilution in distilled water), 1.5µl of each internal primer, 10µl 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 seconds (annealing) and 72 °C for 45 seconds (extension) with a final extension step at 72 °C for 5min.

The PCR products were analyzed by 1.5% agarose gel electrophoresis. Bands created in the gel were visualized 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 a negative control. The predicted size of ITS2 products in the second-round PCR was 247bp for *L. major* (GenBank accession numbers: FJ753394), 206bp for *Leishmania gerbilli* Wang, Qu and Guan, 1964 (GenBank accession numbers: AJ300486), and 141bp for *Leishmania turanica* Strelkova etc, 1990 (GenBank accession numbers: AJ272382).

Also, the ectoparasites collected from PCR positive rodents were tested for detection of *Leishmania* parasites using nested PCR assay as described.

PCR-RFLP and sequencing

The results of the second-round PCR products were confirmed by two methods; sequencing and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) using restriction digestion with MnlI (Jena Bioscience, Germany). PCR-RFLP was done by mixing 1µl of the MnlI enzyme and 1.5µl of G buffer with 12.5µl of the positive nested PCR product in a final volume of 15µl (21). In this technique, 120, 73, 43 and 11bp band lengths were expected for *L. major* (GenBank accession number: FJ753394), 158, 37 and 11bp band lengths for *L. gerbilli* (GenBank accession number: AJ300486), 131 and 10bp band lengths for *L. turanica* (GenBank accession number: AJ272382).

Moreover, 30µl 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 replicates. All the obtained sequences in this study 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. 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 one *Nesoki indica* Gray and Hardwicke, 1832 (one 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 (527) were collected from them. The identified ectoparasites included one tick species (one nymph of *R. sanguineus*), one sucking louse species (*Polyplax* spp. Enderlein, 1904), five flea species (399 *Xenopsylla nuttalli* Ioff, 1930, 46 *Echidnophaga oschanini* Wagner, 1930, 5 *Nosopsyllus ziarus* Klein, 1963, 5 *Coptopsylla mesghalii* Farhang-Azad, 1966 and one *Nosopsyllus turkmenicus turkmenicus* Vlasov and Ioff, 1937) and two mite species (28 *Hirstionyssus* sp. Fonseca, 1948 and 41 *Dermanyssus sanguineus* Hirst, 1914 (synonym: *Liponyssoides sanguineus*)) (Fig. 1).

The amastigote form of *Leishmania* parasites was seen in 14 (15.2%) out of 92 collected *R. opimus* using microscopic examination. While 77 of *R. opimus* and one *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% of rodents (14) with *L. major* and *L. turanica*, 7.53% of the rodents (7) with *L. major* and *L. gerbilli*, and 6.45% of the rodents (6) with the three *Leishmania* species. *Nesoki indica* was infected with *L. major*. Fisher's exact test showed a significant difference in the leishmanial infection rate between different seasons (P= 0.004). The rodents *Leishmania* spp. infection rate was the highest (94.29%) in the summer and the lowest (45.45%) in the winter. Mixed leishmanial infection of *L. major* and *L. turanica* was observed in all the seasons (Table 1).

Moreover, 39 out of 54 fleas (72.22%), five out of eight mites (75%), and one tick nymph (100%) were found *Leishmania* PCR positive 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*. Of five PCR positive specimens of *X. nuttalli* fleas with gravid abdomen status, one (female) was *L. major* PCR positive for, two (males) were positive for both of *L. major* and *L. turanica*, and two (females) for both of *L. major* and *L. gerbilli*. Moreover, one of two *L. major* PCR positive *Hirstionyssus* sp. mites was gravid 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 two *E. oschanini* (male) and one *N. ziarus* (male) fleas, respectively (Table 2).

Out of ten positive ITS2 nested PCR products, nine were sequenced well. All these sequences were compared with the sequences of ITS2 in the GenBank. The GenBank accession numbers for the ITS2 region 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*),

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MK372252 (*L. major*; host: *R. sanguineus*), MK372253 (*L. major*; host: *C. mesghalii*) and MK372254 (*L. major*; host: *D. 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%).

Leishmania tropica Wright, 1903 ITS2 sequence was obtained from GenBank and used as an out group for construction of phylogenetic tree. All the *L. major* (n=7) sequences obtained in this study clustered with *L. major* from GenBank (accession number: AJ786164) and formed a separate clade in the tree. Also, *L. trunica* obtained in this study was sister taxon with *L. turanica* from GenBank (accession number: HF545838) and both associated with *L. gerbilli* (accession number: HF545839) and formed separate clade (Fig. 2). The genetic distance coefficient showed no difference between the sequences of *L. major* in this study and the sequence recorded in the GenBank. The evolutionary correlation coefficient between the *L. tropica* 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 1. Abundance and rate of seasonal *Leishmania* parasite infection in rodents detected by nested-PCR assay of ITS2-rDNA gene, Segzi area, Esfahan Province, October 2016–October 2017

Season	All captured rodents	Infected rodents with *Leishmania* spp.					Total infected rodents (%)
		L. major (%)	*L. turanica* (%)	*L. major* + *L. turanica* (%)	*L. major* + *L. gerbilli* (%)	*L. major* + *L. turanica* + *L. gerbilli* (%)	
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)

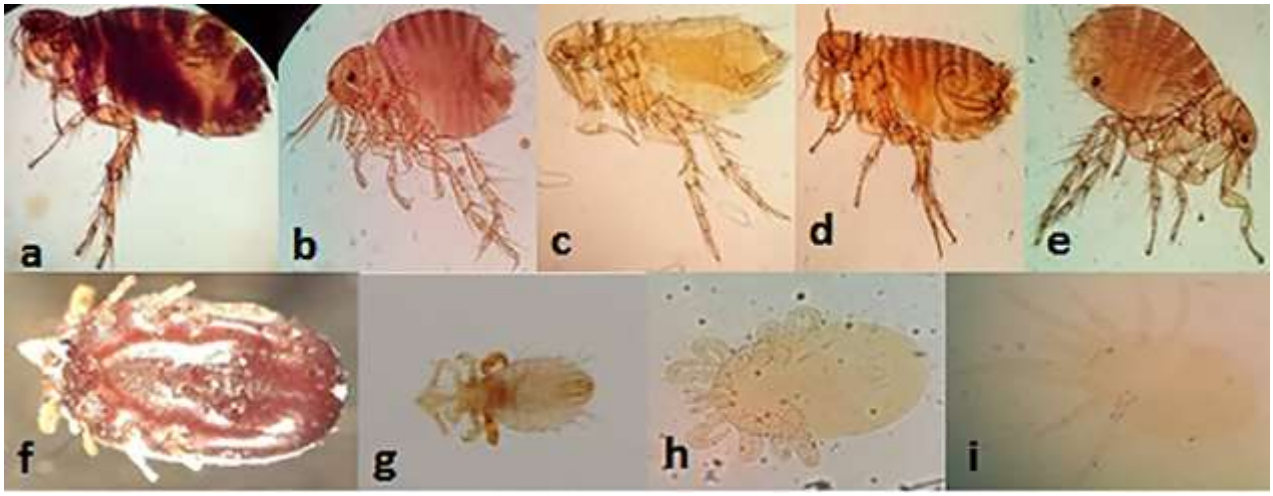

Fig. 1. Different species of collected Ectoparasites collected from rodents in Segzi, Esfahan province, 2016-2017; a: *Coptopsylla mesghalii*, b: *Echidnophaga oschanini*, *Nosopsyllus turkmenicus turkmenicus* (c), *Nosopsyllus ziarus* (d), *Xenopsylla nuttalli* (e), *Rhipicephalus sanguineus* (f), *Polyplax* spp. (g), *Hirstionyssus* sp. (h) and *Dermanyssus sanguineus* (i) (Original photos)

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Table 2. Abdominal status and *Leishmania* parasite infection in rodents' ectoparasites detected by nested-PCR assay of ITS2-rDNA gene, 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*
Xenopsylla nuttalli	40	27	22	**5**	23	0	2	2	0
Echidnophaga oschanini	8	6	6	0	4	0	0	0	2
Nosopsyllus ziarus	3	3	3	0	2	1	0	0	0
Coptopsylla mesghalii	3	3	3	0	3	0	0	0	0
Dermanyssus sanguineus	5	4	4	0	4	0	0	0	0
***Hirstionyssus* sp.**	3	2	1	**1**	2	0	0	0	0
Rhipicephalus sanguineus	1	1	1	0	1	0	0	0	0
Total	63	46	40	6	39	1	2	2	2

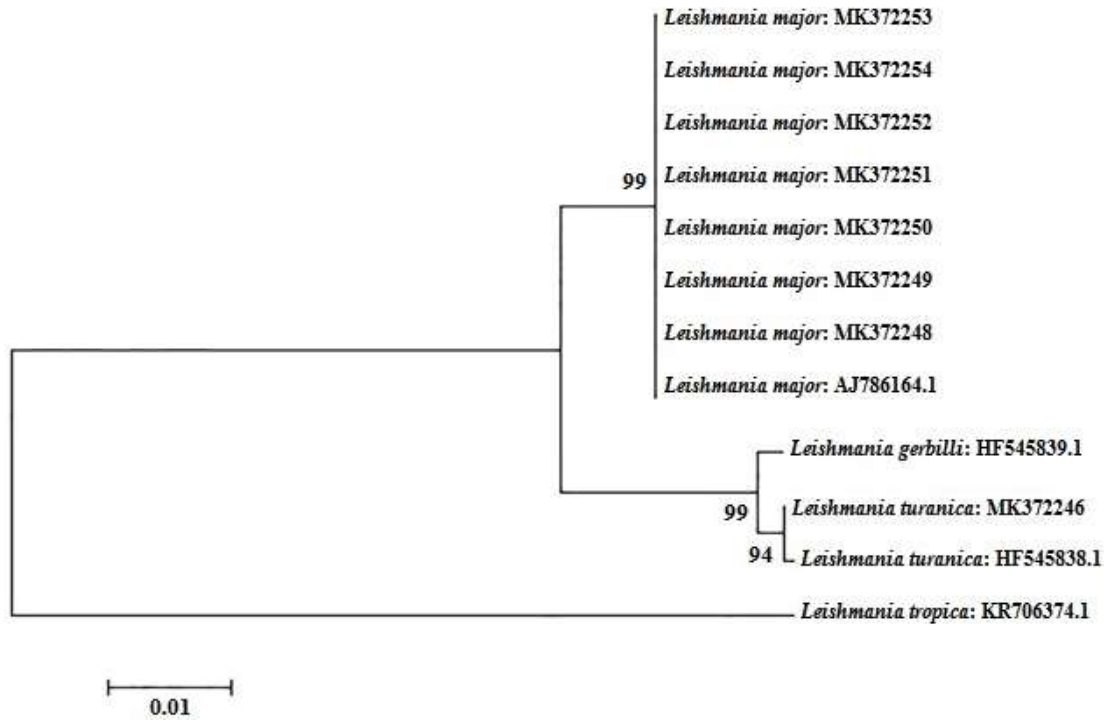

Fig. 2. Phylogenetic tree of the partial ITS2 gene sequences of the isolated *Leishmania* parasites from ectoparasites (MK372248-49 and MK372251-54) and smear of rodents' ear lobes (MK372246-50) and the ITS2 sequences obtained from GenBank. Bootstrap values that are reported as percentages at the nodes were obtained from 1000 bootstrap replicates. Scale bar corresponds to 0.1 change per nucleotide

Table 3. Comparison of the ITS2 sequences of the isolated *Leishmania* parasites from ectoparasites and smears of ear lobes of rodents, Segzi area, Esfahan Province, October 2016–October 2017

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

Note. These sequences were compared with the available ITS2 sequences in 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

The current study showed that *R. opimus* was 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 (23). 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. (20) 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. The results of our and other recent studies (20, 21, 24) 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 summer and spring respectively, and the highest percentage of *L. major* and *L. turanica* coinfections was seen in 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 (25). *Leishmania turanica* raises the persistence of *L. major* infection in *R. opimus* (26). Therefore, mixed natural infections with *L. major* and *L. turanica* help to preserve leishmaniasis in rodent populations. According to the results of this study, young *R. opimus* gerbils matured after the winter which more than 40% of them were infected (Table 1) at the emerging of *P. papatasi* and probable vectors in late May. Due to the suitable environmental and climatic conditions and the consequent abundance of food resources, gerbils reproduce twice or more per year (19, 27). In the Segzi region, which receives the most rainfall in late winter and early spring, a significant increase in population rates was observed in spring. From early spring to summer, the increasing trend in the numbers of *R. opimus* declined (27) and the rate of bites per rodent increased. So, it is expected that the chances of parasite ingestion by *P. papatasi* and ectoparasites from infected rodents are then greater. In our study, phylogenetic analysis results showed no significant difference between the *L. major* sequences and *L. major* isolated from rodents and ectoparasites were located in the same clade or monophyletic group. It means that *L. major* parasite extracted from rodents were not genetically different from those extracted from ectoparasites and were similar (Fig. 2 and Table 3).

Zoonotic cutaneous leishmaniosis is increasing Esfahan Province, central Iran (28), while the leishmanial infection rates of the main vector are low in the endemic areas (29). Therefore, it could be suggested that other vectors could have a role in disease transmission. In Aran and Bidgol city in Esfahan Province, Doroodgar et al. (29) reported that 17.8% of *R. opimus*, 71.4% of human isolates and 1.9% of *P. papatasi* infected with *L. major*. The possibility of the transmission of *Leishmania* parasites by ectoparasites has long been discussed (6, 11, 30) and has been already proved in laboratory conditions (5, 13, 31). 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 vectors 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 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 (6) demonstrated that the collected *R. sanguineus* from naturally infected dogs can inject *Leishmania* parasites into the healthy dogs during blood-feeding and infect them. Ferreira et al. (7) demonstrated that *C. felis* fleas collected from infected dogs could transmit *Leishmania* spp. noninfected laboratory hamsters and it was observed that 18.1% of the hamsters were positive by both methods of PCR and enzyme-linked immunosorbent assay (ELISA). Coutinho et al. (5), found that six *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. 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

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out of 144 (29.9%) fleas by PCR (13).

In a study in northwest of Iran by Azarm, in which three species of fleas; *C. canis* Curtis, 1826, *C. felis* and *Pulex irritans* Linnaeus, 1758 were collected from dogs, 75% of *C. canis* and 66.7% of *C. felis* collected from infected dogs were PCR-positive for *L. infantum*. But *Leishmania* DNA was not detected from *P. irritans* (32). In fact, Coutinho et al. (5), McKenzie (6), Ferreira et al. (7), and Coutinho and Linardi (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 were gravid, were found *Leishmania* DNA PCR positive (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 (33). In a study by Colombo, *L. infantum* was detected in 23% of the fleas and 50% of the ticks collected from the infected dogs by reverse transcription PCR (RT-PCR), real time PCR, and ELISA. In addition, RNA analysis of the tick specimens collected from infected dogs after seven to ten days showed that the parasites were alive. Moreover, the alive parasites were isolated from adult ticks that had molted recently (34). In a study conducted in Brazil, the results of immunohistochemistry (IHC) and real time PCR showed *Leishmania* spp. promastigotes in the intestine, ovaries, and salivary glands of the *R. sanguineus* ticks collected from infected dogs (35). Probably *Leishmania* spp. may remain in these ectoparasites such as *X. nuttalli* and *Hirstionyssus* sp., at least until full digestion of blood meal. 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* Milne-Edwards, 1867 were examined by RT-PCR. The results showed that none of the pools was infected with *L. major* (36). However, in this study the main reservoir has not been tested for the potency of ticks in the transmission of *L. major*. A study by Rakhshanpour et al. (37), 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.

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 (38). 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*) (39, 40) 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. ma-*

jor 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. 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.

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Ethical considerations

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).

Conflict of interest statement

Authors declare that there is no conflict of interest.

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