Original Article

First Report of Natural Infection of *Phlebotomus mongolensis* to *Leishmania major* and *Leishmania turanica* in the Endemic Foci of Zoonotic Cutaneous Leishmaniasis in Iran

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Abstract

Background: The primary aim of this study is to determine infection to *Leishmania* parasites in the wild population of *Phlebotomus caucasicus* and *Phlebotomus mongolensis* using molecular methods in some important zoonotic cutaneous leishmaniasis foci in Iran.

Methods: Sand flies were collected from active colonies of rodent burrows from 16 trapping sites using sticky trap paper. In order to detect and identify of *Leishmania* parasites in females *Ph. caucasicus* and *Ph. mongolensis*, the Nested–PCR amplification of ITS2-rDNA region was performed to generate amplicon with 245bp for *Leishmania major*, 206bp for *L. gerbilli* and 141bp for *L. turanica*.

Results: In the current study we found DNA of different gerbil parasites such as *L. major* and *L. turanica*, and mixed infection of *L. major/L. turanica* in *Ph. caucasicus* and *Ph. mongolensis.* It should be noted that, in Iran, natural infection with *Leishmania* parasites is recorded for the first time in this study in *Ph. mongolensis*.

Conclusion: Both species of *Ph. caucasicus* and *Ph. mongolensis* not only may participate in the ZCL transmission cycle between reservoir hosts, but also results of this study support the role of these species as secondary vectors in the transmission of leishmaniasis to humans.

Keywords: Leishmaniasis; *Phlebotomus caucasicus*; *Phlebotomus mongolensis*; *Leishmania major*; *Leishmania turanica*

Introduction

Leishmaniasis is a group of vector-borne diseases caused by a protozoan parasite belonging more than 20 *Leishmania* species. The disease spreads to tropics, subtropics, and the Mediterranean basin, as well as to 98 tropical countries in Asia (Middle East), Europe (Southern Europe and the Mediterranean), Africa (Tropics, North, West, and East Africa), and the United States (Mexico, Central and South America). More than 1 billion people are at risk for leishmaniasis in endemic areas. The prevalence of this disease is 12 million cases worldwide and it is estimated that 700 000 to one million new cases occur annually (1, 2).

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There are three main forms of leishmaniases, including cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), also known as Kala azar, and mucocutaneous leishmaniasis (MCL) (2). Cutaneous leishmaniasis is the most common form of the disease and in 2021 over 85 % of new CL cases occurred in 10 countries: Afghanistan, Algeria, Brazil, Colombia, Syria, Libya, Tunisia, Pakistan, Iraq, and Iran (2, 3). There are two epidemiological types of cutaneous leishmaniasis in Iran: anthroponotic cutaneous leishmaniasis (ACL) or urban/ dry form and zoonotic cutaneous leishmaniasis (ZCL) or rural/wet form. Zoonotic cutaneous leishmaniasis is a major public health problem in Iran which is endemic in many rural regions in 19 out of 31 provinces and about 85% of confirmed leishmaniasis cases in the country are of ZCL type. The causative agent of ZCL in Iran is *Leishmania major* and the main animal reservoirs of disease are rodents of the subfamily Gerbillinae (4).

Of more than 1000 species of identified phlebotomine sand flies, 31 species of *Phlebotomus* (Old World) and 47 species of *Lutzomyia* (New World) are proven vectors of human leishmaniasis (5–7). According to recent studies, to date 48 confirmed phlebotomine sand flies have been reported from Iran, including 30 species of the genus *Phlebotomus* and 18 species of the genus *Sergentomyia* (8–12). *Phlebotomus* (*Phlebotomus*) *papatasi* is the proven and main vector of *L. major* to human in endemic foci of ZCL in Iran. *Phlebotomus caucasicus* group belongs to the subgenus *Paraphlebotomus*, which has been considered as species group including *Ph. caucasicus*, *Ph. mongolensis* and *Ph. andrejevi*, playing a main role in maintenance of enzootic cycle of *L. major* among rodent reservoir hosts (8). *Phlebotomus caucasicus* and *Ph. mongolensis* not only participate in the transmission cycle of ZCL among reservoir hosts but also play an important role as secondary vectors in the transmission of leishmaniasis to humans (8). The females of these species have similar taxonomic characteristics and are isomorphic but based on recent study, morphometric analysis and morphological characters have been used for discrimination of these closely related species (13). Natural promastigote infection was isolated from *Ph. caucasicus* collected from gerbil and jird burrows in the focus of Esfahan Province in Iran and typed by isoenzymes assays as *L. major* zymodeme MON-26 (14). The traditional or classical methods such as sand fly dissection and culture of parasite have been used for *Leishmania* detection, but these techniques are time consuming and requires many sand fly specimens and also are less sensitive than molecular techniques and are not able to differentiate *Leishmania* parasite species (15). The present study has used a Nested-PCR method, which able to differentiate *Leishmania* parasite species and is a specific alternative method to classical techniques (16).

In recent years, molecular techniques are frequently used in epidemiological studies specifically on phlebotomine sand flies as vectors of ZCL in endemic foci of Iran for detection and identification of *Leishmania* infection in phlebotomine sand flies (17–27). The objective of present study was to use molecular methods for the first time to detect and identify of *Leishmania* infection within wild caught *Ph. caucasicus* and *Ph. mongolensis* in some important zoonotic cutaneous leishmaniasis foci in Iran.

Materials and Methods

Sand flies' collection and species identification

Sand flies were collected from the different allopatric locations in the provinces of Esfahan and Fars (central and southern Iran, respectively) and sympatric locations in Golestan Province (northeastern Iran). From June through October 2016, sand flies from the active rodent burrow colonies were collected using sticky trap papers (castor oil coated white papers, 21×30cm) from 16 collecting sites. Col-

lected sand flies were stored in 96% ethanol and kept in -20 °C for morphological and molecular assays. At first, for removing castor oil on specimen's body surface, collected specimens were washed twice in 1% detergent and sterile distilled water and then was dissected in a drop of sterile normal saline by sterilized forceps. The head and the last two abdominal segments were cut off and slide mounted in Puris' medium for species identification and identified after 24–72 hours using valid identification keys (13, 28–30). The remaining body (abdomen, wings, and legs) were preserved in 1.5ml sterile micro-tubes containing 96% ethanol for DNA extraction and detection of *Leishmania* parasite.

Molecular detection and identification of *Leishmania* species

GeneAll® Exgene™ Tissue Kit (GeneAll Biotechnology Company, South Korea) was used to extract genomic DNA. To detect and to identify *Leishmania* parasites we used the Nested-PCR assay developed by Akhavan et al. (16). Nested-PCR method has been used to amplify the *Leishmania* spp. regions of ITS2, primers as follows: Leish out F (5′-AAA CTC CTC TCTGGT GCT TGC-3′), Leish out R (5′-AAA CAA AGG TTG TCG GGG G-3′), Leish in F (5′-AAT TCA ACT TCG CGT TGG CC-3′) and Leish in R (5′-CCT CTCTTT TTT CTC TGT GC-3′). PCR products were separated by 1.5% (w/v) agarose gel electrophoresis in TBE buffer (0.09mM Tris, 0.09mM boric acid and 20mM EDTA, pH 8.3), visualized under ultraviolet light after staining with Safe Stein (0.5µg/ml) and photographed. Reference strains of *L. major* (MRHO/IR/75/ER), *L. gerbilli* (MRHO/CN/60/GERBILLI) and *L. turanica* (MRHO/SU/1983/MARZ-051) were used as positive controls. Also, double distilled water was included in each run as a negative control (16).

In order to sequencing, the PCR products of the second-round (nested) PCR for all positive samples were purified using the Gel Purification Kit (Expin™ PCR SV, GeneAll Biotechnology Company, South Korea). Both forward and reverse strands of amplified DNA were sequenced with the PCR primers. Nucleotide homologies of the sequenced products were evaluated with *Leishmania* spp. sequences available in GenBank and then checked by using Basic Local Alignment Search Tool (BLAST) analysis software (http://www.ncbi.nlm.nih.gov/BLAST)

Results

A total of 176 female sand flies were selected in this study (64 specimens of *Ph. caucasicus* and 112 specimens of *Ph. mongolensis*), and extraction of genomic DNA was conducted to identify *Leishmania* parasites in these phlebotomine sand flies. In 17 female sand fly specimens including 6 specimens of *Ph. caucasicus* and 11 specimens of *Ph. mongolensis*, *Leishmania* parasites were detected. Out of these 17 specimens of *Leishmania*-infected sand flies, seven, eight, and two specimens were infected to *L. major*, *L. turanica*, and mixed infection of both *L. major* and *L. turanica* respectively. The positive specimens produced species-specific band/s corresponding to *L. major* (245 and 233bp), and *L. turanica* (141bp) (Fig. 1). It is important to note that *L. major* and *L. turanica* parasites were detected in Esfahan and Golestan provinces in both *Ph. caucasicus* and *Ph. mongolensis*, but in Fars Province (Sadegh abad) only *L. major* parasite from *Ph. mongolensis* was detected in one case (Table 1). In this study, the infection rate for *Leishmania* parasites were estimated to be 9.3 % for *Ph. caucasicus* and 9.8% for *Ph. mongolensis*. Details of *Leishmania* parasites detected in six specimens of *Ph. caucasicus* and 11 specimens of *Ph. mongolensis* in different collection sites with their abdominal status mentioned in tables 1 and 2.

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Table 1. *Leishmania* parasite positive PCR detected in specimens of *Phlebotomus caucasicus* and *Ph. mongolensis* based on collection sites in Esfahan, Golestan and Fars provinces, Iran, 2016

Species (*Leishmania* positive samples)	Collection sites	No of Examined specimens	Positive samples			
			Total	*L. major*	*L. turanica*	Mix of *L. major* + *L. turanica*
Ph. caucasicus (6 specimens)	Habib Abad (Esfahan)	32	4	2	2	-
	Ali Abad (Esfahan)	14	-	-	-	-
	Nik Abad (Esfahan)	12	-	-	-	-
	Ezhiyeh (Esfahan)	1	1	-	1	-
	Agh Tagheh (Golestan)	5	1	1	-	-
Ph. mongolensis (11 specimens)	Sian (Esfahan)	8	1	-	1	-
	Abbas Abad (Esfahan)	3	1	-	1	-
	Nik Abad (Esfahan)	2	-	-	-	-
	Heydar Abad (Esfahan)	4	-	-	-	-
	Ezhiyeh (Esfahan)	3	-	-	-	-
	Raja Abad (Fars)	16	-	-	-	-
	Sadegh Abad (Fars)	7	1	1	-	-
	Kouh Sabz (Fars)	3	-	-	-	-
	Band Amir (Fars)	2	-	-	-	-
	Ghareh Gol (Golestan)	8	-	-	-	-
	Ouch Quee (Golestan)	12	1	-	1	-
	Agh Tagheh (Golestan)	26	4	3	-	1
	Narlidagh (Golestan)	18	3	-	2	1
Total		176	17	7	8	2

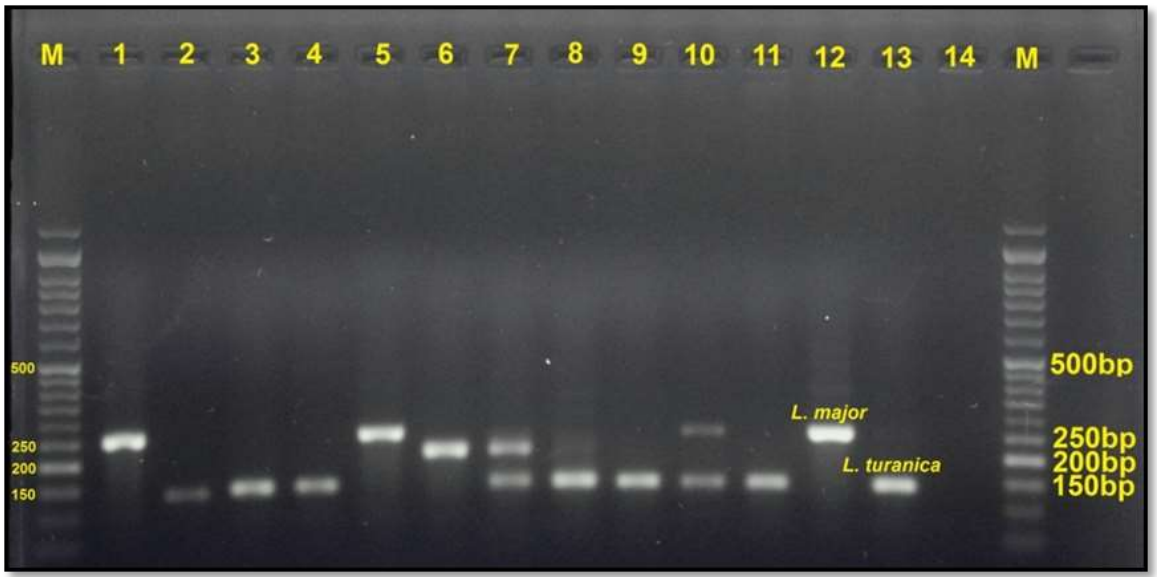

Fig. 1. Agarose (1.5%) gel electrophoresis of Nested-PCR products for *Leishmania* parasite infection in *Phlebotomus caucasicus* and *Ph. mongolensis* in Esfahan, Golestan and Fars provinces. Lanes M, 50 bp Ladder (ExcelBand™, SMOBIO Technology); Lane 1, *L. major* (245 bp, detected in *Ph. caucasicus*); Lane 2, *L. turanica* (detected in *Ph. caucasicus*); Lanes 3-4-8-9-11, *L. turanica* (detected in *Ph. mongolensis*); Lane 5, *L. major* (245bp, detected in *Ph. mongolensis*); Lane 6, *L. major* (233 bp, detected in *Ph. mongolensis*); Lane 7, mix infection with *L. major* (233 bp) and *L. turanica* (detected in *Ph. mongolensis*); Lane 10, mix infection with *L. major* (245 bp) and *L. turanica* (detected in *Ph. mongolensis*); Lane 12, *L. major* (positive control); Lane 13, *L. turanica* (positive control); Lane 14, negative control (distilled water)

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Table 2. *Leishmania* parasite positive PCR detected in *Phlebotomus caucasicus* and *Ph. mongolensis* specimens based on abdominal status in Esfahan, Golestan and Fars provinces, Iran, 2016

Species	Specimens	*Leishmania* positive samples				Abdominal status of specimens			
		Total	*L. major*	*L. turanica*	*L. major* + *L. turanica*	UF	FF	SG	G
Ph. caucasicus	64	6	3	3	-	4	1	1	-
Ph. mongolensis	112	11	4	5	2	9	-	-	2
Total	176	17	7	8	2	13	1	1	2

UF= Unfed, FF= Fresh fed, SG= Semi gravid, G= Gravid

Discussion

Identification of phlebotomine sand flies as vectors of leishmaniasis is very important and crucial for leishmaniasis control programs. In this study, molecular detection, and identification of *Leishmania* parasites based on Nested-PCR method allowed us to detect more *Leishmania* infections than previously in Iranian sand flies. Numerous natural sand fly promastigote infections in ZCL foci in Iran have been reported based on parasitological methods and direct examinations (31–35), identification of *Leishmania* parasite using isoenzyme electrophoresis (14, 36), and molecular methods based on polymerase chain reactions (17–27). In the present study two species of *Leishmania* parasites including *L. major* and *L. turanica* as well as *L. major* + *L. turanica* mixed infection in *Ph. caucasicus* and *Ph. mongolensis* were detected using Nested-PCR of ITS2-rDNA region.

These parasites are in concordance with identified gerbils' parasites and *Ph. caucasicus* and *Ph. mongolensis* belongs to Kazakhstan, Uzbekistan, Turkmenistan and China (37, 38) and Iran (23, 39). *Phlebotomus caucasicus* is an Asiatic species, first recorded from the Transcaucasia region and is distributed from the geographical area of Iran to China. It is a common species in sandy deserts and hills, and usually lives in rodent and bird nests, bites rarely humans (40).

Various transmissions of gerbil parasites have occurred in Iran's northern neighbors in Central Asia, including *L. major*, *L. turanica*, and *L. gerbilli*, within or near the nest of the great gerbil of *Rhombomys opimus* (41). Rodent colonies provide habitat for many species of sand flies, increasing the risk of the *Leishmania* parasite being spread to mammals (42). *Phlebotomus caucasicus* was first introduced by Adler and Theodor in 1957 and was identified among Central Asian rodents as a suspected vector of *L. major* and *L. gerbilli* parasites (43). It is also considered an *L. turanica* vector in Turkmenistan (37, 44), and *L. donovani* vector in Central Asia and Kazakhstan (45).

In this research, three samples of *L. major* parasite (Esfahan and Golestan provinces) and three samples of *L. turanica* parasite (Esfahan province) were identified from six *Ph. caucasicus* specimens. Typing of parasites isolated from this sand fly species by isoenzyme method led to the precise diagnosis of parasite as *L. major* Mon-26 and proved to be the same type of human parasite, also *Ph. papatasi* as vector and *Rh. opimus* as reservoir (14). *Phlebotomus caucasicus* is also confirmed to be 12.5 and 7.5% natural leptomonad infection in the Nikabad and Borkhar regions of Esfahan Province respectively (14). In 2008, Parvizi and Ready identified two species of *L. major* and *L. gerbilli* from *Ph. caucasicus* in

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Esfahan Province and *L. gerbilli* in Golestan Province based on Nested-PCR method by sequencing of ITS1-5.8S rDNA region (17). Using the RAPD-PCR method, *L. major* parasites were reported from four *Ph. caucasicus* group specimens (4.2%) caught from rodent burrows in Shahroud County (Semnan Province) in Iran (39). Once again, using the Nested-PCR method of kDNA genome and using the RFLP method of ITS1-rDNA region, the *L. major* parasite was reported from *Ph. caucasicus* group of sand flies collected in Damghan city (Semnan province) in Iran (46).

According to these reports, along with the 20% anthropohilic index for *Ph. caucasicus* (14, 47), strong evidence shows that *Ph. caucasicus* is a natural vector of *L. major*, *L. turanica* and *L. gerbilli* parasites among reservoir rodents as well as a secondary or suspected vector of *L. major* for humans in ZCL foci in Iran (8).

In the current study, four samples of *L. major* (Golestan and Fars provinces), five samples of *L. turanica* (Esfahan and Golestan provinces) and two mixed infection samples of both *L. major* and *L. turanica* (Golestan Province) were found from 11 collected *Ph. mongolensis* specimens. It should be noted that, in Iran, natural infection with *Leishmania* parasites is recorded for the first time in this study in *Ph. mongolensis*. *Phlebotomus mongolensis* is also an Asiatic species and is a dominant species in the sandy deserts and hills and usually lives in rodents burrow (41). It is also known to be *L. turanica* and *L. gerbilli* vector in Turkmenistan (37, 44), *L. turanica* in China (38), *L. donovani* and *L. major* in Central Asia and Kazakhstan (45). Based on the findings of this study, it is possible that both *Ph. caucasicus* and *Ph. mongolensis* are potential vectors of the gerbil *Leishmania* parasites. However, the detection of genomic DNA of *Leishmania* in sand flies does not confirm that they have been vector and PCR-based methods can not differentiate *Leishmania* promastigotes in both infectious and non-infectious forms. The preference of sand flies to human blood, and the growth and development of parasites in the external cycle and experimental bite transmission have been the most important criteria for disease parasite transmission (48–50).

Considering that another criterion for the definition of a sand fly as a vector is the presence of the infective form of metacyclic promastigotes in the thoracic areas of midgut, foregut and mouth sections of sand flies, the position of the sand flies' abdomen is also very critical in determining the vector. In this study, among the 17 *Ph. caucasicus* and *Ph. mongolensis* sand flies' specimens with *Leishmania* infection, 13 specimens with empty (unfed) abdominal status, indicating that the parasite of *Leishmania* was successfully developed in midgut areas. The transmission dynamics of *Leishmania* parasites depend not only on the *Leishmania* species diversity but also on the parasite's intra-species strain diversity.

In this research, according to the results of molecular identification of *Leishmania* parasites, it can be concluded that *Ph. caucasicus* and *Ph. mongolensis* were considered as potential vectors of *L. major* and *L. turanica* parasites and allowing them to circulate of *Leishmania* parasites among rodents and probably humans.

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Ethical considerations

This experiment was carried out under the guidance of the Ethics Committee of the Tehran University of Medical Sciences (IR.TUMS.REC.1394.144).

Conflict of interest statement

Authors declare that there is no conflict of interest.

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