Original Article

Molecular Identification of Cutaneous Leishmaniasis Vectors in Alborz Province, North of Iran

Nouroozi Kouh, T[1], Hoghooghi Rad, N[1 *], Navidpour, Sh[2], Shirali, S[1, 3], Esmailnia, K[2]

1. *Department of Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, Iran*
2. *Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran*
3. *Department of Biotechnology, Ahvaz Branch, Islamic Azad University, Ahvaz, Iran*

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Corresponding Author's E-Mail:
hoghooghiradnasser@yahoo.com

ABSTRACT

Cutaneous leishmaniasis (CL) is a vector-borne disease widely distributed in tropical and subtropical areas of North and South America, Europe, Asia, and Africa. Considering the increasing number of CL cases in recent years and the fact that no study has been conducted to identify CL fauna and vectors in Alborz province, this study was carried out to identify sand flies and CL vectors in this region. Sand flies were collected from August to October 2021 from plain and mountainous indoor and outdoor areas of the region using sticky paper traps and were detected morphologically. DNA was extracted from the midguts of female sand flies. In this study, 1157 sand flies were collected and identified. The number of sand flies caught from indoor and outdoor places was 367 (31.72%) and 790 (68.28%), respectively. Overall, six species of flies were of the genus *Phlebotomus* (Raynal, 1937), including *Phlebotomus papatasi* (*P. papatasi*, 695 [60.07%]; Scopoli, 1786), *P. kandelakii* (13 [1.12%]; Shchurenkova, 1926), *P. sergenti* (232 [20.05%]; Parrot, 1917), *P. major* (14 [1.21%]; Annandale, 1910), *P. caucasicus* (4 [0.35%]; Marzinowsky, 1917), *P. alexandri* (18 [1.56%]; Alexandri Sinton, 1920), and four were of the genus *Sergentomyia* (Artemiev, 1978), including *Sergentomyia tiberiadis* (109 [9.42%]; Adler, Theodor & Lourie, 1930), *Sergentomyia baghdadis* (53 [4.58%]), *Sergentomyia sintoni* (14 [1.21%]; Sintoni Pringle, 1933), *Sergentomyia clydei* (5 [0.43%]). *P. papatasi* spp. were dominant in indoor and outdoor places, with a prevalence of 695 (60.07%). The *Leishmania major* (*L. major*) gene was identified in five samples of *P. papatasi* spp. This suggests that *P. papatasi* is the potential vector spp. in the study area. Moreover, *L. major* was confirmed as the aetiological agent of CL cases in Alborz province. The identification of vectors and parasite spp. is very important for the treatment and operational planning of disease vectors.

Keywords: kDNA, *Leishmania major*, Nested-PCR, *Phlebotomus*

1. Introduction

Leishmaniasis is a zoonotic disease and a health problem worldwide. However, over 90% of new cases occur in just 13 countries, including Afghanistan, Algeria, Bangladesh, Bolivia, Brazil, Columbia, Ethiopia, India, Iran, Peru, South Sudan, Sudan, and Syria. It is estimated that between 0.9 and 1.7 million people are newly infected every year, but only a small fraction of them develop the disease, and 20,000-30,000 eventually die (1).

The ecology of leishmaniasis varies widely in different geographic zones. In central Asia, it occurs in semiarid and arid locations. In the Mediterranean region and the Middle East, it is typically urban, and in Africa, it is primarily rural. In the American tropics, it has traditionally been a forest disease but is advancing into urban areas. In Peru, it occurs in small towns and farms in high mountain valleys. Worldwide, about 200 million people in 98 countries are at risk of leishmaniasis, with an estimated incidence of 12 million infections and 2 million additional cases each year (2).

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania* and occurs in three principal clinical forms known as cutaneous, visceral, and mucocutaneous leishmaniasis. Twenty species (spp.) of the genus *Leishmania* have been recognized based on genetic or immunologic criteria. The identification of *Leishmania* spp. usually requires culture of the pathogen followed by immunological, biochemical, and molecular assays, although polymerase chain reaction (PCR) and other diagnostics are under development. Cutaneous leishmaniasis (CL) ulcer may be localized and self-limiting, which heals with or without medication, or it may be chronic and diffuse, which is resistant to treatment. Co-infections of *Leishmania* and Human Immunodeficiency Syndrome (HIV) have been reported in 35 countries, most notably Spain, Portugal, France, and Italy. Individuals co-infected with *Leishmania* and HIV can infect sand flies and act as human reservoirs in zoonotic foci to shift the local epidemiology toward a human-to-human transmission pattern (3).

Iran is considered one of the most important foci of CL in the world. There are two forms: the rural CL caused by *Leishmania major* (*L. major*) and the urban CL caused by *L. tropica*. This disease has emerged as a health problem in many provinces of the country, and despite measures taken to control it, the incidence and new foci of the disease are increasing. There are 19 centers of the rural type and eight centers of the urban type. According to the report of the disease management center, the number of people suffering from leishmaniasis in Iran is about 20,000 annually, whereas the actual cases are four to five times more (4, 5).

In order to implement control measures to reduce the incidence rate of the disease, the identification of the disease vectors is very important. Finding a sand fly infected with parasites is a basic step in the identification of vector spp. and the potential for disease transmission in indigenous regions (6). There are two classical methods for estimating infection rates in reservoir hosts or vectors, including microscopic analysis and isolation in culture, both of which are laborious and inaccurate, as many spp. and subspecies of flagellate protozoa are often morphologically indistinguishable. The above methods are time-consuming and difficult and require high levels of experience and skill. They also have limitations, such as polluting the cultivation environment, and to determine the identity of the isolated parasite, complementary methods are needed, such as isozymes or molecular assays. Diagnostic assays for leishmaniasis have been developed based on the amplification of several Deoxyribonucleic acids (DNAs), targets such as the minicircle of Kinetoplastid DNA (kDNA), ribosomal ribonucleic acid (rRNA) genes, the mini-exon-derived RNA, and repeated genomic sequences. The minicircle (0.8 to 1 kb in length) of kDNA is an ideal target since it is present in 10,000 copies per cell that are distributed among about 10 different sequence classes. In addition, the minicircle sequence is known for most *Leishmania* spp. and possesses a variable region that offers accurate discrimination between spp. (7).

Currently, the annual incidence of CL has increased in endemic and non-endemic foci in Iran, most of which are caused by *L. major*. Considering the increasing number of CL cases in recent years and the fact that no study has been conducted to identify CL fauna and vectors in Alborz province, the present study aimed to morphologically classify sand flies spp. and identify disease vectors using special primers designed by Noyes, Reyburn (8) and Nested-PCR techniques. It also aimed to detect sequences of positive spp. and compare their phylogenetic relationship to that of those available and registered in GenBank.

2. Materials and Methods

2.1. Study Area

This study was conducted in Alborz province, located in the north of Iran, between latitudes 35°28´ to 36°30´ N and longitudes 50°10´ to 51°30´ E. It has a special place in the country due to its industrial and agricultural value, tourism, ecotourism, cultural heritage, and academic centers. It has a population of approximately 2,712,400 people and an area of 5,125 km^2. Geographically, the province is surrounded from the north by Mazandaran province, from the east and southeast by Tehran province, from the southeast by Central province, and from the west by Qazvin province. The climate is influenced by the Alborz Mountain ranges, with cold winters and mild summers, as well as a mean annual rainfall of 354 mm, a relative humidity of 51%, and an annual temperature of between -17.8°C and +21.1°C.

2.2. Sand Fly Collection

In this cross-sectional study, sand flies were caught and collected biweekly from August to October 2021 from plain and mountainous regions, as well as indoor and outdoor areas, in Alborz province, using sticky paper traps. After recording the sampling data and locations, they were gently separated from sticky papers using a dissecting needle, defatted, placed in vials containing 96% ethanol, and stored in a refrigerator at 4°C (9). To identify spp., sand flies were mounted in Puri's medium (10) and identified after 24 h using the keys of (4), Lewis (11).

2.3. DNA Extraction

For molecular detection in sand fly specimens, DNA was extracted using the method of Coen, Thoday (12). The DNA samples were stored at -20°C before use in the PCR assay (13, 14).

2.4. DNA Amplification

The Nested-PCR was performed to detect *Leishmania* spp. using specific primers. In this study, specific primers were used for the detection of *Leishmania* parasites in *Phlebotomus papatasi* (*P. papatasi*) and *P. sergenti* based on kDNA for *L. major* and *L. tropica*, according to Noyes, Reyburn (8) (Table 1).

Table 1. Specific primers for molecular identification of *Lieshmania*

Primer	Target location	Sequence 5'-3'
CSB2XF	kDNA	CGAGTAGCAGAAACTCCCGTTCA
CSB1X R	kDNA	ATTTTTCGCGATTTTCGCAGAACG
13Z	kDNA	ACTGGGGGTTGGTGTAAAATAG
LiR	kDNA	TCGCAGAACGCCCCT

The first round of PCR for each sample included the following: Taq DNA polymerase 0.75 µl, dNTPs 2.5 mM, PCR buffer 2.5 µl, MgCl2 2 mM, 5 µl of DNA sample, and 40 ng of CSB2XF and CSB1XR (External primers). The PCR premix tube was then completed by deionizing (ddH2O) PCR water into 20 µl. The second-round primers (Internal) were 13Z and LIR and the same PCR master mix. In this step, the mentioned reaction mixture was used in a total reaction volume of 30 ml, containing 2 ml of the DNA product of the first round. The reaction was amplified in a programmable thermocycler (PeqSTAR, Peqlab Comp., Germany) for 5 min at 94°C (1 cycle), followed by 30 cycles at 94°C for 30 sec, 55°C for 60 sec, and 72°C for 90 sec, and a final extension at 72°C for 5 min. A total of 15 µl of each PCR product were resolved in 5 µl of loading buffer, then electrophoresed in a 1.5% agarose LE gel in TBE buffer (0.089 M Tris-HCl, 0.089 M boric acid, and 0.02 M EDTA) containing 0.75% ethidium

bromide, and visualized under ultraviolet transilluminator (3UVP, Transilluminator, USA). *Leishmania* spp. were identified by comparing the PCR products of specimens to the reference strains and molecular weight markers (13, 15).

2.5. kDNA Sequencing and GenBank Accession Numbers

The PCR products for the kDNA gene of *L. major* isolated from *P. papatasi* were sent to Pishgah Biotech Company, Tehran, Iran, for sequencing, and then the results were submitted to GenBank and registered under the accession number OQ185291.

3. Results

3.1. Morphological Study

A total of 1,157 sand flies were collected from plain and mountainous indoor and outdoor areas of the regions in Alborz province. All specimens were identified morphologically according to the keys of (4), Lewis (11). The number of sand flies caught from indoor and outdoor places was 367 (31.72%) and 790 (68.28%), respectively. As a result of this study, 10 spp. of Phlebotomine were identified in two *Phlebotomus* and *Sergentomyia* genera. Six of them were of the genus *Phlebotomus*, including *P. papatasi* (695 [60.07%]), *P. kandelakii* (13 [1.12%]), *P. sergenti* (232 [20.05%]), *P. major* (14 [1.21%]), *P. caucasicus* (4 [0.35%]), *P. alexandri* (18 [1.56%]), and four were of the genus *Sergentomyia*, including *S. tiberiadis* (109 [9.42%]), *S. baghdadis* (53 [4.58%]), *S. sintoni* (14 [1.21%]), and *S. clydei* (5 [0.43%]). *P. papatasi* was the most dominant spp. in indoor and outdoor places, with a prevalence of 60.07% (Table 2).

3.2. Molecular Study

Leishmania kDNA was found in five out of 129 (3.9%) specimens and only in *P. papatasi* spp. There was no *Leishmania* infection in other spp. of sand flies. The obtained visualized bands in the infected specimens were similar to the standard strain of *L. major*, which was equal to 560 bp (Figure 1). All of the infected sand flies had been collected from outdoor places.

3.3. Results of kDNA Sequencing and Phylogenetic Analysis

The 560 bp kDNA sequence of the *L. major* gene was compared to 21 *L. major* and 15 *L. donovai*, *L. Chagasi*, and *L. infantum* from Iran and kinetoplast gene sequences from other countries in GenBank database. Nucleotide BLAST analysis showed a high percentage of similarity (100%) to *L. major* HQ7555 and *L. tarentolae* kinetoplast minicircles and partial identity to MN423166. IQM–kut No.1, MN313420. IQM-kut No.2, and MN313421-23. IQM-kut No.3-5 kinetoplast gene sequences. Phylogenetic analysis was performed based on the partial-length sequence of the kDNA gene. This tree shows that *L. major* isolated from Iran is located close to six other *Leishmania* spp. (Figure 2).

Table 2. Abundance of sand flies species in Alborz province, North of Iran, 2021

Species	Number		Collection sites				gender			
	No.	%	Indoor		Outdoor		Female		Male	
			No.	%	No.	%	No.	%	No.	%
P. papatasi	695	60.07	236	20.40	459	39.67	129	11.15	566	48.92
P. sergenti	232	20.05	112	9.68	120	10.37	63	5.45	169	14.61
P. caucasicus	4	0.35	0	0.00	4	0.35	0	0.00	4	0.35
P. kandelakii	13	1.12	3	0.26	10	0.86	5	0.43	8	0.69
P. major	14	1.21	2	0.17	12	1.04	2	0.17	12	1.04
P. alexandri	18	1.56	0	0.00	18	1.56	2	0.17	16	1.38
S. tiberiadis	109	9.42	9	0.78	100	8.64	12	1.04	97	8.38
S. baghdadis	53	4.58	5	0.43	48	4.15	9	0.78	44	3.80
S.sintoni	14	1.21	0	0.00	14	1.21	0	0.00	14	1.21
S. clydei	5	0.43	0	0.00	5	0.43	1	0.08	4	o.35
Total	1157	100	367	31.72	790	68.28	223	19.27	934	80.73

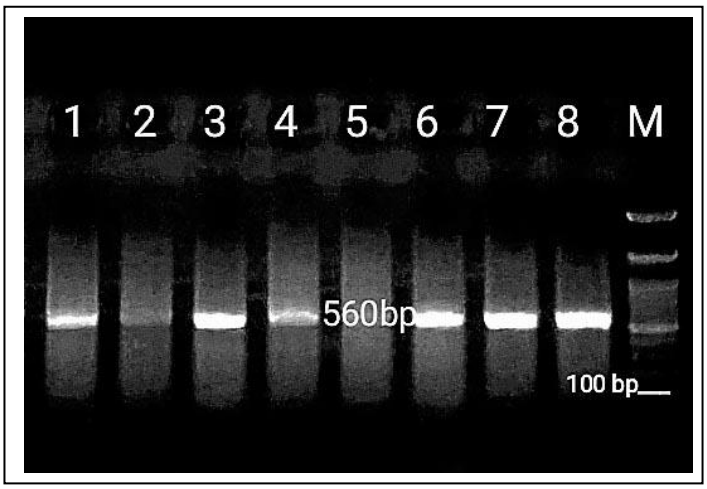

Figure 1. Agarose gel electrophoresis of kDNA *Leishmania* spp., lane 1,3,4,6,7, *Leishmania major* isolates from *Phlebotomus papatasi* 560 bp; lane 2, negative control (male sand fly DNA); lane 8, positive control (*L. major* strain MHOM/IR/54/LV39) 560bp; lane M, 100bp DNA ladder

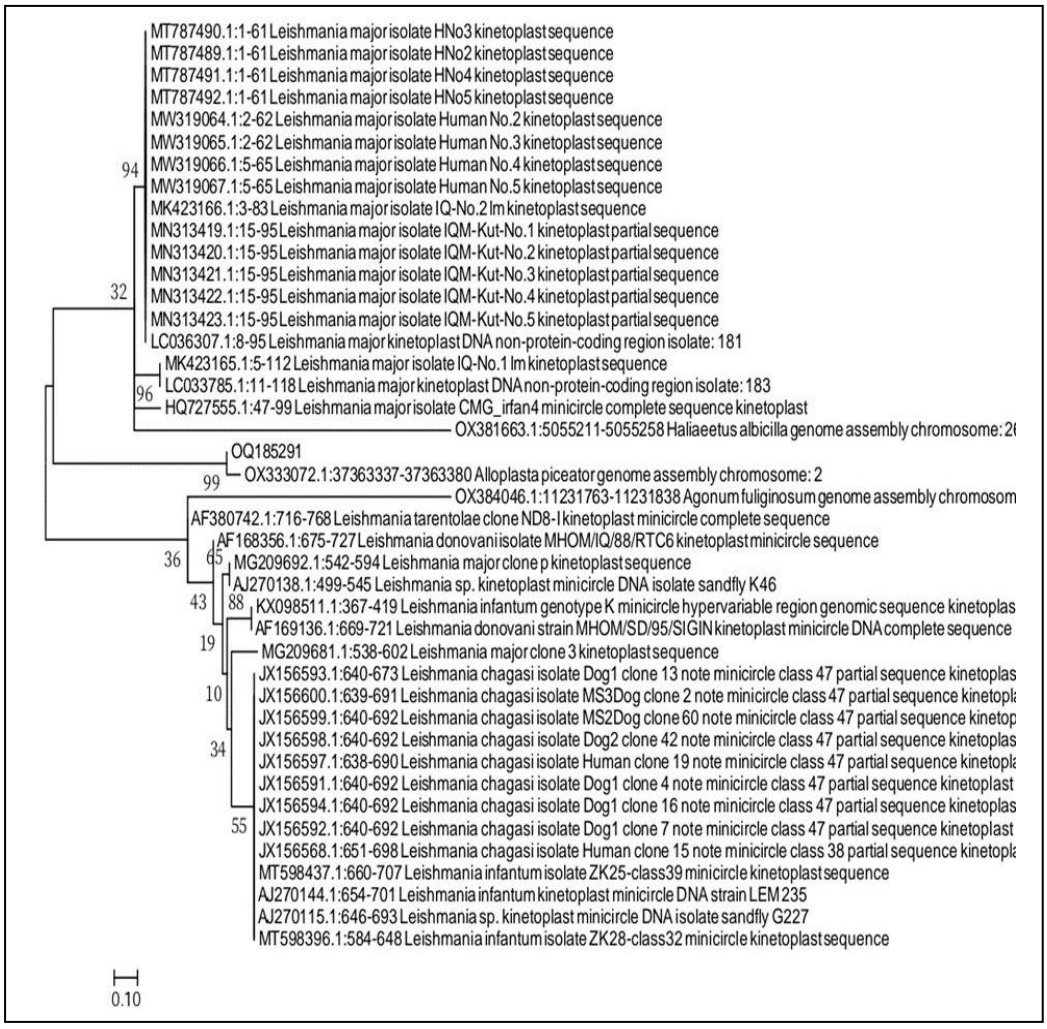

Figure 2. Phylogenetic relationships among *Leishmania* spp., based on kDNA gene sequences

3.4. Statistical Analysis

The statistical analysis was performed using the Chi-squared test in SPSS (version 18.0) for Windows (SPSS Inc., Chicago, IL, USA), and a *P*-value of ≤0.05 was considered statistically significant ($P \leq 0.05$)

4. Discussion

Leishmaniasis is a global health problem due to an insufficient understanding of sand fly ecology and unsuccessful control measures (16). Information and knowledge about the ecology and epidemiology of leishmaniasis are essential to controlling the disease. This knowledge could be focused on the identification of reservoir hosts or the identification and detection of parasites and vectors (17). This study is the first to report on the identification of sand flies fauna and the diagnosis of *L. major* in Alborz province, North of Iran. Ten spp. were identified, six from the *Phlebotomus* genus and four from the *Sergentomyia* genus. *P. papatasi*, a proven vector of *L. major* in Iran (6, 18), was the most abundant spp. in Alborz, especially in rodent burrows. The results of the current study agree with previous findings by Vahabi, Rassi (19) in the West of Iran, which used semi-Nested PCR techniques of kDNA and ITS1-rDNA, followed by restriction fragment length polymorphism, for the identification of the DNA of *Leishmania* parasites in infected sand flies. Our findings suggest that *L. major* is the cause and *P. papatasi* is the primary vector of CL in Alborz. It is noticeable that *P. papatasi* is the main proven vector of CL in Iran (18). This study identified CL vectors using Nested-PCR. This method is widely used by many researchers in Iran and other countries as an alternative method for the detection of *Leishmania* and other protozoa (13, 20-24). Based on our findings, the prevalence of leishmaniasis has increased in Alborz province in recent years, which is consistent with the findings of previous studies in other regions (13, 23). This study has shown that all of the infected sand flies were collected from outdoor places. According to a previous study by Oshaghi, Yaghobi-Ershadi (17), this indicates that there has been sufficient time for the parasites to develop and transform into promastigote, the infective form, which in turn could be transmitted to a new host through bites and cause an outbreak. This study used Nested-PCR for the identification of sand flies for the first time in Alborz province by the minicircle of the kDNA gene. Nested-PCR has significantly improved the detection of leishmanial kDNA in sand flies in endemic areas, as the performance of kDNA PCR for the detection of *L. major* (560 bp), *L. tropica* (750 bp), and *L. infantum* (680 bp) has been confirmed on sand fly samples using specific primers. However, further studies with more samples from other endemic areas are needed to evaluate the use of N-PCR for kDNA detection in sand fly samples.

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Authors' Contribution

Study concept and design: N. H. R. and Sh. N.
Acquisition of data: T. N. K.
Analysis and interpretation of data: S. Sh. and K. E.
Critical revision of the manuscript for important intellectual content: N. H. R. and Sh. N.
Statistical analysis: S. Sh. and K. E.
Drafting of the manuscript: T. N. K and S. Sh.
Administrative, technical, and material support: T. N. K.
Study supervision: N. H. R. and Sh. N.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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