Original Article

Leishmania Infection in *Phlebotomus* Species in Mehran city, Ilam Province, Iran

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ABSTRACT

Ilam province has been identified as a major center for zoonotic cutaneous *Leishmania*sis (ZCL) in western Iran. The present study was conducted with the objective of investigating the infectivity of *Phlebotomus* spp. with *Leishmania major* in Mehran city of Ilam province, Iran. The present study was conducted during the two seasons of the peak mosquito activity, i.e. summer and autumn of 2019. The sticky papers method was utilised for the collection of sandflies. The installation of 400 sticky paper traps resulted in the collection of 2,860 sandflies (950 females and 1,910 males) over the course of two seasons. The female *Phlebotomus* genus and species were identified using the Iranian standard identification key. Subsequently, *Leishmania* DNA was extracted from the female *Phlebotomus* specimen using the phenol-chloroform method, and the ITS1 gene was amplified by PCR. Subsequently, a comparison was made between the genome sequence and the sequence of other samples in the GenBank database, utilizing bioinformatics software. In conclusion, the species of the samples under investigation were determined according to the results of the phylogenetic tree. Furthermore, the parasite species was determined by utilizing the HaeIII restriction enzyme. Of the 617 Phlebotomus female samples collected, 34 were found to be infected with the *Leishmania* parasite. Of these, 32 (5.18%) of *Ph. papatasi* and 2 (0.32%) of *Ph. sergenti* were found to be infected. The results of the RFLP method and sequencing indicated that these mosquitoes were infected with only *L. major*. It is evident, based on the findings of this study, that ZCL type of Leishmaniasis is prevalent in Mehran city. It is imperative that the findings of this study be given greater consideration by the health officials of the province.

Keywords: sandflies; *L. major*; ITS1 Gene; Mehran; Iran

1. Introduction

Leishmania is a protozoan of the genus *Leishmania*, which is transmitted by sandflies (1) and is the causative agent of cutaneous Leishmaniasis. *Leishmania* is a protozoan parasite that can infect both humans and animals. There are two forms of the parasite: the first is a small, round form called an amastigote, which lives inside the cells of the vertebrate host. The second form is elongated and has flagella, and is called a promastigote (2). This form lives inside the body of the insect that transmits the disease. To date, approximately 30 species of parasites have been documented, of which a mere 20 have been determined to be pathogenic to humans (3, 4). The protozoan parasite *Leishmania* is transmitted through the bite of infected female Phlebotomine mosquitoes, which feed on blood to produce eggs. The epidemiology of Leishmaniasis is contingent upon the characteristics of the parasite and the mosquito species, the environmental characteristics of the transmission sites, the exposure of the human population to the parasite, and the behavior and habits of humans. It is estimated that approximately 70 species of animal, in addition to humans, act as natural reservoir hosts for *Leishmania* parasites (5). The feeding habits of sandflies are diverse, with some species exhibiting a wide range of hosts, including canids, rodents, and blood-sucking reptiles. In contrast, other species have a pronounced anthropophilia, feeding primarily on humans. Consequently, human Leishmaniasis exhibits comparable patterns of disease transmission between animals and humans or between humans (6). It is important to note that *Phlebotomus* may be infected with different species of *Leishmania* during the process of blood feeding, whether from humans or animals. During the process of blood feeding, the parasite may transfer *Leishmania* to a new host. The epidemiological and clinical manifestations of Leishmaniasis vary depending on the specific *Phlebotomus* species. It is evident that two cutaneous anthroponotic (ACL) and zoonotic (ZCL) forms of human Leishmaniasis are prevalent in Iran. In the anthropontic form, Ph. sergenti and *Phlebotomus* tropica and humans usually play the main role, and in the zoonotic form of Leis
hmaniasis, Ph. papatasi and *Phlebotomus* major and rodents play the main role. The frequency of these forms varies across different regions of Iran. In the province of Ilam, the zoonic form is typically documented. In the following section, a number of recent studies conducted in Ilam province regarding Leishmaniasis will be mentioned. These include the studies of Asgari Nezhad et al. (2012); Yazdanpanah & Rostamianpur (2013); Roghani et al. (2012); Gholami Parizad et al. (2015); Kassiri et al. (2012); and Kermanjani et al. (2017) (7-12). To date, no research has been conducted on the *Phlebotomus* population of Mehran city, Ilam province, with regard to its potential for transmitting *Leishmania*. The objective of the present study was twofold: firstly, to ascertain the prevalence of *Leishmania* infection in *Phlebotomus* in Mehran city; and secondly, to determine the species of said infection by molecular method.

2. Materials and Methods

2.1. Area of Study

Ilam province is located in western Iran, adjacent to the following provinces: Kermanshah to the north, Khuzestan to the south, Lorestan to the east, and Iraq to the west (Figure 1). The most prominent cities of Ilam province are as follows: The subjects in this study were Ivan, Dehlran, Mehran, and Shirvan. Mehran city is located on the left bank of the Kanjan Cham river and is less than a few kilometers away from the Iraqi border. The city's population is 46,981 individuals, and it is divided into three districts. Mehran, Saleh Abad, and Malekshahi. Mehran city is located at an altitude of 155 meters above sea level. Mehran is considered one of the most fertile areas of the province during years with high precipitation. (13).

2.2. Sampling

The sticky papers method was utilised for the collection of sandflies. The papers were divided into two seasons, with 100 published in the summer and 300 in the autumn. The installation of 400 sticky paper traps resulted in the collection of 2,860 sandflies (950 females and 1,910 males) over the course of two seasons. The sticky papers were installed in indoor locations, such as houses and stables, as well as in outdoor and open areas in the vicinity of these structures, spanning a total of 17 regions during both the summer and autumn seasons. Subsequently, the sand flies were extracted from the adhesive papers using entomological needles or fine brushes. Thereafter, they were washed multiple times with 75% ethanol to eliminate any residual oil. Following this step, the flies were preserved in 70% ethanol and stored in micro tubes prior to identification.

2.3. Microscopic Study

In order to identify the species of sand fly, the head and the posterior two abdominal segments of female sand flies were detached, mounted in Puri's media, and the species were identified using a valid morphological identification key for adult sandflies. (14, 15). In order to ascertain whether the sandfly had been infected by the *Leishmania* parasite, the remainder of the sandfly's body was preserved in 85% ethanol for the purpose of DNA extraction.

Figure 1. Geographical location of Mehran city, Ilam province, Iran.

2.4. DNA Extraction

The phenol-chloroform method was utilized for the extraction of deoxyribonucleic acid (DNA) from the body of *Phlebotomus* . The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique was utilized in order to conduct an investigation into the infection of female *Phlebotomus* and to ascertain the parasite species.

2.5. Polymerase Chain Reaction

The ITS1 region of the *Leishmania* parasite was amplified using the following primers: forward, 5'-CTGGATCATTTTCCGATGT-3'; reverse, 5'-TGATACCACTTATCGCACTT-3'. (12, 15). The reaction mixtures were then adjusted to a final volume of 20 µL, consisting of Taq Master Mix (9.5 µL), 10 pmol of each primer (forward primer: 1 µM; reverse primer: 1 µM), template DNA (4 µL), and sterile deionized water (4.5 µL). The initial denaturation process was conducted at a temperature of 94°C for a duration of 5 minutes. This was followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 30 seconds. The final extension step at 72°C for 10 minutes was subsequently followed by cooling to 4°C. Subsequently, the final product from each reaction was subjected to electrophoresis and analysis on a 2% agarose gel with safe stain.

2.6. RFLP

In order to determine the species of *Leishmania*, HaeIII enzyme was used for cutting the bands in RFLP assay. The anticipated band pattern for *L. major* comprises two fragments of 132 and 203 base pairs, respectively, while for L. tropica, four fragments of 185, 53 and 57 base pairs are expected.

2.7. Sequencing

A total of 20 µl of PCR products, 10 µl of forward primer and 10 µl of reverse primer were dispatched to the Niagen Noor Company (Iran). To facilitate this, the sequences were compared to homologous sequences in GenBank using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST: www.ncbi.nlm.nih.gov/BLAST). The identification of the parasite species was conducted through a meticulous comparison of their respective sequences with those already deposited in GenBank.

2.8. Statistical Analysis

The statistical analysis of the variables was conducted using SPSS software version 16. The data were subjected to a chi-squared test at a 95% confidence level, with a P value of less than or equal to 0.05. A result was deemed to be statistically significant if the P value was less than or equal to 0.05.

3. Results

3.1. General Result

During the course of two seasons, a total of 400 sticky papers were placed in 34 districts of Mehran city, and a total of 2,860 *Phlebotomus* were collected. The results obtained from two sampling seasons indicate that 950 samples are female, of which 617 are from the genus *Phlebotomus* and 333 are from the genus Sargentomia. As indicated in Table 1, the proportion of female *Phlebotomus* and Sergentomyia that had fed on blood was 8.75% and 3.30%, respectively. As indicated by the data presented in the table, 10.75%, 8.39%, and 8.75% of the *Phlebotomus* females, and 2.04%, 12.5%, and 3.30% of the female Sergentomyia, respectively, had blood-feeding activity during the summer, autumn, and throughout the year.

3.2. PCR-RFLP results

Among the 617 617 *Phlebotomus* female samples collected, 34 samples were found to be infected with the *Leishmania* parasite (Table 2). A total of 32 (5.18%) of Ph. papatasi and 2 (0.32%) of Ph. sergenti were identified as infected. After the secondary amplification of the ITS1 gene in PCR assay, the desired band was observed in the fragment ~320 base pairs on the gel (Figure 2). Following the analysis of the data, it was determined that the obtained results exhibited 99-100% homology with the isolates registered as *L. major* species in GenBank. The phylogenetic tree of the identified isolates is depicted in Figure 3. Preliminary analysis of the OMEGA CLUSTAL multiple alignment results from the EBI site indicates that the isolates designated t25 and t27 are likely to be *L. major* (Figure 3).

4. Discussion

The primary objective of the present study was to investigate the infection of *Phlebotomus* with the *Leishmania* parasite in the Mehran region. However, in this region, two species of *Leishmania* have been documented: *L. major* and L. tropica. In the present study, however, only *L.major* was identified from *Phlebotomus* samples collected from Mehran City. Earlier studies in Iran have reported the predominance of *L. major* as the causative agent of Leishmaniasis (12, 16, 17). In the study by Gholami Parizad et al. (2015), the molecular identification of *Leishmania* parasites in smears prepared from skin lesions of patients referred to health centers in Ilam province was performed using the PCR-RFLP method. The study detected *L. major* species (10). In this regard, the findings of the study by Saberi et al. (2018) also demonstrated that the primary cause of CL in Ilam *L. major* (18) was identified. However, it is noteworthy that other Iranian researchers have reported *L. major* based on molecular assay using various genes in Ilam province (9, 11, 12). Kassiri et al. (2012) conducted a study from 2000 to 2007 on Leishmaniasis in humans, rodents, and vectors. The study found *L. major* to be the dominant species in Ilam province, with a rate of 1.2 per 1,000 individuals (11). In the period spanning from 2011 to 2012, Roghani et al. (2012) undertook a descriptive study of individuals afflicted with Leishmaniasis in Ilam province. In this study, the cities of Dehloran and Mehran demonstrated the highest rate of infection and *L. major* as the dominant species (9). In a study conducted by Kermanjani et al. (2017) on cutaneous Leishmaniasis species in Ilam province, it was determined that among a total of 61 patient samples that exhibited clinical symptoms, 64% of them were found to be infected with *Leishmania* species. As indicated by the findings derived from molecular methods, the specified species were identified as *L. major* and *L. tropica* (12). According to certain reports, in areas where Leishmaniasis has been reported to be endemic for a considerable duration, there is a possibility of its abrupt transformation into an epidemic. In addition, the possibility exists of their appearance in an area where no case has been reported in the past. The difficulty of predicting the occurrence of an epidemic of this disease is well documented. The factors that may influence the epidemic include environmental changes in the area where the vector is present, mass migration of people, and weakened immunity (malnutrition). However, the life cycle of Leishmaniasis is of such complexity that effective control measures must be implemented across multiple domains. A pivotal action to consider is the investigation of the parasite's life cycle, the means by which it is transmitted, and the control of the vectors of this disease, which facilitate transmission between different reservoirs and from reservoirs to humans (19). In the present study, two species of *Ph. papatasi* and *Ph. sergenti* were collected, both of which are vectors of zoonotic and anthropogenic cutaneous Leishmaniasis (ZCL, ACL) in Iran. The abundance of these species, especially *Ph. papatasi*, which is known as the main vector of cutaneous leishmaniasis in Iran, can be a risk for the spread of the disease in Mehran city. *Ph. papatasi* was caught in most of the trapped areas, and this finding shows that there is a possibility of ZCL transmission in this city. In conclusion, the results of this study indicate that ZCL type of Leishmaniasis is prevalent in Mehran City. In order to corroborate the findings of preceding studies in this is a possibility of ZCL transmission in this city provided that attention to the results of this study by health officials of the province.

Table 1. The total number and frequency of female sand flies caught in two seasons in Mehran city that fed on blood.

		Phlebotomus		***Sergentomyia***	
		Females	fed on blood	Females	fed on blood
Summer	No.	93	10	293	6
	%		10.75		2.04
Autumn	No.	524	44	40	5
	%		8.39		12.5
Total	No.	617	54	333	11
	%		8.75		3.30

Table 2. The number and frequency of genus and species of Phlebtomine infected and non- infected with *Leishmania* at two seasons in Mehran city.

Genus and species	**Summer**		**Autumn**		**Total**	
	Non-infected	infected	Non-infected	infected	Non-infected	infected
Ph. papatasi	80	10(12.04%)	494	22(4.40%)	606	32(5.18%)
Ph. sergenti	3	0(0.00%)	6	20(0.4%)	11	2(0.32%)
Total	83	10(12.04%)	500	24(4.8%)	617	34(5.51%)

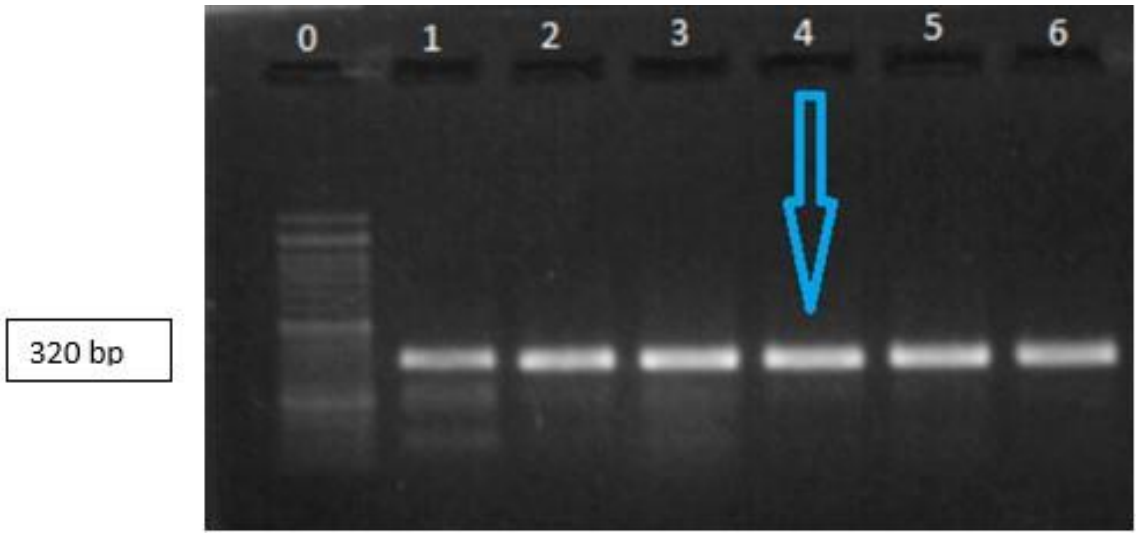

Figure 2. Electrophoresis results from PCR amplification of ITS1 fragment gene of positive *Phlebotomus* samples. From left to right: 0: 100 bp Ladder, Lane1 to 6: *Phlebotomus* samples.

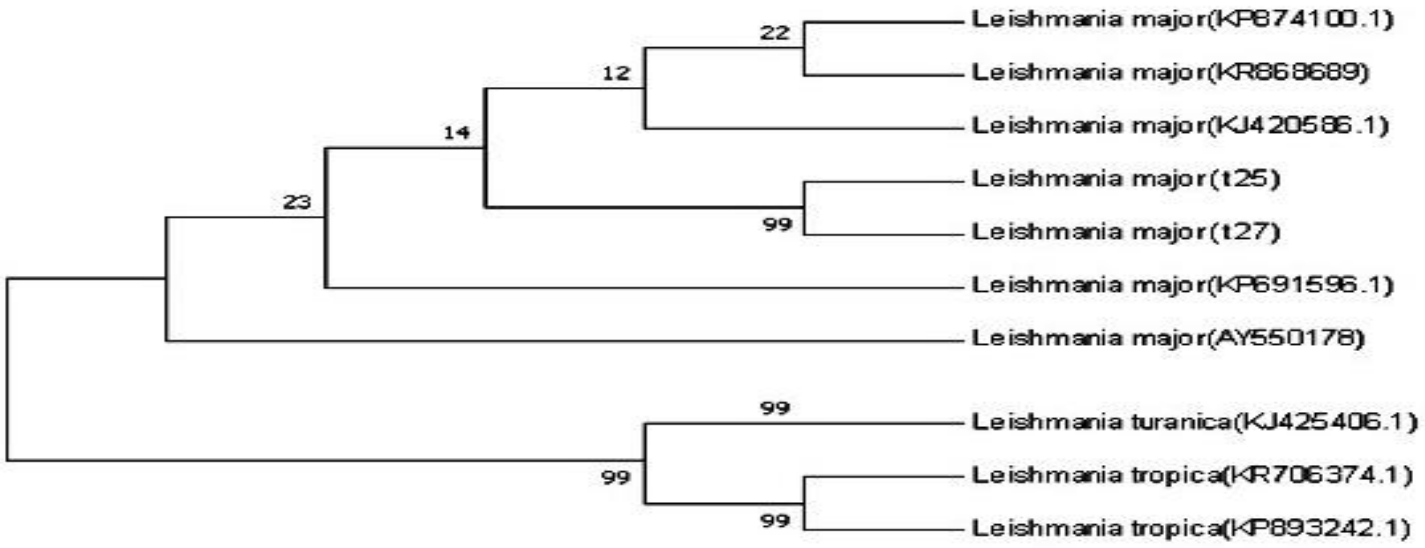

Figure 3. Phylogenetic tree inferred of ITS1 gene sequences of *L. major* isolates from *Phelebotomus* of the present study and other *Leishmania* species obtained from GenBank using MEGA software and maximum likelihood algorithm and bootstrap 500. The genotypes of this study are identified with isolates t25 and t27.

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Authors' Contribution
Study concept and design: T.G. and A.D.
Acquisition of data: T.G.
Analysis and interpretation of data: A.D.
Drafting of the manuscript: A.D. and T.G.
Critical revision of the manuscript for important intellectual content: A.D.
Statistical analysis: A.D.
Administrative, technical, and material support: A.D.

Ethics
This study was confirmed by the Medical Ethics Committee of the Faculty of Medical Sciences of Tarbiat Modares University with code No. IR.MODARES.REC.1397.172.

Conflict of Interest
The authors do not have any conflict of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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