Leishmania infection in *Phlebotomus* species in Mehran city, Ilam province, Iran, Iran

Abstract

Ilam province is one of the most important centers of Zoonotic Cutaneous Leishmaniasis (ZCL) disease in the west of Iran. This research was conducted to investigate the infectivity of *Phlebotomus* spp. with *Leishmania major* in Mehran city of Ilam province, Iran. This study was carried out in the two seasons of the peak of mosquito activity, i.e. summer and autumn of 2019. The sticky papers method was used to collect sandflies. By installing 400 sticky paper traps, 2860 sandflies (950 females and 1910 males) were collected during these two seasons. The female Phlebotomus genus and species were identified using Iranian standard identification key. Then, *Leishmania* DNA was extracted from the body of the female Phlebotomus using the phenol-chloroform method and amplified by PCR of the ITS1 gene. Then, the genome sequence was compared with the sequence of other samples in the Genbank using bioinformatics software. Finally, based on the phylogenetic tree, the species of the samples of this study was determined. In addition, the parasite species was also determined by using *HaeIII* restriction enzyme. Among the 617 *Phlebotomus* female samples collected, 34 phlebotomus female samples were found to be infected with the *Leishmania* parasite. Of which 32(5.18%) of *Ph. papatasi* and 2(0.32%) of *Ph. sergenti* were found to be infected. The results of RFLP method and sequencing indicated that these mosquitoes were infected with only *L. major*. Based on our results, ZCL type of leishmaniasis is prevalent in Mehran city. It is necessary to pay more attention to the results of this study by health officials of the province.

Key word: sandflies, *L. major*, ITS1 gene, Mehran, Iran

1. Introduction

Leishmania is a protozoan of the genus *Leishmania*, which is transmitted by sandflies (1) and it is the causative agent of cutaneous leishmaniasis. *Leishmania* has two forms: a small, round form called amastigote and lives inside the cells of the vertebrate host, the other form is elongated and has flagella and is movable, which is called promastigote (2) and lives inside the body of the insect that transmits the disease. To date, about 30 species of parasites are known, of which only 20 are pathogenic for humans (3, 4).

Leishmania is transmitted through the bite of infected female Phlebotomine mosquitoes, which feed on blood to produce eggs. The epidemiology of leishmaniasis depends on the characteristics of the parasite and the mosquito species, the environmental characteristics of the transmission sites,

the exposure of the human population exposed to the parasite, and the behavior and habits of humans. About 70 animal species, as well as humans, are known as natural reservoir hosts of *Leishmania* parasites (5). Some sandflies feed on a wide range of hosts, including canids, rodents, and blood-sucking reptiles, while others feed mainly on humans. Accordingly, human leishmaniasis shares as patterns of disease transmission between animals and humans or between humans and humans (6).

Phlebotomus may infected with different species of *Leishmania* during blood feeding from humans or animals. In its blood feeding, may transfers *Leishmania* to a new host. Depending on the Phlebotomus species, leishmaniasis has different epidemiological and clinical forms. Two cutaneous anthropontic (ACL) and zoonotic (ZCL) forms of human leishmaniasis are common in Iran. In the anthropontic form, *Ph. sergenti* and *phlebotomus tropica* and humans usually play the main role, and in the zoonotic form of leishmaniasis, *Ph. papatasi* and *phlebotomus major* and rodents play the main role. The frequency of these forms is different in various parts of Iran. In Ilam province, the zoonic form is usually reported.

Among the new studies conducted in Ilam province regarding leishmaniasis, we can mention the studies of Asgari Nezhad et al. 2012(7); Yazdanpanah & Rostamianpur 2013(8); Roghani et al. 2012(9); Gholami Parizad et al. 2015 (10); Kassiri et al. 2012(11); Roghani et al. 2013(12); Kermanjani et al. 2017(13).

So far, the *Phlebotomus* of Mehran city in Ilam province have not been investigated in terms of *Leishmania* infectivity. Therefore, this study was designed and implemented in order to determine the *Leishmania* infection of *phlebotomus* in Mehran city and to determine its species by molecular method.

2. Materials and Methods

2.1.Area of study

Ilam province is in the west of Iran and is adjacent to Kermanshah province to the north, Khuzestan province to the south, Lorestan to the east and Iraq to the west (Fig 1). The most important cities of Ilam province are: Ivan, Dehlran, Mehran and Shirvan. Mehran city is located on the left bank of the Kanjan Cham river and is not more than a few kilometers away from the Iraqi border. This city has a population of 46,981 individuals and has three districts: Mehran, Saleh Abad and Malekshahi. Mehran city is located at an altitude of 155 meters above sea level. Mehran in rainy years, it is considered one of the fertile areas of the province. (14).

Fig 1: Geographical location of Mehran city, Ilam province, Iran

2.2.Sampling

The sticky papers method was used to collect sandflies. The papers were set in two seasons, 100 in summer and 300 in autumn. By installing 400 sticky paper traps, 2860 sandflies (950 females and 1910 males) were collected during these two seasons. The sticky papers were installed for indoor places such as houses and stables and outdoor and open places around houses and stables in 17 regions in the summer and 17 regions in the autumn.

Then collected sand flies were removed from sticky papers using entomological needles or fine brushes, washed several times with 75% ethanol to remove oil, preserved in 70% ethanol, and kept in micro tubes before identification.

2.3.Microscopic study

For identifying sand fly species, head and the last 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. (17, 18). In order to determine the sandfly infection to *Leishmania* parasite, the rest of the sand flies' body was kept in 85% Ethanol and used for DNA extraction.

2.4.DNA extraction

Phenol-chloroform methods were used to extract DNA from *Phlebotomus* body. The PCR-RFLP method was used to investigate the infection of female *phlebotomus* and to determine the parasite species.

2.5.Polymerase Chain Reaction

The ITS1 region of *Leishmania* parasite was amplified, using the following primers (Forward: 5'-CTGGATCATTTTCCGATG-3' Reverse:5' – TGATACCACTTATCGCACTT-3) (15, 16). The reaction mixtures were adjusted to a final volume of 20 µL, and consisted of Taq Master Mix 9.5 µL, 10 pmol of each primer (forward primer 1 µM, reverse primer 1 µM), template DNA 4 µL, sterile deionized water 4.5 µL. Polymerase chain reaction initially denatured at 94 °C for 5 minutes followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 54 °C for 30 s, with extension at 72 °C for 30 s. The final extension at 72 °C for 10 minutes was followed by cooling to 4 °C. Then 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 expected band pattern for *L. major* consists of two fragments of 132 and 203 bp, and for *L. tropica* four fragments of 185, 53 and 57, 24 bp.

2.7.Sequencing

20µl of PCR products, 10µl forward primer and 10µl reverse primer were sent to Niagen Noor Company (Iran). Sequences were compared to homologous sequences in GenBank to the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST: www.ncbi.nlm.nih.gov/BLAST). The parasite species were identified based on their sequence compare to the sequences deposited in GenBank.

2.8.Statistical analysis

SPSS software version 16 was used for the statistical analysis of the variables. All data were compared using chi-square test with a 95% confidence level and P value less than or equal to 0.05 statistically significant was considered.

3. Results

3.1.General result

During two seasons, a total of 400 sticky papers were laid in 34 districts of Mehran city, and a total of 2860 *Phlebotomus* were collected. According to the results obtained from two sampling seasons, 950 samples are female, of which 617 are from the genus *Phlebotomus* and 333 were from the genus *Sargentomia*. Among them, there were 8.75% of females of *Phlebotomus* as well as 3.30% of females of *Sergentomyia* had fed on blood (Table 1). According to the table, 10.75%, 8.39%, and

8.75% of *Phlebotomus* females and 2.04%, 12.5%, and 3.30% of female *Sergentomyia* caught in summer, autumn, and throughout the year, respectively, had blood feeding.

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

3.2 PCR-RFLP results

Among the 617 *Phlebotomus* female samples collected, 34 *phlebotomus* female samples were found to be infected with the *Leishmania* parasite (Table 2). Of which 32(5.18%) of *Ph. papatasi* and 2(0.32%) of *Ph. Sergenti* were found to be infected.

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%)

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).

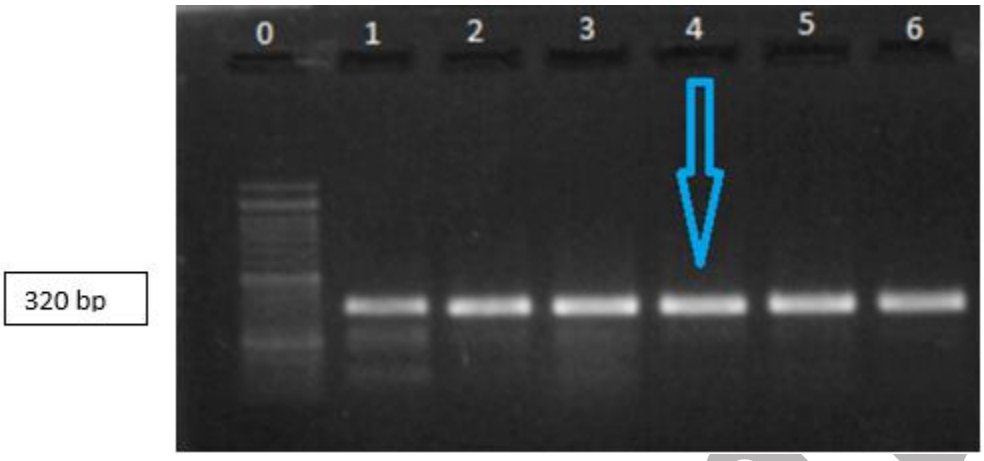

Figure 2. Electrophoresis results from PCR amplification of ITS1 fragment gene of positive *phlebotomus* samples. From left to right: 0: 100 bp Ladder, Lane1 to 6: *phlebotomus* samples.

After blasting the data, the obtained results had 99-100% homology with the isolates registered as *L.major* species in the Genbank. Also, the phylogenetic tree of the identified isolates was drawn in Figure 3. Based on the OMEGA CLUSTAL multiple alignment results of the EBI site, it was determined that the isolates numbered t25 and t27 belonged to *L.major* (Fig. 3).

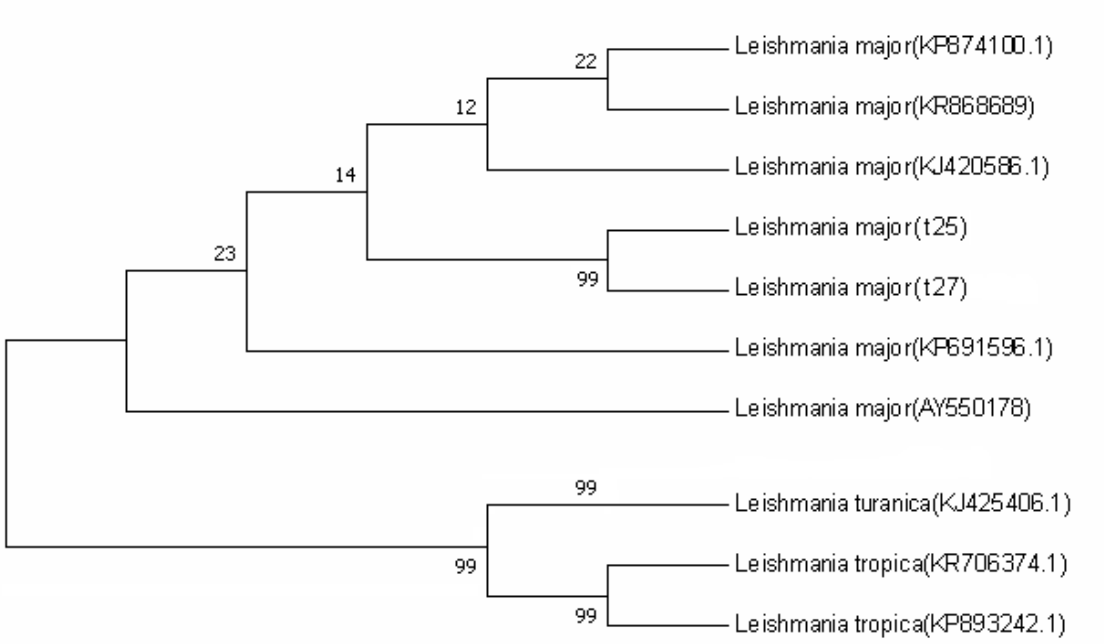

Fig. 3: Phylogenetic tree inferred of ITS1 gene sequences of *L. major* isolates from *phelebotomus* of the present study and other *Leishmania* species obtained from GenBank using MEGA software and maximum likelihood algorithm and bootstrap 500. The genotypes of this study are identified with isolates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

4. Discussion

The main goal of the present study was to investigate the infection of *phlebotomus* with *Leishmania* parasite in Mehran region. However in this region, two species of *Leishmania, L. major* and *L. tropica* have been reported but in the present study, only *L. major* was identified from *phlebotomus* samples collected from Mehran city.

Earlier studies in Ilam have reported the dominancy of *L. major* as the causative agent of leishmaniasis (17, 18, 19). In the study of Gholami Parizad et al. (2015) regarding the molecular identification of *Leishmania* parasites in smears prepared from skin lesions of patients referred to health centers in Ilam province by PCR-RFLP method, *L. major* species was detected (63). In this regard, the findings of the study by Saberi et al. (2018) also showed that the main cause of CL in Ilam *L. Major* (20).

However, it can be noted that other Iranian researchers have reported *L. major* based on molecular assay using various genes in Ilam province (11, 12, 13). Kassiri et al. (2012), in a study conducted during 2000-2007 on leishmaniasis in humans, rodents and vectors, found the *L. major* as the dominant species in Ilam province and its rate was reported 1.2 per 1000 individuals (11).

During 2011 to 2012, Roghani et al. (2013) conducted a descriptive study on people suffering from leishmaniasis in Ilam province. In this study, the cities of Dehlran and Mehran showed the highest rate of infection and *L. major* as the dominant species (12). Kermanjani et al. (2017) during a study on cutaneous leishmaniasis species in Ilam province found that among 61 patient samples that had clinical symptoms, 64% of them were infected with *Leishmania* species. According to the results of molecular methods *L. major* and *L. tropica* were specified species (13).

According to some reports, usually in areas where leishmaniasis has been reported endemic for a long time, it may be suddenly converted to epidemic. Or appear in an area where no case has been reported in the past. It is difficult to predict the occurrence of an epidemic of this disease. Some of the factors that may influence the epidemic include environmental changes in the place where the vector lives, mass migration of people, and weakened immunity (malnutrition). However, leishmaniasis has an extremely complex life cycle, the control of which depends on the implementation of various measures in many fields. One of these key and important actions is to investigate the life cycle of the parasite, how it is transmitted, and control the vectors of this disease, which transmit the disease between different reservoirs and also from reservoirs to humans (21).

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 this 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 provided that there In conclusion**,** based on our results, ZCL type of leishmaniasis is prevalent in Mehran city. While confirming the previous studies in this field in Ilam province, it is necessary to pay more attention to the results of this study by health officials of the province.

Authors' Contribution

TG (first author), methodologist/principal researcher ;AD (second author), supervisor, manuscript writer/methodologist/principal researcher/statistical analyst/discussion writer.

Ethic

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.

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