

Original Research Paper

# Molecular Identification of Leishmania Species in Patient with Cutaneous Leishmaniasis in an Endemic Area of Fars Province, Southern Iran

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**Abstract:** The Cutaneous Leishmaniasis (CL) contains a range of factors, which provide different patterns of transmission that make it difficult to control. The aim of this study was to identify the different species of *Leishmania* in the Jahrom district of Fars province, southern Iran. This descriptive study was done on CL suspected cases who were referential to the Reference of Jahrom Health Center laboratory in 2017 from all different zones mentioned. Demographic variables (age, sex, location, number and place of wounds) was recorded by a questionnaire. The blood smears were checked for the existence of amastigotes by light microscopy. After microscopic examination smears were extract DNA and used in the Polymerase Chain Reaction (PCR) method. Totally 165 slide samples checked were found to be positive for amastigotes via microscopy and positive for leishmanial DNA in the PCR. two (1.2%) of the slide samples gave the 750-bp indicative of *Leishmania tropica* and 163 samples (98.8%) gave the 560-bp indicative of *Leishmania major*. Although the actually that both species of CL are common in Iran, but our results showed that in Jahrom district the main specie was *L. major*. Thus, this fact displays the importance of reservoir rodents in programs of CL control in this County.

**Keywords:** Species Identification, *L. Major*, *L. Tropica*, PCR, Iran

## Introduction

*Leishmaniasis* is a neglected of vector-borne diseases produced by many *Leishmania* species which can infect both humans and other mammalian animals. *Leishmaniasis* is can be seen in three main forms such as cutaneous, mucosal and visceral (Rahmadian *et al.*, 2021).

It is estimated that 350 million population, are at risk of Cutaneous *leishmaniasis* (CL) and 1 million patient have been described in the past five years (Laurent *et al.*, 2017).

In Islamic republic of Iran, two forms of CL are occurs; Zoonotic Cutaneous Leishmaniasis (ZCL) caused by *Leishmania major* (*L. major*) and the rodents including *Rhombomys opimus*, *Meriones libycus* and *Meriones nesokia* are considered as the main reservoir (Shirzadi *et al.*, 2015; Mohammadiha *et al.*, 2018). The Anthroponotic Cutaneous Leishmaniasis (ACL) caused by *Leishmania tropica* (*L. tropica*) whose main reservoir

is human and it is considered to be an excessive significance in many regions of Iran, including some big and middle sized cities such as Mashhad, Tehran, Sabzevar and Neishabur in the north-east, Fars in the south, Kerman and Bam in the southeast, Kashan, Yazd and parts of the city of Esfahan in the central region (Yaghoobi-Ershadi *et al.*, 2013; 2015). Atypical mucosal infections with leishmanial parasites have also been detected in Iran, although infrequently.

Approximately 20,000 cases of CL are reported yearly from provinces of Iran, but it is estimated that the number of actual cases has doubled (Karami *et al.*, 2013; Moein *et al.*, 2018). CL is endemic in 15 provinces of IR and in the recent years. In addition, in recent years, climatic and environmental variables have created suitable conditions for the activity of reservoirs and vectors of CL in some new foci (Moein *et al.*, 2018; Shirzadi *et al.*, 2015).

Epidemiological studies, taxonomic and population genetics surveys, measuring a specific chemotherapeutic treatment, correct identification of the parasite species and the investigation of the genetic variation of the parasites are necessary for leishmaniasis control program in endemic regions (Leite *et al.*, 2010; Van der Auwera *et al.*, 2013).

In Iran, diagnosis commonly depends on detection of the species in the Giemsa-stained smear that is ready with the aspiration material of lesion. Furthermore, culture in Novy-MacNealNicolle (NNN) medium, ELISA, IFA and histopathological examination are used for investigation. On the other hand, diagnostic ways based on Deoxyribonucleic Acid (DNA) have, nevertheless, now made the sensitive and quick recognition of many germs potential. In recent years, for instance, analyses based on PCR have showed to be valid tools for the identification of *Leishmania*, without the need to culture the organisms (Mohammadiha *et al.*, 2013).

Fars Province is an endemic cutaneous and visceral leishmaniasis, so that the cumulative incidence of cutaneous and visceral *leishmaniasis* was reported to be between 59.9-98.8 and 0.58-0.73 cases per 100000 inhabitants, respectively (Shirzadi *et al.*, 2015). The National Leishmaniasis Control Program in Iran due to differences in reservoir, differences in the methods of control and prevention of *Leishmania* species highlights the essential of determining molecular identification of species in endemic regions once every five years. On the other hand, (Rahmaniian *et al.*, 2018), showed in a one

study an increasing trend in number of CL cases in Jahrom county, 90 cases in 2010 to 309 cases in 2014 and due to the lack of information about the kinds of species of CL in the county, this study was to identify the different species of *Leishmania* in the Jahrom district of Fars province, southern Iran.

## Methodology

### Study Site

The Fars province is situated in southern Iran and has a distinct of 122, 400 km<sup>2</sup>. The climate is moderately dusty and dry. The study has been carried out in Jahrom of Fars province. This district is located at ~53°33'E, 28°30'N and 1050 m above the sea level. Due to the appropriate ecological situations for *leishmaniasis* (reservoir hosts, vectors variety and weather conditions), Jahrom has permanently been considered as one of the Cutaneous and Visceral *leishmaniasis* (CL, VL) and focus district (Davami *et al.*, 2011), (Fig. 1).

### Study Design and Sampling Method

In this descriptive study, sampling lasted in a laboratory for 12 months from January to December 2017 in Jahrom leishmaniasis national surveillance system. The inclusion criteria for entering the study were all patients referred by the physician (imported or autochthonous cases) to the reference laboratory in the surveillance system for the diagnosis of leishmaniasis.



**Fig. 1:** The location of Jahrom County in Fars province, Iran

First, infection foci were determined according to the cumulative incidence of the previous year. Then, the samples were aseptically taken from all individuals with suspected CL lesions and referred to the laboratory by the physician after completing the survey form. Two slides were prepared from each of the 165 suspected patients and stained with Giemsa stain. The samples were taken by scalpel from the deep wound through harvesting the tissue containing macrophages, stained with Giemsa stain and explored with a magnification of  $\times 100$  to observe the parasite. The surface of positive the samples was scraped from the slides and used for PCR.

### DNA Extraction

The DNA extraction was made via phenol-chloroform and proteinase K (Motazedian *et al.*, 2002). Thus, the stained sample on the slide surface was poured into 1.5 mM plus 200  $\mu$ L of lysis buffer and 5  $\mu$ L of proteinase K and incubated at 56°C for 2 h. Then, the contents of the microtube were mixed with 100  $\mu$ L of the phenol/chloroform/isoamyl-alcohol mixture and centrifuged at 15,000 rpm for 5 min. At this stage, three layers were observed; the supernatant containing DNA, the middle layer containing lipids and proteins and the bottom layer containing phenol/chloroform/isoamyl-alcohol mixture. The supernatant (about 100-150  $\mu$ L) was carefully separated and poured into a new microtube and added with about 200-300  $\mu$ L of isopropanol and 10-15  $\mu$ L of 3 M sodium acetate. The samples after mixing in the microtubes were frozen at -20°C for at least 40 min. After defreezing, the samples were centrifuged for 15 min at 12000 rpm. The supernatant was discarded, the resulting precipitate was dried and mixed with 50  $\mu$ L of distilled water and used for the PCR process.

### PCR Process

The primers of CSB1XR (ATTTTCGCGATTTTCGCA GAACG) and CSB2XF (CGAGTAGCAGAACTCCCGTTCA) were used in the first step and the primers of 13Z (ACTGGGGGTTGGTGTAATAG) and LIR (TCGCAGAACGCCCT) (The products of Cinna Gen Company)(Davami *et al.*, 2011) in the second step. In the first step, each 25  $\mu$ L of the reaction solution contained 5  $\mu$ L of DNA (with a concentration of 200 ng), 1.5 mM of magnesium chloride, 0.2 mM of deoxynucleotide triphosphates (dNTP mix), 2.5  $\mu$ L PCR buffer (10x), one unit of Taq DNA polymerase and 10  $\mu$ M of CSB1XR and CSB2XF primers (The products of CinnaGen Company). The program used in the thermo cycler (Master cycler gradient; Eppendorf AG, Hamburg, Germany) was as follows: Denaturation

temperature of 90°C for 5 min, followed by 30 cycles including 90°C for 45 sec, 55°C for 1 min and 72°C for 1.5 min and then 72°C for 5 min (Davami *et al.*, 2014).

The produce of this step of the PCR was diluted with pure water. Then, 1  $\mu$ L of this diluted solution was used in the second step of PCR. All steps are like the first step of PCR, except that the primers of LIR and 13Z (ACTGGGGGTTGGTGTAATAG) were used instead of the primers of the first step and the final volume of the reaction solution increased to 100  $\mu$ L (Davami *et al.*, 2011; Azizi *et al.*, 2013).

Next, 5  $\mu$ L of the sample of the second PCR product was poured into the wells onto 1.5% agarose gel marked with ethidium bromide for the implementation of electrophoresis and then explored by UV light (Davami *et al.*, 2011).

The size of each observed product was compared with the ladder marker and the positive control sample. The positive controls were tested using DNA extracted from reference promastigote cultures of parasite *L. major* (MHOM/IR/54/LV39) and *L. tropica* (MHOM/IR/89/ARA2), created on any gel (Pourmohammadi *et al.*, 2010).

## Results

165 slide samples checked were positive for amastigotes via microscopy and positive for *leishmanial* DNA in the PCR. 2(1.2%) of the slide samples presented the 750-bp second-round amplicon indicative of *L. tropica* and 163 samples (98.8%) presented the 560-bp indicative of *L. major* (Fig. 2).

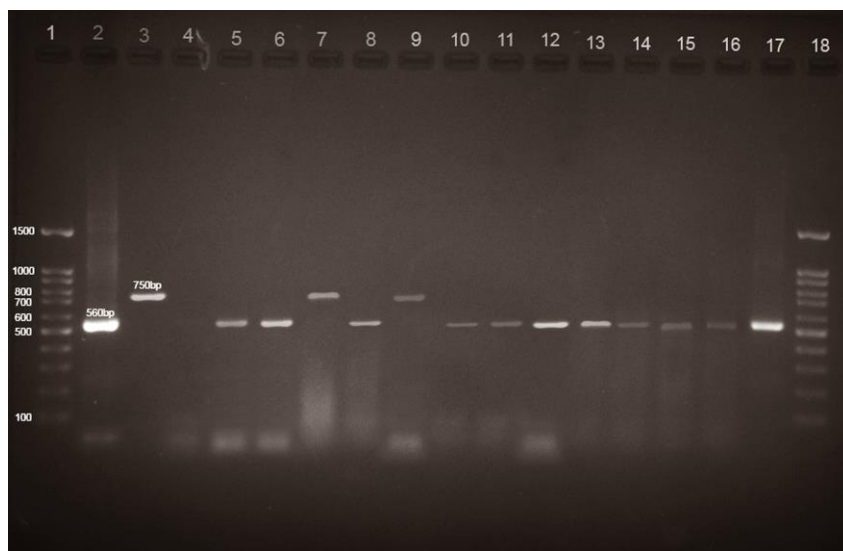
The average age of the patients was 23.36 $\pm$ 16.13 (rang,1-87) years. The male to female ratio was 1.4. However, according to Fig. 4, the peak occurrence of CL was documented in October and January and the lowest was documented in May (Fig. 3).

In total, 114 (69.09%) of the patients were villagers and the rest lived in the urban regions.

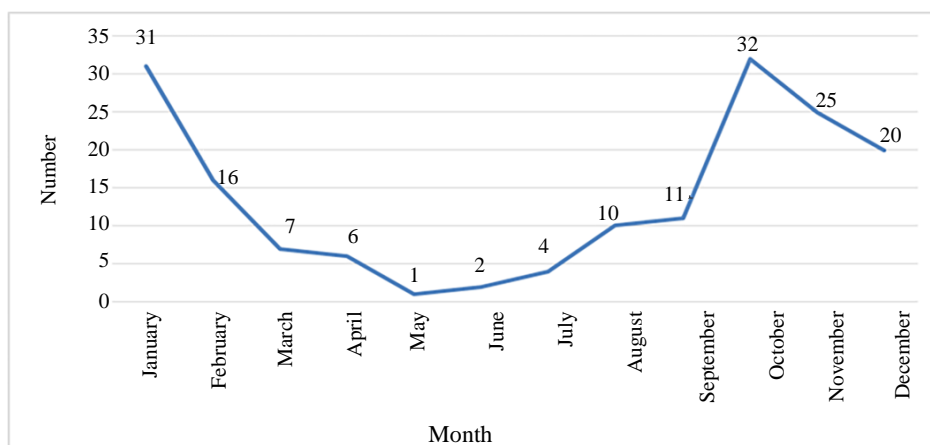
The average diameter of the wounds was 1.83 $\pm$ 1.37 cm. Hands and face were the most affected body parts with 65 and 38 cases, respectively (Fig. 4).

When we interviewed the cases recognized with *L. tropica*, it was indicated that the patient had previously travelled to an endemic area of ACL in the past year. It has been observed that the patient had one lesion on his right hand and two lesions in the face. It has been considered that the cases can be assessed as an imported case since he has come from Marvdasht in Fars province, lately.

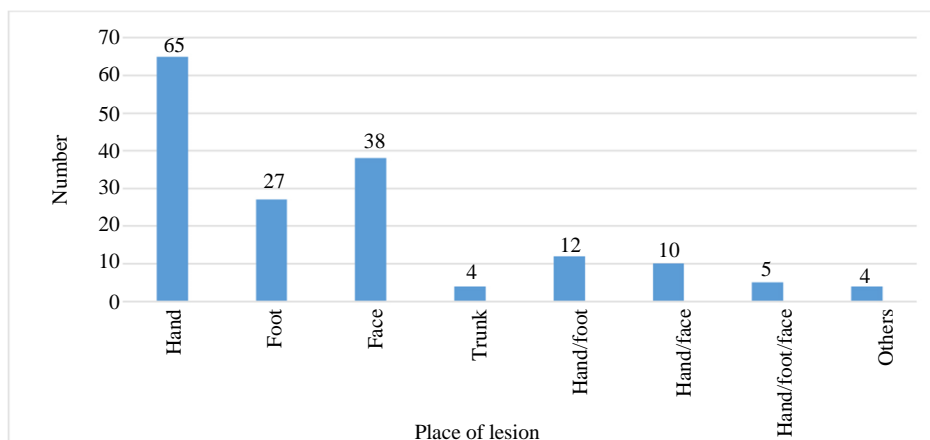
It has been perceived that the second case who has been living in Ghaleini rural had four lesions on his face. Since this case had no travel history to an endemic ACL district, it has been assessed as a native case.



**Fig. 2:** PCR test results of samples isolated from Jahrom patient’s foci. The molecular-weight ‘marker’ (lane 1), The *L. major* positive control (lane 2) and The *L. tropica* positive control (lane 3) and test samples identified as *L. tropica* (lane 7,9) and *L. major* (lanes 5, 6, 8 and 10-17).



**Fig. 3:** Frequency of patients with CL in term of months



**Fig. 4:** Frequency of cases with CL in term of place lesion

## Discussion

Before the emergence of new methods, the identification and isolation of parasites were difficult. Clinical signs, epidemiology of the disease, carriers' examination, ability to cause disease in laboratory animals and growth in the culture medium were factors that were used to detect parasitic species. But nowadays, recognizing genes and using molecular diagnostic methods and using the DNA of organisms, it is easy to determine the species of the parasite (Ghasemian *et al.*, 2011).

In this study, *L. major* was found to be responsible for CL in Jahrom County. It seems that, in 2017, *L. major* was the cause of practically all the CL in Jahrom with *L. tropica*-attributable disease existing but comparatively infrequent. Thus, specific control programs for this species of *Leishmania* is important including rodents elimination as the main reservoir of *L. major* in Iran, however, this program should be prioritized as it is one of the most important strategic ways to control and prevent this disease.

Detecting the *Leishmania* species in each endemic area, can be essential for control and prevention program in disease surveillance system (Alvar *et al.*, 2012).

Hatam *et al.* (2009), examined a number of patients with leishmaniasis in Fars province using iso-enzyme electrophoresis and PCR methods. The finding of this study showed that, all patients were diagnosed with *L. major* and this suggests an increasing in the spread of this type of *leishmania* in the Fars province.

Previous studies have identified *Phlebotomus papatasi* and *Phlebotomus salehi* as a vector of *L. major* and wild rodents and dogs serve as the reservoir of infection in Jahrom district. On the other hand, there are important sandflies such as *P. Sergeantomyiasintoni*, *S. theodori*, *S. palestinensis*, *S. baghdadis*, *S. clydei*, *S. squamipleuris*, *S. tiberiadis* and *S. antennata*, which are considered as potential vectors of leishmaniasis, have been proven in this district (Davami *et al.*, 2011).

Other finding showed that the place of residence, 69.09% of the patients were rural, which could be a good habitat for the native vectors and reservoir hosts of *L. major*.

In the present study, both genders were vulnerable to the CL. However, the frequency of CL cases was more prevalent in males than females. Other research from, Fars and Kermanshah Province, stated similar results (Norouzinezhad *et al.*, 2016; Fattahi Bafghi *et al.*, 2016). High incidence of CL in males could be due to various factors such as behaviors of wearing clothes, occupational, travel to endemic areas, poor health literacy, ecological and vector associated variables.

Due to the seasonal orientation, we found that the peak prevalence happened in autumn and winter. This finding is similar with studies in Esfahan, Yazd, Kermanshah and Qom Provinces (Karami *et al.*, 2013;

Yaghoobi-Ershadi *et al.*, 2015; Hamzavi and Khademi, 2015). Increase the number of CL patients in autumn and winter can be related by the incubation period of the CL, weather conditions, sand-fly highest activities and blood sucking of the victims. Therefore, the major control activities of any *Leishmania* species such as control reservoir, increasing the awareness of the public at risk about the transmission routes and preventive approaches should occur before peak disease incidence.

The CL lesions can occur in any part of the body, however, there are different reports about unusual sites (Doroodgar *et al.*, 2017a-b). In this study, the most common infected sites were hands and face. Our results are similar to the results of some other studies in Iran (Yaghoobi-Ershadi *et al.*, 2015; Norouzinezhad *et al.*, 2016; Karami *et al.*, 2013; Rahmaniian *et al.*, 2018).

Since hands are often uncoated and simply exposed to infected sand-fly bites, hands and faces show a higher occurrence of wounds than other parts of the body. Furthermore, sandflies have a short proboscis; therefore, they cannot bite through dresses.

## Conclusion

The samples of *leishmaniasis* obtained at Jahrom County are mainly of *L. major*, in which the reservoir of wild rodents, especially rats. Therefore, in order to control and prevent CL, rodent control program as one of the most important prevention strategies should be prioritized in this County.

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## Authors' Contributions

**Vahid Rahmaniian:** Organized the study, proposal of study writing, data gathering, manuscript preparation, data analysis and interpretation of data, literature search and final revision of the study.

**Nader Sharifi:** Analysis and interpretation of data, manuscript editing, final revision of the study content, final approval of the revision the manuscript.

**Zahra Kargar:** Design of study, DNA extraction and PCR process, interpretation of data and final approval of the version the manuscript.

**Hayedeh Parvin:** Data gathering, Design of study, literature search and final approval of the version of the manuscript.

**Narges Rahmadian and Hekmatollah Khoubfekr:** Manuscript editing, literature search, analysis and interpretation of data, final approval of the version of the manuscript.

## Ethical Considerations

The protocol of this study was approved by the Ethics Committee of Jahrom University of Medical Sciences (Cod: IR.JUMS.REC.1395.051).

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