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MOLECULAR PREVALENCE OF LEISHMANIA MAJOR AND LEISHMANIA TROPICA IN HUMANS FROM THE ENDEMIC REGION OF FARS, IRAN

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ABSTRACT

The objective of this study was to determine the molecular prevalence of *Leishmania major* and *Leishmaniatropica*in the urban and rural areas of Fars province, Iran.A total of 560 Giemsa-stained slides were collected from confirmed cases of cutaneous leishmaniasis (CL) who attended local health centers of study areas. 72% confirmed CL cases were males and 28% were females. 67% and 33% patientslived in the rural and urban areas respectively. DNA extracted from Giemsa-stained smears was amplified using universal PCR primers. Amplicons were subjected to RFLP analysis.In Shiraz County the prevalence of *L.major* and *L.tropica* was 94% (134 out of 141) and 6% (9 out of 141) respectively. 96% (132 out of 137) of the causative agent of CL in MarvdashtCounty was *L.major* and 4% (5 out of 137) was *L.tropica*. The prevalence of *L.major* and *L.tropica* in Larestan County was 99% (154 out of 156) and 1% (2 out of 156) respectively. 120 out of 124 (97%) isolates of *L.major* and 4 out of 124 (3%) of *L.tropica* isolates were diagnosed in the FarashbandCounty. Our results showed the prevalence of *L.major* and *L.tropica* in rural areas were higher than those in urban areas. There was a significant difference between the percentages of males (72%) and females (28%) infected with *L.major* and *L.tropica* (P< 0.001).Our results provide important information about the vast majority of

L.major infections in Fars province and will likely be very beneficial for management and control programs of the disease.

Keywords: Molecular Prevalence; Leishmania; South of Iran; Human

INTRODUCTION

Genus Leishmania is the obligate intracellular protozoan parasite causing leishmaniasis. These zoonotic diseases are endemic in 88 countries of Africa, Asia, Europe, South and North America. According to WHO, 350 million people are currently at risk of acquiring the infection, more than 12 million people are estimated to be affected by different forms of the disease and 2 million new cases occur annually worldwide, namely 1.5 million of CL and 0.5 million of visceral leishmaniasis [1]. Approximately 90% of all CL cases are reported from seven countries, including Iran [2]. There are more than 30 known species of Leishmania that vary by region [3].*L.tropica* is related to urban cutaneous leishmaniasis, is a disease of man and causes dry and crusted lesionsand*L.major*, endemic in rural areas with a reservoir in rodents, causes exsudative ulcers.Phlebotomus sand flies are a well-known vector for leishmaniasis [4]. In Iran, two species of medical importance are *Phlebotomuspapatasi* and*Phlebotomussergenti*. *L.tropica* and *L.major* are transmitted by *P.sergenti* and *P.papatasi* respectively. Fat sand rat

Psammomysobesus and several Meriones species have been incriminated as putative reservoir hosts of the *L.major*[5]. Dogs accidentally infected with *L.tropica* thus could be a role in the transmission of this parasite to man [6].

CL is a great health problem in Iran. In Iran the potential spread of leishmaniasis has been reported in previous years [7]. The prevalence of CL has been reported as 1.8% to 37.9% in different provinces of Iran [8].

Fars province is an important focus of CL in Iran where *L. major* and *L. tropica*are circulating [6,9]. According to the report of Iranian Ministry of Health, 23% (N=5280) of annually reported cases of CL in Iran in 2009 were recorded from FarsProvince (Iran Ministry of Health).

Traditionally, diagnosis of cutaneous leishmaniasis in Iran involves taking a scraping from a lesion, staining it with Giemsa or Wright and examined microscopically at 1000× magnification. Parasites are seen intracellularly in macrophages, and extracellularly because of rupture of the macrophages during processing. However, the Leishmania species

identification is not possible using these methods because all Leishmania parasites are morphologically similar [10]. Diagnosis of the species level is important for prognosis, epidemiological and therapeutic reasons [11].PCR-based methods proved to be sensitive and powerful tools for direct detection of Leishmania in clinical samples, without the need to culture the parasites [12,13]. However, conventional PCR assays provide results in one or two working days [14].Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified sequences of multi-copy genes such as internal transcribed spacers (ITS) have been proven to be a crucial method for Leishmania species [15,16]

The objective of this study was to determine the molecular prevalence of *L.major* and *L.tropica*in the urban and rural areas of Shiraz, Marvdasht, Larestan and Farashband counties of Fars province, during 2012-2014.

MATERIALS AND METHODS

Study area

The survey was conducted in Fars province, which serves as an endemic region of CLIn Iran. Fars province (Latitude: 27° 2' N to 31° 42' N and 50° 42' E to 55° 36'E) is located in south of Iran and covers an area of 133,000 km2, with a total population of about 4.86 million at the time of the study. Shiraz is the center of Fars province. Field studies were carried out in urban and rural regions of the four infected counties of Fars province. They included: Shiraz, Marvdasht, Larestan and Farashband counties (Figure 1).

In Fars province, under the influence oftopographicfeaturesthreedistinctclimateshave emerged: 1. Mountainous north, northwest and west, with cold winters and temperatevegetationare remarkable. Rainfall in the region of 400to 600 mm per year has been reported.2. Central area: This area is relatively temperate climate with rainfall in winter and in summer; the air is dry and hot. The region between 200 and 400 mm of rain per year. Shiraz, Marvdasht and Farashband Counties located in this area.3. The south and southeast due to the height and width of the geographical distribution of mountains, winter rainfall in the area is less than the spring and fall. The region's climate mild winters and very hot in summer and annual rainfall of 100 to 200mm. Larestan County, located in this dry region.

Samples

A total of 560 Giemsa-stained slides were collected from confirmed cases of CL who attended local health centers of Study areas between September 2012 and August 2014. 403 (72%) confirmed CL cases were males and 157 (28%) were females. 186 (33%) and

374 (67%) patientslived in the urban and rural areas of study counties respectively. All the Giemsa-stained slides were examined under light microscope with magnification (1000×) and the positive smears were divided into five groups according to the average number of amastigotes counted in 10 oil-immersion fields (OIF) scanned randomly by two different blinded persons using the same light microscopy. The quantitative grading of parasite density in the slides is shown in Table 1.

DNA Extraction

All the Giemsa-stained slides were cleaned with xylol, covered with 250 ml lysis buffer and smears scraped completely with a scalpel [17]. DNA was extracted and purified using DNA extraction kit (MBST, Iran) according to manufacture protocol. The concentration of extracting DNA was measured by NanoDrop (Thermo Fisher Scientific, USA).

PCR Amplification

The internal transcribed region (ITS1) of the small subunit ribosomal DNA was amplified from samples by conventional PCR using the universal primers L5.8S (5'-TGATACCACTTATCGCACTT-3') and LITSR (5'-CTGGATCATTTTCCGATG-3') [18]. The PCR was performed in the PCR reactions (total volume of 50 micro liter including one time PCR buffer) containing 2

U Taq DNA polymerase (Cinnagen, Iran), 200 μM dNTPs (Fermentas), 20 pM primers each (cinnagen, Iran), 2 mM MgCl2 (cinnagen, Iran) and 2 μl DNA solution was used as a template in the PCR. The amplification was carried out in an automated thermocycle (Eppendorf, Germany) with the following program: 95 °C for 5 min (initial denaturation), followed by 35 cycles of 94°C, 1 min (denaturation), 56 °C, 45 s (annealing), 72°C, 1 min (extension) and a final extension of 72 °C for 10 min. The sensitivity of the PCR for these parasites was tested for the amplification of the fragments from a negative control (without DNA) and positive controls *L.tropica* (MHOM/IR/99) and *L.major* (MHOM/ IR/75/ER) DNA. Amplification products were subjected to electrophoresis in 2% agarose (cinnagen, Iran) at 100V in 1× TAE (40 mMTris-acetate, 1 mM EDTA, pH 8.3) buffer, stained with ethidium bromide (5 mg/100 ml), and visualized and photographed using a UV transilluminator. Expected PCR products based on the published sequences for *L.major* and *L.tropica* are 300-350 base pairs [19].

RFLP Analysis of Amplified ITS1

Restriction endonuclease digestion was performed in a volume 30μl include PCR product 10μl, HaeIII enzyme (Fermentas) 2 μl, 10× buffer 2 μl and distilled water 16 μl

for 4 hours at 37˚C. The fragments were analyzed using electrophoresis on agarose gel 3%, stained with ethidium bromide (5 mg/100 ml) and visualized on a UV transilluminator. The expected fragments for *L.major* are 220 and 140 bp and for *L.tropica*are 200 and 60 bp [19].

Data Analysis

The Chi-square test was used to compare the prevalence of *L.tropica* and *L.major* among different groups. *P* value less than 0.05 was considered statistically significant. The statistical analyses were carried out with SPSS version 16.0 for windows.

RESULTS

A total of 560 smears in which amastigotes had been detected were collected from the local health centers of Shiraz, Marvdasht, Larestan and Farashband counties and examined through PCR-RFLP technique. All samples showed positive results through PCR-RFLP that reconfirmed microscopy examination. An expected 300-350 bp DNA fragment of *L.major* and *L.tropica* ITS1 rRNA gene was amplified. These expected fragments were also generated from positive control DNA obtained from reference strains, but not in negative control samples. PCR-RFLP band pattern with restriction enzyme HaeIII*L.tropica* revealed two fragments of 60 and 200 bp size and *L.major* yielded 220 and

140 bp sized fragments which are correlated to *L.tropica*(MHOM/ir/89/ARAZ) and *L.major* (MHOM/ IR/54 lv39) reference strains Pattern respectively [20] (**Figure 2**).

According to table 2 In Shiraz County the prevalence of *L.major* and *L.tropica* was 94% (134 out of 141) and 6% (9 out of 141) respectively. 96% (132 out of 137) of the causative agent of CL in Marvdasht County was *L.major* and 4% (5 out of 137) was *L.tropica*. The prevalence of *L.major* and *L.tropica* in Larestan County was 99% (154 out of 156) and 1% (2 out of 156) respectively. 120 out of 124 (97%) isolates of *L.major* and 4 out of 124 (3%) of *L.tropica* isolates were diagnosed in the Farashband County (Table 2). More than 96% of detecting parasites in the study counties were *L.major*. Our results showed the prevalence of *L.major* and *L.tropica* in rural areas were higher than those in urban areas (67% vs 33%, *P*<0.001). There was a significant difference between the percentage of males (72%) and females (28%) infected with *L.major* and *L.tropica* (P< 0.001).

DISCUSSION

In the present study, we investigated the prevalence of *L.tropica*and *L.major* infections in Giemsa-stained slides using the PCR-RFLP assay in the endemic region of Fars, Iran. Our data showed that Giemsa-stained slides that

were stored more than 3 years and contain less than five parasites in each OIF, could be used for Leishmania DNA extraction and analysis by PCR-RFLP method, similarly several reports have indicated the use of archived Giemsa-stained smears for molecular diagnosis and differentiation of the Leishmania species [19,21,22]. The traditional technique based on Giemsa stained smear can be problematic since Leishmania species have similar morphological characteristics [10]. Besides being unable to detecting low parasite load on a slide [21]. Accurate diagnosis technique is important for the prevalence surveys. The prevalence survey is useful for Leishmania control and prevention strategies due to factors such as reduce the vectors and reservoir hosts. The prevalence of a disease in the population has an important influence on the positive predictive value of a diagnostic test.

CL has been reported as an important endemic disease in Iran since 1971 [6]. This parasitic disease was previously reported to be highly prevalent in Iran [8]. Both species of *L.tropica* and *L.major* are causative agents of CL in different provinces of Iran and have a wide geographical distribution in the country. Our results revealed *L.major* was the most prevalent species in Fars province. This finding is consistent with previous epidemiological and clinical reports in Iran [21,23,24].Our finding showed the 96% of the causative agent of CL in urban and rural areas of Shiraz, Marvdasht, Larestan and Farshband counties were *L.major*. Similarly, it reported in some previous studies [7,25]. The dominance of *L.major* in Urban areas as well as in rural areas may be due to the expansion of cities into rural areas where the colonies of the rodents that act as reservoir hosts in zoonotic CL are found [26]. It may also be due to ineffective control of reservoirs (gerbil populations) and vectors (*P.papatasi*) of the areas. In contrast to our finding, Fata et al. [27]using PCR technique reported that *L.tropica*was more prevalent than *L.major* in Khaf district, Khorasan-e-Razavi province, Iran. The prevalence of *L.major* in the present study was also higher than that in the Poldokhtar city, Lorestan Province, Iran, which described the prevalence of *L.tropica* and *L.major* were 72.47% and 27.53% respectively [28]. However the difference between our result and two earlier studies is likely to be due to changes in sample size, population, geographic locations and techniques used to collect data. It should be noted that the prevalence of Leishmania spp. also related to differences in lifestyle and occupational status.

Our data showed the significant difference between males and females infected with *L.major* and *L.tropica*. Which was in accordance with some previous studies [24,29].

The lower prevalence of the infections in Females, as compared to males, could be explained by the way of dressing and more staying home of the females in Iran. We observed more CL in rural areas of study counties that correlated to a previous study [7]. We believe that the lifestyle in the rural area in Fars province may account for this result. In rural areas within the province, the majority of residents take part in outdoor farming work and agricultural income is the exclusive economic source for rural in the rural area in Fars province. The highly educatedurbanpeople in Fars province may lead to qualified jobs and superior socioeconomic status; therefore, those subjects may not spend most of their time working outdoors.

In conclusion ITS1 PCR-RFLP based techniques represent a very sensitive and specific method for the investigating the occurrence of *L.tropica* and *L.major*. The sensitivity of the PCR test was assessed with samples from the patients with confirmed cutaneous leishmaniasis. Our results provide important information about the vast majority of *L.major* infections in Fars province and will likely be very beneficial for management and control programs of the disease.

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Table 1: Quantitative grading of *Leishmania* amastigotes on Giemsa-Stained slides were used for DNA extraction

Grade	Number of slides	Average parasite density per OIF[*]
G1	114	>100
G2	218	50 – 100
G3	145	10 - <50
G4	72	5 - <10
G5	11	<5

[*]**10 oil-immersion fields**

Table 2: Molecular prevalence of *L.major* and *L.tropica* from four counties as identified by PCR-RFLP technique

Risk Factors	Urban areas		Rural areas	
	No. of *L.major* (%)	No. of *L.tropica* (%)	No. of *L.major* (%)	No. of *L.tropica* (%)
County Shiraz	39 (85)	7 (15)	95 (98)	2 (2)
Marvdasht	55 (92)	5 (8)	77 (100)	0 (0)

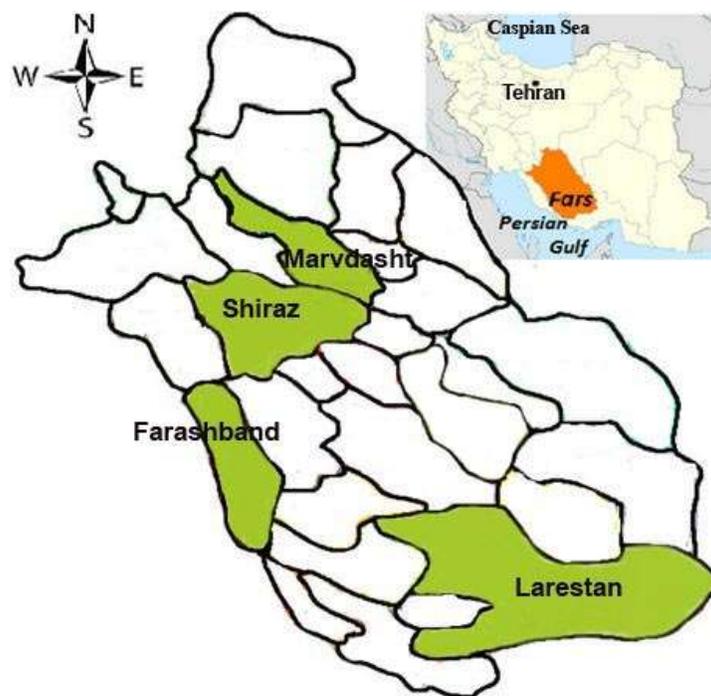|				
Larestan	**32 (94)**	**2 (6)**	**122 (100)**	**0 (0)**
Farashband	**42 (91)**	**4 (9)**	**78 (100)**	**0 (0)**
Gender				
Male	**134 (90)**	**15 (10)**	**252 (99)**	**2 (1)**
Female	34 (92)	3 (8)	120 (100)	0 (0)

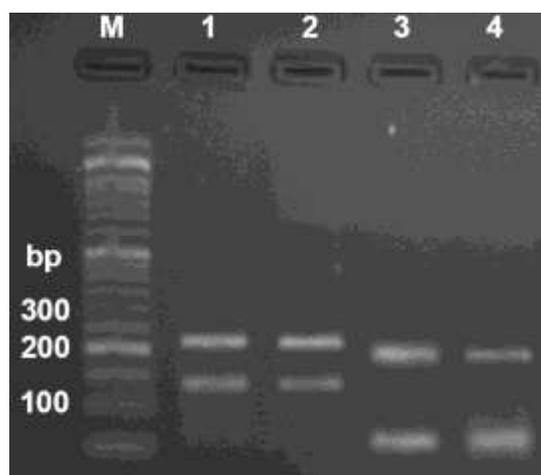**Figure 1: Map of Fars Province and the geographic location of the study sites**

Figure 2: RFLP (Restriction fragment length polymorphism) of PCR products of *L.major* and *L.tropica*from test samples and standard isolates,digested with HaeIII. Lane M, 50 bp DNA size marker; lane 1 and lane 3, standard isolates of *L.major* and *L.tropica*, respectively. Lane 2 and lane 4 represent test samples identified as *L.major* and *L.tropica*, respectively