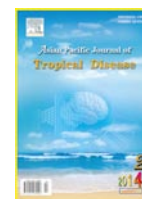




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Molecular identification of *Leishmania* species in Taybad district, IranSalehi Ghodrattollah¹, Fata Abdolmajid^{1,2*}, Mohaghegh Mohammad Ali¹, Mousavi Bazzaz Sayyed Mojtaba³, Rafatpanah Hushang⁴, Movahedi Abdolghayom¹¹Department of Medical Parasitology and Mycology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran²Cutaneous Leishmaniasis Research Center, Emam Reza Hospital, Mashhad University of Medical Sciences, Mashhad, Iran³Department of Community Medicine, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran⁴Department of Immunology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

Objective: To identify *Leishmania* species in patients with cutaneous leishmaniasis in the city of Taybad in Razavi Khorasan Province from April 2012 to March 2013.**Methods:** Among 52 persons who referred to Health Center of Taybad with suspected skin lesions, stained slide smears of 35 patients showed positive result for *Leishmania*. Also polymerase chain reaction assay performed using specific kDNA primers. Data of patients were analyzed with SPSS.**Results:** Of 35 positive smears for *Leishmania*, 21 (60%) belonged to males and 14 (40%) belonged to females. Polymerase chain reaction bands were observed in all 35 samples of which 31 (88.6%) samples showed *Leishmania tropica* and 4 (11.4%) showed *Leishmania major*. The highest infected age group was 11–20 years old.**Conclusions:** Both anthroponotic cutaneous leishmaniasis and zoonotic cutaneous leishmaniasis are present in Taybad. *Leishmania tropica* is the dominant causative species for anthroponotic cutaneous leishmaniasis. Further study is recommended to discover probable reservoir and vector for *Leishmania major* in Taybad.

1. Introduction

Leishmaniasis is a vector-borne disease of human beings and animals caused by protozoan parasites of the genus *Leishmania*[1]. Over 21 species of *Leishmania* cause infection in humans worldwide, resulting in three clinical phenotypes: cutaneous, mucocutaneous, and visceral disease[2]. It is estimated that more than 15 million people are infected with this disease with 1.5–2.0 million new cases per year[3]. Leishmaniasis is still one of the most

important neglected diseases in developing countries[4]. Two species of *Leishmania* are involved in cutaneous leishmaniasis (CL) infections in Iran. *Leishmania major* (*L. major*) is causative agent of zoonotic cutaneous leishmaniasis (ZCL) and *Leishmania tropica* (*L. tropica*) causes anthroponotic cutaneous leishmaniasis (ACL). Both *L. major* and *L. tropica* are intracellular parasites transmitted through the bite of the female phlebotomine sand flies[5].

L. tropica usually leads to dry ulcers on the skin and *L. major* often produces severely inflamed and ulcerated lesions. Both ZCL and ACL are endemic in different parts of Iran[6].

ZCL is found in many rural foci of Isfahan, Khuzestan and Khorasan Provinces, while ACL is endemic in many

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large and small cities including Shiraz in the south, Kerman in the southeast, and Mashhad in northeastern of Iran[6–8].

Despite the extensive efforts by the health authorities to control it, new focuses have appeared in different parts of the country. The disease is a major health problem that causes serious psychological as well as social and economic burden to the community. In national CL control program, the identification and description of the disease have been emphasized[9].

In the last few years, polymerase chain reaction (PCR) has been widely used due to high sensitivity as a parasitological diagnostic test[10–12]. This study aimed to identify *Leishmania* species in patients with CL in the city of Taybad in Razavi Khorasan Province from April 2012 to March 2013.

2. Materials and methods

2.1. Study area

Taybad is located in the east of Khorasan Razavi near the border with Afghanistan. It is situated 225 km from southeast of Mashhad. The county's population is reported 143 205 at 2006 census. Taybad's altitude is 806 m above sea level with warm and dry climates.

2.2. Sampling

In this cross-sectional study, patients with suspicious lesions referred to health center in Taybad city. First, agreement of patients to participate in this study was acquired. Then personal and epidemiological information such as name, sex, age, job, location of lesion and habitat were recorded in data collection forms. Samples were collected using a sterile scalpel by scraping from the edge and the center of the lesion on the clean microscopic slides. Then microscopic slides were air dried and fixed with methanol and stained with Giemsa and examined by light microscope carefully. At least 2 slides for each patient were prepared and kept for DNA extraction.

2.3. DNA extraction

DNA was extracted from the prepared slides of patient's lesions[10–13]. It was performed with Genet Bio DNA extraction kit. According to manufacturer's instructions, the tissues on the slides should be suspended in lysis

buffer. For this purpose, poured 200 μ L of lysis buffer on the slides and specimen are dissolved in it and moved in a 1.5 mL sterile microtube. Then it was added with 20 μ L proteinase K and incubated at 56 °C for 1–3 h. Then 200 μ L GB buffer was added, which helped to further dissolution of tissues, and incubated at 56 °C for 10 min. The DNA was precipitated in 200 μ L of absolute ethanol and carefully transferred the lysate into the spin column and centrifugated at 10 000 r/m for 1 min. Then spin column was washed at 2 stages with 0.5 mL of GW1 and GW2 buffers and centrifugated at 10 000 r/m for 1 min after each step. At the last stage, 200 μ L of GE buffer (D.W. or elute buffer) was added to the spin column. Eluted DNA was isolated by centrifugation at 10 000 r/m for 1 min. DNA was stored in the freezer until the PCR test.

2.4. PCR amplification

PCR reaction was provided for each sample, using 20 μ L PCR mix (containing reagents and *Leishmania* kDNA primers), and 5 μ L DNA template in 0.2 mL microtubes. A positive control reaction using standard *L. major* DNA and a negative control using distilled water were established in every round of PCR.

PCR was performed using specific primers F : (5' T C G C A G A A C G C C C C T A C C 3') and R : (5' A G G G T T G G T G T A A A A T A G G 3') dependent to kDNA of *Leishmania*.

Amplification of the target DNA was performed using thermocycler ASTEC under the following conditions: one cycle of 5 min initial denaturation at 95 °C, followed by 38 cycles of 94 °C for 30 seconds, 60 °C for 45 seconds, 72 °C for 60 seconds, ending by one cycle of final extension at 72 °C for 7 min. Electrophoresis of the PCR products was performed using 10 μ L of each amplicon, on 2% agarose gel in 0.5 \times TAE buffer for 50 min at 100 mA, followed by staining of the gels in 1 mg ethidium bromide in 100 mL TAE buffer and consequent observation of amplified DNAs by Gel grab under UV light. Exactly 100 bp DNA Ladder was also used as the DNA size marker.

3. Results

This descriptive study was carried out on 35 patients with active lesions from April 2012 to March 2013 that were positive by PCR. Single fragments of 615 bp and 744 bp are indicative of the species *L. major* and *L. tropica*, respectively (Figure 1).

Table 1Frequency of *Leishmania* species in different sex and clinical presentation in patients from Taybad with cutaneous leishmaniasis.

Data	Sex		Living place		Type of lesion		Number of lesion			
	Male (%)	Female (%)	Urban (%)	Rural (%)	Papule & nodule (%)	Ulcer (%)	1 (%)	2 (%)	3 (%)	≥4 (%)
<i>L. tropica</i>	17 (48.6)	14 (40)	14 (40)	17 (48.6)	22 (62.9)	9 (25.7)	24 (68.6)	4 (11.4)	2 (5.7)	1 (2.9)
<i>L. major</i>	4 (11.4)	0 (0.0)	3 (8.5)	1 (2.9)	1 (2.9)	3 (8.5)	2 (5.7)	2 (5.7)	0 (0.0)	0 (0.0)
Total	21 (60)	14 (40)	17 (48.5)	18 (51.5)	23 (65.8)	12 (34.2)	26 (74.3)	6 (17.1)	2 (5.7)	1 (2.9)

All of the samples were examined by PCR. PCR bands were observed in all of cases included 31 cases of *L. tropica* and 4 cases of *L. major*.

The most highly infected age group was 11–20 years old with a rate of 37.1% and children under 10 years old with the rate of 14.3% (Figure 2). *Chi*-square analysis indicates that there are significant differences in number of positive cases among different age groups ($P < 0.05$).

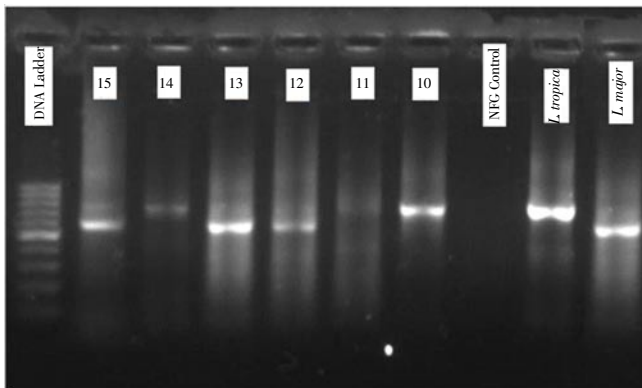


Figure 1. Gel electrophoresis of PCR amplification for identification of *Leishmania* species on Giemsa-stained slides of patients from Taybad. Lanes 10 and 14 represent *L. tropica* and Lanes 12, 13 and 15 are *L. major*.

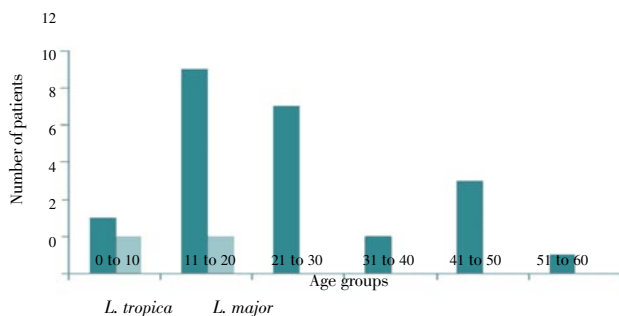


Figure 2. Distribution of cutaneous leishmaniasis in different age groups of patients from Taybad according to species of *Leishmania*.

The proportion positive cases of males and females were 60% and 40% respectively. Despite of the most of studies, nearly half of the patients infected with *L. tropica* (54.8%) were lived in rural zones and 75% of ZCL patients lived in urban zones. Lesions in the most of the patients infected with *L. tropica* (71%) were dry and the most of patients infected with *L. major* (75%) represented exudative ulcers (Table 1).

Most of the patients had one active lesion (74.3%) and most of them were observed on the hands (40%), face (25.7%) and legs (22.9%) (Figure 3).

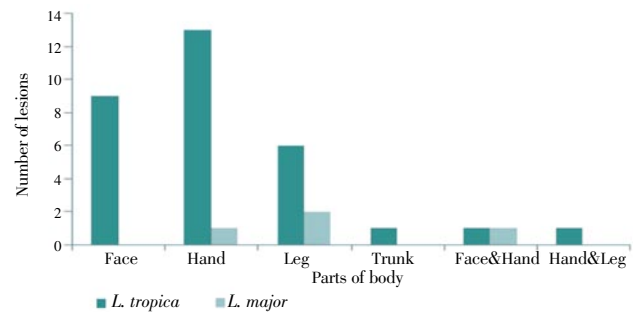


Figure 3. Distribution of lesions in different parts of body in patients from Taybad with cutaneous leishmaniasis according to species of *Leishmania*.

The ACL cases were equally distributed during the year but seasonal distribution of the ZCL was followed from July till November (Figure 4).

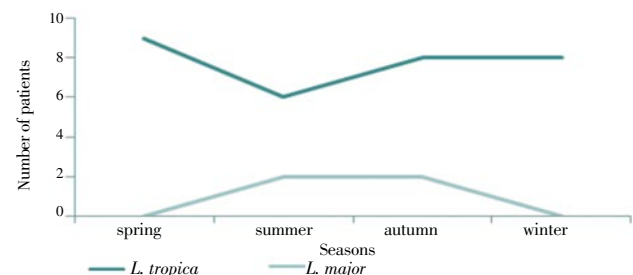


Figure 4. Seasonal distribution of ACL & ZCL in Taybad according to species of *Leishmania*.

4. Discussion

Management and planning for disease control need identification of *Leishmania* species. Knowing epidemiology of leishmaniasis as an important measure is quite helpful. Infection due to different species may require separate treatment regimens. The epidemiological survey is a major component for combating against disease[10].

This is the first formal study which has been performed in rural and urban areas of Taybad, located at Razavi Khorasan Province, northeastern of Iran. Based on previous experiments, we used PCR method for diagnosis

and characterization of *Leishmania* species by extracted DNA obtained from Giemsa stained smears^[10–12]. Also in some studies, filter papers used to extract DNA^[13,14]. *Leishmania* minicircle kinetoplast DNA is one of the genetic targets that has been applied and proved to be useful for detection of parasites in clinical specimens and isolate characterization by many researchers^[15–18].

PCR-based methods as a powerful tool for diagnosis of cutaneous leishmaniasis seem to be accepted as a gold standard status because of advantages in the collection and transport of specimens and DNA extraction procedures are more efficient in individual and field-based protocols^[19–22]. Many researchers have reported 100% specificity with increasing sensitivity which in overall is between 92% and 98%, appearing to be the most sensitive single diagnostic test for each form of leishmaniasis^[19].

In this study, all of patients suffering from *L. major* infection had a travel history to endemic areas. This may indicate that the native and original cause of leishmaniasis is *L. tropica* in the study area. Regarding to high prevalence of CL in Afghanistan and travelling to this country by inhabitants of the study area, the presence of potential vectors and reservoir hosts can lead to the emergence of new ZCL foci in this area. So it needs serious consideration by public health centers.

The main risk factors related to ZCL are urbanization, agricultural development and new irrigation schemes that provide capacity in favor of the parasite life cycle. The most of the ACL foci restricted to urban and suburban. Migration from rural to urban areas is the major risk factor for ACL due to reside in the poor suburb^[23].

Children under 10 years old had a rate of 14.3%. This may be due to several factors, such as children's outdoor activities or sleeping outdoors or indoors without a bed net, which increases exposure to sand fly bites.

Some patients with ACL presented exudative ulcers (29%) and one patient with ZCL didn't have exudative ulcers. So the clinical presentation of the skin lesions does not determine the clinical form of CL.

Similar to the findings of some other studies, the majority of patients had a single lesion, and the most affected part of the body was hand and face^[24,25]. This is one of the usual characteristics of the ACL whereas in the ZCL the most commonly involved site is hand and foot. However, this is not always consistent, and there are some reports indicating predominant involvement of the face in ZCL^[24].

ACL occurred in all seasons of the year whereas

occurrence of the ZCL increased in summer and fall. This is a property of rural CL because of seasonal activity of sand flies and the prepatent period of infection with *L. major*^[24].

However, both *L. tropica* and *L. major* isolated from the residence of Taybad district, but we believe that the dominant and original species of *Leishmania* is *L. tropica*. All patients infected by *L. major* are the residents who have travelled to ZCL foci. Due to proximity Afghanistan as a ZCL focus^[26], for prevention of the emergence of new ZCL foci in Taybad, serious consideration is necessary.

Rate of CL is high in Taybad city, Razavi Khorasan Province. It is necessary to take strong steps to control the CL and prevent its extension to neighboring cities. It is also critical to provide rapid treatment of patients. The control of CL needs to close cooperation between the research centers of medical sciences, health centers and the government.

Conflict of interest statement

We declare that we have no conflict of interest.

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