Identification of Species Causing Cutaneous Leishmaniasis by PCR in Chahbahar, Iran

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ABSTRACT

Background and Objective: Chabahar is in Southern Iran located near the Iran-Pakistan border. Since leishmaniasis is an emerging disease in this region, this study aimed to diagnose the disease and identify different species of *Leishmania* parasite in the patients referred to the central laboratory.

Methods: This descriptive cross-sectional study was conducted in 2011-2012 on patients referred to the central laboratory in the city of Chabahar. The sampling of lesions, slide preparation, culture and PCR specific for kinetoplast DNA (kDNA), extracted from the media and slides, were performed. The data collected by a questionnaire were analyzed by the SPSS software.

Results: The resulted bands from the 48 tested cutaneous leishmaniasis isolates were compared with the standard strains of *Leishmania tropica*, *L. infantum* and *L. major*. All 48 investigated bands were in the 620bp region, which is related to *L. major*.

Conclusion: Since PCR has high sensitivity and specificity, it is recommended to use kDNA (present in a unique organelle called kinetoplast) for the routine diagnosis and treatment of the disease.

Keywords: Leishmaniasis, Cutaneous, Polymerase Chain Reaction, Iran.

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INTRODUCTION

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*. Its vector is female sandflies from the *Phlebotomus* genus. From the epidemiologic perspective, the causes of cutaneous leishmaniasis or leishmaniasis in Iran are *Leishmania major*, *L. tropica* and less likely *L. infantume* (1, 2).

Cutaneous leishmaniasis is one of the most important public health problems in many parts of Iran (3). Typically, diagnosis of cutaneous leishmaniasis is based on clinical symptoms found in patients and its confirmation by parasitological methods (4).

Although the accurate identification of species are of great epidemiological and clinical importance, it is not yet possible to do so due to the similarity of morphological and clinical manifestations (1,3). Sometimes the phenotype does not represent the genotype and lesions caused by *L.tropica* along with *Crithidia* would cause *L. major*-like moist wounds (2). Lesions also manifest in multiple atypical forms as demonstrated by a study in Isfahan, clinical leishmaniasis were observed in 7.1% of cases in unusual shapes (1, 3). Thus, the distinction between species requires molecular techniques, enzyme electrophoresis, DNA hybridization probes and polymerase chain reaction (PCR) (5). The high sensitivity and specificity are some advantages of DNA replication-dependent methods (1). kDNA have multiple copies of minicircles which makes it a suitable target for diagnosis (6).

Nowadays, due to the advances in molecular methods including numerous techniques, different molecular markers have been developed to assess and distinguish different species of *Leishmania* (7, 8). So far, ribosomal ssuRNA (9), internal transcribed spacers (10), tubulin gene (11), gp63 gene (12), microsatellite DNA (13), extrachromosomal DNA and kDNA minicircles (14) have been used as target genes for the detection of *Leishmania*. Therefore, shizodeme analysis and kDNA were used in this study to determine the species and evaluate the population of *Leishmania*. The main aim of this study was to determine the species causing cutaneous leishmaniasis using the PCR method in the city of Chabahar.

MATERIAL AND METHODS

This was a descriptive and cross sectional study on 48 patients with cutaneous leishmaniasis who were referred to the central laboratory of Chabahar during 2011-2012. Data were collected from patients with cutaneous leishmaniasis through a prepared questionnaire. This study was conducted to determine the cutaneous leishmaniasis causing species in the city of Chabahar. The samples were obtained from the lesions of the patients with cutaneous leishmaniasis referred to the central laboratory during 2011-2012. Data were gathered using a questionnaire.

First, the patients' lesions were disinfected with ethanol and the help of scalpel or vaccinostyle, steriled on flame. After cooling, the samples were obtained from the edge and border of the swollen wounds, in depth and from the floor of the wound. The resulting serosity from each isolate was transferred onto N.N.N medium. In addition, two microscopic slides were prepared from thin layer spreads of some of the lesion samples from each patient. The obtained samples were kept in special flasks containing ice (Icebag) with temperature of 4-6 ^{o}C during transportation.

DNA extraction was done using kits in order to obtain sufficient amount of pure DNA with no contamination. At this stage, amastigotes were collected from the slides and the standard medium. High Pure PCR Template Preparation kit (Roche co.) was used for DNA extraction. The prepared slides from the patients were also used for DNA extraction. The steps were performed according to the kit manufacturer's protocol.

After DNA extraction from the obtained samples based on the above procedures, PCR was done for DNA amplification and determination of cutaneous leishmaniasis causing specie using Leishmania PCR determination and detection Kit (CinnaGen Co.) in the city of Chabahar. In order to identify the *Leishmania* species causing cutaneous leishmaniasis, specific genome of specie and specific kDNA markers were used. The pair of primers used in this experiment included an upstream 5' TCG CAG AAC GCC

CCT ACC 3' and downstream 5' AGG GGT TGG TGT AAA ATA GGC 3' primer, which were used to find the *Leishmania* kDNA sequence (14). The thermocycler temperature program included: an initial denaturation cycle at 95 °C for 3 minutes, then 34 cycles of primers annealing at 63 °C for 30 seconds, elongation at 72 °C for 60 seconds and secondary denaturation at 93 °C for 40 seconds and finally, a cycle at 72 °C for 5 minutes. After PCR amplification of kDNA of the samples from the patients with cutaneous leishmaniasis, the PCR products were electrophoresed on 1% agarose gel along with the standard strains of *L. tropica* (MHOM / SU / 79 / K27), *L. major* (MHON / IR75 / ER), *L. infantum* (MHON / IR03 / MASH878) and negative control (distilled water). The profiles or the pattern of resulted bands was recorded using a gel documentation system (Gel Doc.) and identified by comparing the size of the bands related to the patients' samples with the marker bands (Figure 1).

At this stage, 1X Tris/Borate/EDTA and 1% agarose gel were prepared. After staining with GelRed, the PCR products were loaded onto the horizontal electrophoresis apparatus and next to the 1000 bp DNA ladder. The results were studied and imaging was done using a transilluminator and Gel Doc.

RESULTS

In this study, 60.4% of the infected individuals were male and the rest were female. The X^2 test showed no significant difference between the two genders in terms of parasitic infection. The demographic characteristics of the participants are shown in Table 1. Table 2 represents the frequency of ulcers in different parts of the body. The results showed that the purified DNA of all 48 patients tested had the 620bp band, which is associated with *L. major*.

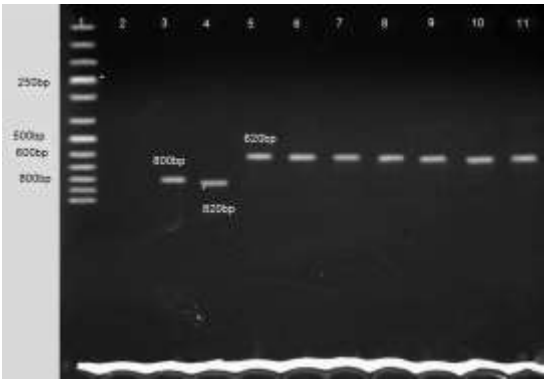
Table 1- Demographic characteristics of the subjects

Percentage	Number		
39.6	19	Under 10 years	Age
29.1	14	11-20 years	
16.6	8	21-30 years	
14.7	7	Over 30 years	
60.4	29	male	Gender
39.6	19	female	
4.1	2	High	Socioeconomic status
8.2	4	Upper Middle	
52.2	25	Middle	
35.5	17	Lower Middle	
52.1	25	Chahbahar	Residential site
8.2	4	Dashtyari	
6.3	3	Tis	
8.2	4	Plan	
25.2	12	Other sites	

Table 2- Frequency of ulcers in patients with cutaneous leishmaniasis in terms of the wound site

Total	Hand, foot, trunk	Foot and face	Hand and foot	Foot and body	Hand and face	face	feet	hands	Ulcer site
48	1	2	2	1	1	10	18	13	Number
100	2.2	4.4	4.4	2.2	2.2	20.2	37.5	27.1	Percentage

Figure 1- PCR products on (1%) gel electrophoresis, imaged by the transilluminator



Column 1: DNA Ladder & Marker (1000bp), Column 2: Negative control (distilled water), Column 3: *L. tropica* control MHON/IR03/MASH878 (800bp), Column 4: *L. infantum* control MCAN/IR07/MOHEB-GH (820bp), Column 5: *L. major* control MHON/IR75/ER (620bp), Column 6-11: PCR products related to the patients' samples (620bp)

DISCUSSION

Several studies have been conducted by researchers in different parts of Iran. Similar to the present study, the studies in Mirjaveh and Damghan (15, 16) have reported *L. major* as the cause of cutaneous leishmaniasis. However, the studies in endemic areas of Iran including Shush, Shiraz, Sabzevar and Mashhad found both species of *L. major* and *L. tropica* as the causes of cutaneous leishmaniasis (6, 17-19).

A pair of specific primers against kDNA was used in this study. The parasite's kDNA and circular DNA were good targets for molecular diagnostic studies and identification of the species due to having thousands of variable and conserved copies. On the other hand, this region has many taxonomic applications due to its high number of copies and repetitive sequences in the parasite genome (20). Similar to some studies, the present study used kDNA (present in a unique organelle called kinetoplast) that is both specific and highly sensitive for diagnosis.

In the present study, only *L. major* was found as the cause of cutaneous leishmaniasis. The prevalence of ulcers in the extremities such as hands and feet was higher, which is consistent with a study in Damghan (16).

Although many patients stated that they have never left the villages of Chabahar, the disease was still present in villages outside Chabahar. This indicates the presence of disease reservoir and potential vectors, as well as the increasing trend of the disease in this region. Considering the evolutionary process of the parasite and different times of the disease incidence in this

The statistical analysis also showed that the highest prevalence of infection was related to both genders in the individuals under 10 years of age (39.6%) and the lowest prevalence was observed in the age group of over 31-40 years (2.1%), while it was similar or evenly distributed in the rest of the age groups. The highest rate of infection was related to the patients less than 10 years of age, while it decreased in older age groups.

Other studies have also reported that the incidence of rural cutaneous leishmaniasis was higher in children in comparison to adults. In agreement with our findings, Sharifi and Zamani study on the epidemiology of cutaneous leishmaniasis in Southern areas of the city Baft reported the highest prevalence of the disease in the age group of 10-11 years (23).

The highest infection rates were observed in the participants with low or average socioeconomic status, living in poor houses. In the study of Ebadi et al., 93% of the patients were living in poor houses and areas with low socioeconomic status. The development of urbanization, city expansion and construction, high load of construction waste and unsanitary disposal of waste may be related to the risk of outbreak and spread of disease (24).

Such diseases are thought to be controlled or eradicated in the developed countries that have high levels of health facilities. The city of Chabahar and its surroundings still have wastelands that seem to be the living area of rodents that can act as reservoirs of the disease.

In this study, the extent of the area and numerous migrations to this area as a free zone should be considered. The geographical factors such as climate, agricultural development, development of cities and commerce are associated with the spread of the disease in this region. On the other hand, these also allow the possibility of developing new hybrids that could cause further problems for the treatment and prevention of this disease. Considering the short distance with Pakistan, it is expected to find the hybrid strains that have the pathological features of any specie in Iran and Pakistan. The hybrid parasites have been reported in Saudi Arabia and Ecuador (26, 25).
In this study, DNA was obtained from promastigotes, the culture medium and slides. There is an issue with diagnosis of *Leishmania* and the role of each parasite in pathogenicity in humans. It must be said that the selective cell culture eliminates a specie or strain. This issue has been demonstrated in a study conducted in Bolivia (27).

CONCLUSION

Cutaneous leishmaniasis is an important health problem in the province of Chabahar and planning for controlling this disease should be taken into consideration. Some efective measures should be taken for prevention of the disease by considering the side effects, cost of treatment and prevention of emotional and psychological damages caused by the disease. Moreover, using lace, mosquito nets and insect repellent creams will be effective in preventing the disease. It is suggested to conduct a study on the entire region to identify the cause of cutaneous leishmaniasis in this region. Furthermore, some epidemiological studies are recommended for identification of the exact type of rodent (as the disease reservoir) and specie of sandflies (as vectors) in this region, as well as molecular studies to identify the parasite vectors and reservoirs.

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CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

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