

Original Article

Cutaneous Leishmaniasis in Suspected Patients Referred to the Health Centers of Abarkouh, Ardakan, Bafgh and Khatam, Yazd Province, Iran

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Background and Aims: Cutaneous Leishmaniasis is a significant health problem in many parts of Iran. Management of the disease and its treatment is a global dilemma. In this study, the status and the proportions of Cutaneous Leishmaniasis induced by *Leishmania major* and *tropica* among suspected patients referred to the Health Centers of Abarkouh, Ardakan, Bafgh, and Khatam cities, Yazd Province, Iran were investigated.

Materials and Methods: The lesion was diagnosed using direct smear microscopy and conventional polymerase chain reaction.

Results: A total of 90 samples were prepared of which 64 (71.1%) were male and 26 (27.9%) were female. Also, 30 (33.3%) samples came from Ardakan, 29 (32.2%) samples from Bafgh, 21 (23.3%) samples from Abarkouh, and 10 (11.1%) samples from Khatam city. Ninety samples with 112 lesions were recruited and parasitologically examined. The results showed that, in macroscopic examination, 90 of the patients had 59 lesions: patients with lesion(s) induced by *Leishmania major* = 52 (M=35, 67.3%, F=17, 32.7%), lesion(s) induced by *Leishmania tropica* =4 (M=2, 50.0%, F=2, 50.0%), and lesion(s) induced by false positive =33 (36.4%). Besides, in microscopic method, 90 of the patients had 59 lesions: patients with lesion(s) induced by *Leishmania major* =44 (M=29, 65.9%, F=15, 34.09%), lesion(s) induced by *Leishmania tropica* =9 (M=8, 88.8%, F=1, 11.2%), and lesion(s) induced by false positive =37 (M=28, 75.7%, F=9, 24.3%).

Conclusion: Although travel history to an endemic area is important for diagnosis, parasitological confirmation is necessary to initiate treatment.

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Introduction

Leishmaniasis is a worldwide, wide-range disorder without any available definitive cure, vaccine, or pesticide. It has no sterile immunity, and efforts in this field have not been successful placing it in the sixth rank of most important infectious diseases of the world and tropical regions by the World Health Organization (WHO). Cutaneous leishmaniasis (CL) is one of the zoonotic parasite diseases that is endemic in 88 countries of the four continents of the world (22 countries in Europe and America, and 66 countries in Asia and Africa). It is regarded as the most important disease of tropical and subtropical diseases after Malaria [1, 2]. With an average incidence of more than 150 per 100 000 population, it has the highest rates of infectious diseases. The number of reported CL cases increased from 13729 in 2002 to more than 24000 in 2006. Thereafter, the disease prevalence increased, and new CL foci emerged in Iran [3-5]. Although the diagnosis of CL in its typical form in sporadic areas is not a difficult task, atypical forms of the disease resemble various skin disorders such as eczema, lupus erythematosus, furuncle, atypical mycobacterium infections, deep mycosis, sarcoidosis, leprosy, syphilis, foreign body granuloma, and even sometimes malignant skin tumors which justify initiating the treatment only when the lesion is parasitologically confirmed. Routine diagnosis of CL is based on the presence of *Leishmania* amastigote in a direct smear prepared by scratching the periphery of a suspected lesion. Usually, research facilities in endemic areas perform culture on Novy–Nicolle–McNeal

(NNN) medium in addition to direct smear [6]. Surveys of molecular epidemiology suggest that sensitivity and specificity identification of the type of CL in the area has increased the scope of the problem and the type of treatment. This study, explored for the first time the status and the proportions of CL induced by *Leishmania major* (*L. major*) and *Leishmania Tropica* (*L. Tropica*) among CL suspected patients referred to the Health Centers of Abarkouh, Ardakan, Bafgh, and Khatam cities, Yazd Province, Iran.

Materials and Methods

Sample collection

Sample collection was based on national guidelines and was equivalent to the diagnosis and treatment method of cutaneous leishmaniasis ordered by the WHO. Each patient was interviewed, and the characteristics of his or her largest wound were selected. The wound and surrounding skin were cleansed and sterilized using 70-degree ethyl alcohol. Samples were collected by shaving the skin from the margin of the lesion, and three samples were used for direct diagnosis.

Direct smear

The sample was smeared on a glass slide and stained using Giemsa (10%) stain, and then the slide was checked under the microscope in search for *Leishmania* Amastigote; sometimes, sampling was done more than once, twice or thrice when the first attempt failed to see the parasite, when the slide was not taken appropriately or when the sample showed superinfection.

DNA extraction

All positive and negative slides were

considered to extract DNA using the Gen All Exgene Cell SV (#106-101, Gen All, Korea) according to the manufacturer's instructions. The extracted DNA was analyzed using a spectrophotometer and electrophoresis.

Detection and identification

Genus detection was done using ITS1-polymerase chain reaction (PCR) by the specific primers of LITSR (5'-CTG GAT CAT TTT CCG ATG- 3') and L58S (5'-TGA TAC CAC TTA TCG CAC TT-3'). The reaction PCR included the final concentration of 1x PCR buffer, 0.5 nm each primer, 0.2 mm dNTP, 1.5 mm MgCl₂, and 5 mm DNA. The condition of amplification was done by the first denaturation of 94°C and followed by 40 cycles of denaturation at 94°C, annealing at 50°C, and extension at 72°C, each in 45 s. The final extension was done at 72°C for 5 min. The PCR product was assessed using 1% agarose gel electrophoresis alongside with 50 bp DNA ladder. The species identification was performed using restriction fragment length polymorphism (RFLP) analysis using Hae III restriction enzyme with the concentration of 10 U in each reaction of 20 μ l volume. The digestive assessment was done using 3% agarose gel electrophoresis alongside with 50 bp DNA ladder. This research was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran (IR.SSU.REC.1394.13).

Statistical analysis

Statistical analysis was performed using SPSS16 and the related statistical tests for each type of analysis ($p<0.05$).

Results

RFLP results: after species identification, all PCR products of *L. major* and *L. tropica* from the skin smears of CL patients were digested by Taq1 restriction enzyme to detect potential genetic polymorphism. Digestion products showed that all isolates displayed two DNA bands of approximately 300 and 350 bp (Fig. 1 & 2). If bands 220 and 140 bp were observed after digestion of PCR products with enzyme HaeIII, it was related to *L. major*, and if bands 200 and 60 bp were observed, it was related to *L. tropica*. A total of 90 samples that were suspected patients were referred to the health centers of Abarkouh, Ardakan, Bafgh, and Khatam, Yazd Province, Iran (during 2015-2016). Of these, 64 (71.1%) samples were male, and 26 (27.9%) samples were female (Fig. 3). Thirty (33.3%) samples were from Ardakan, 29 samples from Bafgh, 21 from Abarkouh, and 10 from Khatam. The results showed that, in macroscopic examination, 90 of the patients had 59 lesions: patients with lesion(s) induced by *L. major* =52 (M=35, 67.3%, F=17, 32.7%), lesion(s) induced by *L. tropica* =4 (M=2, 50.0%, F=2, 50.0%) and lesion(s) induced by false-positive =33(36.4%). Moreover, in microscopic method, 90 of the patients showed 59 lesions: patients with lesion(s) induced by *L. major* =44 (M=29, 65.9%, F=15, 34.1%), lesion(s) induced by *L. tropica* =9 (M =8, 88.8%, F=1, 11.2%) and lesion(s) induced by false-positive =37 (M=28, 73.6%, F=9, 24.4%) (Figure 5).

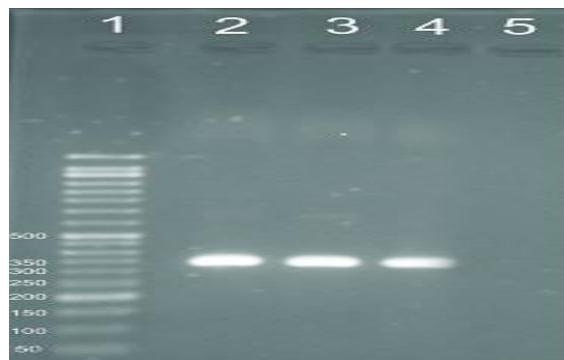


Fig. 1. Leishmania species. Lane 1: 50 bp DNA ladder; lane 2: *L. major* (MRHO/IR/75/ER); lane 3: *L. tropica* (MHOM/IR/NADIM3); lane 4: unknown sample detected as *Leishmania* spp.; lane 5: negative control.

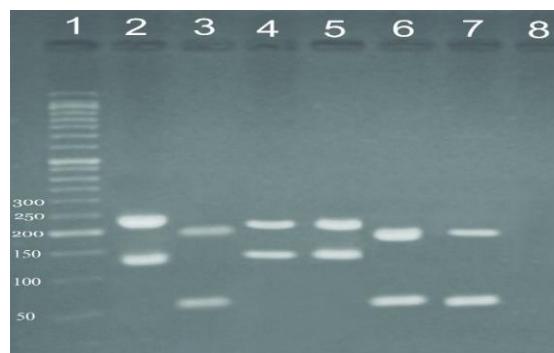


Fig. 2. Leishmania species. Lane 1: 50 bp DNA ladder; lane 2: *L. major* (MRHO/IR/75/ER); lane 3: *L. tropica* (MHOM/IR/NADIM3), lanes 4-5: unknown sample which was detected as *L. major*, lanes 6-7: unknown sample which was detected as *L. tropica*; lane 8: negative control.



Fig. 3. Frequency distribution of *L. major* and *L. tropica* according to the method of PCR-RFLP in terms of sex

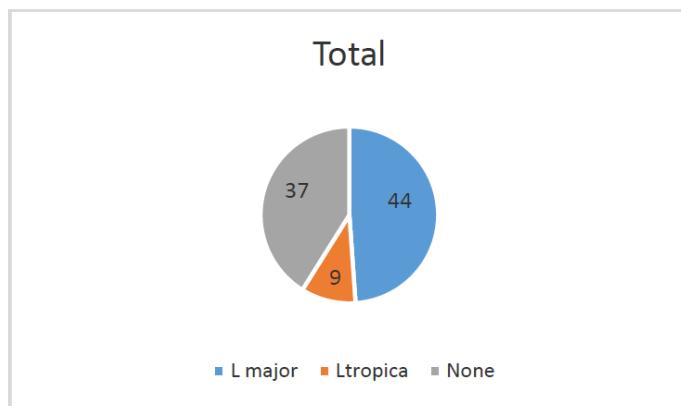


Fig. 4. Frequency distribution of *L. major* and *L. tropica* according to the method of PCR-RFLP

The comparison of mean *L. major* and *L. tropica* species between microscopic and molecular methods was conducted by chi-square test, which was significant ($P=0.000$). The history of travel to the endemic area was not always correlated with isolated *Leishmania* species. A total of 90 samples consisting of suspected patients who were referred to the health centers of Abarkouh, Ardakan, Bafgh, and Khatam, Yazd Province, Iran (during 2014-2015) were selected according to month and age group (Figs. 6 and 7).

Discussion

Leishmaniasis is an important disease that has been investigated by many researchers in various aspects [7-10]. The maintenance of this disease has been reported from various parts of the world due to immigration, population growth, presence of infected

Phlebotomus spp., and suitable environment [11-13]. Analysis of clinical data obtained from referrals to health centers in Yazd showed that out of 897 people with leishmaniasis, 457 were men and 439 were women, and this rate is high for Yazd. Although various methods have been used for diagnosis, PCR is a sensitive and specific method used to identify leishmaniasis in Iran and this study [14-19]. The main purpose of this study was to identify different species, including *L. major* and *L. tropica* molecularly. In this study, out of 372 samples, 159 samples were positive by PCR, including 87 cases (54.7%) of *L. major* species and 73 cases (45.3%) of *L. tropica* species [20-21]. The prevalence of the two main cutaneous leishmaniases, i.e., urban and rural leishmaniasis, was almost identical in Yazd. Although *L. major* was higher in rural areas, *L. tropica* was higher in urban areas.



Fig. 5. Frequency distribution of *L. major* and *L. tropica* by PCR-RFLP method according to the number of lesions



Fig. 6. Frequency distribution of *L. major* and *L. tropica* by PCR-RFLP method according to month

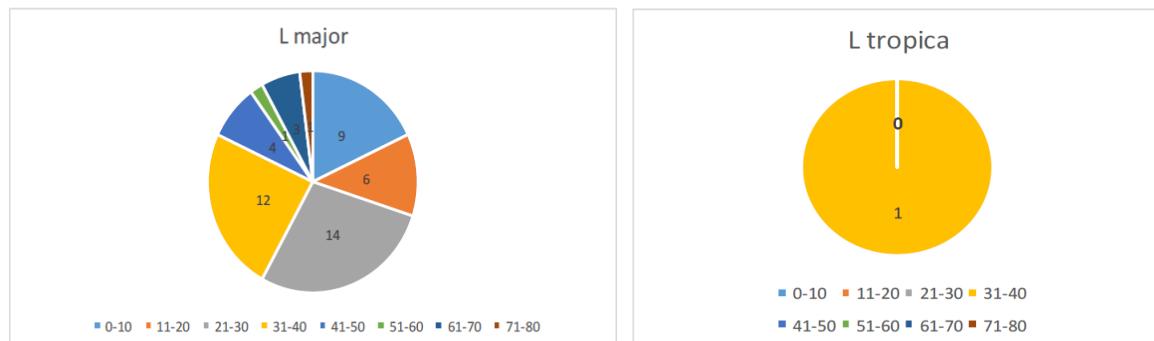


Fig. 7. Frequency distribution of *L. major* and *L. tropica* according to PCR-RFLP method in terms of age group

Our data and results are consistent with most results in Iran [22-30]. CL in Yazd Province follows climatic and ecological features. In the suburbs and villages, more proximity to desert areas and the availability of rodents as a reservoir, and the lack of drugs, pesticides, and ineffective parasite control programs cause the spread of rural leishmaniasis. Urban species of CL in developed urban areas due to continuous traffic from other provinces and neighboring

countries (Afghanistan, Pakistan, Iraq, and others) cause a steady outbreak of leishmaniasis. Additionally, indigenous and immigrated populations, whether domestic or foreign, lack of appropriate drugs to treat patients, lack of pesticides to control vector mosquitoes, and lack of coherent planning for further control and prevention, insecticides, and ineffective parasite control programs cause the spread of rural leishmaniasis.

Conclusion

Confirmation of the parasite's diagnosis is the only reliable evidence for starting treatment, even though a history of travel to or from a known native area is an important factor in suspecting a lesion known as cutaneous leishmaniasis. Cutaneous leishmaniasis is a combination of urban and rural leishmaniasis in Yazd Province. However, the distribution of *Indus* is different. In rural and less developed areas, the predominant rural species is dominant whereas, in urban and developed areas, urban-type is prevalent. Nevertheless, in

total, 50% of leishmaniasis in Yazd Province is rural and 50% is urban. Planning to control and reduce the incidence and prevalence of cutaneous leishmaniasis and combating reservoirs in Yazd province can be done by specifying the extent and type of the problem.

Conflicts of Interest

There was no conflict of interest.

Acknowledgments

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