ORIGINAL ARTICLE

An overview of a diagnostic and epidemiologic reappraisal of cutaneous leishmaniasis in Iran

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ABSTRACT

Cutaneous leishmaniasis (CL) is a widespread tropical infection which has a high incidence rate in Iran. *Leishmania tropica*, the causative agent of anthroponotic cutaneous leishmaniasis (ACL), and *Leishmania major*, which causes zoonotic cutaneous leishmaniasis (ZCL), are endemic in various parts of Iran with a high incidence rate. The aim of this study was to evaluate the reappraisal of the diagnosis and epidemiology of CL in Iran, by different clinical, parasitological and molecular assays among patients suspected of CL referred to the Department of Parasitology, at the Pasteur Institute of Iran during 2006-2009. Two hundred samples from patients with ulcerative skin lesions were collected, clinical analyses were applied, data questionnaire was completed and samples were examined for CL by using both direct microscopic and culture methods. Moreover, PCR assay was applied for detection of *Leishmania* species in CL isolates resulting from parasitological assay. Clinical observation revealed that the majority (58%) of lesions was single; double lesions were observed in 22% of patients, and only 20% of CL had multiple lesions. Out of 200 patients, Leishman body was observed in 77 samples (38.5%) by direct smear and 40% by cultivation assay. Most patients (21.3%) had a travel history to the Isfahan province, one of the most important endemic areas of CL located in center of Iran. PCR assay by kDNA indicated 32 and 18 out of 50 isolates respectively had similar patterns with standard *L. major* and *L. tropica*. In conclusion, clinical manifestations and an appropriate diagnostic assay with a parallel molecular characterization of CL may lead to a screening evaluation of disease, prognosis, treatment and control strategies.

Keywords: parasitological analysis; molecular biology; leishmaniasis; cutaneous; Iran; epidemiology.

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INTRODUCTION

Leishmaniasis is endemic in 88 countries throughout Africa, Asia, Europe, and North and South America. There are estimated 12 million cases worldwide, with one million new cases each year.[1,2] At least 21 species and subspecies of *Leishmania* have been recorded as being infective to humans, many of which cause extensive morbidity and are responsible for a wide spectrum of clinical symptoms. Cutaneous leishmaniasis (CL) is a common skin disease in the Middle East region affecting all ages and both sexes.[3]

Both CL and visceral leishmaniasis (VL) occur in different parts of Iran.[4] *Leishmania (L.) tropica*, the causative agent of anthroponotic cutaneous leishmaniasis (ACL), and *L. major*, which causes zoonotic cutaneous leishmaniasis (ZCL), are endemic in various regions of Iran with a high incidence rate.[5,6] CL is still considered an important health problem in many areas of the world, especially the Eastern Mediterranean region, and almost all countries of the Middle East, including Iran.[7,8] In Iran the disease prevalence is high in some foci, including Isfahan,[9] Shiraz,[10] Khorasan,[11] Khuzestan and Kerman[12] provinces.

Correct diagnosis and characterization of the particular parasite is important for prognostic evaluation and for prescribing appropriate treatment.[13] According to routine assays, diagnosis is based primarily on clinical symptoms, microscopic observation of parasites in stained smears, and/or cultivation of promastigotes.[14] A timely and definitive diagnosis of CL is important for initiating appropriate clinical management and treatment of this disease. Microscopy may be the simplest method of achieving this, but the high rate of undiagnosed

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cases associated with this method underscores its deficiency. The only rapid method for the diagnosis of CL is the PCR; however, it is not yet available outside the research settings and remains expensive for field deployment. Diagnosis of CL by cultivation in liquid media has several advantages, mainly the possibility of examining the entire sample collected in a closed system for the emergence of a few motile promastigotes. For decades, blood-based biphasic media of various formulations have been used for this purpose with variable degrees of success. The culture often turns out positive when inoculated with lesion aspirates from CL suspected patients having a large number of amastigotes. Also, a prolonged incubation is often required for a positive result. Efficient culture techniques for diagnosis of leishmaniasis require an *in vitro* environment for rapid conversion of a small number of amastigotes into a population of motile promastigotes visible by microscopy.[15]

This study was performed to evaluate the reappraisal of the diagnosis and epidemiology of CL in Iran, by different clinical, parasitological and molecular assays among CL patients suspected referred to the Department of Parasitology, at the Pasteur Institute of Iran, Tehran, from 2006 to 2009. The parasitological techniques (microscopy and culture) will be applied as gold standard for CL diagnosis; PCR assay was also used for detection of CL species in collected samples.

MATERIALS AND METHODS

Study population

This descriptive study was carried out with patients clinically suspected of having CL, who were referred to the Department of Parasitology, at the Pasteur Institute of Iran, Tehran, for laboratory confirmation. The diagnosis of CL was based on clinical observation, positive smear and culture assay. For each case having cutaneous lesions, a questionnaire was completed to record the necessary information including name, age, gender, address, location of ulcer on the body, data and place of acquiring the disease, previous travel history and work address. A total number of 200 patients with skin lesions, suspected of CL were examined during the period of 2006-2009.

Samples and smears

Generally, samples were obtained from those ulcers which showed the most indurate margins. The lesions were cleaned from debris with normal saline. Purulent or necrotic ulcers were treated with particular care and debris removed before sampling. According to clinical history, none of the patients had received any antileishmanial chemotherapy prior to diagnosis. Samples for parasitological diagnosis were dermal scraping of the active indurate margins of lesions or dermal scraping of the bottoms of the ulcer. Skin scrapings from the lesion were obtained and smears prepared on a slide, stained with Giemsa and examined microscopically for presence of amastigotes. Bacterial contamination of *Leishmania* cultures was minimized by cleaning lesions with 70% methanol and local debridement before obtaining specimens. At least two Giemsa-stained slides for each patient were prepared for microscopic examination.

Culture method

The samples were aspirated from the edge of the skin lesions and cultured in liquid phase (normal saline) of Novy Macneal Nicolle (NNN) media. The culture was incubated at 25°C and checked for growth of *Leishmania* promastigotes and supervised every day using an inverted microscope for 28 days. Penicillin-G and Streptomycin were added to the phosphate buffered saline (PBS) solution utilized in the NNN media culture.[16-18]

DNA extraction

DNA extraction and PCR assay were performed on promastigotes isolated from NNN media. The mass cultured promastigotes were harvested by centrifugation (3,000 rpm) at 4°C for 10 min and washed three times in cold sterile PBS (pH 7.2). DNA was extracted by DNG-plus extraction Kit (Cinnagen Co., Iran) according to the manufacturer's manual. Briefly, the pellet was mixed in 400 µL DNG-plus solution and vortexed for 15-20 s. Three hundred microliters of isopropanol were added and mixed by vortexing, and the specimen was centrifuged at 12,000 rpm for 10 min. The tube was decanted by gently inverting and placed on tissue paper for 2-3 s downward. One milliliter of 75% ethanol was added to the pellet, mixed by 3-5 s vortexing and centrifuged at 12,000 rpm tor 5 min. The ethanol was poured off completely, and the pellet dried at 65°C for 5 min. The DNA pellet was dissolved in 50 µL of sterile distilled water and incubated in a water bath at 65°C for 5 min and stored at 4°C until use.[19]

PCR assay

Leishmania isolates were detected according to the kit manufacturer company by using *Leishmania* spp. PCR Determination kit (Cinnagen Co, Iran). This kit is designed for qualitative detection of *Leishmania* spp. kinetoplast DNA by PCR. Assay was performed according to the manufacturer's protocol with the final volume of 25 µL of each PCR reaction. PCR amplification was carried out in a DNA Thermal Cycler (Master cycler gradient, Eppendorf, Hamburg, Germany) based on the following conditions: initial denaturation (95°C, 3 min; 63°C, 30 s; 72°C, 60 s) 1 cycle followed by 35 cycles including denaturation (93°C, 20 s), annealing (63°C, 20 s) and extension (72°C, 40 s). Finally, 10 µL of amplified samples without adding loading buffer were loaded in a 2% agarose gel containing 0.5 mg/mL ethidium bromide in electrophoresis and the products were visualized by UV transillumination.

RESULTS

Frequency of cutaneous leishmaniasis according to age groups and gender

Leishmania amastigotes were detected by microscopic observation in 77 cases (38.5%) out of 200 patients; however the NNN culture led to the growth of promastigotes in 80 samples (40%). Association of CL infection and gender was observed in 63.8% (51) males and 36.2% (29) females. Although the highest rate (31.3%) of infection was recorded in 21-30 years age group, the lowest rate (10%) was represented by the 31-40 years age group.

Frequency of CL lesion number

Number of lesions varied with single lesions being observed in the majority of patients (58%), appearing as a round papular plaque with a 4-80 mm diameter. In addition, double lesions were seen in 22% of cases; finally, 20% of patients presented multiple lesions between 3-7 mm (Figure 1).

Frequency of CL by place of residence and travel history

Residence, workplace, travel history of patients were important data for determining the location where the infections might have taken place. The highest proportion of infections (21.25%) was recorded in patients who were traveling or living in Isfahan and Kashan. Lowest percentage (1.25%) was reported in patients living in Hormozgan, Ilam and Khuzestan provinces (Table 1).

Figure 1: Single and multiple lesions in patients with CL in Iran.

Table 1. Frequency of CL by place of residence and travel history

Name of province	Endemic area	Travelling	Residence	Number of isolates	%
Fars	Shiraz	5	1	6	7.5
Ilam	Dehloran	2	-	2	2.5
Isfahan	Isfahan & Kashan	17	-	17	21.25
Kerman	Kerman & Bam	7	3	10	12.5
Khorasan	Mashhad & Sabzevar	9	3	12	15
Khuzestan	Ahwaz	1	1	2	2.5
Semnan	Damghan	8	1	9	11.25
Tehran	Tehran & Varamin	4	2	6	7.5
Yazd	Yazd	5	-	5	6.25
Hormozgan	Bandar Abass	1	-	1	1.25
Other countries	Afghanistan	10	-	10	12.5
Total 11 Locations	**11 Endemic areas**	**69**	**11**	**80**	**100**

CL lesion location in body

The samples were taken from different sites of suspected lesions. Hand and arm were the most commonly affected sites (40.0%), while other major sites of lesion location were face and neck (37.5%). Detailed location of lesions is shown in Table 2.

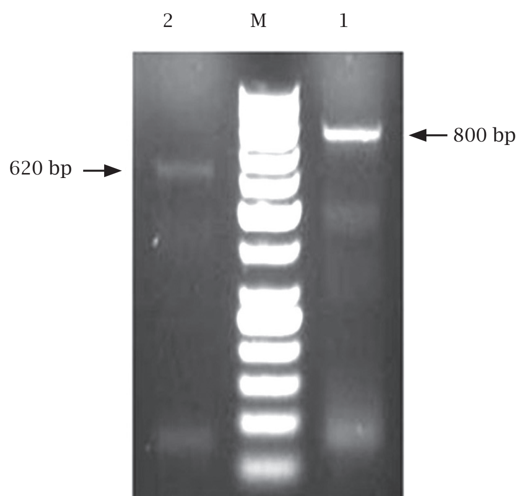
Table 2. Body location of lesions among cutaneous leishmaniasis suspected patients in Iran

%	Number of patients	Lesion location
37.5	30	Face and neck
40	32	Hand and arm
20	16	Legs
2.5	2	Other parts of body
100	**80**	**Total**

Electrophoretic patterns of PCR products from genomic DNA

Electrophoresis patterns from each isolates were compared with reference strains of Iranian *L. tropica* and *L. major*. In this study, a single 620 bp band for identification of *L. major* and an 800 bp band for detection of *L. tropica* were evidenced. Thirty and twenty samples out of 50 were identified as *L. major* (64%) and *L. tropica* (36%), respectively (Figure 2).

Figure 2: Electrophoretic patterns of PCR products obtained from crude parasite genomic DNAs for Leishmania species detection 1; *L. tropica*, MHOM/IR/09/Mash-F 2; *L. major* MRHO/IR/75/ER, M; marker.



DISCUSSION

Findings of this research revealed that *Leishmania* parasite was less detected by microscopic observation (38.5%) than by culture method (40%). This is in agreement with authors' previous report,[18] but not with Kumar *et al*. who indicated that direct microscopy or parasite culture alone detected respectively 65.5% and 48.2% of the positive samples.[20]

CL infection was more prevalent among males (63.8%) than females (36.2%). Previous reports confirmed the same results indicating that males are more commonly infected than females, most likely because of their exposure, possibly as a result of occupational contact with the outdoor sand fly vectors.[18-21] Moreover, the highest proportion of infection (31.3%) was recorded in 21-30 years age group, and the lowest (10%) was in the 31-40 years age group, which is in agreement with previous reports indicating more exposure as a result of educational and occupational situations.[18-21]

According to the number of lesions in the patients, single lesions were more common (58%) than both double (22%) or multiple lesions (20%), which is consistent with a previous report by Talari *et al.*[7] that showed 69.7% had only one lesion.[22] Multiple biting habits by the sand fly could explain the finding of about 42% of patients having more than one skin lesion.

Knowing the geographical location of infection acquisition is important in order to focus on epidemiological pattern of disease. The majority of patients were resident or at least had a travel history to Isfahan and Kashan (21.25%), central Iran, while a few patients were traveling or living in Hormozgan (1.5%) and Khuzestan provinces (2.5%), south Iran or in Ilam province (2.5%), west part Iran. There is an agreement between our findings with previous reports which confirm disease dissemination for many years in the rural areas of Kashan, specially in Abouzeidabad. This might be due to the high transmission rate maintained by the presence of both vector (*Phlebotomus papatasi*) and reservoir (*Rhombomys opimus*) in those areas.[7,18] However some studies have indicated different results.[23] Two species of *Leishmania* are involved in CL infections in Iran. *L. major* for ZCL and *L. tropica* for ACL. ACL has a disperse frequency whereas ZCL is found in many rural *foci* in the north, east and south of Iran.[7,24]

Hand and arm were the most commonly affected sites of suspected lesions (40%), followed by face and neck (37.5%), legs (20%) and other parts of the body (2.5%). Due to sand fly attack to the exposed body areas to have a blood meal, the most of the lesions appear in the hands, face, and legs, and upper parts of the body, which may be due to the outdoor sleeping on the terraces or yards without using bed net during the summer. Previous report by Talari *et al.*[7] confirms this finding.[22]

According to PCR findings, two strains of *L. tropica* and *L. major* are the causative agents of CL in Iran. Most of isolates collected from patients infected in the Mashhad area and the majority of isolates from Kashan were characterized as *L. tropica*. These findings lend support to previous publications about *Leishmania* species in Iran.[12,24,25] Most of the isolates characterized as *L. major* species were collected from patients with a travel history to endemic *foci* in Shiraz, Chabahar and Sabzevar, as previously shown. Razmjou *et al*. found a 23.2% prevalence rate among 1,000 inhabitants of the three villages of Shiraz, with *L. major* being identified in the majority of cases in rural areas of Shiraz.[26] However, these findings have not been confirmed by other researchers. In the study by Rahbarian *et al*. all isolates detected by PCR method in Gonbad-e Qavus county, north Iran were of *L. major.*[27] Parvizi *et al* (2010) also reported *L. major* in Natanz, Isfahan province in centre of Iran, in a rural ZCL *focus*.[28] Conclusively, there are indication that Isfahan has been a major endemic *focus* of ZCL in Iran for several decades.[24]

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