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A molecular and parasitological survey on cutaneous leishmaniasis patients from historical city of Kashan in Isfahan province, center of Iran

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ABSTRACT

Objective: To study cutaneous leishmaniasis (CL) for identifying the dominant *Leishmania* species on CL patients referred to medical health centers of historical Kashan city and suburbs located in Isfahan province in central part of Iran during 2010 to 2011. **Methods:** From 137 CL cases, were microscopically positive, the skin lesion serosity materials of 103 cases were cultured in monophasic culture media (RPMI 1640). We used the PCR-RFLP method for characterization the *Leishmania* isolates, by using specific internal transcribed spacer (ITS1) primers and HAEIII as the restriction fast enzyme. DNA was extracted from 63 samples. **Results:** *L. tropica* is main species in 58 (92.1%) cases and *L. major* is identified in 5 (7.9%) cases. Indeed randomly two isolates were the species characterized as *L. major* produced ulcer at the base tail of BALB/c mice after 3 weeks but from three *L. tropica* isolates none of them produced any lesion during 6 months post inoculation. **Conclusions:** The parasitological, epidemiological aspect and molecular methods of this study showed that, Kashan and suburb are anthroponetic CL area despite this city located in Isfahan province as an ancient focus of zoonotic CL in Iran.

1. Introduction

Cutaneous leishmaniasis (CL) is counted as one of the most important major medical public health issues of the world especially in tropical and sub-tropical countries. More than 90 percent of CL cases live in the following countries: Afghanistan, Saudi-Arabia, Aljazeera, Brazil, Iran, Iraq, and Syria. Meanwhile 350 million people are exposed to the parasite. The number of new cases of CL has reached to 1.5 million people in the word[1]. In Iran, CL distributes in some geographical locations such as north-east[2], center[3], west, east and south[4,5]. In Iran, there are two forms of CL including Anthroponotic CL (ACL) and Zoonotic CL (ZCL). In ACL, the causative agent is *L. tropica* and phlebotomus sergenti is the main vector, infected human and dogs have the reservoir role. In ZCL, *L. major* is the principal agent; *P. papatasi* and rodents (Gerbillidae) are the main vector and reservoir of disease respectively[6]. Isfahan province in center of Iran is one of the main foci of the ZCL and meanwhile the disease exists as a medical health problem in the following cities: Isfahan, Natanz, Badroud, Ardestan, Aran-Bidgol and Kashan[7,8]. Kashan is an ancient and historical city which is located in Isfahan province[9]. Due to its historical background, it has turned to be one of the tourist cities of the world. So it requires a thorough and consistent study for detecting the abundance and dispersion

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of different species of *Leishmania* parasites. By gathering proper information we could take comprehensive and proper actions for controlling the disease in the area. The methods of preventing and controlling of leishmaniasis vary with the *Leishmania* species and we could detect the existence of infection and parasite presence by microscopically observing stained smears. Due to morphological similarities of the *Leishmania* parasites, accurate species identification is not possible. Characterization is based on the golden standard iso-enzyme method which requires an abundant number of parasites. Despite the cost, this method is time-consuming. In recent years, molecular DNA based methods such as PCR- RFLP (restriction fragment length polymorphism), RAPD-PCR (Random Amplified Polymorphic DNA), nested-PCR and Real time PCR as a high tech method, have been widely used in diagnosing and identifying the parasite species[10,11]. One of the main advantages of using these methods is that we could detect the infection, parasite species and even the load of parasite by small amount of sample. One of the genome fragments which has a lot of applications is the ribosome gene (rDNA) which is used for the amplification of ITS1 (internal transcribed spacer1) part. Since the length of ITS1 fragments are equal for all cutaneous and visceral Leishmania species, we used the specific restriction enzyme for differentiating these species[12]. For reconfirmation we used sequencing for some cases. This study has been conducted with the purpose of identifying dominant *Leishmania* parasite species in Kashan city using the PCR-RFLP method.

2. Materials and methods

2.1. Study area

Kashan is a city with a population of about 248 789 people and a hot and dry climate located in the central part of Iran. It is located in the north part of Isfahan province. As an ancient and historical city, it gathers hundreds of tourists every year. Kashan district consists of six cities (Kashan, Meshkat, Ghamsar, Niasar, Joshaghan and Barzok). Figure 1 shows geographical situation of Kashan and suburbs. The mean temperature is 18 ℃ and the mean of annually raining is 116 mm[9].

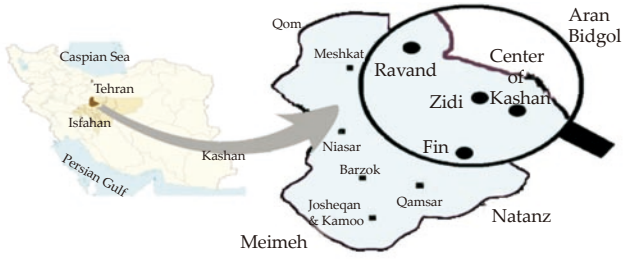

Figure 1. The map of endemic Leishmaniasis foci in Kashan city and suburb.

2.2. Sample collection and culture

This study committed as a cross sectional study. The studied group composed of the CL infected patients referred to Kashan health centers that had positive results in the parasitological tests. The area in which these patients were infected included Kashan and suburbs (central Kashan, Fin, Ravand, Zidi) in Isfahan province (Figure 1). After obtaining a complete clinical history, the samples were taken from the swollen edge of the lesion by injecting normal saline method[13]. A direct smear for microscopical examination was prepared and a little of cerosites cultured into RPMI 1 640 medium (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco), the media, incubated at 26 ℃. All *Leishmania* isolates in promastigotes form were harvested at stationary phase of growth after 5 to 7 days.

2.3. Animal inoculation

For further study, the promastigotes form early stationary five *Leishmania* isolates (about 2×10^6 promastigotes) were inoculated subcutaneous into the base of the tail of the 10 Balb/C mice. Each isolate inoculated to 2 mice, which were examined weekly for appearance of lesion at the injection site up to 6 months.

2.4. DNA extraction

DNA template was extracted from 1.5 mL early stationary phase of *Leishmania* promastigotes cultured isolates or Geimsa stained slides by using the High Pure PCR Template Purification (Roche, Germany) as recommended by the manufacturer.

2.5. PCR- RFLP method

We used the ITS1 region, using the primers LITSR (5′-CTGGATCATTTTCCGATG-3′) and L5.8S (5′-TGATACCACTTATCGCACTT-3′) (Bioneer, Korea). Amplification reactions were performed with ready to use the master mixed (Roche, Germany). Amplification was performed with 35 cycles including (30″ at 94 ℃, 30″ at 49 ℃, and 45″ at 72 ℃) in a thermocycler (PeQlab Biotechnology GmbH). For RFLP analysis, the PCR product including the amplified ITS1 were digested with Fast digestion HaeIII (BsuR1) (Fermentas, Life Sciences, Germany) as recommended by the manufacturer. Digestion products were separated by using 3% agarose gels in TAE buffer and visualized after staining by gel red[13]. The electrophoresis pattern of the all samples was evaluated with the pattern of Iranian *Leishmania* reference strains including with the Accession numbers: *L. tropica* (EU727198), *L. major*

(EF653269), and *L. infantum* (FJ497004).

2.6. DNA sequencing

For sequencing, the PCR products of a number of isolates were used with forward primer (LITSR) following the manufacturer recommendations (Bioneer, Korea).

3. Results

In this study totally 137 CL infected patients were selected (Table 1). These cases were confirmed by both microscopy (1000×) and culture exams.

Table 1
The frequency of CL patients based on study site who referred to different public health centers in Kashan and suburb for following the leishmaniasis during 2010 to 2011.

Location	Frequency	Percent
Ravand	65	47.5
Fin	23	16.8
Zidi	21	15.3
Center of kashan	28	20.4
Total	137	100

The patient characteristics according to gender, number of lesions, ulcers duration, distribution of the skin lesions on the bodies and the kind of the lesion wet or dry were shown in (Table 2).

Table 2
The main characteristics of 137 confirmed cutaneous leishmaniasis positive patients.

Characteristic	
Age (year)*	32.3±21.1
Gender	
Male	74 (54%)
Female	63 (46%)
Average number of lesions*	2.15±1.8
Lesion duration*(day)	96.7±122
Lesion site on	
Hand	86 (62.8%)
Leg	14 (10.2%)
Face and head	12 (8.8%)
Hand and leg	14 (10.2%)
Other sites	11(8%)
Leishmania species identification	
L. major	5 (7.9%)
L. tropica	58 (92.1%)
lesion appearance	
Dry	124 (90.5%)
Wet	13 (9.5%)
Parasitemia rate**	
<+4	61 (44.5%)
≥+4	76 (55.5%)

* Mean±SD, ** Grading of *Leishmania* parasites was obtained by average parasite density using ×10 eyepiece and ×100 oil-immersion [4+(1-10) parasites/fields, 3+(1-10) parasites/10 fields, 2+(1-10) parasites/100 fields, 1+(1-10) parasites/1 000 fields].

From mentioned 137 cases 74 (54%) were male and 63 cases (46%) were female. The mean age was 32 years old. The average number of lesions was about 2 and the lesion duration was 97 days. In 62.8% cases the lesions was on hands and the appearance of the lesions in 124 cases (90.5%) was dry. Half (50%) of cases had one lesion with mean lesion duration 96.7 days. Seasonal distribution of the samples were equaled so in spring was 32 cases (23.3%), in summer was 31 cases (22.7%) in autumn 42 cases (30.7%) and in winter was 32 cases (23.3%).

In direct smear examination the parasitemia rate were from 1+ to 4+ and more, and in 76 cases (55.5%) this rate was more than 4+. From 103 cases that lesion cerosites cultured in RPMI1640, 54 (52.4%) cases the result was positive and the promastigotes appeared after 10 days. DNA extracted successfully from above cultured samples and also in 9 cases that the cultured media was contaminated, DNA extracted from Geimsa stained slides. Totally 63 clinical samples were positive by PCR and the PCR products for species characterization digested with *HAE*III as the restriction enzyme. Fifty eight (92.1%) of samples patterns identical to that of *L. tropica* and 5 (7.9%) to that of *L. major* in compared to reference strain (Figure 2).

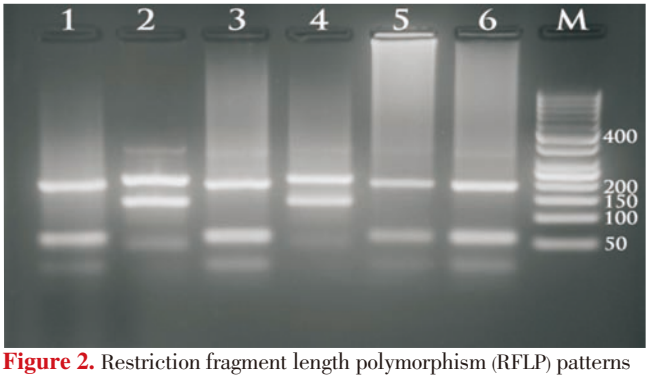

Figure 2. Restriction fragment length polymorphism (RFLP) patterns obtained from *Leishmania* stocks and patients samples. Lane 1 (*L. tropica* stock), Lane 2 (*L. major* stock), Lane 3, 5, 6 *L. tropica*, and 4 *L. major* M: 50-bp size marker (Fermentas).

Table 3 showed the distribution of characterized *Leishmania* isolates by PCR-RFLP based on patients infected location area in Kashan and suburb.

Table 3
Distribution of Leishmania species using PCR-RFLP based on patients infected study site in Kashan and suburb during 2010-2011.

Leishmania species	Study site				Total
	Ravand	Fin	Zidi	Center of kashan	
L. tropica	33 (56.9%)	11 (19.0%)	9 (15.5%)	5 (8.6%)	58 (100.0%)
L. major	2 (40.0%)	1 (20.0%)	1 (20.0%)	1 (20.0%)	5 (100.0%)
Total	35 (55.6%)	12 (19.0%)	10 (15.9%)	6 (9.5%)	63 (100.0%)

The most *L. tropica* infected sites were Ravand (56.9%), Fin (19%), Zidi (15.5%) and central Kashan (8.6%) respectively. Two cases that the species were characterized as *L. major* could produced ulcer after 3 weeks incubation time and none of

three of *L. tropica* isolates inoculated, produced lesion at the base tail of mice after 6 months. In relation to this study we submitted a number of samples in Gene bank with the Accession Numbers: JN860715 (*L. tropica*), JN860716 (*L. tropica*), JN860717 (*L. tropica*), JN860718 (*L. major*), JN860719 (*L. tropica*), JN860720 (*L. major*).

4. Discussion

Leishmaniasis is a parasitological disease, which is common between human and animals. This disease always causes serious health problems for the human communities[14]. Leishmaniasis is prevalent in many parts of Iran, including Kashan in Isfahan province. Previous study based on clinical examination determined the CL incidence in rural and urban districts of Kashan was approximately 37.6 cases in 100 000 people in Kashan during 2007-2008. CL has been increased in Kashan in recent years and altogether 664 CL cases were reported in these areas during 3 years ago[15,16]. Kashan and suburbs are located between CL endemic areas such as Badrood and Natanz in the south, Aran-Bidgol in the north, but principal species of the disease were not well known in the studied areas so, we decided to determine *Leishmania* species for designing appreciate control program. Species identification is of major importance because it is the spotter of the infection type in a region. Accurate identification of the *Leishmania* sp. parasites with the Giemsa stained smears is not possible due to morphological similarities of these parasites. According to DNA based methods such as PCR the existence of infection, parasite species and the type of infection could be detected[17,18]. Based on experiments, for identification of *Leishmania* species isolates from Kashan and suburbs we used ITS1 as an ideal target for characterization of different *Leishmania* species through the PCR-RFLP method. This technique was fast and easy to run and had a potential value for diagnosis and identification of *Leishmania*. Indeed it could be useful for field work with different samples like lesion serosites, filter paper imprints, Geimsa stained smear and blood in different human patients, vectors and leishmania reservoirs[13,19,20]. In previous studies on patients, reservoirs, and vectors in Natanz, Qom, and Aran-Bidgol, the dominant species was announced to be *L. major*[21-23]. In spite of the studied region are so close to Kashan focus so it was expected that the dominant species in Kashan would be *L. major* as well, but according to the results 58 (92.1%) of samples were *L. tropica* and 5 (7.9%) were *L. major*. Also the rest of the results of this study prove the state of leishmaniasis in Kashan city and suburbs similar to those of anthroponetic *leishmania* focuses (ACL) in Iran. In the molecular studies committed in Mashhad city more than 95% of the cases were *L. tropica* and the others were *L. major*[2].

In this study the rate of infection remained approximately the same in different seasons. Results showed most of the lesions belonged to the dry kind and the most involved organ were hands with the percentage of 61%. These results are consistent with the other urban leishmaniosis focuses of our country. In this study 51.8% of the patients were younger than 30 years old and 50% of the patients had only one lesion whereas 25 % had two lesions. These results were similar to the epidemiological study that Doroodgar *et al*[15,23] committed in 2008 in Kashan city. In 76 (55.5%) cases, grading of *Leishmania* parasites in Geimsa stained slides were more than 4 positive, and this is similar to that we expected in slides belongs to ACL form. However this is a valuable point for DNA extraction from slides in cases that the culture media infected to bacteria or fungi contamination. We used this extracted method for nine cases with good results. Finally five *L. major* cases were identified, in which 2 of them belonged to ravand, and one case in each cites Zidi and fin and Kashan center. Leishmaniasis cases in Ravand area were higher than the other parts. These isolates could produce ulcer in Balb/C mice. Dabirzadeh *et al* found a highly polymorphic species among clinical *L. major* samples in Isfahan, Iran[24,25].

Epidemiological aspects and improbable conditions like old houses, muddy walls, muddy streets, unhygienic and traditional way of living, and generally low economy level of families were the main causes of Leishmaniasis outbreak in this region with respect to the other surveyed parts. Despite the increasing number of reported CL cases from Kashan, to our knowledge no molecular studies of the species distribution have been performed so far. Results of this study showed that *L. tropica* is the predominant agent of CL and is distributed in the most endemic areas of the city and its suburbs. Moreover, this study revealed that PCR-RFLP method is an appropriate tool for *Leishmania* species characterization. Further studies need to be done to determine other epidemiological aspects of CL in the studied areas.

Conflict of interest statement

We declare that we have no conflict of interest.

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