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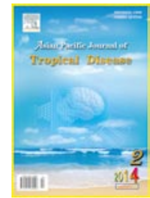

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Molecular epidemiological study of cutaneous leishmaniasis in the east north of Iran

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ABSTRACT

Objective: To identify and study the epidemiology of *Leishmania* species isolated from patients with leishmaniasis by PCR method in Sabzevar, Khorasan Razavi Province, Iran.
Methods: Aspirated samples from the lesions of 86 patients with confirmed leishmaniasis used for direct smear preparation. The samples are prepared and cultured in the Novy-MacNeal-Nicolle and Roswell Park Memorial Institute-1640 culture mediums. After extraction of DNA by using phenol-chloroform, parasite kintoplast DNA gene amplification was done by using PCR. The electrophoresis pattern of each species was compared with standard species of *Leishmania tropica* (*L. tropica*) and *Leishmania major* (*L. major*).
Results: Results of PCR patterns of kintoplast DNA gene suggested that two types of *L. tropica* and *L. major* isolated from leishmaniasis patients in Sabzevar and their findings indicated that both species of parasites, *L. tropica* and *L. major* are prevalent in Sabzevar.
Conclusions: According to this information, Sabzevar can be divided in two focal points of dry and dry-wet in terms of leishmaniasis, while it was previously known as the dry focal point. Likewise a significant relationship observed between *Leishmania* species to time distribution, type of lesion, amount of parasites and infection districts.

1. Introduction

Cutaneous leishmaniasis is a parasitic infection that is caused by various species of *Leishmania*. The disease occurs due to *Leishmania* inoculation to human skin via the bite of sand fly infected with promastigotes of the parasite. After infection, promastigotes enter into macrophages[1-3]. In macrophages, promastigotes convert to amastigotes which will induce nodules after proliferation and gradually the progressive lesion turns into papules and vesicles. After the lesion got well, it leaves a scar. Clinically, the lesions are divided into dry and wet forms[4-7]. *Leishmania major* (*L. major*) and *Leishmania tropica* (*L. tropica*) are responsible for wet and dry forms of leishmaniasis, respectively. There are different reservoirs for these parasites. Mice and wild-type rodents are reserviores of *L. major*, while human and dog (accidental hosts) are as reservoir hosts of *L. tropica*[8].

The disease is considered as a public health problem in some parts of Iran, particularly Sabzevar in Khorasan Province (Figure 1). Since reservoirs of the disease are

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Figure 1. Map of the city of Sabzevar in Khorasan Razavi Province, north eastern of Iran showing the geographical location and study sites.

different in the two species of parasites, in order to eradicate the disease, the species need to be identified. All forms of cutaneous leishmaniasis have the same morphology and it is not possible to differentiate species of *Leishmania* by clinical signs or microscopic methods. Among the existing approaches, molecular methods, such as PCR are very sensitive and rapid for identification of infectious agents. Since Sabzevar is one of the important focal points of the disease in Iran, we decided to study the epidemiology of cutaneous leishmaniasis using PCR method there[9-12].

2. Materials and methods

In this study 86 biopsies were collected from cutaneous leishmaniasis suspected persons in Sabzevar from May 2010 to May 2011. Biopsy was done with a scalpel under the lesion through picking up the macrophages. Two slides of each patient's lesions were prepared and stained with Giemsa. For amostigote cultivation, aspiration was performed from lesion using an insulin syringe containing 0.5 mL sterile saline buffer. The stained slides were examined by microscope with 40× and 100× magnification for observing amastigotes. If amastigotes were observed, the sample obtained from aspiration of the lesion would be entered into the Novy-MacNeal-Nicolle medium. To reduce microbial contamination potential, 250 to 500 IU of penicillin and 2 mg streptomycin per mililiter of culture was added to Novy-MacNeal-Nicolle medium. The culture mediums were incubated at 25 °C. After 3 to 4 d, the wet slides were prepared from the culture medium for observation of the promastigotes forms of parasites. Promastigotes with flagella were counted using a Neobar slide. If they reached one to two million per mililiter, they would be transferred to Roswell Park Memorial Institute-1640 culture medium containing fetal bovine serum to mass cultivation of promastigotes. After proliferation adjusted (1 000 promastigotes per mililiter) in culture medium, the promastigotes were centrifuged and washed with phosphate buffered saline for DNA extraction. DNA extraction was performed with phenol-chloroform method[13-15].

2.1. Kintoplast DNA (kDNA) amplification by PCR

A pair of primers for conserved sequences of *Leishmania*, kDNA including forward: (5′ TCG CAG AAC GCC CCT ACC 3′) and reverse: (5′AGG GGT TGG TGT AAA ATA GG3′ were

designed. Amplification of kDNA using these primers led to 600 bp and 800 bp bands for *L. major* and *L. tropica*, respectively[16].

PCR for amplification of kDNA gene was performed according to the following stages (Table 1). A total of 20 μL reactions were prepared by using 2 μL 10× PCR buffer adjusted to 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP (Invitrogen, Carlsbad, CA, USA), 20 pmol of each primer, 1 IU *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 5 ng of DNA template. Two positive control (*L. major* strain MRHO/IR/75/ER and *L. tropica* strain MHOM/IR/01/yaza) and a negative control sample were used[17].

Table 1
PCR to amplify kDNA of *Leishmania* species.

Cycle	Process	Temperature (°C)	Time (seconds)
1	First denaturation	95.0	300
31	Denaturation	94.0	30
31	Annealing	60.6	60
31	Extension	72.0	60
1	Final extension	72.0	300

3. Results

3.1. Leishmania species

The results showed from a total of 86 isolates, 32 samples (37%) were *L. tropica* and 54 samples (63%) were *L. major* (Figure 2).

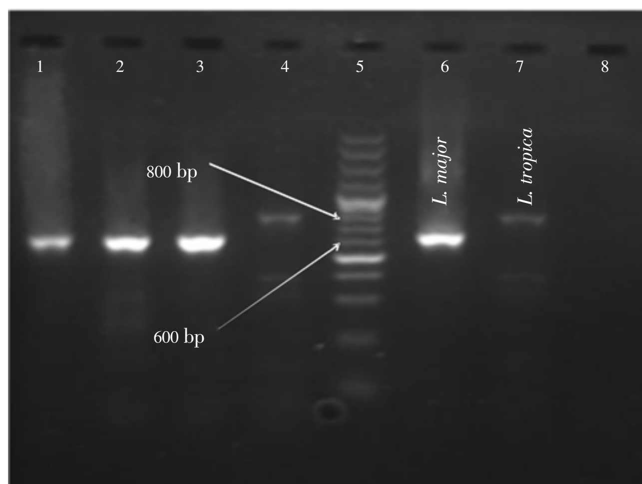

Figure 2. The pattern of PCR of kDNA gene with corresponding primers. Lanes 1 to 3: PCR product from patients infected with *L. major* species with molecular weight 600 bp; Line 4: PCR product from patients infected with *L. tropica* with molecular weight of 800 bp; Lane 5: 100 bp DNA marker; Lane 6: Standard *L. major*; Lane 7: Standard *L. tropica*; Lane 8: Negative control.

3.2. The mean size of the lesions

Most of isolates had 11–20 mm lesions and a few of them had lesions with more than 40 mm in size (Table 2). There was not any significant relationship between the size of lesions and the parasite species (P>0.05).

Table 2
Mean size of the lesions in patients with cutaneous leishmaniasis according to species.

Mean size of lesions (mm)	*L. tropica*		*L. major*		Sum	
	Number	Percent	Number	Percent	Number	Percent
0–10	10	11.8	22	25.5	32	37.3
11–20	16	18.8	25	29.0	41	47.8
21–30	5	5.9	6	6.8	11	12.7
>40	1	1.1	1	1.1	2	2.2
Sum	32	37.6	54	62.4	86	100.0

3.3. Type of lesion

The comparison between the two species revealed that among *L. tropica* species dry type is dominant, while the highest frequency is related to secretory type in *L. major* species. Moreover, 8 patients had two types of lesions on their body (Table 3). These information indicated a significant relationship between the type of lesions and parasite species (P<0.05).

Table 3
Type of lesion in patients with cutaneous leishmaniasis according to species.

Type of lesions	*L. tropica*		*L. major*		Sum	
	Number	Percent	Number	Percent	Number	Percent
Dry	22	25.5	10	11.6	32	37.1
Secretory	8	9.3	21	24.4	29	34.7
Purulent	0	0.0	17	20.0	17	20.0
Two types of lesions	2	2.4	6	6.8	8	9.2
Sum	32	37.2	54	61.8	86	100.0

3.4. Correlation between Leishmania species and season

In patients with *L. tropica* infection, the highest frequency was in the spring with 12 cases and the lowest one was in the winter season with 4 cases. In patients with *L. major*, the highest frequency was in the summer season with 38 cases and the lowest frequency was in winter season with 1 case (Figure 3). It revealed a significant correlation between onset of lesions and the parasite species (P<0.05).

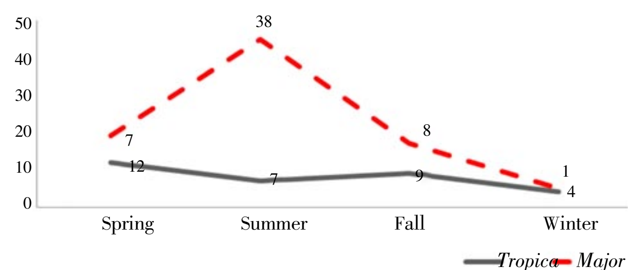

Figure 3. Time distribution of onset of lesions in patients with cutaneous leishmaniasis according to species.

3.5. Amount of parasites

In patients with *L. tropica* infection, severe slides with 19.6% had the highest percentage, while none of the patients had rare slides. Among slides of patients infected to *L. major*, a few slides with 31.3% had the highest percentage, while no severe slide was observed in this species (Table 4). The results illustrated a significant relationship between amount of parasites on the slides taken from patients and the parasite species ($P<0.05$).

Table 4
Amount of parasites in patients with cutaneous leishmaniasis according to species.

Parasite amount	*L. tropica*		*L. major*		Sum	
	Number	Percent	Number	Percent	Number	Percent
Rare	0	0.0	8	9.3	8	9.3
Few	3	3.6	27	31.3	31	36.0
Moderate	11	13.0	19	22.0	30	35.0
Severe	17	19.6	0	0.0	17	19.6
Sum	32	37.2	53	62.8	86	100.0

3.6. Distribution of species according to districts

Nevertheless, the largest number of patients came from Hokm Abad town with 54 patients while the smallest number came from Rudab town with only 1 patient. Maximum number of *L. tropica* was for Sabzevar City with 12 cases and maximum number of *L. major* was for Hokm Abad town with 43 cases. There was a significant relationship between patient's locations and the species ($P<0.05$).

4. Discussion

At the past, studies on detection and differentiation of parasite species are accompanied with many problems and need information about clinical symptoms, epidemiology of the disease, induced disease in animals and growth in culture medium. Identification of the *Leishmania* species seems to be necessary for especial treatment and also for programmers to design controlling strategies against *Leishmania* reservoirs[12,18]. Applying biochemical methods and using isoenzymes can partially overcame the problems of identification of *Leishmania* parasite species. Usage of the methods mentioned above required multiple enzyme systems and special skills, even though lack of parasite identification would reduce the value of those approaches[19]. Nowadays, with powerful, fast and sensitive tools such as molecular diagnostic methods, the conventional methods are eliminated. Different PCR techniques have been commonly used as potential tools for this purpose[20,21]. In this study we used a consensus sequence of kDNA gene for designing especial PCR for identification of different *Leishmania* species. The number of parasites among slides of the patient's lesions varied according to the species. Among the slides taken from infected patients with *L. tropica*, "severe" with 17 cases had the highest frequency, while "rare" slides accounted for the smallest number. Besides, among the patients' slides infected by *L. major*, the "few" slides accounted for most of slides, but no "severe" slide was observed in this species. Furthermore, statistical tests represented that there was a significant relationship between the parasite species and number of parasites in stained slides ($P<0.05$), so the slides with high parasites (severe) were observed in lesions of infected patients with *L. tropica*. However, slides with low parasites (few) were seen in lesions of infected patients with *L. major* species.

According to acquired information in the present study, Sabzevar cannot be presented as a dry core because it includes both focuses. The epidemiology variation could be due to immethodical construction in towns and villages, accumulation of construction debris, dumping waste near houses which cause the growth of *L. major* reservoirs and carriers of both species and agriculture in the habitat which provides the situation for the growth of parasite reservoirs. Outbreak time of the disease is different in each species, in leishmaniasis with *L. tropica*, the highest frequency was related to spring season with 12 cases, while in leishmaniasis with *L. major*, the highest frequency observed in summer season with 38 cases. Lesions size was different between two species, the lesions of *L. major* were larger than the lesions of *L. tropica*. Types of lesions also were different between them so that *L. tropica*-induced lesions were often generally dry, while *L. major*-induced lesions were wet and secreted. In conclusion, based on the findings of this study, Sabzevar can be divided into two focal points: one focal point where merely *L. tropica* is observed including Sabzevar City and Rudab; and the other where both species are seen including Hokm Abad, Qazalgharshy, Jaghatay, Rivadeh and Mohammad Abad. Our study proved that both parasites were seen in Sabzevar. Therefore, Sabzevar cannot be considered as the dry focal point. Characteristics of the collected *Leishmania* isolates from Sabzevar showed that regions in western and south eastern of Iran, *L. major* is a predominant parasite of cutaneous leishmaniasis, thus significant relationship reported among *Leishmania* species to time distribution, type of lesion, amount of parasites and infection districts[22,23].

Conflict of interest statement

We declare that we have no conflict of interest.

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