Characterization of *Leishmania* parasites isolated from provinces of the Islamic Republic of Iran

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التعرُّف على خصائص طفيليات الليشمانية المستفردة من عدد من ولايات جمهورية إيران الإسلامية

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الخلاصة: تمت دراسة طفيليات الليشمانية المستفردة من جمهورية إيران الإسلامية بإجراء التفاعل التسلسلي المضخَّم لبوليميراز الدنا المتعدِّد الأشكال، بشكل عشوائي. وقد كان 80 من المستفردات من مجمل 82 مستفردة من آفات الليشمانيات الجلدية فيما كانت مستفردة واحدة من آفة لحنجرة بشرية ومستفردة واحدة من كلب. ومن مجمل المستفردات كانت 42 مستفردة تحتوي على الليشمانية المدارية و36 مستفردة تحتوي على الليشمانية الكبيرة ومستفردتان تحتويان على الليشمانية الطفلية، وكان هناك مستفردتان لم يمكن استعرافهما (وهما تلك المستفردة من آفة حنجرية وتلك المستفردة من الكلب)، وقد أظهرتا 52٪ من التشابه مع الليشمانية المدارية و48٪ من التشابه مع الليشمانية الطفلية. وقد استفردت كلٌّ من الليشمانية المدارية والليشمانية الكبيرة من أربع ولايات مما يشير إلى التغيير الذي يجري حالياً في وبائيات داء الليشمانيات الجلدية، كما استفردت الليشمانية المدارية من ثلاث ولايات، واستفردت الليشمانية الكبيرة من ولاية واحدة، في حين استفردت الليشمانية الطفلية من آفة جلدية بشرية ومن كلب في ولاية بوشهر.

ABSTRACT *Leishmania* parasites isolated in the Islamic of Iran were studied by a random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). Of 82 isolates, 80 were from cutaneous lesions, 1 from a human throat lesion and 1 from a dog. Of these, 42 isolates were *L. tropica*, 36 were *L. major* and 2 were *L. infantum*. There were 2 unidentified isolates (from the throat lesion and a cutaneous lesion) and these demonstrated 52% and 48% similarity with *L. tropica* and *L. infantum*. Both *L. tropica* and *L. major* were isolated from four provinces indicating a recent change in the epidemiology of cutaneous leishmaniasis. *L. tropica* was isolated from three provinces; *L. major* from one province. *L. infantum* was isolated from a human cutaneous lesion and from a dog in Bushehr province.

Caractérisation des parasites *Leishmania* isolés dans différentes provinces de la République islamique d'Iran

RESUME Les parasites *Leishmania* isolés en République islamique d'Iran ont fait l'objet d'une étude de polymorphisme aléatoire de l'ADN amplifié par PCR (RAPD-PCR). Sur les 82 isolats, 80 provenaient de lésions cutanées, un (1) d'une lésion pharyngée chez l'homme et un (1) d'un chien. Parmi ces derniers, 42 isolats appartenaient à l'espèce *L. tropica*, 36 à *L. major* et 2 à *L. infantum*. Il y avait deux isolats non identifiés (provenant de la lésion pharyngée et d'une lésion cutanée) ; ceux-ci présentaient une similitude avec *L. tropica* et *L. infantum* à 52 % et 48 %. *L. tropica* et *L. major* ont tous deux été isolés dans quatre provinces, ce qui indique une évolution récente de l'épidémiologie de la leishmaniose cutanée. *L. tropica* a été isolé dans trois provinces, *L. major* dans une province. *L. infantum* a été isolé dans une lésion cutanée chez l'homme et sur un chien dans la province de Bushehr.

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Received: 03/05/01; accepted: 28/08/01

Introduction

Leishmaniasis is a major public health problem in the Islamic Republic of Iran. There are over 30 000 new cases every year. Both forms of cutaneous leishmaniasis (CL) are present. Zoonotic cutaneous leishmaniasis (ZCL) is found in many rural foci of the country. Wild rodents, i.e. *Rhombomys opimus*, *Nesokia indica* and *Meriones libycus* are the reservoir hosts [*1*]. Anthroponotic cutaneous leishmaniasis (ACL) is also endemic in many large and medium-sized cities. *Phelebotomus papatasi* is the main vector of disease [*1,2*]. The spectrum of clinical manifestations varies from a simple nodule to erysipeloid and lupoid forms [*3,4*]. Visceral leishmaniasis caused by *Leishmania infantum* is endemic in Fars province and the north-western part of the country [2]. CL due to *L. infantum* has also been reported recently [*5*]. From early 1990 there have been several outbreaks of CL in different parts of the Islamic Republic of Iran. In spite of reports of various clinical manifestations by one species, *in vivo* resistance to antimonial treatment and different reservoir hosts in nature, further knowledge of causative agents seems to be needed. Identification of parasites in man and in animals is also necessary for treatment and control of the infection.

A random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) technique has been found to be a simple and sensitive method for discrimination of *Leishmania* organisms [*6*]. In this study this technique was applied for characterization of organisms isolated mostly from patients with clinically suspected CL, and also from some other sources.

Methods

A total of 52 isolates were recovered from patients clinically suspected of CL. These patients were referred by dermatologists to the relevant laboratories in the different provinces for diagnosis. In addition, 20 isolates of *Leishmania* were received from Kerman where vaccination projects using killed *L. major* and BCG were in progress. These isolates were also from patients suspected of CL. Another 8 isolates studied were from Teheran from patients from various parts of the country. One isolate was from Afghanistan and another one was isolated from throat lesions of a patient in Shiraz. Finally, one of the parasites studied was isolated from a dog in Bushehr. Numbers and sources of various isolates are given in Table 1.

Slit-skin technique was used for obtaining samples, which were spread on precleaned slides and with sterile swabs specimens were taken for culture on Novy–MacNeal–Nicolle (NNN) medium. The prepared smear was stained with Giemsa and the culture was incubated at 25 °C and checked for growth of *Leishmania* promastigotes.

To prepare DNA, organisms were grown in RPMI-164 (Sigma, Dorset, United Kingdom) or brain–heart infusion broth plus 10%–20% heat inactivated fetal calf serum. Cells were harvested and washed with phosphate buffered saline (PBS). Then 150 mL of lysis buffer (50 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8, 1% Tween 20, 8.5 mL proteinase K from 19 mg/mL) were added and incubated for 2 hours at 55 °C. The lysate was extracted with phenol–chloroform. DNA was precipitated with absolute ethanol, washed with 70% ethanol, dried and dissolved in Tris-EDTA (TE) buffer as described elsewhere [*7*]. However, the DNA of some parasites was extracted with TELT (Tris-HCl 0.5 M, EDTA 0.5 M, LiCl 212 g, 2%Triton X-100 in 20 mL) as described by Medina-Acosta et al. [*8*]. The DNA was amplified in 25 mL

Table 1 **Distribution of *Leishmania* organisms isolated and characterized from various parts of the Islamic Republic of Iran**

Province	No. of isolates	*L. tropica*	*L. major*	*L. infantum*	Unknown
Fars	34	16	16	–	2
Kerman	20	13	7	–	–
Yazed	2	2	–	–	–
Isfahan	7	1	6	–	–
Teheran	8	5	3	–	–
Bushehr	2	–	–	2	–
Golestan	2	2	–	–	–
Khorasan	3	–	–	–	
Khuzestan	4	–	4	–	–
Total	82	42	36	2	2

of PCR consisting of 1 unit taq DNA polymerase (Boehringer Mannheim), 2.5 mL of PCR buffer, 200 mL of each deoxyribonucleotide (Boehringer Mannheim), 1 mM random primer and 10–20 ng of DNA. The reaction was carried out in a thermocycler (Techne, Cambridge, United Kingdom) programmed at one cycle at 94 °C for 3 minutes followed by 30 cycles at 94 °C for 30 seconds, 36 °C for 1 minute and 72 °C for 2 minutes. Then 12 mL of each reaction was run on 1.2% agarose gels and visualized under ultraviolet light with ethidium bromide. Parasites were identified by comparison to reference strains *L. tropica* (MHOM/IR/89/ARD 2), *L. major* (MHOM/IR/54/LV 39) and *L. infantum* (MHOM/IR/59/LEM 188) as described elsewhere [7]. Four primers were used for identification (Table 2).

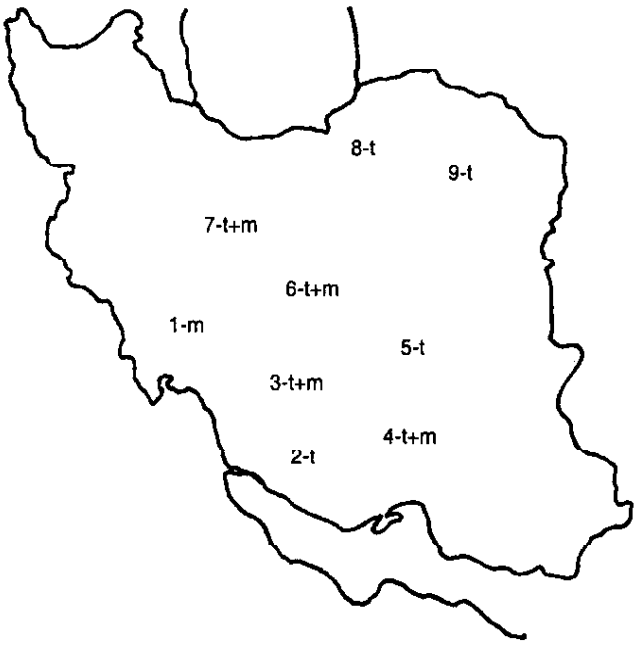
Results

The geographical locations of the 82 *Leishmania* isolates are given in Figure 1. The 42 *L. tropica* isolates were found in the provinces of Fars and Kerman (southern Islamic Republic of Iran), Esfahan, Yazd and Teheran (central Islamic Republic of Iran), and Golestan and Khorasan (northern Islamic Republic of Iran). The 36 *L. major* isolates were from the provinces of Fars, Kerman, Teheran and Khuzestan (southwestern Islamic Republic of Iran). The two *L. infantum* isolates, one from a human a cutaneous lesion and another from a dog, were from Bushehr province (southern Islamic Republic of Iran). Two strains from Fars province could not be identified; one

Table 2 **Primers used for identification**

Primers	Sequences
AB1-05	TGCGCCCTTC
AB1-07	GGTGACGCAG
AB1-14	TTCCCCCGCT
AB1-17	AGGGAACGAG

All primers came from Advance Biotechnologies, United Kingdom.



Provinces: 1-Khuzestan; 2-Bushehr; 3-Fars; 4-Kerman; 5-Yazd; 6-Isfahan; 7-Teheran; 8-Golestan; 9-Khorasan.
Parasites: i = *L. infantum*; t+m = *L. tropica* and *L. major*; t = *L. tropica*; m = *L. major*.

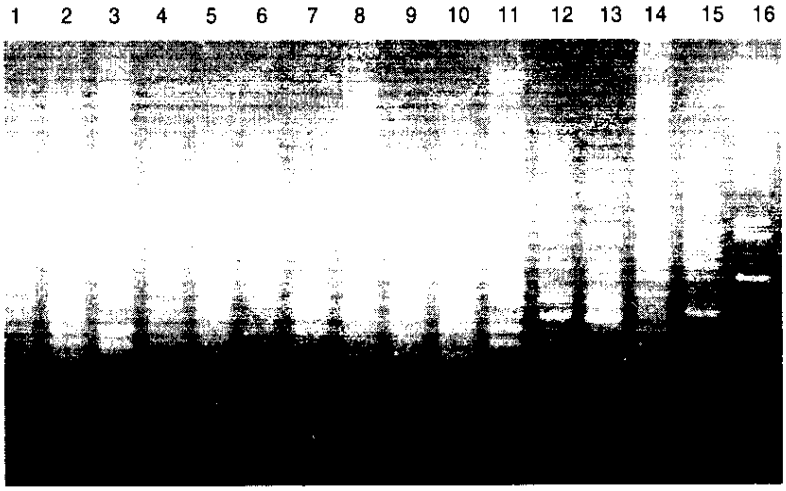
Figure 1 **Distribution of isolated parasites among the provinces of Islamic Republic of Iran**

was isolated from a human throat and the other from a chronic cutaneous lesion.

In four provinces (Fars, Kerman, Esfahan and Teheran) both *L. tropica* and *L. major* were found. In three provinces, *L. tropica* alone was found. In only one province was *L. major* alone found to be the causative agent of CL. Also *L. infantum* was the responsible agent for a visceral form of the disease only in one province (Table 1). The isolated parasite from the Afghan patient was *L. major*. Gel electrophoresis of some isolates by AB1-07 primer is shown in Figure 2.

Discussion

Identification of parasites is necessary for the control of disease because of different reservoir hosts and methods of treatment. According to World Health Organization recommendations, CL caused by *L. major* should not be treated by antimonial treatment except in severe cases. Patients with *L. tropica* however should be treated this way because lesions caused by this parasite remain a potential source for transmission of infection.



Nos. 1, 2, 4–6 and 8–10 were identified as *L. tropica* by 1145 and 890 kb polymorphic bands.
Nos. 3, 7, 11, 12 were identified as *L. major* by 610 and 460 polymorphic bands.
Nos. 13–15 were identified as *L. major*, *L. tropica* and *L. infantum* (1480,810 polymorphic bands).
Reference strains and marker, no. 16 (5148,4268,3530,2027,1904,1584,1375,947,831,554 kbs from the top to the bottom).

Figure 2 **Electrophoresis of RAPD-PCR product of isolated parasites from the provinces by AB1-07 primer on 1.2% agarose gel**

Characterization of parasites in the past was mainly based on the clinical manifestations, geographical foci of distribution of the disease in humans, and biological characteristics of the parasites in laboratory animals. Recently the new methods, such as mAbs and isoenzymes have been used for characterization [*9*]. These techniques have clarified some aspects of the disease, e.g. that one species can elicit a spectrum of clinical manifestations [*3–5*]. RAPD-PCR is a genomic based method. It can demonstrate the genomic diversity among species and between species. Genomic variation and hybrids can also be identified by this method [*10,11*].

In the present study 82 isolates from different provinces were studied and all but 2 were identified. These 2 isolates were from two patients in Fars province, southern Islamic Republic of Iran where all clinical forms of the disease have been reported. The two unidentified parasites showed 52% and 48% similarity with *L. tropica* and *L. infantum*; the isolates showed more similarity to *L. tropica* than *L. infantum* but the reference strain of *L. donovani donovani* was not available for comparative studies. In these two cases, there may have been mixed infections or a hybrid isolate. Ardehali et al. reported cross-reaction with four isolates of *L. tropica* and *L. infantum* in this area. Therefore the possibility of mixed infections cannot be ruled out. Cloning from the culture might answer the question of mixed infec-

tion. Mixed infection has been reported in humans in Sudan and also from *Rhombomys opimus* in the former Soviet Union as both *L. major* and *L. gerbili* were isolated from the same animal. Hybrid parasites have been reported from Saudi Arabia and Ecuador [*12,15*]. In such cases it is necessary to use other techniques, such as pulsed field gel electrophoresis, sequencing of kinetoplast and isoenzyme studies, to answer these questions.

In the past there were limited foci of CL in different parts of the Islamic Republic of Iran. However, CL is now one of the most prevalent diseases and a public health problem. Outbreaks of the disease are being reported from most parts of central, southern and eastern regions of the country. In these areas natural and geographical factors, such as weather, agricultural development and the migration of refugees from Afghanistan, have provided suitable conditions for further spread of the disease.

ZCL, which had previously been observed in only a few areas, now appears to have spread to most parts of the country [*2*]. This could be attributed to leishmanization of soldiers during the Iran–Iraq war, which resulted in 2% having unhealed lesions, and also to population growth which resulted in urban development spreading to the countryside where wild rodents are prevalent.

Our study demonstrates the coexistence of two different species (*L. tropica* and *L. major*) in four provinces: Fars, Kerman, Isfahan and Teheran. Given these facts, the use of new methods for the characterization of parasites is important and efforts are needed to clearly identify vectors, reservoir hosts and parasites in order to control the disease.

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