The PCR-based detection and identification of the parasites causing human cutaneous leishmaniasis in the Iranian city of Ahvaz

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In Iran, *Leishmania major* or *L. tropica* cause almost all of the human cutaneous leishmaniasis (CL). Unfortunately, the detection methods frequently used for CL (the microscopical examination of direct smears or the culture of biopsies) are not very sensitive and the *Leishmania* species causing each case of CL in Iran is usually only tentatively identified from extrinsic factors, such as the case's clinical manifestations and region of residence. Recently, however, a nested PCR that targets the parasites' kinetoplast DNA has been used in the city of Ahvaz (the capital of the province of Khuzestan, in south-western Iran) to confirm the microscopical diagnosis of CL and to identify the causative parasites, to species level. Smears from the lesions on 100 suspected cases of CL were fixed, stained with Wright's eosin-methylene blue, and checked for amastigotes under a light microscope. Scrapings from the same smears were then tested for leishmanial DNA, using a nested PCR that allows the DNA from *L. tropica* to be identified and distinguished from that of *L. major*. The 100 smears investigated were all found amastigote-positive by microscopy and PCR-positive for either *L. major* DNA (97 smears) or *L. tropica* DNA (three smears). The predominant species causing CL in Ahvaz is therefore *L. major*.

The various forms of human leishmaniasis are an important public-health problem in many tropical and subtropical countries (WHO, 1990; Razmjou *et al.*, 2009). The cutaneous form of the disease has a wide spectrum of clinical manifestations, depending on the causative species (WHO, 1990; Dowlati, 1996). Although such cutaneous leishmaniasis (CL) rarely causes severe morbidity, the lesions may take several months to heal and often leave ugly scars on the face or other exposed areas of skin. At least two species of Old World *Leishmania* are responsible for the CL seen in Iran, which usually presents as

self-healing ulcerative lesions but occasionally takes on a metastatic form (Javadian *et al.*, 1976; Nadim *et al.*, 1977; Motazedian *et al.*, 2006). Atypical, mucosal infections with leishmanial parasites have also been observed in Iran, albeit rarely (Yaghoobi, 2001).

Both zoonotic cutaneous leishmaniasis (ZCL) caused by *Leishmania major* and anthroponotic cutaneous leishmaniasis (ACL) caused by *L. tropica* occur in Iran, ZCL being endemic in rural areas in the centre, north-east, south-west and south-east of the country and ACL being largely confined to some large and medium-sized cities (Nadim *et al.*, 1994). In the subtropical province of Khuzestan, which lies in south-western Iran and shares a border with Iraq, the annual incidence of CL

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has risen worryingly over the last decade, with major outbreaks of the disease in 2003 and 2004 and an increase in the percentage of CL cases attributed to *L. major* (Maraghi *et al.*, 2007).

In areas where two or more species of CL-causing *Leishmania* co-occur, the accurate identification of the parasite(s) causing each case of CL is necessary if the local measures for the control of the disease are to be effective (Grimaldi and Tesh, 1993). The detection methods frequently used for CL (i.e. the microscopical examination of direct smears and/or the culture of biopsies) are not very sensitive and the *Leishmania* species causing each case of CL in Iran is usually only tentatively identified from extrinsic factors, such as the case's clinical manifestations and region of residence. Diagnostic methods based on deoxyribonucleic acid (DNA) have, however, now made the sensitive and rapid detection and identification of many micro-organisms possible. Over the last couple of decades, for example, assays based on PCR have proved to be sensitive and powerful tools for the detection and identification of *Leishmania* in clinical samples, without the need to culture the parasites (Rodgers *et al.*, 1990; Smyth *et al.*, 1992; Lopez *et al.*, 1993; Noyes *et al.*, 1998). In the present study, based in Ahvaz (the capital city of Khuzestan), a nested PCR was used to confirm the microscopical diagnosis of CL and identify the causative parasites to species level.

PATIENTS AND METHODS

Study Area

The city of Ahvaz (31°19'39"N, 48°41'38"E; Fig. 1) lies at a mean elevation of 20 m above sea level and has long, hot summers and short, mild winters. Summer temperatures often exceed 50°C.

Preparation of Slit Smears

Slit biopsies were collected, with a sterile vaccinostyle, from the edge of a cutaneous

lesion on each of 100 suspected cases of CL who presented, consecutively, in Ahvaz in 2003–2004. Each biopsy was spread on a glass slide, allowed to dry, fixed in methanol, stained with Wright's eosin–methylene blue (Merck, Darmstadt, Germany) and checked for amastigotes, under oil immersion at $\times 1000$, by light microscopy. For each suspected case of CL, the case's age and gender, the time between the lesion being first noticed and presentation at a health facility, the number of skin lesions and the location of each lesion, on the patient's body, were recorded.

DNA Extraction

After the microscopy, the immersion oil on each smear was gently wiped off with xylol on a paper tissue. The entire smear was then scraped off the slide, with a sterile scalpel, so that the total DNA in the smear could be extracted by digestion, in a 1.5-ml microcentrifuge tube, with 200 μ l lysis buffer [50 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1% (v/v) Tween 20] containing 8.5 μ l of a proteinase-K solution that had 19 mg enzyme/ml (Yokota *et al.*, 1995; Motazedian *et al.*, 2002). The tube was incubated overnight, at 37°C, before 200 μ l phenol/chloroform/isomyl alcohol (25:24:1, by vol.) were added to it. The tube was then shaken vigorously and centrifuged at 6000 $\times g$ for 10 min. The resultant supernatant solution was transferred to another tube and mixed with 400 μ l absolute ethanol. The DNA that precipitated was centrifuged down (at 6000 $\times g$ for 20 min), dried, dissolved in 50 μ l ultrapure water [produced in a Purelab® UHQ system (Siemens Water Technologies, Warrendale, PA)], and stored at 4°C (Motazedian *et al.*, 2002) before use in the PCR-based assay.

Nested-PCR-based Assay

The variable segments on minicircles of kinetoplast DNA from any *Leishmania* parasites present in the smear scraping were amplified with two rounds of nested PCR (Noyes *et al.*, 1998). The primers were

FIG. 1. Map of Iran, showing the locations of Khuzestan province and the city of Ahvaz.

CSB1XR (ATT TTT CGC GAT TTT CGC AGA ACG) and CSB2XF (CGA GTA GCA GAA ACT CCC GTT CA) for the first round, and LiR (TCG CAG AAC GCC CCT) and 13Z (ACT GGG GGT TGG TGT AAA ATAG) for the second. Each 25- μ l, first-round reaction mixture contained 5 μ l template DNA, 200 μ M of each deoxynucleoside triphosphate (Roche, Penzberg, Germany), 1.5 mM $MgCl_2$, 1.0 U *Taq* polymerase, 50 mM Tris-HCl (pH 7.6), 10 μ M CSB1XR and 10 μ M CSB2XF. The thermocycler used (MyCycler; Bio-Rad, Hercules, CA) was set to give 5 min at 94°C, followed by 30 cycles, each of 30 s at

94°C, 1 min at 55°C and 1.5 min at 72°C, and then a final extension for 5 min at 72°C. The product of this first round of PCR was diluted 1:9 with ultrapure water and then 1 μ l of this dilution was used as the template for the second round of PCR, which used the same conditions and reaction mixture as the first round except that LiR and 13Z were used as the primers. A 5- μ l sample of the second-round product was subjected to electrophoresis in 1.5% (w/w) agarose gel, stained with ethidium bromide and visualized by ultraviolet trans-illumination (Moemenbellah-Fard *et al.*, 2003). The size of each amplicon detected was estimated by comparison with a

FIG. 2. The results of the electrophoresis of the products of the nested-PCR-based amplification of DNA extracted from the stained smears. The nine lanes contained a molecular-weight 'ladder' (lane 1), a negative control (lane 2) and the products from reference strains of *Leishmania tropica* (lane 3) and *L. major* (lane 4) and test samples identified as *L. tropica* (lane 8) or *L. major* (lanes 5, 6, 7 and 9).

100-bp–1500-bp molecular-weight 'ladder' (Roche) run on the same gel.

As positive controls, DNA extracted from promastigote cultures of reference strains of *L. major* (MHOM/IR/54/LV39) and *L. tropica* (MHOM/IR/89/ARA2) was run on each gel (Pourmohammadi *et al.*, 2008). These strains, which were routinely maintained on Novy–MacNeal–Nicolle medium, were transferred to Roswell Park Memorial Institute medium 1640 (Invitrogen, Carlsbad, CA) enriched with 20% (v/v) foetal calf serum (Invitrogen) prior to the extraction of their DNA. Negative controls (the products of PCR in which ultrapure water replaced the template DNA) were also run.

RESULTS

All 100 smears investigated were found positive for amastigotes by microscopy and

positive for leishmanial DNA in the nested PCR. Three (3%) of the smears gave the 750-bp second-round amplicon indicative of *L. tropica* and the rest (97%) gave the 560-bp product indicative of *L. major* (Fig. 2). No other sizes of second-round amplicon were seen, no sample gave more than one amplicon (indicating a mixed infection), and no amplicons were detected in the negative-control samples.

Most (57) of the suspected cases of CL investigated were aged <21 years but the number of male cases was similar to the number of female cases (see Table). The time between the appearance of the lesion and presentation was 1–3 months for the 97 cases infected with *L. major* but >3 months for the three cases infected with *L. tropica*. Although most of the lesions caused by *L. major* were seen on the hand, whereas those caused by *L. tropica* were equally

FIG. 3. The lesions of cutaneous leishmaniasis seen on two patients from Ahvaz, in south-western Iran, who were found to be infected with *Leishmania tropica* (a) or *L. major* (b).

likely to occur on the face, neck and hands, this difference did not reach statistical significance. The number of lesions/case tended to be greater with *L. major* than with *L. tropica*, the greatest number of lesions seen on a case (14) occurring on a patient infected with *L. major*. In the present study, although (as expected) ‘wet’ lesions were mainly attributed to *L. major* and ‘dry’ lesions to *L. tropica* (Fig. 3), dry lesions were seen in 15 of the 97 patients found PCR-positive for *L. major* DNA while one of the three cases found PCR-positive for *L. tropica* DNA had a wet lesion.

DISCUSSION

With the incidence of the disease in the city increasing over the last decade, many local clinicians now consider CL to be the most important public-health problem in Ahvaz (unpubl. obs.). Identifying the *Leishmania* species that is responsible for each case of CL or, at least, for the vast majority of cases (if any species predominates) can be important for effective treatment and the design of an appropriate and effective programme for the control of the disease (WHO, 1990). Lesions caused by *L. major*, for example, should not be treated with antimonial compounds except in severe cases (Markle and Makhoul, 2004). Until now, the *Leishmania* parasites causing CL in Iran (and in many other areas where the disease is endemic) — which, whether in their promastigote or amastigote forms, are morphologically quite similar — have only been tentatively identified to species level, on the basis of extrinsic factors such as clinical manifestations and the existing (and often outdated) knowledge of the geographical distributions of each *Leishmania* species. Although such factors may improve the accuracy of any parasite identification, they

TABLE. The numbers of male and female cases of cutaneous leishmaniasis who were investigated, split by age-group

Age (years)	No. of cases:		
	Male	Female	Investigated
<10	18	13	31
11–20	9	17	26
21–30	6	9	15
31–40	4	11	15
>40	5	8	13

are insufficient for the definitive identification of the causative agents of CL.

Various molecular, immunological and biochemical methods have been developed to identify and characterise *Leishmania* species (Pourmohammadi *et al.*, 2010). Currently, the most commonly used methods involve iso-enzyme electrophoresis, monoclonal antibodies, specific DNA probes or PCR with or without RAPD analysis (Jaffe and McMahon-Pratt, 1983; Rioux *et al.*, 1990). Several PCR-based assays have been developed to detect and identify leishmanial parasites (Barker, 1989; De Bruijn and Barker, 1992; Piarroux *et al.*, 1994; Osman *et al.*, 1997; Noyes *et al.*, 1998; Fakhar *et al.*, 2008). The results of the present study were encouragingly clear-cut; every test sample produced a single amplicon (that matched, in size, the single amplicon produced from the reference strains of *L. major* or *L. tropica*) and the absence of amplicons from the negative-control samples indicated the absence of contamination that could have given false-positive results.

In the present study, curiously, patients infected with *L. major* presented more quickly (after their lesion appeared) than the patients infected with *L. tropica*. Although Maraghi *et al.* (2007) made the same observation in another study in Khuzestan, the reasons behind this difference remain unclear.

It appears that, in 2003–2004, *L. major* was the cause of almost all the CL in Ahvaz with *L. tropica*-attributable disease present but relatively rare. Although *L. major* and the ZCL that it causes are generally considered rural, it appears that the citizens of Ahvaz are much more likely to be infected with *L. major* than with (the usually more urban) *L. tropica*, perhaps because the local development of a sugar-cane industry and irrigation systems and the outward spread of the city's human populations have increasingly brought the city's residents into the habitats of the local vectors and reservoir hosts of *L. major* (Javadian *et al.*, 1998). Maraghi *et al.* (2007) made similar observations in the city

of Shoush, which lies, in Khuzestan, to the north-west of Ahvaz. It appears that the epidemiological pattern of CL in several endemic areas of Iran has changed in recent years, with *L. major* becoming the predominant causative agent of CL in urban areas as many cities expand into rural areas where the colonies of the rodents that act as reservoir hosts in ZCL are found (Davami *et al.*, 2010).

In conclusion, the CL found in Ahvaz is now predominantly ZCL. The PCR-based assay used in the present study appears to be a suitable and powerful tool for the characterisation of at least two *Leishmania* species and the confirmation of CL.

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