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Determination of *Leishmania* species causing cutaneous leishmaniasis in Mashhad by PCR-RFLP method

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ABSTRACT

Three species of *L. tropica, L. major* and *L. infantum* are known as main causal agents of leishmaniasis have been reported in Iran. Since cutaneous leishmaniasis (CL) is endemic in North East of Iran, in the present work, 50 *Leishmania* positive isolates from human cases in Mashhad (Center of Razavi province, North East of Iran), were genotyped by means of polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) of the mini-exon gene. Our results showed that the *Leishmania tropica* was more prevalent in Mashhad where among 50 isolates, 17 were detected *Leishmania major* (34%) and 38 samples were *Leishmania tropica* (66%).

Keywords: *Leishmania major*, *Leishmania tropica* , PCR-RFLP , mini-exon gene

INTRODUCTION

Leishmaniasis is a parasitic disease which is associated with a wide spectrum of clinical manifestations prevalent in the some parts of Iran. Three species of *L. tropica, L. major* and *L. infantum* are known as main causes of leishmaniasis have been reported in Iran (Ardehali *et al* 1994). Cutaneous Leishmaniasis (CL) is endemic in some parts as well as North East of our country (Javadian *et al* 1976). Correct diagnosis of a *Leishmania* species is essential to determine the clinical prognosis and a species-specific therapeutic approach (Romero *et al* 2001). Specification of different species of genus *Leishmania* depends on several factors such as the geographical distribution of an isolate, the clinical finding of the disease and the epidemiology of the vector and the animal reservoir (Lainson & Shaw 1987, Pearson *et al* 2001). Diagnosis of the parasite species based on geographic location or the site of infection is not satisfactory. Up to now, a variety of biochemical, immunological or molecular criteria such as characterization by isoenzyme electrophoresis (zymodeme analysis) (Kreutzer & Christensen 1980) or monoclonal antibodies (Grimaldi *et al* 1987, Valizadeh *et al* 2004) or by hybridization with species-specific probes such as minicircle DNA probes (Wirth *et al* 1989) have been introduced for classification of pathogenic species. In addition, several PCR based assays for species differentiation of genus *Leishmania* were developed. The gene targets for amplification used either nuclear DNA, such as the SSU rRNA gene (van Eys *et al* 1992), repetitive sequences (Piarroux *et al* 1995), internal transcribed spacer (ITS) regions (Cupolillo *et al*

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1995, Eisenberger & Jaffe 1999), the tubulin gene (Luis *et al* 1998), the GP63 gene locus (Victoir *et al* 1998), microsatellite DNA (Russell et al., 1999), or extra-chromosomal DNA, such as the repetitive kinetoplast DNA (kDNA) minicircles (De Bruijn & Barker 1992, Belli *et al* 1998, Felger *et al* 2003). Most molecular diagnostic methods developed for genotyping *Leishmania* species were based on the polymorphic kDNA minicircle, which is considered a prime candidate for a sensitive assay because of the presence of 10000 to 20000 minicircles per cell. The mini-exon gene, which is involved in the trans-splicing process in kintoplastid protozoa, is present 100 to 200 times per nuclear genome as randomly repeated copies, and it is absent from the vertebrate host or invertebrate vector. A detailed study on sequence variation in the mini-exon gene repeat of human pathogenic *Leishmania* species had previously shown that the diversity detected in the non-transcribed spacers represents an informative phylogenetic marker (Fernandes *et al* 1994).

Like Felger *et al* (2003), we have used a PCR approach similar to that of Fernandes *et al* (1994), but we applied restriction enzymes to digest the PCR product. The resulting patterns of restriction fragments were characterized for each species causing cutaneous Leishmaniasis in Mashhad, North East of Iran.

MATERIALS AND METHODS

Study area. The study was conducted over a period of 2 years (from 2006 to 2007) in North East of Iran (Mashhad), where CL is endemic. Samples were collected from patients visiting the Dermatology Department of the Imam Reza Hospital in Mashhad, with lesions suspected to cutaneous leishmaniasis. Fifty positive samples were collected directly from skin scars of the patients on filter paper as well as on slides. In addition aspirated samples were taken from lesions for culturing in NNN (Novy-MacNeal-Nicolle medium).

Microscopical examination. Smears on glass slides, air dried and fixed with methanol for a few seconds. The smears stained with Giemsa were examined by using light microscope.

In vitro cultivation of *Leishmania* .

Cultures were obtained by needle aspiration from the border of the lesions. Usually it was necessary to inject about 0.1 ml of sterile 0.9% saline into the lesion in order to aspirate a drop of fluid (Herwaldt 1999). The aspirated fluid was discharged into the culture tubes, NNN medium, supplemented with 10% fetal calf serum, 200 µg/ml streptomycin, and 200 U/ml penicillin. The cultures were incubated at 24-28 °C. After a few days one drop of media was examined microscopically. If promastigotes were found the cultures were transferred into $RPMI_{1640}$ for further culturing and counted with the Cell Counter. The cells were washed twice with 0.15 M phosphate-buffered saline (PBS) pH 7.4, pelleted by centrifugation.

DNA extraction. Promastigote forms of the parasite were transferred into 500 µl digestion buffer (10 mM Tris/HCl pH 8; 5 mM EDTA; 0.5% SDS). Proteinase K (Sigma-Aldrich, Inc., CH) was added to a final concentration of 5 mg/ml and the sample was incubated over night at 56 °C. DNA was extracted successively with one volume of phenol pH 8.0, one volume of phenol/chloroform, and one volume of chloroform according to standard procedures (Sambrook & Russell 2001). DNA was precipitated from the aqueous phase with ethanol, the pellet washed with 75% ethanol, air dried and finally resuspended in 35 µl H_2O.

PCR. Primers were designed based on the mini-exon gene repeat that identified by Felger *et al* (2003), including: forward primer Fme (5'-TAT TGGTAT GCG AAA CTT CCG-3') and reverse primer Rme (5'-ACA GAA ACT GAT ACT TAT ATA GCG-3'). PCR was performed by using the PCR mixture contained 2 µl of DNA solution were

amplified in a 100µl reaction containing 50 mM KCl, 20 mM Tris-HCl pH 8.4 (CinaGen), 0.2mM dNTPs (CinnaGen),12% DMSO, 40 mM tetramethylammonium chloride,1.5 mM MgCl2 (CinaGen), 0.5µM of each primer (CinnaGen), and 1U Taq DNA polymerase. The PCR conditions were 5 min at 94 °C followed by 25 to 35 cycles of 30 sec at 94 °C, 30 sec at 54 °C, and 45 sec at 72 °C. The number of cycles depended on the origin and concentration of the template. PCR products were separated on a 1.5% agarose gel.

Restriction digestion. RFLP were performed according to methods described by Felger *et al* (2003). 10 µl of the PCR products was digested in a 20 µl reaction mixture containing 10U of *HaeIII* (Fermentas) and 2µl of the appropriate restriction buffer at 37ºC water bath for overnight. All 20µl of the digest was used for electrophoresis on 2.5% agarose gel. Fragment sizes were estimated by comparison with bands of a DNA length standard (100 bp ladder DNA size marker, CinnaGen Co).

RESULTS

Mini-exon PCR products from different *Leishmania* species (CL) in Mashhad is shown in Figure 1. Old World species (*L. major* (lane 1,2), *L. tropica* (lane 3, 4)) gave rise to amplification products of 435 bp (Figure 1). The RFLP pattern of two species on an agarose gel after diagnostic restriction digests with *Hae III*. Digestion produced one fragment (127 bp) from *Leishmania major* (lane 5, 6), while two different sized fragment (225 and 178 bp) were detected in *Leishmania tropica* (lane 2, 3) (Figure 2).

Of 50 samples have been studied in this assay, 17 (34%) samples were detected *Leishmania major* and 37 (66%) samples were *Leishmania tropica* by specific PCR-RFLP method.

DISCUSSION

Correct diagnosis of *Leishmania* species is essential to determine the clinical prognosis and a species-specific therapeutic approach (Romero *et al* 2001), contrary wrong diagnosis may lead to mistreatment. Most commonly used methods for the direct detection of the parasite (e.g., microscopic examination of Giemsa-stained smears and in vitro cultivation) lack sensitivity because of the scarcity of *Leishmania* parasites in some specimens (Weigle *et al* 1987) or the parasites may be scanty and are mostly extracellular in the slide preparations, or are hampered by the problem of contamination (Berman 1997). Indirect methods such as serological methods are also limited in sensitivity and are not able to differentiate between the species or past and current infections (Felger *et al* 2003). The PCR technique has opened new windows in the diagnosis of Leishmaniasis, and several approaches have been developed during the last two decades (Wirth *et al* 1989). Most of these assays are based on highly repetitive genomic gene loci or extra-chromosomal kinetoplast DNA sequences. In fact, kinetoplastids possess several unusual features that differentiate them from other eukaryotes. The structure of kinetoplast DNA (kDNA) is unique for a mitochondrial genome (Shlomai 1994), kDNA also exhibits a unique form of post-transcriptional RNA processing termed ' RNA editing' (Seiwert 1995). The 25-50 identical maxicircle DNA molecules of each kinetoplast encode various mitochondarial proteins, but the primary RNA transcripts from these are not directly translatable. When compared to the final transcript the gene sequences are found to contain insertions and deletions of uridine residues (Ashford & Bates 1998). According to the Fleger *et al* (2003) study, the detection limit was 50 parasites for *Leishmania* species producing amplification products ranging from 300 to 450 bp. It has been shown that the assay is specific to the

Leishmania genus (CL) (Felger *et al* 2003). Regarding epidemiological point of view, our results showed that the *Leishmania tropica* was major species in Mashhad. The study of Hajjaran *et al* in Mashhad also showed that *L. tropica* was the main cause of CL which is accordance with our result. Moreover, the Zahraei-Ramazani *et al* Study revealed that Anthroponotic Cutaneous Leishmaniasis was epidemic in some non-endemic quarters in the city of Isfahan. But Tashakori *et al* in their study indicated that *L. major* was the major cause of the disease in Isfahan, Dehloran and Kashan. The results of Maraghi *et al* study by PCR technique indicated that the predominant species in Shush City (Khuzestan Province) was *L. major*. In addition, according to the Fazaeli *et al* study *L. major* was the species responsible for CL in Mirjaveh. *Leishmania tropica* usually causes dry lesions on face, observed in four seasons and its vector is *Phelebotomus sergenti* in Khorasan Razavi. *Leishmania major* causes wet lesions and was observed in autumn and winter and its vector is *Phlebotomus papatasi* in the region.

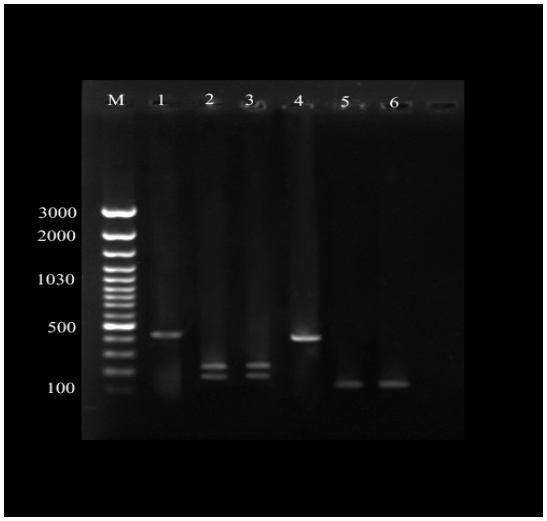

Figure 1. Ethidium-bromide-stained agarose gel of PCR products of mini-exon gene of *Leishmania* species (CL) from different isolates. M molecular marker (100 bp), Lanes 1 & 2 *L. major*; Lanes 3 & 4 *L. Tropica*

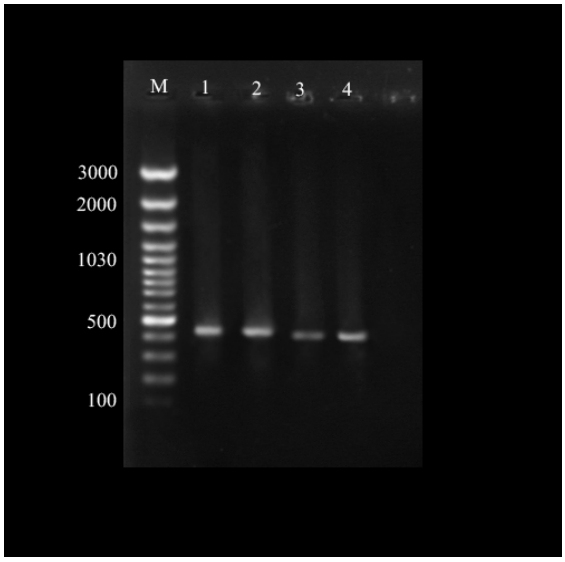

Figure 2. Ethidium-bromide-stained agarose gel of *HaeIII* digested PCR products of mini-exon gene of *Leishmania* species (CL) from different isolates. M molecular marker (100 bp), Lanes 1 & 4 non-digested, *Lanes 2 & 3 L. tropica;* Lanes 5 & 6 *L. major.*

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