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# IDENTIFICATION OF *LEISHMANIA* SPECIES FROM DIFFERENT PARTS OF IRAN USING A RANDOM AMPLIFIED POLYMORPHIC DNA IN HUMANS, ANIMAL RESERVOIRS AND VECTORS

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## ABSTRACT

In this study, we used Random Amplified Polymorphic DNA (RAPD) for identification of 17 isolates of *Leishmania* from the skin and reticuloendothelial system of humans, animal reservoirs (rodent and dog) and sandflies in various parts of Iran in the last decade.

Fifteen species have been confirmed by isoenzyme characterization by the London School of Hygiene and Tropical Medicine and Shiraz University of Medical Sciences. In this study, strong confirmation has been observed between random amplified polymorphic DNA with isoenzyme characterization.

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**Keywords:** *Leishmania* species, RAPD-PCR, Iran.

## INTRODUCTION

Leishmaniosis is a major public health problem. It is estimated that there are 12 million human cases worldwide, and approximately 350 million to be at risk.[7] In Iran at least 20,000 cases per year have been reported. About 20 species of *Leishmania* are known to infect humans, causing a spectrum of symptoms ranging from simple self-healing skin ulcers to disseminated cutaneous lesions and life-threatening visceral involvement. Previous study has shown that different clinical forms of leishmaniosis can be produced by morphologically the same parasites, and a major problem over the past decade has been to identify and differentiate the species of parasites responsible for the various clinical forms of disease. Precise taxonomic identification of parasites is essential for epidemiologic studies.[2,6] A number of biochemical methods are available for identification and classification of *Leishmania* but none of them are ideal.[4] Recently, the random amplified polymorphic DNA (RAPD-PCR) method has provoked a great deal of interest.[4,5] It is relatively simple to perform, requires no prior knowledge of the parasite genome, and requires a minimal number of parasites. Previous studies have shown that RAPD can be useful also for identifying members of a sandfly species complex,[5] and for identifying and classifying members of new and old *Leishmania* species.[4,5] In this study we used RAPD-PCR technique for identification of *Leishmania* species isolated from humans, animal reservoirs and vectors from various parts of Iran.

## MATERIAL AND METHODS

In this study, all of the *Leishmania* species have been isolated from cutaneous and mucosal lesions and the reticuloendothelial system of humans, animal reservoirs (rodent, dog) and sandflies by Evans's method.[3] DNA was extracted from the promastigote cultured at 20°C in NNN+LIT* medium. Parasites ($10^4$ in 10 mL) were har-

vested by centrifugation (1000g, 10min) and washed with Lock's solution (150 mM NaCl, 6mM KCl, 4mM $CaCl_2$, 2mM $NaHCO_3$ and 5mM glucose). The pellet was resuspended in 100μL lysis buffer (50 mM Tris-HCl (pH8), EDTA (pH4) 62.5 mM, LiCl 2.5M, Triton X-100 4% V/V). The lysate was extracted once with equal volumes of 1:1 (V/V) phenol: chloroform and once with 24:1 (V/V) chloroform: isoamyl alcohol and precipitated by ethanol. The DNA was resuspended in 100 mL of 10 mM Tris-HCl, 1mM EDTA, and a 1:10 dilution was prepared in PCR grade water. Amplification reactions were done in a total volume of 25 μL containing 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl, pH9, 0.01% (W/V) Tween 20, 2mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, 1mM primer and 1 unit of taq polymerase. One microliter of DNA (20 ng/μL) was added by centrifugation through the mineral oil overlay and the reaction was carried out in a thermocycler (Genus) programmed at one cycle of 94°C for 2 min, followed by 30 cycles of 94°C, 30 sec., 36°C, 1min. and 72°C, 2 min. Twelve microliters of each reaction was run on 1.5% agarose gel and visualized under UV light with ethidium bromide. Primers used in this study are listed in Table I.

**Table I.** The type of primers used in this study.

| No. | Code | Sequence | %CG |
|---|---|---|---|
| 1 | AB1-07 | GGT GAC GCA G | 70 |
| 2 | 327 | ATA CGG CGT C | 60 |
| 3 | 329 | GCG AAC CTC C | 70 |
| 4 | 333 | GAA TGC GAC G | 60 |
| 5 | 335 | TGG ACC ACC C | 70 |

## RESULTS

Leishmania species that were isolated and characterized are as follows:

Ten specimens were isolated from humans (7 from skin, 2 bone-marrow and 1 from the mouth and tongue). Two specimens were isolated from the skin and viscera of infected gerbils, five specimens were isolated from the liver and spleen of infected dogs and two of them were isolated from the phlebotomus midgut. Two species of the isolates originally were from Sudan and Pakistan which were used as the control group (Table II).

Fifteen of the *Leishmania* isolates have also been characterized by isoenzyme method, 6 of which were carried out in London school by Evans and Mazloumi and the others in Shiraz University of Medical Sciences by Hatan and Ardehali.[3]

Table I shows the primers that reliably amplified *Leishmania* species that had been isolated in hu-

**Table II.** Characteristics of 19 isolates of *Leishmania* species concerning locality, hosts, specimens and the results.

| No. | Location | Specimen | Host | Isoenzyme result | RAPD-PCR result |
|---|---|---|---|---|---|
| 1 | Meshkin-Shahr | spleen | Human | *L. infantum* | *L. donovani complex* |
| 2 | Meshkin-Shahr | spleen | Rodent(*Meriones persicus*) | *L. donovani* | *L. donovani complex* |
| 3 | Isfahan | skin | human | *L. major* | *L. major* |
| 4 | Isfahan (Natanz) | skin | human | *L. major* | *L. major* |
| 5 | Sudan* | spleen | human | *L. donovani* | *L. donovani complex* |
| 6 | Karadj (Kordan) | spleen-liver | dog | *L. infantum* | *L. donovani complex* |
| 7 | Baluchestan(Iran) | skin | Rodent(*Meriones hurriane*) | *L. major* | *L. major* |
| 8 | Kerman (Bam) | skin | human | *L. infantum* | *L. donovani complex* |
| 9 | Tehran | spleen | dog | *L. infantum* | *L. donovani complex* |
| 10 | Pakistan (Karachi)* | skin | human | *L. major* | *L. major* |
| 11 | East Azarbaijan | spleen | dog | *L. infantum* | *L. donovani complex* |
| 12 | Tehran | skin | human | *L. major* | *L. major* |
| 13 | Tehran | ulcer of leishmanization | human | *L. major* | *L. major* |
| 14 | Tehran | skin | human | *L. major* | *L. major* |
| 15 | Baluchestan(Iran) | mid-gut | sandfly (*P. salehi*) | - | *L. major* |
| 16 | Baluchestan(Iran) | mid-gut | sandfly (*P. papatasi*) | - | *L. major* |
| 17 | Tehran | mouth and tongue | human | - | *L. major* |
| 18 | Meshkin-Shahr | spleen | dog | *L. infantum* | *L. donovani complex* |
| 19 | Bushehr | spleen | dog | *L. infantum* | *L. donovani complex* |

*Novy MacNeal and Nicolle + Liver Infusion broth tryptose

mans, animal reservoirs and sandfly. Primers 329 and 327 can clearly differentiate *L. donovani* complex from *L. major* and *L. tropica* species as shown in Fig. 1. With primer 329, *L. donovani* complex shares a strong band at 433 bp and *L. major* shows 6 bands from 653 bp to 2176 bp.

## DISCUSSION

Isoenzyme analysis is a standard method for identification of *Leishmania* but practically it needs a bulk of *in vitro* culture of parasites ($7 \times 10^5$ parasites/mL) and is an expensive, slow, laborious method and consequently is not suitable for large scale application. Methods using DNA amplification have the potential for exquisite sensitivity without sacrificing specificity or simplicity. RAPD-PCR has already proved useful in eco-epidemiological studies of *Leishmania* species in the new and old world.[4,5] It needs a small number of parasites ($10^4$ in 10 mL), requires no knowledge of the parasite genome and one unspecific primer, so it is simple to use and can be used in any laboratory. RAPD-PCR has been used in this study for identification of *Leishmania* species and compared to the results of isoenzyme characterization. Five of the 13 primers tested yielded reproducible multiple banding patterns with these specimens that were suitable for numerical analysis, as shown in Table I. The 329 and 327 primers gave finger prints with little similarity between *L. major* and *L. donovani* complex (Fig. 1). All of the species identified with both techniques were the same, with two exceptions of *Leishmania donovani* as shown in Table II. Therefore, RAPD can not distinguish between *L. donovani* and *L. infantum* and needs more experience for discriminating between the *L. donovani* complex. This study showed that RAPD can be used in the field trial and is suitable for epidemiological analysis.

Although RAPD-PCR fingerprints can only be obtained from cultured parasites, sufficient parasites are available in the supernatant of the biphasic media that is obtained after parasite isolation. As a single primer is sufficient for identification pruposes, RAPD has considerable potential for epidemiological surveys in which it is necessary to combine for identification at the species level with the capacity to process large numbers of samples.

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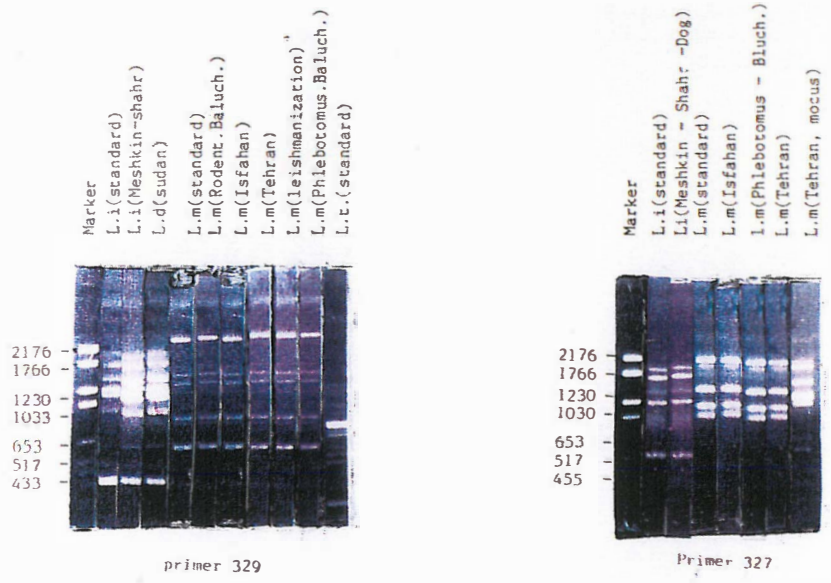


**Fig. 1.** RAPD products of primers 327 and 329 with Leishmania reference strains and parasites isolated from humans, animal reservoirs, and phlebotomus. L.i, L. infantum; L.m, *L. major*; L.t, *L. tropica*; L.d, *L. donovani*.

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