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Comparative molecular epidemiology of *Leishmania major* and *Leishmania tropica* by PCR-RFLP technique in hyper endemic cities of Isfahan and Bam, Iran

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: *Leishmania* is an obligate intracellular protozoa, and the sandfly, as a vector, transmits infectious forms of the parasite to the vertebrate host. The etiologic agents of cutaneous leishmaniasis (CL), *Leishmania major* and *Leishmania tropica,* are the most prevalent factor in Iran, especially in the Isfahan and Bam regions. Because of the importance of CL in endemic regions and the interaction of species diversity factors in developing control strategies, several isolated *Leishmania* species from 2 hyperendemic regions of Iran, Isfahan and Bam cities, were examined in this study by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP).

Material/Methods: In this study, 340 samples were taken from clinically suspected CL patients to prepare slides for direct microscopy and cultures for promastigotes by PCR-RFLP. The internal transcribed spacer 1 (ITS1) region of genomic DNA was extracted and amplified with LITSr and L5.8s primers. Amplification by PCR-RFLP was performed to determine 4 kinds of genotype pattern of the species in the 2 main cities of Isfahan and Bam. Some of the product samples were sequenced and analyzed.

Results: Two genotypic groups were detected from *L. major* isolates, LmA and LmB; also *L. tropica* showed 2 patterns, LtA and LtB, in comparison with standard species. The most prevalent genotypes related to isolates of Isfahan were LmA and of Bam were LtA. These 2 genotypes were recorded as major etiologic factors of CL in these 2 regions.

Conclusions: *Leishmania major* and *L. tropica,* the causative agents of zoonotic CL and anthroponotic CL, respectively, in Isfahan and Bam, are genetically highly polymorphic species, and a correlation may exist between genetic heterogeneous and clinical manifestation and geographic regions of the disease in humans.

key words: **cutaneous leishmaniasis • ITS1 • PCR • RFLP • molecular epidemiology**

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Background

Leishmaniasis is a serious complication for public health and pertains to different diseases caused mainly from manifestations such as cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, and visceral leishmaniasis. These diseases threaten approximately 350 million people in more than 88 countries around the world, with an increasing annual incidence mostly manifested in the CL form [1–4].

Cutaneous leishmaniosis in Iran has 2 forms, zoonotic cutaneous leishmaniasis (ZCL), which is mainly caused by *Leishmania major,* and anthroponotic cutaneous leishmaniasis (ACL), which is mainly caused by *Leishmania tropica*. Hyperendemic Iranian-based areas reported for *L. major* include the provinces of Isfahan, Golestan, Khuzestan, Ilam, Booshehr, and Semnan, whereas *L. tropica* occurs in metropolitan endemic foci including Tehran, Shiraz, Mashhad, Kerman, and small cities such as Bam. Also there are some overlapping regions for the 2 species [5,6].

Since, there are differences in clinical manifestations between ZCL and ACL along a diversity of other atypical infectious and skin lesions from *L. major* and *L. tropica* [1,7], proper identification of *Leishmania* species is important for correct diagnosis, prognosis, disease prevention, and controlling strategies, as well as management decisions [8–10].

In recent decades conventional identification and taxonomic procedures for *Leishmania* microscopic examination slides, geographic distribution, clinical manifestations, pathogenic features, and culturing patterns were identified and categorized. These lack the necessary precision because there is a high similarity among species that makes the morphologic identification difficult, and also there are epidemiologic distributions for multiple *Leishmania* species coexisting in both nonendemic and endemic areas [11].

The aforementioned methods with regard to molecular methods are not useful today, and isoenzyme analysis and parasite DNA studies are the gold standards for species differentiation [9]. Clinical signs and symptoms cannot actually be used for identification of *Leishmania* species because a broad differential diagnosis makes for diverse clinical manifestations [12]. Advanced polymerase chain reaction (PCR) -based methods including microsatellite, kinetoplast DNA, telomeric sequences, or gp63, hsp70, mini-exon, β-tubulin, or rRNA genes are used for differentiation of *Leishmania* isolates [1,13–16].

Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified sequences of multicopy genes like internal transcribed spacers (ITS) has been proven to be a crucial method especially for New World *Leishmania* species [2,7,17–19]. However, this method has not been used in identifying Old World *Leishmania* species. The specificity of sequencing ITS1 is very high because there is the lowest conservation among species [20].

Therefore, ITS1-RFLP analysis was performed for comparative phylogenetic epidemiology of *L. major* and *L. tropica* in 2 hyperendemic parts of Iran (Isfahan and Bam), which gathered 340 samples on both species. The DNA profiles were different in the current study, and as a result the genetic sequences were identified.

Material and Methods

Patients and samples

During June 2008 to March 2009, 450 cases were examined for CL and among them 340 isolates were obtained by culture (n=215 for CL from Isfahan city; n=125 for CL from Bam city). The skin lesions presented different clinical morphologic manifestations ranging from an erythematous nodule to ulcerated lesions including nodular, noduloulcerative, and nonulcerative diffuse types with or without crusting.

Isolation and parasite culture

Two microscopic smears were taken from each patient by scraping the internal border of skin lesions with a surgical blade; one sample was fixed only with 100% methanol for molecular study and the other was fixed with 100% methanol and also stained with Giemsa for microscopy. The punch skin biopsy specimens were taken under sterile conditions and local anesthesia (Xylocaine), transferred to Brain Heart Infusion (BHI; Merck, Germany) medium, inoculated into modified medium Novy-Nicole-MacNeal (NNN) followed by incubation at 24±1°C, and microscopically observed every 3 days for 4 weeks before they were reported as negative. If the cultures were positive for mass production, the growth promastigotes were run in RPMI 1640 supplemented with 10% fetal calf serum. Parasites were harvested in the late log phase, washed 3 times, and kept at –70°C for PCR.

Standard species

Three reference strains established as cryopreserved lines were used in the study: the Iranian reference strain of *L. major* (MRHO/IR/75/ER), *L. tropica* (MHOM/IR/99/YAZ1), and *Leishmania infantum* (MCAN/IR/97/LON49).

DNA extraction

All the slides were soaked through sterile phosphate-buffered saline (PBS; pH=7.4). The smears were removed completely with surgical blades and transferred to a 1.5-mL reaction tube [8] and were washed 3 times in ice-cold sterile PBS and centrifuged (3000 rpm, 5 minutes, 4°C). Then the pellets were resuspended in 200 µL of TE (10 mM Tris, 1 mM EDTA, pH 8.0), 200 µL of binding buffer, and 20 µL of proteinase K, and incubated for 2 to 4 hours at 72°C or overnight at 56°C. Finally all DNA extractions were conducted using the High Pure PCR Template Preparation Kit (Cat.No.11796828001; Roche, Germany) as recommended by the manufacturer. The DNA from the cultured promastigotes suspension was extracted as follows: Parasites (5×10^6) from –70°C frozen stocks were thawed, resuspended in 200 µL of sterile PBS, 200 µL of binding buffer, and 40 µL of proteinase K, and incubated at 70°C for 10 minutes. The DNA was dissolved in 200 µL of distilled water or 200 µL of elution buffer, and all DNA extracted was run in a 0.8% agarose gel at 80 V in Tris/borate/EDTA (TBE) 1× buffer for 20 to 40 minutes and visualized under UV light after staining in a 0.7-µg/mL solution of ethidium bromide. The DNA concentration was measured spectrophotometrically and was stored at 4°C.

Table 1. Frequency distribution of isolates related to Trypanosomatidae family from direct microscopy or cultures in Isfahan and Bam, Iran.

Genus	Isfahan	Bam	Total
	No (%)	No (%)	No (%)
Leishmania major	205 (95.3)	50 (40)	255 (75)
Leishmania tropica	4 (1.9)	72 (57.6)	76 (22.4)
Crithidia	6 (2.8)	3 (2.4)	9 (2.6)
Total	215	125	340

PCR amplification of ITS1

Small subunit (SSU) ribosomal RNA (rRNA) and 5.8S rRNA regions that are related to ribosomal ITS1 were amplified using the primers LITSr (5'-CTGGATCATTTTCCGATG-3') and L5.8s (5'TGATACCACTTATCGCACTT-3') [20–22]. Amplification of the DNA was performed in a 50-µL reaction composed of 200 µM deoxyribonucleotide triphosphates (dNTPs) mix, 2.0 mM $MgCl_2$, 2 U of *Taq* polymerase (Roche Biotech, Germany), 10 pmol of each primer, and 100 ng of DNA from culture isolates.

Amplification stages were as follows in a Corbett thermocycler: initial denaturation at 95°C for 5 minutes was performed, followed by 30 cycles of 95°C for 20 seconds, 50°C for 30 seconds, and 72°C for 1 minute, with an additional 72°C for 6 minutes for post-extension. Ten microliters of PCR product was run along with a 50-base pair (bp) ladder on a 1% agarose gel containing ethidium bromide for 25 to 45 minutes at 80 V. The gel was observed on a UV transilluminator and was evaluated with the 3 *Leishmania* standards [8,21].

RFLP analysis of amplified ITS1

The PCR products were digested with *Hae*III (Fermentas, Leon-Rot, Germany) and its related buffer at 37°C for 2 hours to analyze the species. For the detection of strains, *Taq*I and Tanq buffer were used at 65°C for 3 hours. Restriction fragments were separated in 2% agarose gels in 1×TBE buffer and visualized by staining with ethidium bromide on a UV transilluminator [8,9,21].

ITS sequencing

The PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Chatsworth, CA) according to the manufacturer's protocol. After that, PCR products of isolates that displayed different profiles were directly sequenced. Nucleotide sequence data reported in this article have been submitted to the GenBank database with accession numbers AY550178 (LmA) and AY573187 (LmB) for *L. major,* as well as HM101131 (LtA) and GQ913688 (LtB) for *L. tropica.*

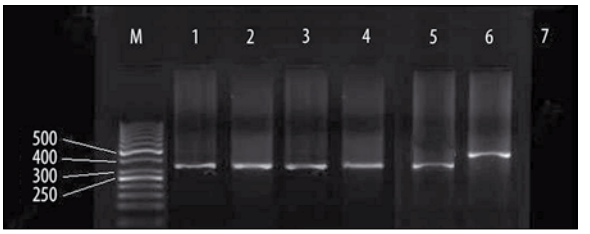

Figure 1. Electrophoresis results of ITS1-PCR from Leishmania cultures or Giemsa-stained smears. M: Molecular marker (50 bp); Lane 1: Standard Leishmania major (MRHO/IR/75/ER); Lane 2: Standard Leishmania tropica (MHOM/IR/99/YAZ1); Lane 3: Leishmania infantum (MCAN/IR/97/LON49); Lane 4: PCR product according to standard sample from Isfahan; Lane 5: PCR product according to standard sample from Bam; Lane 6: PCR product with no accordance with standard sample from Isfahan and Bam; Lane 7: negative control.

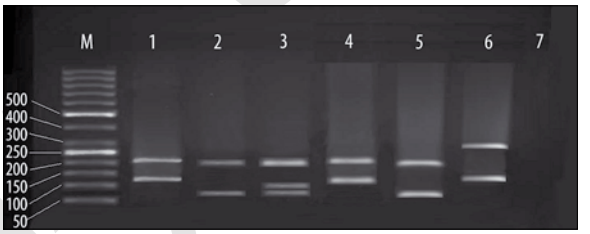

Figure 2. Restriction enzyme digestion profile of amplified ITS1 region form Leishmania cultures or Giemsa-stained smears with the enzyme HaeIII. M: Molecular marker (50 bp); Lane 1: Standard Leishmania major; Lane 2: Standard Leishmania tropica; Lane 3: Standard Leishmania infantum; Lane 4: Leishmania major according to standard sample from Isfahan and Bam; Lane 5: Leishmania tropica according to standard sample from Isfahan and Bam; Lane 6: Crithidia; Lane 7: negative control.

Statistical analysis

Through the χ^2 test, the frequency distributions of both *Leishmania* species and their genotypes were analyzed in Isfahan and Bam ($P<.001$).

RESULTS

In this study, among 450 smears and cultures collected, 350 isolates, 215 from Isfahan and 125 from Bam, were positive for *Leishmania.* The negative smears and cultures contaminated with bacteria or fungi, including 110 samples, were discarded. The 340 positive specimens were confirmed by ITS1-PCR method. From the Isfahan collection, 6 (2.8%) and 209 (97.2%) samples exhibited the 450-bp and 350-bp fragments, respectively. Among the samples collected from Bam, 3 (2.4%) and 122 (97.6%) showed 450-vp and 350-bp fragments, respectively (Table 1, Figure 1).

A few of the isolates yielded apparent variability as an ITS1-PCR product size of 100 bp compared with the standard specimens using agarose gel. The ITS1-PCR amplicons were digested by *Hae*III; in the nonstandard pattern of CL, there were 6 (2.8%) and 3 (2.4%) cases from the Isfahan and Bam collections with fragments of 153 and 296 bp, respectively, but in the standard pattern of *L. major,* 205 (95.3%) and 50 (40%)

Table 2. The frequency distribution of isolated genotypes of Leishmania major and Leishmania tropica from direct microscopy or cultures in Isfahan and Bam, Iran.

Genotypes	Isfahan	Bam	Total
	No (%)	No (%)	No (%)
LmA	201 (96.2)	49 (40.2)	250 (75.5)
LmB	4 (1.9)	1 (0.8)	5 (1.5)
LtA	3 (1.4)	71 (58.2)	74 (22.4)
LtB	1 (0.5)	1 (0.8)	2 (0.6)
Total	209	122	331

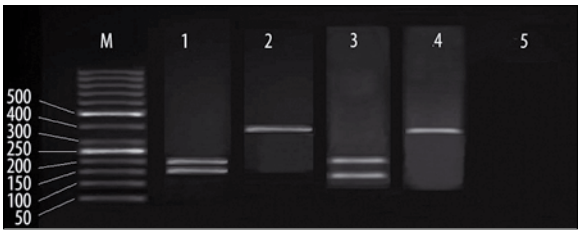

Figure 3. Restriction enzyme digestion profile of amplified ITS1 region from Leishmania major and Leishmania tropica cultures or Giemsa-stained smears with the enzyme TaqI. M: Molecular marker (50 bp); Lane 1: LmA; Lane 2: LmB; Lane 3: LtA; Lane 4: LtB; Lane 5: negative control.

Table 3. Genotypic groups of the ITS1 sequences variable parts of the different profiles detected within *L. major* and *L. tropica* isolates in Isfahan and Bam, Iran.

Genotypic groups	Strains	Region	Accession numbers
LmALmB	MHOM/IR/00/PII 1MHOM/IR/00/PII 2	ITS1ITS1	AY550178AY573187
LtALtB	MHOM/IR/10/Bam.163MHOM/AF/88/KK 27	ITS1ITS1	HM101131GQ913688.1

cases from the Isfahan and Bam collections showed fragments of 140 and 210 bp, respectively. In the standard pattern of *L. tropica,* 4 (1.9%) and 72 (57.6%) cases from the Isfahan and Bam collections showed fragments of 60 and 200 bp, respectively (Table 1, Figure 2). After species diagnosis, PCR products were further digested with *Taq*I enzyme, and fragments of 150 and 200 bp appeared in 201 (96.2%) and 49 (40.2%) cases from Isfahan and Bam cities, respectively, related to the genotypic pattern of LmA. However, the pattern related to LmB with no fragments was observed in 4 (1.9%) and 1 (0.8%) cases with *L. major* from Isfahan and Bam cities, respectively (Table 2, Figure 3). Furthermore, in cases diagnosed with *L. tropica,* 3 (1.4%) and 71 (58.2%) samples from Isfahan and Bam cities showed fragments of 135 and 200 bp, respectively, related to LtA. However, the pattern related to LtB with no fragments was observed in 1 (0.5%) and 1 (0.8%) cases with *L. tropica* from Isfahan and Bam cities, respectively (Table 2, Figure 3).

Finally, 2 polymorphic profiles were determined for *L. major* (LmA and LmB), as well as 2 for *L. tropica* (LtA and LtB) after comparison with standard species. A few diagnosed species in Isfahan and Bam foci did not show any similarities with standard *L. major* and *L. tropica* species.

Some of the LmA, LmB, LtA, and LtB genotypes were then transmitted to be sequenced. The related results, based on molecular analysis with Blast software, showed that the LmA genotype was similar to MHOM/IR/00/PII1 strain (accession number AY550178), the LmB genotype was similar to MHOM/IR/00/PII2 strain (accession number AY573187), the LtA genotype was similar to MHOM/IR/10/Bam-163 strain (accession number HM101131), which was reported in Iran for the first time, and the LtB genotype was similar to MHOM/AF/88/KK27 strain (accession number GQ913688; Table 3).

These results showed that a few of the Isfahan and Bam isolates are not similar to *L. major* and *L. tropica* species. As a result, based on molecular analysis with the Blast software, it was identified that it was very similar to *Crithidia,* and the sequence of this gene was submitted for the first time to the GenBank database with an accession number of GQ331988.

Discussion

In the past decades, DNA-based molecular techniques have improved the identification of *Leishmania* parasites at both the species and strain levels. The differential diagnosis of species and strains as well as suitable therapeutic strategies for the disease can be determined through precise protocols for identification of isolates from endemic areas where more than 1 *Leishmania* species is present. Treatments for *Leishmania* are expensive and have side effects. Strains that show resistance to a drug of choice, meglumine antimonate (Glucantime), were reported, and therefore species detection can be made accurately using protocols in this study. This information can be used for ecologic, clinical, and epidemiologic studies on *Leishmania* species. Study of the life cycle of the vector and species distribution can help us to design prophylactic strategies for disease control and preparing an effective vaccine based on strains [9,23].

In the present study, PCR amplification of ITS1 from all isolates yielded fragments of about 350 and 450 bp, whereas most standard strains show a similar 350-bp fragment [10,21,24]. This substantial difference in the PCR product sizes directed us to sequence some of the products. Molecular analysis by Blast software showed that the selected sequence had a close similarity with 97% *Crithidia fasciculata,* and 90% *Crithidia luciliae,* and a similarity of 40% with *L. infantum* (MCAN/IR/97/LON49). It was submitted to GenBank database with an accession number of GQ331988. As a result of digestion with *Hae*III, ITS1-PCR products yielded 140-bp and 220-bp fragments corresponding with *L. major,* and 60-bp and 200-bp fragments corresponding with *L. tropica,* as

well as fragments of 60, 80, and 200 bp corresponding with *L. infantum* [9,10,21,24]. In *L. tropica,* with regard to the restriction map of the enzyme used, 4 fragments with approximate sizes of 20, 55, 64, and 200 bp should be observed in this case. However, because of the small size of the first fragment (20 bp) and overlapping of 2 fragments with similar molecular weights (55 and 64 bp), we only observed 60-bp and 200-bp fragments for *L. tropica* [21].

In this study, we applied ITS1-RFLP as a tool for identification of *Leishmania* species. For a further characterization of DNA polymorphisms within *L. major* and *L. tropica* isolates from Isfahan and Bam, we used sequencing of the amplified ITS1 region of representative strains of each RFLP pattern. Through PCR-RFLP, a genetic polymorphism was determined for *L. major* as LmA and LmB and for *L. tropica* as LtA and LtB for a number of samples. This may be related to either strain heterozygosity or mixed strains, as isolates were not cloned.

The sequenced results for the LmA, LmB, LtA, and LtB genotypes showed LmA is related to the MHOM/IR/00/PII1 strain; this strain not only had the highest frequency in the Isfahan zone, but also based on reports of Tashakori and colleagues it was found in other geographic regions of Iran, such as Kashan, Tehran, Dezfool, and Dehloran [5]. The LmB genotype related to the MHOM/IR/00/PII2 strain has the lowest frequency in the Bam zone. From the report of Tashakori and associates, this genotype is only found in Kashan [5]; as we did not have any information about patients traveling inside Iran or abroad, this genotype seems to have resulted from transporting the patient from Kashan to Isfahan and Bam. The highest frequency in the MHOM/IR/10/Bam-163 strain, which was reported in Iran for the first time and was special for the Bam region, is related to the LtA genotype. This genotype, based on molecular analysis, showed that the ITS1 locus in the MHOM/IR/02/Mash-2, MHOM/IR/03/Mash-878, and MHOM/IR/02/Mash 10 strains that were isolated in Mashhad had differences in 3, 4, and 7 nucleotides, respectively. Also, the MHOM/IR/07/Kermanshah strain isolated in the Kermanshah zone had differences in 6 nucleotides. However, the LtB genotype, which is related to the MHOM/AF/88/KK27 strain, had a low frequency in both Isfahan and Bam that seems to be related to Afghan immigrants. Based on our study, most of the exclusive genotypes of some of the geographic regions are special as it seems that the LmA genotype is related to *L. major* and the LtA genotype is related to *L. tropica* in Isfahan and Bam, respectively, which may be from the oldest genotype (and the others evolved from it) or may be the most successful genotype from an adaptive aspect within new areas. The continuity of these kinds of genotypes with different zones and regions may represent the differences of conformity with vectors and animal reservoirs in one special region [17].

In some studies, the theory of genetic exchange and recombination between species was proposed; for example, *Leishmania peruviana, Leishmania guyanensis,* and *Leishmania braziliensis* exist in hybridized forms in the New World [27,28]. Kreutzer and colleagues showed via quantitative microspectrophotometry that nuclear fusion and sexual reproduction occurs among amastigotes inside of cells [25]. Youssef and associates demonstrated this matter in amastigotes and promastigotes nuclear DNA using computer imaging [26]. Gene exchange and hybridization among *Leishmania* species could lead to developing new strains with new clinical manifestations [29,30]. We can hypothesize that their disease may be transmitted by sandflies with a new chance for hybridization among strains in geographic areas with new lesions [31]. Most genotypes assessed in this study were specific to some geographic areas with heterogeneity as a result of adaption to environmental changes. Some strains like LmA in Isfahan and LtA in Bam manifested with high frequency in the present study. In a certain area, the consistency of new genotypes for adapting with environmental changes indicates the differences in adapting with the vectors and animal reservoirs [17]. Schönian and coworkers proposed the hypothesis of heterozygosity in DNA strands among different transcripts of rDNA [9,24,32]. Also, Tashakori and associates found a genetic polymorphism of 2 ITS1 and ITS2 amplicons in isolates from Iran by PCR-RFLP and single-strand conformation polymorphism analysis [6].

Conclusions

The present study showed an intensive polymorphism not only among *L. major* species in Isfahan but also among *L. tropica* in Bam. Also, it indicates that although the clinical manifestation of CL and disease pathogenesis are influenced by both host characteristics and disease pathogenesis, the genetic properties of a species can play an important role in the clinical manifestations, pathogenesis, epidemiology, and classification of the parasites. Therefore, it is necessary the further investigations with more samples from patients, vectors, and reservoir hosts from different geographic areas in Iran be performed.

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