

Genetic analysis of clinical isolates of *Leishmania major* from Isfahan, Iran

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ABSTRACT

Background & objectives: Leishmaniasis is a geographically widespread severe disease which includes visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL). There are 350 million people at risk in over 80 countries. In the Old World, CL is usually caused by *Leishmania major*, *L. tropica*, and *L. aetiopica* complex of which 90% of cases occur in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil and Peru. Recently, Eslami *et al* (2011) reported a novel *TRYP6* gene encoding trypanedoxin peroxidase from an Iranian *L. major* strain exhibiting homology with the related gene in a divergent genus of Kinetoplastida, the *Crithidia*. This prompted us to analyze the mentioned gene in 100 isolates obtained from patients with suspected CL. Consequently, we analyzed internal transcribed spacer 1 (*ITS1*) region, RNA polymerase II largest subunit (*RPOIILS*) and the mitochondrial DNA polymerase beta (*DPOLB*).

Methods: After obtaining samples from 100 patients, DNA extraction was performed and *TRYP6* was analyzed using conventional PCR. All samples harbouring *TRYP6* with smaller size (555 bp) were analysed based on three other regions: *ITS1*, *RPOIILS* and *DPOLB* genes.

Results: Results showed that 10% of the isolates have the same character as observed in our previous study. The *ITS1*-RFLP-PCR of this 10% isolates showed their similarity to the one from *Crithidia fasciculata*. RNA polymerase II largest subunit (*RPOIILS*) showed genetic diversity but the mitochondrial DNA polymerase beta (*DPOLB*) did not show any genetic diversity.

Conclusion: This study might also help in solving the problems concerning Leishmaniasis outbreaks currently reported in Iran and some other endemic regions of the world.

Key words *DPOLB*; *ITS1*; *Leishmania major*; *RPOIILS*; *TRYP6*

INTRODUCTION

The leishmaniasis are the worldwide severe diseases with an increasing incidence likely because of the population migration or travel into endemic areas, the movement of infected people into non-endemic regions, the global warming and the other environmental factors^{1,2}. Leishmaniasis caused by more than 20 species of *Leishmania* is a geographically widespread severe disease, which include visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis^{3,4}. The increasing incidence of 0.5 million cases of VL and 1.5 million cases of CL and 350 million people at risk in over 80 countries^{1,3}, creates great concern regarding this infection. Cutaneous leishmaniasis is usually caused by *Leishmania mexicana* and *L. braziliensis* complex in the New World and by *L. major*, *L. tropica*, and *L. aetiopica* complex in the Old World, however, 90% of the cases occur in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil, and Peru⁵.

In Iran, the most prevalent CL is a zoonotic which caused by *L. major* and is endemic in the north, north-

eastern, west, south-western and central parts of Iran^{6–8}. One of the important hyper-endemic foci in Iran is Isfahan.

Zoonotic cutaneous leishmaniasis (ZCL) is a polymorphic disease ranging from asymptomatic infection to benign self-limited cutaneous sore or to more protracted and extensive lesion. This clinical polymorphism may reflect variability either in the host immune response or in the parasite diversity. It is thought that this diversity resulted from gradual accumulation of divergent mutations, sexual recombination^{9–11}, genetic exchange and hybridization^{12,13}.

Recently, Eslami *et al*¹⁴ reported that *TRYP6* encoding trypanedoxin peroxidase from an Iranian *L. major* strain exhibiting homology with the related gene in a divergent genus of Kinetoplastida, the *Crithidia*. This prompted us to analyze the mentioned gene in 100 isolates obtained from patients with suspected CL. Consequently, analysis of internal transcribed spacer 1 (*ITS1*) region and two important genes named RNA polymerase II largest subunit (*RPOIILS*) and the mitochondrial DNA polymerase beta (*DPOLB*), encoding critical biologic functional pro-

teins in kinetoplastida were performed. *RPOIILS* (LmjF31.2610) and *DPOLB* (LmjF08.0890) genes present on chromosomes 31 and 8, respectively. This study might also help in solving the problems concerning leishmaniasis outbreaks currently facing in Iran and some other endemic regions of the world.

MATERIAL & METHODS

Sampling and DNA extraction

The samples were obtained from 100 patients with suspected CL from Isfahan, Iran between September 2009 and December 2010. This study was approved by the Ethical Committee of Shahid Sadoughi University of Medical Sciences and the Ethical Review Committee of the Isfahan University of Medical Sciences, Isfahan, Iran.

Sampling from patient lesions, staining tissue smears with Wright's Giemsa stain and culturing parasites were performed based on the recent study¹⁵. The specimens were extracted using either the DNA extraction kit (Roche #11796828001) or method described by Eslami *et al*¹⁴. The extracted DNA sample was quantified using spectrophotometer and analyzed by agarose gel electrophoresis.

PCR amplification and analysis of TRYP6

The complete coding region of *TRYP6* gene was amplified as described previously¹⁴ (Table 1). PCR amplification was performed using PCR master mix containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each dNTPs, 20 pmol of each primer and 0.5 unit of Taq polymerase (Fermentas). Thermal cycling was applied as described in Table 1. The PCR products were analyzed by agarose gel electrophoresis.

PCR amplification and analysis of ITS1

Small subunit (SSU) ribosomal RNA (rRNA) and 5.8S rRNA regions that are related to ribosomal *ITS1* were amplified using the specific primers¹⁶⁻¹⁹ (Table 1). Amplification of the DNA was performed in a 50 µl reaction composed of 0.2 mM deoxyribonucleotide triphosphates (dNTPs) mix, 1.5 mM MgCl₂, 1U of Taq DNA polymerase (Fermentas), 10 pmol of each primer, and 100 ng of DNA from culture isolates. Amplification stages were applied as shown in Table 1. The PCR products were analyzed on a 1% agarose gel containing ethidium bromide.

PCR amplification and analysis of RPOIILS

RPOIILS coding sequence of *L. major* was retrieved from the GenBank database, aligned and used to design primers by Primer3 software. The primers used in this study are present in Table 1. For this study, a partial sequence with 252 bp of the coding region of *RPOIILS* was amplified in a 50 µl reaction composed of 0.2 mM deoxyribonucleotide triphosphates (dNTPs) mix, 1.5 mM MgCl₂, 1U of Taq DNA polymerase (Fermentas), 10 pmol of each primer, and 100 ng of DNA from culture isolates. Amplification stages are as shown in Table 1. The PCR products were analyzed on a 1% agarose gel containing ethidium bromide. The PCR products were subjected to *Ava*II and *Bam*HI digestion for *Crithidia* and *L. major* identification based on standard sequences in databases.

PCR amplification and analysis of DPOLB

Similarly, primers were designed for DNA polymerase beta (LmjF08.0890) with product size of 845 bp. The primers used in this study are present in Table 1. Amplification of the DNA was performed in a 50 µl reaction com-

Table 1. Primers and PCR conditions used in this study

Gene name	Primers	PCR condition
<i>TRYP6</i>	Forward: ATGTCCTGCGGTAACGCCAAG Reverse: TTACTIONTGTGTGGTCGACCTTCATGC	Initial denaturation at 94°C for 5 min, 30 cycles with 94°C for 45 sec, 63°C for 45 sec and 72°C for 45 sec and a final elongation at 72°C for 5 min.
<i>ITS1</i>	<i>LITSr</i> : CTGGATCATTTTCCGATG L5.8s: TGATACCACTTATCGCACTT	Initial denaturation at 95°C for 5 min, 40 cycles with 95°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec and a final elongation at 72°C for 5 min.
<i>RPOIILS</i>	Forward: CATCCTCGTCGACACCATG Reverse: CTGCAGCTTGCTCATGTCA	Initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 45 sec, 55°C for 45 sec, and 72°C for 45 sec and a final elongation at 72°C for 5 min.
<i>DPOLB</i>	Forward: GAACATCATCCGCATCTTCC Reverse: CGTTGAAGTTCTTGCTGCC	Initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 60 sec, 58°C for 60 sec, and 72°C for 60 sec and a final elongation at 72°C for 5 min.

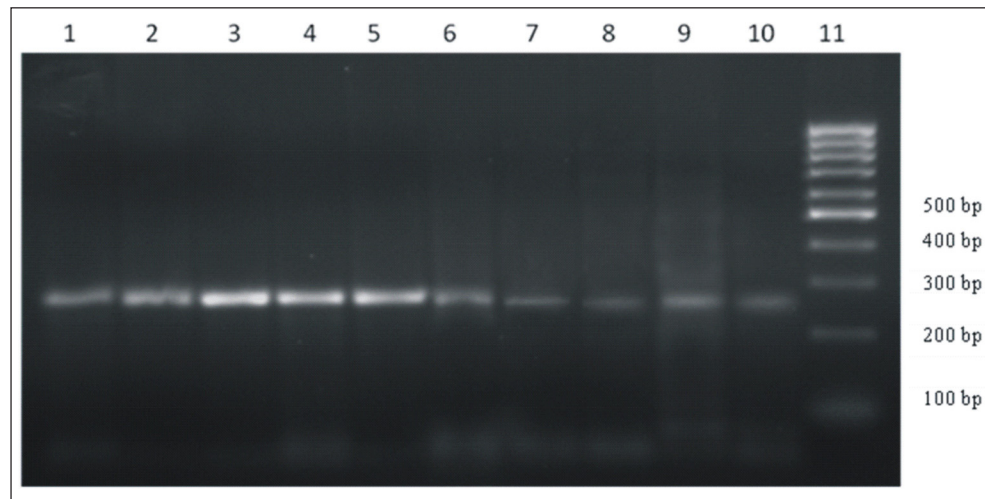


Fig. 1: Agarose gel electrophoresis of PCR products of the *RPOIILS* gene in all strains used in this study (lanes 1 to 10); lane 11: 100 bp DNA ladder.

posed of 0.2 mM deoxyribonucleotide triphosphates (dNTPs) mix, 1.5 mM MgCl₂, 1U of Taq DNA polymerase (Fermentas), 10 pmol of each primer, and 100 ng of DNA from culture isolates. Amplification stages were done as Table 1. Initial denaturation was performed at 95°C for 5 min, followed by 30 cycles of 95°C for 60 sec, 58°C for 60 sec, and 72°C for 60 sec, with a final elongation at 72°C for 5 min. The PCR products were analyzed on a 1% agarose gel containing ethidium bromide. *Ava*II digestion of the products was used for typing. Based on the sequences present in databases, fragments with length of 474, 237 and 134 bp determined *L. major* and fragments with length of 134, 170 and 541 bp would be revealed this gene identity with *Crithidia*.

RESULTS

Amplification and analysis of *TRYP6*

Analysis of the *TRYP6*-PCR products was performed on 100 isolates obtained from patients suspected with CL. We clustered the results obtained by PCR amplification of *TRYP6* in two patterns: the isolates harboring *TRYP6* with 555 bp in length named *TRYP*-A group and the isolates harboring *TRYP6* with 600 bp in length named *TRYP*-B group. *TRYP6*-A was found in just 10 isolates. After sequencing of an isolate from each group, BLAST analysis indicated *TRYP6*-A group has 97% identity with *TRYP* gene from *Crithidia fasciculata* and 82% identity with *L. major*. The isolates in *TRYP6*-A group were selected and analyzed based on *ITS1*, *RPOIILS* and *DPOLB* genes.

Amplification and analysis of *ITS1*

Analysis of *ITS1*-RFLP-PCR was performed on *TRYP*-A group. This analysis showed that all the isolates in this

group exhibited *ITS1* region with 450 bp in length which after digestion, they showed 310 and 240 bp fragments (data not shown).

Amplification and analysis of *RPOIILS*

Analysis of the *RPOIILS*-PCR products revealed identical PCR products with 250 bp in length in all the iso-

Table 2. Isolates with *tryparedoxin peroxidase* gene with > 90% similarity with *Crithidia*

No.	Strain	<i>RPOIILS</i> gene		<i>DPOLB</i> gene
		BamHI	AvaII	AvaII
1	EUM15	Pattern A	Pattern B	Pattern A
2	EUM21	Pattern B	Pattern B	Pattern A
3	EUM36	Pattern B	Pattern B	Pattern A
4	EUM43	Pattern B	Pattern A	Pattern A
5	EUM52	Pattern B	Pattern A	Pattern A
6	EUM76	Pattern A	Pattern C	Pattern A
7	EUM41	Pattern B	Pattern A	Pattern A
8	EUM92	Pattern B	Pattern A	Pattern A
9	EUM35	Pattern B	Pattern A	Pattern A
10	EUM66	Pattern B	Pattern A	Pattern A

EUM: Esfahan University of Medical Sciences (Esfahan); *RPOIILS* gene: *RNA polymerase II largest subunit* gene, RFLP analysis with BamHI showed two patterns A and B. Pattern A: Based on the sequences from databases, the fragments of 100 and 152 bp in length, similar to the related gene in *L. major*; Pattern B: no digestion. RFLP analysis with *Ava*II showed three patterns, A, B and C. Pattern A: the fragments of 100 and 152 bp in length, similar to the related gene in *Crithidia*; Pattern B: the fragments of 82 and 170bp in length, similar to the related gene in *L. major*; Pattern C: the fragments of 82, 100, 152 and 170 bp in length (comprising two pattern A and B); *DPOLB* gene: *mitochondrial DNA polymerase beta* gene. RFLP analysis of this gene with *Ava*II showed just one pattern A with 134, 237 and 474 bp fragments, similar to *L. major*.

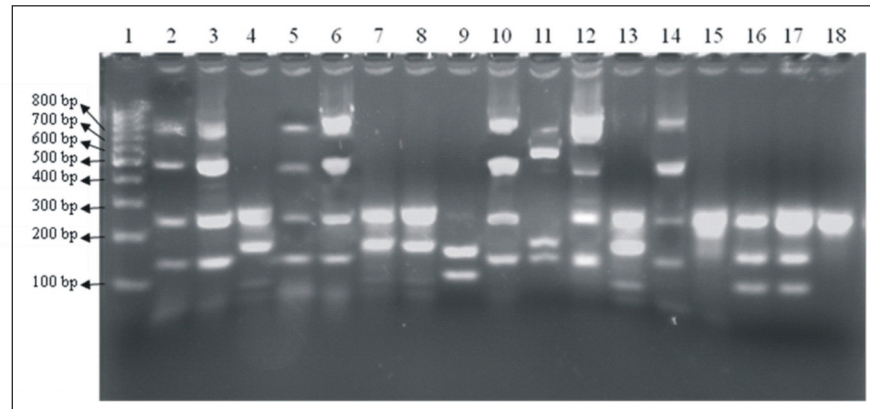


Fig. 2: Agarose gel electrophoresis of some isolates after digestion with *AvaII* for RFLP-*DPOLB* and with either *AvaII* or *BamHI* for RFLP-*RPOIILS* analysis. Lane (1): 100 bp DNA ladder; Lanes (2, 3, 5, 6, 10, 11, 12, 14): RFLP-*DPOLB* analysis with *AvaII* (EUM92, EUM76, EUM41, EUM43, EUM21, *Cr. fasciculata*, EUM52, EUM35, respectively); Lanes (4, 7, 8, 13): RFLP-*RPOIILS* analysis with *AvaII* (EUM36, EUM21, EUM15, *Leishmania major* respectively); Lanes (9, 15, 16, 17, 18): RFLP-*RPOIILS* analysis with *BamHI* (*L. major*, EUM36, EUM15, EUM76, EUM21, respectively).

lates with *TRYP6*-PCR patterns A and C (Fig. 1). Digestion with *BamHI* showed two patterns, *RPOIILS*-*BamHI* pattern A with 98 and 150 bp fragments and *RPOIILS*-*BamHI* pattern B without digestion. Two isolates showed *RPOIILS*-*BamHI* pattern A and remaining showed *RPOIILS*-*BamHI* pattern B (Table 2, Figs. 2 and 3). Also, the isolates with *TRYP6*-PCR pattern A and *TRYP6*-PCR pattern C were digested with *AvaII* which elicit three patterns, *RPOIILS*-*AvaII* pattern A with 98 & 150 bp fragments and *RPOIILS*-*AvaII* pattern B with 82 & 170 bp fragments and *RPOIILS*-*AvaII* pattern C with 82, 98, 150 & 170 bp fragments. Isolate EUM92 showed *RPOIILS*-*AvaII* pattern A, EUM76 showed *RPOIILS*-*AvaII* pattern C and remaining showed *RPOIILS*-*AvaII* pattern B (Table 2;

Figs. 2 and 3). Two isolates produced an additional undigested 250 bp fragment too.

Amplification and analysis *DPOLB*

RFLP analysis of the *DPOLB*-PCR products in parallel with *RPOIILS* was performed on all the isolates with *TRYP6*-PCR patterns A & C. PCR products in these isolates determined identical size of 845 bp (Fig. 4). *AvaII* digestion revealed identical digestion patterns for all isolates with 474, 237 and 134 bp which were predicted as *DPOLB* gene from *L. major*. All other isolates excluding EUM15, EUM41 and EUM92, produced an undigested 845 bp fragment as well (Fig. 2).

As *DPOLB* genes in all the isolates in this study were

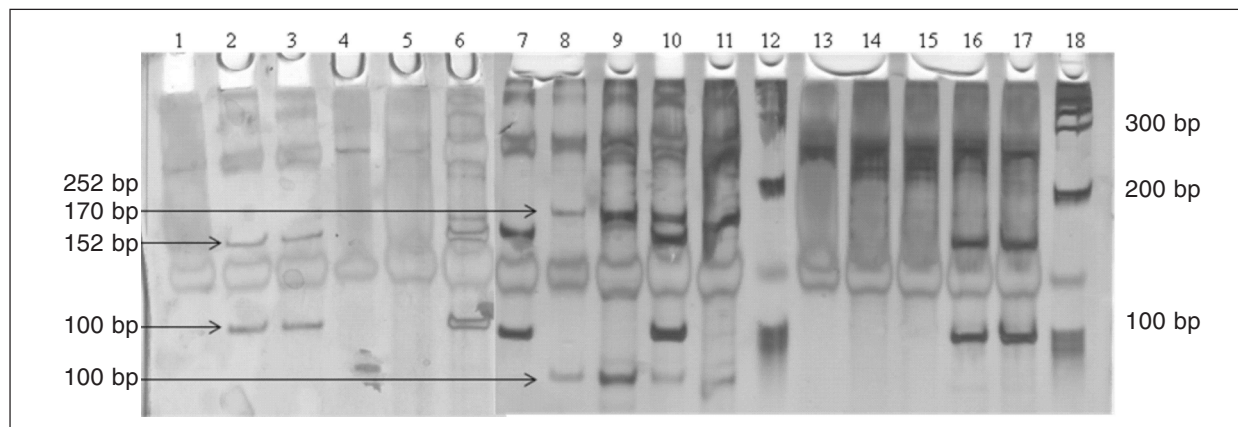


Fig. 3: Acrylamide-Polyacrylamide gel electrophoresis of some strains; Lanes (1, 4, 5, 13, 14, 15, 16, 17): RFLP-*RPOIILS* analysis with *BamHI* (EUM52, EUM41, EUM66, *Cr. fasciculata*, EUM36, EUM21, EUM76, EUM15, respectively); Lanes (2, 3, 6, 7, 8, 9, 10, 11): RFLP-*RPOIILS* analysis with *AvaII* (EUM52, EUM41, EUM66, *Cr. fasciculata*, EUM36, EUM21, EUM76, EUM15, respectively); Lanes (12, 18): 100 bp DNA ladder.

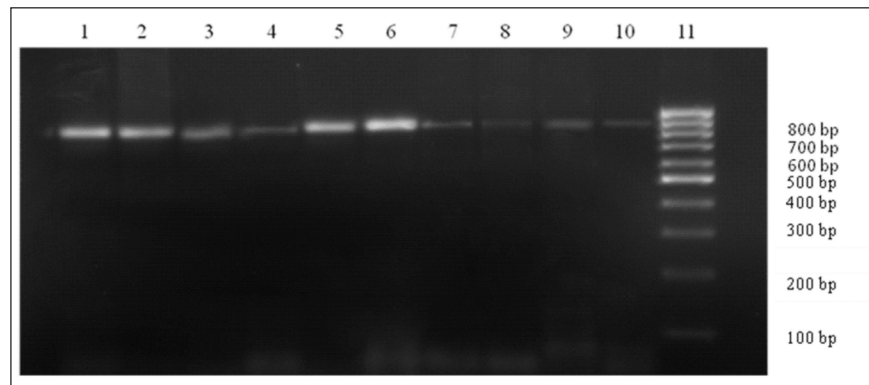


Fig. 4: Agarose gel electrophoresis of the PCR products from *DPOLB* gene. Lanes 1–10: Strains used in this study; Lane 11: 100 bp DNA ladder.

identical, we decided to sub-group isolates based on either *RPOIILS*-AvaII or *RPOIILS*-BamHI analysis. Therefore, those were divided into four sub-groups: isolates with *RPOIILS*-AvaII pattern A and *RPOIILS*-BamHI pattern B, isolates with *RPOIILS*-AvaII pattern B and *RPOIILS*-BamHI pattern A, isolates with *RPOIILS*-AvaII pattern B and *RPOIILS*-BamHI pattern B, isolates with *RPOIILS*-AvaII pattern C and *RPOIILS*-BamHI pattern A (Table 2).

DISCUSSION

In this study, gene analyses were performed directly on infected clinical samples, as patients' tissue aspirates, from 100 patients inhabitant of Isfahan, a highly endemic region in Iran²⁰. Results showed a genetic heterogeneity of *TRYP6* gene among isolates used in this study. *TRYP6*-PCR analysis showed 10 isolates with *TRYP6*-PCR pattern A, and the remaining with *TRYP6*-PCR profile B (Table 2). The last group showed homology with *L. major*, therefore, we decided to analyze all the isolates present in *TRYP6*-PCR profile A which showed homology with the mentioned gene in *Crithidia*¹⁴ based on *ITS1*, *RPOIILS* and *DPOLB*.

ITS1 analysis showed the same results like the recent study by Douidi *et al*²¹. As their proof, *ITS1* in our interested isolates have 450 bp fragment after amplification and 240 and 310 bp fragments after restriction digestion with HaeIII. Molecular analysis by BLAST software showed that the selected sequence had a close similarity with 97% *Cr. fasciculata*, and 90% *Cr. luciliae*, and a similarity of 40% with *L. infantum* (MCAN/IR/97/LON49).

DPOLB-PCR-RFLP with AvaII showed unique pattern A with 474, 237 and 134 bp fragments (Fig. 2), further analysis verified *ITS* homology with one in *L. major*. As shown in the results, the RFLP technique detected more variation in the *RPOIILS* gene. *RPOIILS*-PCR-RFLP with

BamHI showed two patterns A and B. Pattern A with 152 and 98 bp fragments could verify *ITS* homology with the mentioned gene in *Cr. fasciculata*, while in pattern B, fragments remained undigested. The results showed two isolates with *RPOIILS*-PCR-RFLP-BamHI pattern A and remaining with *RPOIILS*-PCR-RFLP-BamHI pattern B. On the other hand, the isolates in the former pattern produced an undigested 250 bp fragment following digestion with BamHI. This finding could be indicative of heterozygosity. It is speculated that as this gene being a house-keeping gene, therefore, it is assumed that it would be diploid^{22,23}.

RPOIILS-PCR-RFLP with AvaII resulted in three patterns A, B and C. Six isolates showed *RPOIILS*-PCR-RFLP-AvaII pattern A, three isolates showed *RPOIILS*-PCR-RFLP-AvaII pattern B and one isolate showed *RPOIILS*-PCR-RFLP-AvaII pattern C. Since we could not present any interpretation for the base on the original sequence accessible through GenBank, therefore, we decided to select one of the isolates from this group for sequence evaluation. BLAST analysis revealed *ITS* homology with *RPOIILS* from *L. major* with a transverse mutation of G→T, creating a restriction site for AvaII. Isolates with *RPOIILS*-PCR-RFLP-AvaII pattern A were indistinguishable from the other strains of *L. major*. Our genetic analysis queries the mentioned gene is separate from *L. major* Friedlin and closely to the mentioned gene from *Crithidia*.

One isolate with *RPOIILS*-PCR-RFLP-AvaII pattern C demonstrated two forms of the mentioned gene, one identical with *L. major* and one related to *Crithidia*. Therefore, this isolate possessing three forms of this gene as digestion with BamHI produced patterns similar to *L. major* and digestion with AvaII resulted in two patterns with similarity to *L. major* and *Crithidia*. Performing double digestion using both the enzymes resulted in an additional fragment of 250 bp. The report from an experimental cross of *Trypanosoma brucei rhodesiense* 058 and

T.b. brucei 196 showed two of the hybrid clones to have DNA contents about 1.5 times of parental values. Molecular karyotyping used to further proving what they have obtained by RFLP in taking place the trisomy events of some chromosomes through evaluation of some genes like phosphoglycerate kinase, tubulin and phospholipase C genes on different chromosomes. They proposed that these chromosomes appear prone to substantial size alterations associated with genetic exchange^{24,25}. There are also some evidences for aneuploidy via nuclear hybridization in *Leishmania* and genetic hybridization in *T. cruzi*^{13, 26, 27}.

To our knowledge, this is the first report of genetic hybridization between two different genera *Leishmania* and *Crithidia* that are so distant phylogenetically and epidemiologically. In the New World, more evidences for hybridization events have been brought^{28,29}. The hypothesis that certain *Leishmania* genotypes correspond to hybrid genotypes between different species has been first proposed by Evans *et al* in the Old World³⁰.

Ravel *et al*¹¹ stated that hybridization between two divergent species, *L. infantum* and *L. major* is a natural hybrids taking place inside the mammalian hosts. On the other hand, Akopyants *et al*²⁷ proposed that hybridization events between these two genera may occur in insect gut. We believe that since close association is an obvious prerequisite for genetic exchange, and the infection of host fly of *Leishmania* by *Crithidia*, so their coexistence inside the insect gut, could prompt the genetic exchange to take place probably through meiosis as it is evident in *T. brucei*²⁴. Moreover, cell fusion probably occurs in the sandfly vector. *Leishmania* can undergo genetic exchange during growth and development in the sand fly vector and can transmit infectious stage hybrids progeny to a mammalian host²⁷. The finding *Leishmania* hybrids' ability to develop in *Phlebotomus papatasi* may have important epidemiological implications. It is a peridomestic and anthropophilic sand fly, reaching high densities in many places. This suggests that in nature, hybrid isolates may circulate by using this sand fly vector, thereby increasing the risk of their spreading into new foci throughout the broad range of *P. papatasi* distribution.

It is considered that hybridization might repair double-strand DNA damage and, therefore, could be a consequence of either a better adaptation of these hybrid isolates to their host environments such as temperature, pH and many other parameters, or a resistance to drugs or to immune response, or even to both which could influence the host response and thus the outcome of infection, and therefore, will be involved in the biology of the host-parasite relationship. Sexual recombination is conventionally believed to play a major role in organism adaptive evolution. There-

fore, one might expect such an event to occur in parasites, such as *Leishmania* spp, to ensure their fitness for survival in varying environments.

Potentially, these hybrids might arose from rare mating events, yielding offspring with a strong selective advantage, and suggested by the clonal propagation of an emergent hybrid mucosal strain in Peru¹⁰.

CONCLUSION

Finally, the PCR-RFLP approaches used in this study detected a genetic variation of *RPOIILS* gene among different isolates of the species *L. major*, therefore, this gene could be used for genotyping of *L. major* at least in one of the hyper endemic foci like Isfahan.

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