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Genetic variation in *RPOIILS* gene encoding RNA polymerase II largest subunit from *Leishmania major*

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Abstract Leishmaniasis is a geographically widespread severe disease which includes visceral leishmaniasis, 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*, *Leishmania tropica*, and *Leishmania aethiopica* complex which 90 % of cases occurring in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil, and Peru. Recently, some reports showed that some strains of *L. major* have internal transcribed space (*ITS-1*) with differential size 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. After obtaining samples from 100 patients, DNA extraction was performed and *ITS-1* was analyzed using PCR–RFLP. These samples were sequenced for verifying their homology. Then, *RPOIILS* gene was analyzed in the samples that their *ITS-1* gene exhibiting homology with the related gene in *Crithidia*. Results showed that 10 % of the isolates have *ITS-1* exhibiting different size with the routine ones. Sequencing of them showed their similarity to the one from *Crithidia fasciculata. RPOIILS* gene encoding RNA polymerase II largest subunit analysis showed genetic diversity. This study might also help in solving the problems concerning Leishmaniasis outbreak currently facing in Iran and some other endemic regions of the world.

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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 nonendemic 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. Visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucoCL are the important types of the disease [3, 4]. The incidence of the disease is decorating a steady increase with presently 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], but based on http://www.cdc.gov/ncidod/dpd/parasites/leishmania/factsht_leishmania.htm, about 90 % of cases occurring in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria, Brazil, and Peru. This alerting situation created a great concern regarding this infection. CL is usually caused by *Leishmania mexicana* and *Leishmania braziliensis* complex in the New World and by *Leishmania major*, *Leishmania tropica*, and *Leishmania aethiopica* complex in the Old World.

In Iran, the most prevalent of CL is a zoonotic which caused by *L. major* in many region of country [5–7], 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

Table 1 Primers and PCR conditions used in this study

Gene name	Primers	PCR condition
ITS1	L*ITS*r: CTGGATCATTTTCCGATG L5.8 s: TGATACCACTTATCGCACTT	Initial denaturation at 95 °C for 5 min, 40 cycles with 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, the final elongation at 72 °C for 5 min
RPOIILS	Forward: CATCCTCGTCGACACCATG Reverse: CTGCAGCTTGCTCATGTCA	Initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, the final elongation at 72 °C for 5 min

resulted from gradual accumulation of divergent mutations, sexual recombination [8–10], and/or genetic exchange and hybridization [11, 12].

In spite of all preventive measures, we faced a serious outbreak of leishmaniasis in Iran, as well as some other important infected countries. However, no one could present a rationale explanation on the main factors affected activation of the disease after many years of quiescent sate.

Recently, Doudi et al. [13] reported that *ITS-1* in some Iranian *L. major* strain exhibiting homology with the related gene in a divergent genus of kinetoplastida, the *Crithidia*. This prompted us to analyze this gene in clinical isolates obtained from patients diagnosed with CL. Consequently, analysis of another important gene named *RPOIILS* (accession number XM_001685196) encoding RNA polymerase II largest subunit present on chromosomes 31 was performed.

Materials and 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 as described previously [13, 14]. Qualitative and quantitative analysis performed on extracted DNA samples using spectrophotometer and agarose gel electrophoresis.

PCR amplification and restriction digestion of *ITS1* and *RPOIILS* genes

Small subunit (SSU) ribosomal RNA (rRNA) and 5.8S rRNA regions that are related to ribosomal *ITS*1 were amplified using the specific primers [15–18]. *RPOIILS* (accession number XM_001685196) 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 presented in 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 $MgCl_2$, 1 U of *Taq* DNA polymerase (Fermentas), 10 pmol of each primer, and 100 ng of DNA (Table 1). The PCR products were analyzed on a 1 % agarose gel containing ethidium bromide.

The PCR products were subjected to *Ava*II (Fermentas, # ER0311) and *Bam*HI (Fermentas, # ER0055) digestion for *Crithidia* and *L. major* identification based on standard sequences in databases. Finally, the digested fragments were analyzed using 10 % polyacrylamide gel electrophoresis (PAGE) and 0.1 % silver nitrate solution was used for staining.

Results

Amplification and analysis of TRYP6

Analysis of the *ITS1*-PCR–RFLP products was performed on 100 isolates obtained from patients suspected with CL. Based on the sizes of *ITS1* amplicons, two categories were recognized: category A with 320–350 bp in length and category B with around 450 bp in length. *ITS1*-B was found in just 10 isolates. After sequencing of all 10 isolates from group B, BLAST analysis indicated that *ITS1*-B group demonstrating high degree of similarity of 97 % to *Crithidia fasciculata,* and 90 % to *Crithidia luciliae,* but only 40 % similarity with *L. infantum* (MCAN/IR/97/LON49).

The isolates in *ITS1*-B group were selected and analyzed based on *RPOIILS* gene. The restriction digestion with *Hae*III showed 310 and 240 bp fragments.

Amplification and analysis of RPOIILS

Analysis of the *RPOIILS*-PCR products, revealed identical PCR products with 250 bp in length in all isolates with *RPOIILS*-PCR patterns A and B (Fig. 1). Digestion with

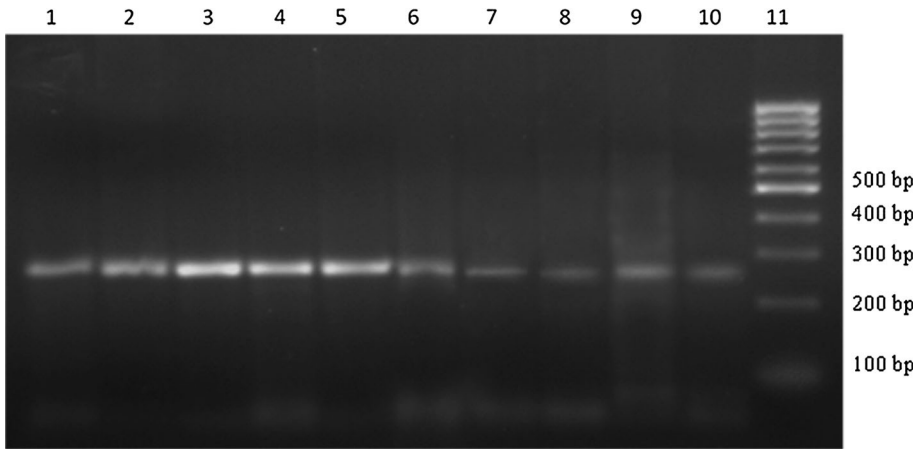

Fig. 1 Agarose gel electrophoresis of PCR products of the *RPOIILS* gene in all strains used in this study (*line 1–10*); *line 11* 100 bp DNA ladder

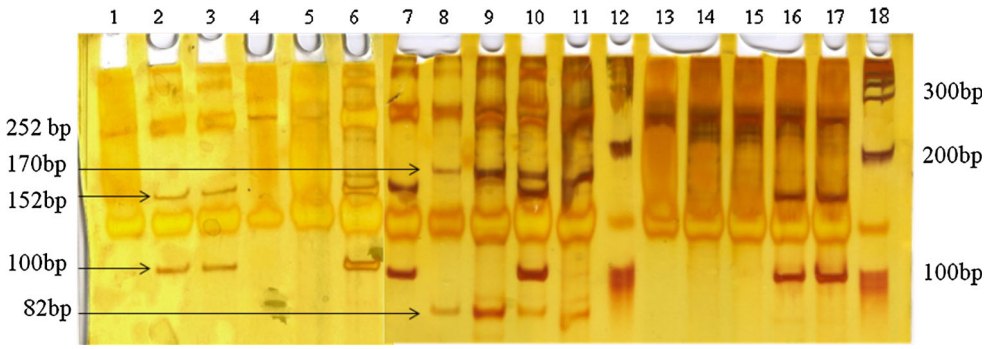

Fig. 2 Acrylamide–polyacrylamide gel electrophoresis of some strains. *Lines* (*1*, *4*, *5*, *13*, *14*, *15*, *16*, *17*): RFLP-*RPOIILS* analysis with *Bam*HI (EUM52, EUM41, EUM66, *Crithidia fasciculate*, EUM36, EUM21, EUM76, EUM15, respectively). *Lines* (*2*, *3*, *6*, *7*, *8*, *9*, *10*, *11*): RFLP-*RPOIILS* analysis with *Ava*II (EUM52, EUM41, EUM66, *Crithidia fasciculate,* EUM36, EUM21, EUM76, EUM15, respectively). *Lines* (*12*, *18*): 100 bp DNA ladder

*Bam*HI showed two patterns, *RPOIILS-Bam*HI pattern A with 100 and 150 bp fragments and *RPOIILS-Bam*HI pattern B without digestion. Two isolates showed *RPOIILS-Bam*HI pattern A and remaining showed *RPOIILS-Bam*HI pattern B (Fig. 2). Also, the isolates with *TRYP6*-PCR pattern A and *TRYP6*-PCR pattern C were digested with *AvaII* which elicit three patterns, *RPOIILS-Ava*II pattern A with 100 and 150 bp fragments and *RPOIILS-Ava*II pattern B with 80 and 170 bp fragments and *RPOIILS-Ava*II pattern C with 80, 100, 150 and 170 bp fragments. Isolate EUM92 showed *RPOIILS-Ava*II pattern A, EUM76 showed *RPOIILS-Ava*II patterns C and remaining showed *RPOIILS-Ava*II pattern B (Fig. 2). Two isolates produced an additional undigested 250 bp fragment too.

Discussion

In this study, gene analyses was performed directly on infected clinical samples, as patients' tissue aspirates, from 100 patients inhabitant of Isfahan, a highly endemic regions in Iran [19].

Results showed a genetic heterogeneity of *RPOIILS* gene among isolates used in this study. *ITS1*-PCR analysis showed ten isolates with *ITS1*-A pattern, and the remaining with *ITS1*-B profile (Fig. 2). The last group showed homology with *L. major* therefore all isolates presented in *ITS1*-B which showed homology with the mentioned gene in *Crithidia* [13] based on *RPOIILS* gene was analyzed. Actually, *ITS1* analysis showed the same results like the recent study by Eslami et al. [20]. As their proof, *ITS1* in the interested isolates have 450 bp fragment after amplification and 240 and 310 bp fragments after restriction digestion with *Hae*III. Molecular analysis by Blast software showed that these strains had a close similarity with 97 % *C. fasciculata,* and 90 % *C. luciliae,* and a similarity of 40 % with *L. infantum* (MCAN/IR/97/LON49).

RPOIILS-PCR–RFLP with *Bam*HI showed two patterns; A and B. Pattern A with 150 and 100 bp fragments could verified *ITS* homology with the mentioned gene in *C. fasciculata*, while in pattern B, fragments remained undigested. The results showed two isolates with *RPOIILS*-PCR–RFLP-*Bam*HI pattern A and remaining with *RPOIILS*-PCR–RFLP–*Bam*HI pattern B. On the other hand, the

isolates in the former pattern produced an undigested 250 bp fragment following digestion with *Bam*HI. This finding could be indicative of heterozygosity. It is speculated that as this gene being a housekeeping gene, therefore, it is assumed that it would be diploid [21, 22].

RPOIILS-PCR–RFLP with *Ava*II resulted in three patterns A, B and C. Six isolates showed *RPOIILS*-PCR–RFLP-*Ava*II pattern A, three isolates showed *RPOIILS*-PCR–RFLP-*Ava*II pattern B and one isolate showed *RPOIILS*-PCR–RFLP-*Ava*II pattern C. Since there is not any interpretation for the base on the original sequence accessible through GenBank, therefore one of the isolates from this group was selected 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 *Ava*II. Isolates with *RPOIILS*-PCR–RFLP-*Ava* II pattern A were indistinguishable from the other strains of *L. major.* The genetic analysis queries the mentioned gene is a separate from *L. major* Friedlin and closely to the mentioned gene from *Crithidia.* One isolate with *RPOIILS*-PCR–RFLP-*Ava*II 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 *Bam*HI produced patterns similar to *L. major* and digestion with *Ava*II 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 [23, 24]. There are also some evidences for aneuploidy via nuclear hybridization in *Leishmania* and genetic hybridization in *T. cruzi* [12, 25, 26].

Based on present knowledge, this is the first report of genetic variation in *RPOIILS* gene among Iranian *L. major* strains. In the New World, more evidences for hybridization events have been brought [27, 28]. The hypothesis that certain *Leishmania* genotypes correspond to hybrid genotypes between different species has been first proposed by Evans et al. [29] in the Old World.

Ravel et al. [10] stated that hybridization between two divergent species, *Leishmania infantum* and *L. major* is a natural hybrids taking place inside the mammalian hosts. On the other hand, Akopyants et al. [26] proposed that hybridization events between these two genuses may occur in insect gut. It is believed 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* [23]. More over cell fusion probably occurs in the sand-fly 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 host [26]. 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 of double-strand DNA damage and therefore could be a consequence of either a better adaptation of these hybrid isolates to their hosts environments such as temperature, pH and many other parameters, or a resistance to drugs or to immune response [30–32], 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. Therefore, 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 Peru [9].

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, Isfahan.

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