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Research Article

Molecular Diagnosis of Clinical Isolates of Cutaneous Leishmaniasis Using ITS1 and KDNA Genes and Genetic Polymorphism of Leishmania in Kashan, Iran

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Abstract

Cutaneous leishmaniasis is a common skin disease caused by leishmania parasite. An accurate diagnosis of parasites species is possible using molecular techniques. This study was carried out to compare internal transcribed spacer (ITS1) and kinetoplast deoxyribonucleic acid (KDNA) genes for identifying Leishmania species by Polymerase Chain Reaction (PCR), furthermore, genetic diversity of isolates was studied. This research examined 130 patients who were suspected of cutaneous leishmaniasis and referred to Kashan's health centers from 2011-2014. After DNA extraction from serosity, PCR were performed using ITS1 and KDNA primers. Cutaneous Leishmaniasis was diagnosed by the observation of 320 bp band in the ITS1-PCR. The PCR products were digested with restriction enzyme HaeIII and then leishmania species were identified by patterns of enzymatic digestion. The diagnostic criteria of Cutaneous Leishmaniasis (CL) in KDNA-PCR were based on the observation of 760 and 650 bp for *Leishmaniasis tropica* and *Leishmaniasis major*, respectively. Twelve isolates of leishmania were sequenced and the phylogenetic tree was traced using the results of sequencing by Mega 4 software. Out of 130 suspected patients to CL, 70 (53.8%) and 98 (75.4%) isolates were positive by Restriction Fragment Length Polymorphism (RFLP) of ITS1 and KDNA, respectively. Using ITS1 PCR, 60 samples (85.7%) and 10 samples (14.3%) were identified as *L. tropica* and *L. major*, respectively, ITS1-PCR had 25.3% false negative, compare to microscopy. While, microscopy had false negative in 13 cases compare to KDNA-PCR. Due to the lower sensitivity of the PCR-RFLP of ITS1, KDNA-PCR is recommended for diagnosis of CL. The *L. tropica* and *L. major* are the causative agents of CL.

Key words: *Leishmania major*, *Leishmania tropica*, ITS1, KDNA, diagnosis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cutaneous leishmaniasis is a skin dermatitis transmitted through the bite of sand flies infected with leishmania parasites. This disease is considered as a major endemic public health problems in 98 countries and 5 continents, particularly in Mediterranean countries, including Iran (Murray *et al.*, 2005; Reithinger *et al.*, 2007). There are two types of cutaneous leishmaniasis in Iran, dry skin ulcer (urban type) caused by *Leishmania tropica* and moist skin (rural type) caused by *L. major* (Collier *et al.*, 2005). The most important endemic foci of zoonotic cutaneous leishmaniasis (rural) in Iran are Isfahan, Golestan, Khuzestan and Fars provinces (Beheshti *et al.*, 2013; Mesgarian *et al.*, 2010; Ghasemian *et al.*, 2011; Baghaei *et al.*, 2012; Karamian *et al.*, 2008). The endemic foci of anthroponotic cutaneous leishmaniasis (urban) in Iran are Mashhad, Bam, Kerman and Lorestan provinces (Shahbazi *et al.*, 2008; Aflatoonian *et al.*, 2013; Kheirandish *et al.*, 2013; Poursmaelian *et al.*, 2011). Furthermore Isfahan province and Kashan city are reported as the foci of endemic rural and urban cutaneous leishmaniasis in Iran (Doroodgar *et al.*, 2009; Shiee *et al.*, 2012; Ghorbanzadeh *et al.*, 2014). The classical methods for diagnosing and identification the CL and species of the parasite is giemsa stained direct smear, which is time-consuming and less sensitive (Collier *et al.*, 2005; Shahbazi *et al.*, 2008). However diagnosis of the leishmania species by genetic-based methods is highly sensitive and specific. Now a days, several molecular techniques such as; PCR, nested PCR, (RAPD-PCR), PCR-restriction fragment length polymorphism (RFLP-PCR) and determination of nucleotide sequence have been used for the diagnosis and identification of Leishmania species (Karamian *et al.*, 2008; Shahbazi *et al.*, 2008; Kheirandish *et al.*, 2013; Poursmaelian *et al.*, 2011; Doroodgar *et al.*, 2009; Shiee *et al.*, 2012; Ghorbanzadeh *et al.*, 2014; Vaeznia *et al.*, 2009). The targets for amplification with PCR serve either nuclear DNA; such as, the Internal Transcribed Spacer 1 (ITS1) or mini-exon regions or extrachromosomal DNA, such as KDNA minicircles (Ghasemian *et al.*, 2011; Baghaei *et al.*, 2012; Karamian *et al.*, 2008; Shahbazi *et al.*, 2008; Kheirandish *et al.*, 2013; Poursmaelian *et al.*, 2011; Shiee *et al.*, 2012; Ghorbanzadeh *et al.*, 2014; Vaeznia *et al.*, 2009; Saki *et al.*, 2010). Genetic diversity of Leishmania species causes cutaneous leishmaniasis Mini Exon RFLP and ITS RFLP and markers of microsatellite were used to analysis genetic diversity of *L. major* and *L. tropica* (Tashakori *et al.*, 2012; Mauricio *et al.*, 2004; Vaeznia *et al.*, 2009; Saki *et al.*, 2010). Cupolillo *et al.* (2003), who applied PCR RFLP on ITS, reported

genetic polymorphism in *L. braziliensis* (Cupolillo *et al.*, 2003). Since fast and accurate identification of species of leishmania is necessary for treatment and control programmes and controversial reports of leishmania species in Kashan, this study was carried out to compare KDNA PCR and RFLP-PCR for identification of leishmania species and genetic diversity of Leishmania isolates in patients with cutaneous leishmaniasis in Kashan, Iran.

MATERIALS AND METHODS

Sample collection: The serosity of ulcer was collected from 130 suspected patients with cutaneous leishmaniasis referred to Zidi and Shaeed Beheshti Hospital of Kashan from 2011-2014. These patients were examined by three methods. A questionnaire including demographic and epidemiological criteria, furthermore clinical symptoms were recorded by interview.

Microscopic examination: All of the prepared smears were stained by 20% Giemsa and then examined for the presence of the amastigote.

DNA extraction: The genomic DNA of samples were extracted directly from serosity using DNA tissue Kit (Bioneer; Korea) according to the manufacturer's instruction and then stored at -20°C.

ITS1 PCR-RFLP: The ITS PCR was performed on all 130 extracted DNA samples. Amplification reaction was performed in volume 20 µL containing PCR Master mix (Bioneer, Korea), primers LITSR (5' CTGGATCATTTCCGATG-3) and L5.8S (5'TGATACCACTTATCGCACTT-3') (10 p mol) and DNA (Davila and Momen, 2000). Also, standard strains of *L. major* (MHOM/IR/75/ER) and *L. tropica* (MHOM/IR/99) were used as positive control. Distillated water was used as negative control. Reaction were amplified in a thermocycler (Appendorf USA) as follows: initial denaturation at 95°C for 5 min was followed by 35 cycles of 94°C for 30 sec, 48°C for 30 sec and 72°C for 1 min and final extension 72°C for 10 min. At the end, PCR products were analyzed using 1.5% gel electrophoresis. The diagnostic criteria of cutaneous leishmaniasis in ITS PCR was based on the observation of expected band 320 bp in PCR product and after enzymatic digestion by the observation of its pattern, species of Leishmania were identified.

RFLP analysis of amplified ITS1: All positive samples of ITS PCR product were digested with restriction enzyme HaeIII and then leishmania species were identified based on pattern of

enzymatic digestion. The PCR products (10 µL) were digested with 1 µL of HaeIII (fast digest Fermentas) at 37°C for 20 min using conditions recommended by the supplier (Fermentas Life Sciences, Germany). The restriction fragments were subjected to electrophoresis in 4% agarose gel.

KDNA PCR: In KDNA PCR, specific primers, LINR4 (forward, GGG GTT GGT GTA AAA TAGGG) and LIN17 (reverse, TTT GAA CGG GAT TTC TG), were carried out as described by Aransay *et al.* (2000) for amplifying the variable region of the leishmania KDNA. The length of this region varies in different leishmania species (Aransay *et al.*, 2000). PCR was carried out in a 20 µL reaction mixture containing Master mix (Bioneer, Korea), 10 pmol LINR4, LIN17 and DNA (2 µL). The reaction mixtures were incubated in a thermocycler (Appendorf, USA) as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 40 sec and final extension at 72°C for 5 min. The PCR products were electrophoresed in 1.2% agarose gel. In the KDNA-PCR, *L. tropica* and *L. major* were determined by the observation of expected bands 760 and 650 bp for *L. tropica* and *L. major*, respectively.

DNA sequencing: Twelve PCR isolated positive samples of *L. major* and *L. tropica* were sent to "Gene Fannavar" company to determine their sequences. Then, after getting the results of sequencing, MEGA 4.1 beta and Sequencher Tm 4.1 were used to analyze them and drawing phylogenetic trees.

Statistical analysis: All the data were recorded in SPSS software version 13.5 (SPSS Inc., Chicago, IL) and were analyzed using the chi-square and exact sig chi-squared test. Three isolate of *L. tropica* and *L. major* were submitted in DDBJ Gen bank. This study was approved by the ethical committee of Kashan University of Medical Sciences, Iran.

RESULTS

Out of 130 suspected cutaneous leishmaniasis patients, 70 (53.8%) and 98 (75.4%) cases were positive by ITS- PCR and KDNA-PCR, respectively (Table 1 and Fig. 1). Twenty two cases (25%) were positive by microscopic method, while these samples were negative by ITS1PCR, furthermore, notable 13 infected persons were positive by KDNAPCR, but were negative by microscopic method. In PCR-RFLP after HaeIII digestion of ITS1-PCR product, two bands 60 and 200 bp were identified as *Leishmania tropica* and two fragments of 140 and 220 bp as *L. major*, respectively (Fig. 2). By using RFLP-PCR method; 60 (85.7%) and 10 (14.3%) of samples were

Table 1: Frequency of positive cutaneous leishmaniasis and species identification based on diagnostic techniques

Assay	Positive No.	%	Negative No.	%	Total	<i>Leishmania</i> sp.		
						<i>L. tropica</i>	<i>L. major</i>	Mix
Microscopic examination	87	66.9	43	33.1	130 (100)	-	-	2 (2.1)
kDNA PCR	98	75.4	32	24.6	130 (100)	70 (71.4)	26 (26.5)	-
ITS1 PCR	70	53.8	60	46.2	130 (100)	60 (85.7)	10 (14.3)	-

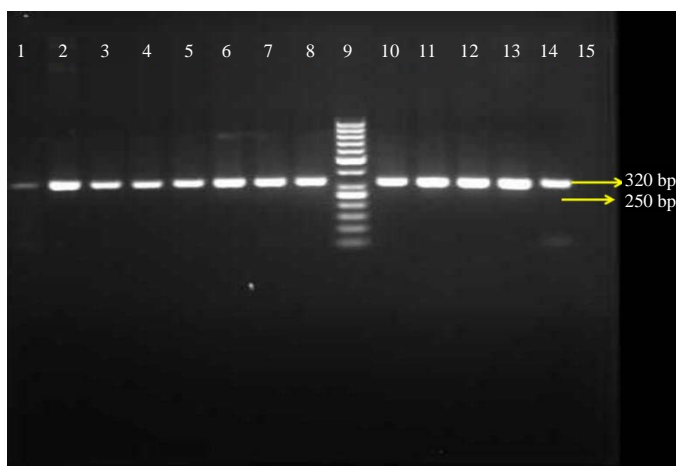


Fig. 1: Gel electrophoresis of ITS PCR products of leishmania isolates using LITSR and L5.8S primers, Lanes 1-8 and 10-12: *Leishmania* isolates, Lane 9: 50 bp DNA ladder marker, Lane 13: Reference strain of *L. tropica* (MHOM/IR/89/AR2), Lane 14: Reference strain of *L. major* (MHOM/IR/54/LV39), Lane 15: Negative control

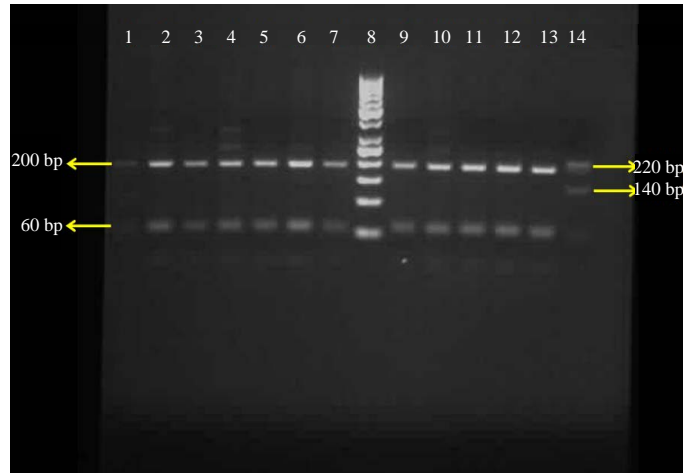


Fig. 2: Gel electrophoresis of PCR RFLP products of leishmania isolates using HaeIII enzyme on 4% gel agarose, Lanes 1-7 and 9-12: *Leishmania tropica* isolates, Lane 8: 50 bp DNA ladder marker, Lane13: Reference strain of *L. tropica* (MHOM/IR/89/AR2), Lane 14: Reference strain of *L. major*(MHOM/IR/54/LV39), Lane 15: Negative control

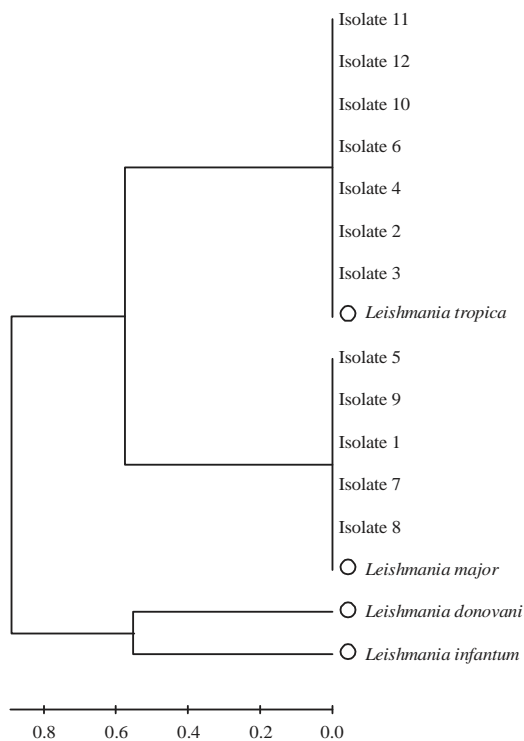


Fig. 3: Neighbor-joining tree showing the relationships of the KDNA gene sequences of *Leishmania major* and *L. tropica*

identified as *L. tropica* and *L. major*, respectively. However by using KDNA-PCR; 71.4 and 26.5% of isolates were determined as *L. tropica* and *L. major* and 2.1% mix, respectively (Table 1). The results of the study showed that 39 (58.2%) and 31 (49.2%) patients were men and women, respectively.

The highest (65%) and lowest (18%) rate of infection were observed in people with primary and college education, respectively. Fifty one cases (67.1%) of wounds were in hand and 29-40% were in face and legs and body.

Sequencing and phylogenetic analysis: Twelve products of isolated *L. tropica* and *L. major* were prepared and sequenced by "Bioneer" Company at South Korea. The sequence for three isolates were recorded in the gene bank of Japan, DDBJ with the Accession numbers of LC033785 (*L. major*), LC036306 (*L. tropica*) and LC036307 (*L. major*).

Phylogenetic analysis and sequences alignment showed no significant intraspecific differences between isolates from patients and all samples were limited to two species and no strain was observed. Phylogenetic tree of leishmania isolates was constructed based on KDNA gene sequencing using the neighbor-Joining algorithm (Fig. 3).

DISCUSSION

The ITS1 and ITS2 are important in molecular diagnoses, because of evolving rapidly and having diverse sequences even among genus and different species of one genus. Thus, the results can help to identify polymorphisms. The KDNA gene has 10000 copies and is suitable for diagnostic purposes (Aransay *et al.*, 2000).

In the current study, in order to compare RFLP-PCR profile ITS1 and KDNA- PCR for identification of leishmania species, 130 patients suspected to CL were examined. In this study serosity of ulcer instead of culture samples was used for DNA extraction and PCR. The culture method is time

consuming, expensive and has false negative (Shiee *et al.*, 2012; Satow *et al.*, 2013) but it can directly extract DNA from serosity is easy and rapid (Hajjaran *et al.*, 2011). According to Ghorbanzadeh *et al.* (2014) studied in kashan, 71.4 and 26.5% were identified as *L. tropica* and *L. major* and 2 cases were mix, respectively. However the results in present study using RFLP-PCR profile ITS1 on the same DNA, showed that 70 (53.8%) of the samples were positive, of which 60 (85.7%) and 10 (14.3%) were identified as *L. tropica* and *L. major*, respectively. According to the results of Shiee in kashan, *L. tropica* and *L. major* were identified as 92.1 and 7.9%, respectively (Shiee *et al.*, 2012). Thus this rate was different from the results of present study. In fact this study sampling was performed on patients in Beheeshti hospital and Zidi center and isolates of CL in the hospital were *L. major*. Therefore the results of the study were probably based on sampling location, type of primer and DNA extraction. In the present study, 22 cases (25.3%) microscopically-positive, RFLP-PCR profile ITS1 were negative. The majority of this false negative isolates belonged to *L. major*. Because in the rare number of parasites in zoonotic cutaneous leishmaniasis, ITS1 primer cannot detect CL. These results are in consistence with previous studies done by Hajjaran *et al.* (2011) and Hayat *et al.* (2013). But Karamian in Birjand, Iran reported high sensitivity of ITS1 than KDNA PCR for detecting CL. In this study 90% of isolates were *L. tropica* (Karamian *et al.*, 2013).

Notable in this study in 13 cases microscopically-negative, were positive using KDNA- PCR. According to the results of previous studies, KDNA is more sensitive than microscopy and ITS1 for detection of CL (Karamian *et al.*, 2008; Satow *et al.*, 2013; Hayat *et al.*, 2013; Bensoussan *et al.*, 2006). Azmi *et al.* (2011) and Akkafa *et al.* (2008) using RFLP PCR reported that the dominant species of CL in Palestine and Turkey were *L. tropica* (Azmi *et al.*, 2011; Akkafa *et al.*, 2008). According to the results of Mesgarian in Konabad, Beheshti in Isfahan, Ghasemian in Ahvaz, Iran, *L. major* were the predominate species of CL (Beheshti *et al.*, 2013; Mesgarian *et al.*, 2010; Ghasemian *et al.*, 2011). *Leishmania tropica* is the most common species in the Bam and Mashhad, respectively (Aflatoonian *et al.*, 2013; Mahmoodi *et al.*, 2010). Sequences alignment showed no differences between isolates and all samples were *L. tropica* and *L. major*. Genetic diversity of 24 isolates of *L. major* from different endemic foci were evaluated by Tashakori *et al.* (2012) using mini exon RFLP and ITS RFLP, three strain of *L. major*, while markers of microsatellites revealed 21 different genotypes (Tashakori *et al.*, 2012). In present study it should be noted that, the rate of infection in individuals with college education was significantly less than ill iterated subjects ($p = 0.003$),

due to the more health care behavior. The CL is affecting all age groups but from 70 positive cases, the highest rate of infection was observed in 20-39 years and the least rate was seen at age 80-100 years ($p = 0.25$). This result is in agreement with the result of study reported by Kheirandish *et al.* (2013) and Ghorbanzadeh *et al.* (2014). This age group is more exposed to the risk of affliction due to the work condition. However, Ghasemian *et al.* (2011) reported that the most rate of the infection was in age group less than 10 years. In the present study, the types of wounds in 85% of CL were dry and 85.8% of positive cases of CL were caused by *L. tropica*. These results are in consistence with a previous study (Mesgarian *et al.*, 2010; Ghorbanzadeh *et al.*, 2014). The clinical spectrum of CL is broad, lacks specificity. Majority of form of wounds of *L. tropica* were papules and pustules. Most wounds in patients infected to *L. major* were papules and ulcer.

CONCLUSION

Due to lower sensitivity of the PCR-RFLP of ITS1, KDNA-PCR is recommended for diagnosis of CL. *Leishmania tropica* and *Leishmania major* are the causative agents of CL.

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