International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2017; 4(2): 44-51

Received: 08-01-2017
Accepted: 09-02-2017

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# The identification of new leishmania using ITS1-rDNA gene

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**Abstract**
**Background and objective*:*** Leishmaniasis is a parasitic disease that is caused by protozoan parasites of the genus *Leishmania*. Cutaneous Leishmaniasis is an important sanitation and public health issue in Iran. The identification of *Leishmania* parasites in human, source, and carriers of the disease through routine laboratory methods, isoenzyme, monoclonal antibodies, and the proliferation of part of the genome of *Leishmania* parasites by polymerase chain reaction (PCR) method is common in Iran. However, certain identification and confirmation of the parasite is always associated with doubt without determining the sequencing of the genome of *Leishmania* parasites. The objective of this research was to search for *Leishmania* species in human isolated bloods.
**Methods:** This research was a descriptive-analytical experiment that consisted of 41 samples provided by Pasteur Institute of Iran. In the present study, in order to identify the species of *Leishmania* parasites in human isolates with wet cutaneous Leishmaniasis, first, culture in biphasic medium was carried out, and then, DNA was extracted from cultured promastigotes. Standard PCR was employed to detect *Leishmania* by amplifying a region of the ribosomal RNA genes (ITS1-rDNA). Amplicons were examined with Gel electrophoresis. PCR products were directly sequenced and molecular software applications were employed to confirm the species of the *Leishmania*.
**Results:** *Leishmania* species were detected using Standard PCR and sequencing of ITS1-rDNA gene. Sequence analysis revealed that 17 out of 39 positive samples were *Lishmania major*. *Crithidia fasciculata* was observed in 5 samples; 16 samples were unnamed *Leishmania*; only one sample was identified as *Leishmania tropica*; and one sequence was unreadable therefore not identified.
**Conclusion:** With its high accuracy and sensitivity, ITS1-rDNA amplification and sequencing is one of the best methods to identify different species of *Leishmania*.

**Keywords:** PCR, ITS1-rDNA, Leishmaniasis, *Leishmania major*, *Leishmania tropica*, Identification

## 1. Introduction
Leishmaniasis is extensively spotted in different region of the world. This disease is referred to as one of the main public health concerns in many countries in the world. The disease agents include species of *Leishmania*, disease vectors, different types of sandflies, and sources like canines and rodents. In Iran, 3 species of *Leishmania* have been introduced as the causes of human Leishmaniasis, which are *Leishmania major*, *Leishmania tropica*, and *Leishmania infantum*, in order of their epidemiological importance [18]. Due to the importance of this disease from a hygienic aspect, it has been paying close attention by the World Health Organization for years. It is considered as one of the 10 most important diseases in tropical regions, and the Tropical Diseases Research Department of the World Health Organization has counted it as one of the three first diseases (African Trypanosomiasis, Dengue fever, and Leishmaniasis) and one of the new and uncontrollable diseases [18].
Leishmaniasis is endemic in 98 countries around the world and 350 million people are at risk of Leishmaniasis [6].
In Iran, this disease is seen in two forms of cutaneous lesions (cutaneous Leishmaniasis) and visceral (kala-azar). Diagnosis of cutaneous Leishmaniasisis usually carried out based on clinical signs observed in patients and its confirmation is through direct examination, culture, or biopsy [11]. Identification of types of *Leishmania* is impossible through shape. Pathogenicity of different species of *Leishmania* depends on the parasite's virulence and the host's characteristics. In the past, classification of parasites was based on clinical characteristics, geographical distribution, and sometimes the size of the parasites. Nowadays,

these criteria are not so acceptable, and it is necessary to determine detailed and scientific specification of *Leishmania*. Over the last few years, criteria like analysis of DNA, monoclonal antibodies, and isoenzyme patterns have extensively been utilized [10].

In most studies, Iranian researchers have reported *rhombomys opimus* as the main source of the disease and *phlebotomus papatasi* as the main carrier of cutaneous Leishmaniasis in rural regions. However, some studies carried out in some regions of Iran have introduced *meriones libycus* as the secondary source and some species of sandflies of sub-genus *para phlebotomus* as the secondary carriers, and species of sandflies of sub-genus *para phlebotomus* have been believed to play the role of keeping the cycle of the parasite in the animal sources of the disease [20].

Identifying *Leishmania* in human, its source, and the carrier of Leishmaniasis through conventional methods, isoenzyme monoclonal antibodies, and the proliferation of part of the genome of *Leishmania* parasites by PCR method is common in Iran. Although identifying this parasite is to some extent confirmed by some specialists as a way to understand the disease status through the above methods, certain identification and confirmation of the parasite is always associated with doubt without determining the sequencing of the genome of *Leishmania* parasites. In Iran, the characteristics of the species of *Leishmania* as the cause of cutaneous Leishmaniasis are determined according to clinical signs, geographical concentration, specific animal sources, laboratory infection to animal sources or carriers, serology, and sometimes monoclonal antibodies in patients [19, 12, 9].

Identifying *Leishmania* infection is carried out by few biochemical or molecular methods. On the other hand, employing molecular methods is sometimes limited to carrying out PCR, observing the related band in the gel and employing advanced methods like determining the nucleotide sequence of the gene under investigation. Molecular and phylogenetic analyses have seldom been used to identify *Leishmania* [19, 12, 9].

In this regard, the present study was carried out in order to identify the species of the existing *Leishmania* through microscopic diagnosis and molecular method, and to target ITS1-rDNA gene in *Leishmania* samples available in Pasteur Institute of Iran.

## 2. Materials and Methods

The present study was a descriptive-analytical investigation whose statistical population consisted of 44 patients suspected of cutaneous Leishmaniasis who had referred to Pasteur Institute from Tehran and other cities.

### 2.1. Preparing the biphasic blood-agar medium for human isolates

In order to prepare the culture medium for the human isolates, which were stored in liquid nitrogen, water was heated to the boiling temperature, and the mentioned amounts of salt and agar were added to it. The boiling process of the solution was continued until the agar seeds were dissolved. Afterwards, the solution was poured into test tubes with screw caps (about one third of the tube). Then, the caps were fixed, and the tubes were sterilized with autoclave (at temperature of 121 ºC for 10 min). They were kept in a refrigerator until their use. At the time of using, the tubes were placed in boiling water so that the mediums became liquid then; they were cooled down to 40-50 ºC. About one third of the medium volume (15% ml10) of defibrinated rabbit blood was added to each tube, and the contents were well mixed. Afterwards, they were placed in room temperature in a slant form so that they became solid, then they were transferred into a refrigerator (4-8 ºC). In order to make sure that they are not contaminated, one of the tubes was placed in 37 ºC incubator for 24 hours. Finally the solid phase was prepared.

The liquid phase included normal physiological saline or sterilized RPMI added to the solid phase, and penicillin and streptomycin with concentrations of 100 g/ml and 100IU/ml respectively were added to prevent the growth of bacteria. During the experiment, the liquid phase was added to the solid phase and covered the slope surface. Promastigotes were placed into the nutrient agar at the depth of 2 mm from the end of the slope surface. After sample was transferred, the culture medium was kept at a temperature of 23 ºC (incubator). Parasites accumulated in the fluid and grew in the slope part of the medium. The culture was examined daily until they had reach late log phase. All of 44 *Leishmania* isolates samples were mass cultivated in biphasic blood-agar medium.

### 2.2. Extraction of DNA from quantified numbers of cultured *Leishmania* promastigotes

DNA was extracted from promastigotes with the TECHLAB Bacterial DNA Kit and Cinna pure cell, tissue DNA kit-50t. The procedure were performed based on the steps mentioned one the DNA extraction kit for all samples. The purified DNA tubes stored at 4 ºC to be used in molecular diagnosis of *Leishmania* species.

### 2.3. Gel Electrophoresis

In order to examine the presence and quality of the extracted *Leishmania's* DNA, gel electrophoresis was carried out.

The material needed for gel electrophoresis included *Leishmania* DNA, agarose powder, Ethidium Bromide (loading dye), TAE 1x, loading buffer, marker, and distilled water. Purified DNA were loaded in 0.8% agarose gel and visualized under UV lightand the gel images were captured (see the results section).

### 2.4. PCR with the purpose of amplifying ITS1-rDNA gene of *Leishmania* species

For all DNA samples extracted from *Leishmania* samples, PCR was performed. A set of specific primers of genus *Leishmania* LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3') used for sequencing and targeted the ITS1 region. After that, the PCR products were run on an agarose gel to check whether the PCR reaction had occurred (7) that is species-specific by DNA sequence. ITS1 is a non-coding region placed at SSUrRNA, which is bounded by the genes 18S and 5.8S that produce a 300-350 bp fragment of *Leishmania*. At the same time, the specificity of the primers was controlled on Blast website. Finally, the best condition with regard to $MGCL_2$ rate, the concentration of the primers, and annealing temperature was chosen in a way that reference strains of *Leishmania tropica* and *L. major* could create a band close to 400 bp.

**2.4.1. Preparing the PCR reaction solution**

A volume of 3 µl of extracted DNA from purified DNA samples was amplified with 1 µl of each forward and revers primers in the presence of 1 µl mgCL2, 2. 5 µl PCR buffer, 1 µl dNTP mix,1 µl Taq DNA polymerase (PCR master mix; Super Taq Company, England) in a final volume of 25µl. Afterwards, the samples were put in a thermal cycler, and the specific program of thermal cycler was used to carry out PCR for ITS1 gene of *Leishmania*.

The PCR was performed for each sample with conditions as follows:

Denaturation for 5 minutes at 94 ºC

**Ten cycles**

1. Denaturation for 1 minute at 94 ºC
2. Annealing for 1 minute at 62 ºC and for 1 minute at 51 ºC
3. Extension for 1 minute and 15 seconds at 72 ºC

**Twenty cycles**

1. Denaturation for 1 minute at 94 ºC
2. Annealing for 1 minute at 55 ºC
3. Extension for 1 minute and 15 seconds at 72 ºC

In the end, extension for 7 minutes at 72 ºC

PCR products were loaded on 1.5% agarose gels and visualized by ethidium bromide staining and using 100 bp ladder DNA as a molecular marker.

**2.4.2. Sequencing**

The samples visualized on the gel were purified and then were sent to Macrogene (Korean Company) by GEN FANAVARAN, where they were sequenced employing the same primers used for the PCR.

**2.5. Data analysis**

After the sequences obtained were typed and aligned to the standard marker strains submitted in GenBank. Homologies with the available sequence data in GenBank was checked by using basic local alignment search tool (BLAST) analysis software (http://www.ncbi.nlm.nih.gov) and *Leishmania* species were identified.

## 3. Results

**3.1. DNA extraction from human samples suspected of different species of *Leishmania***

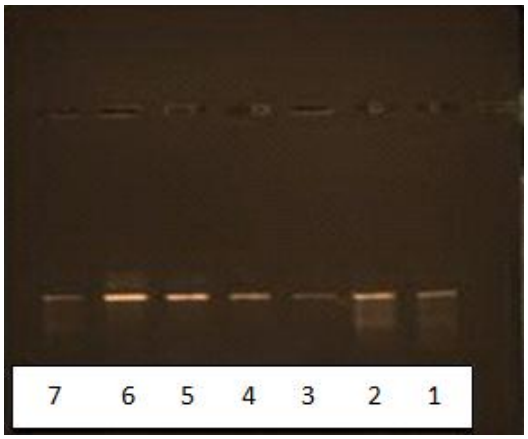


**Fig 1:** Agarosegel electrophoresis of DNA extracted from cutaneous Leishmaniasis culture. Lanes 1 and 6: L. major, lanes 2, 3, 4 and 5: crithidia fasciculata, lane7: no PCR product

In the present study, in order to make sure about the presence of *Leishmania* DNA in the samples after DNA extraction process, each DNA was loaded on agarose gel in order to make sure about the presence of DNA by observing the relevant bands. Some of these images are as follows:

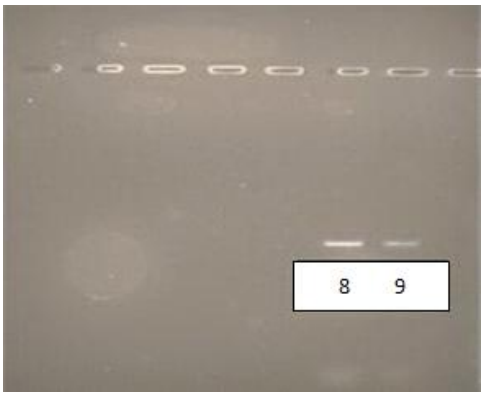


**Fig 2:** Agarose gel electrophoresis of DNA extracted from cutaneous Leishmaniasis culture. Lanes 8 and 9: L. major

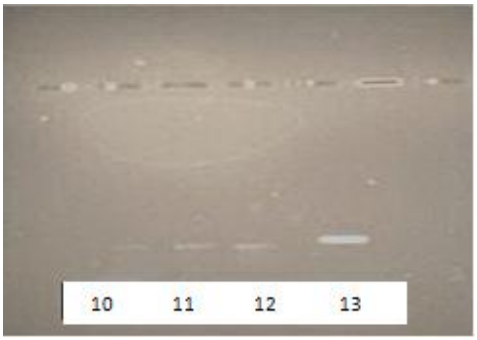


**Fig 3:** Agarose gel electrophoresis of DNA extracted from cutaneous Leishmaniasis culture. Lane 10: deleted, lanes11 and 12: L. major, lane 13: crithidia fasciculate

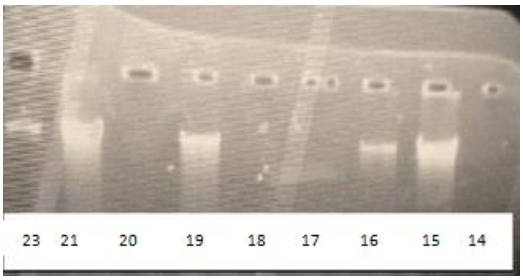


**Fig 4:** Agarose gel electrophoresis of DNA extracted from cutaneous Leishmaniasis culture .Lanes 14, 15, 16, and 17: Unnamed Leishmania, lanes 18, 19, 20, 21, and 23: L. major.

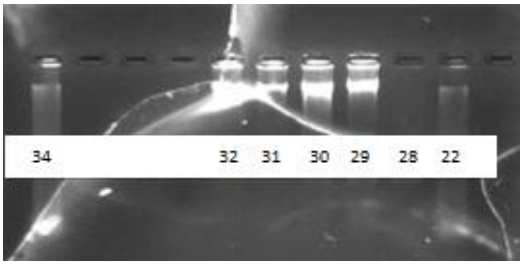


**Fig 5:** Agarose gel electrophoresis of DNA extracted from cutaneous Leishmaniasis culture .Lanes 22 and 28: L. major, lanes 29, 30, 31, and 32: unnamed Leishmania, lane 34: no similar sample was found.

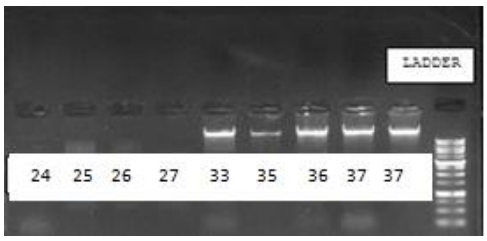


**Fig 6:** Agarose gel electrophoresis of DNA extracted from cutaneous Leishmaniasis culture. Lanes 24, 25, 26, 27, and 33: unnamed Leishmania, Lanes35, 36, (2) 37: L. major.

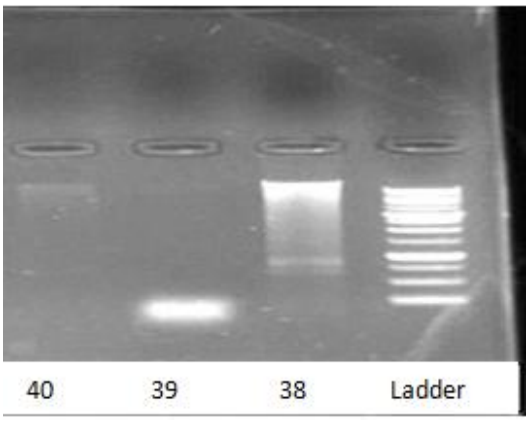


**Fig 7:** Agarose gel electrophoresis of DNA extracted from cutaneous Leishmaniasis culture. Lanes 38 and 39: Negative for Leishmania, lane 40: L. major.

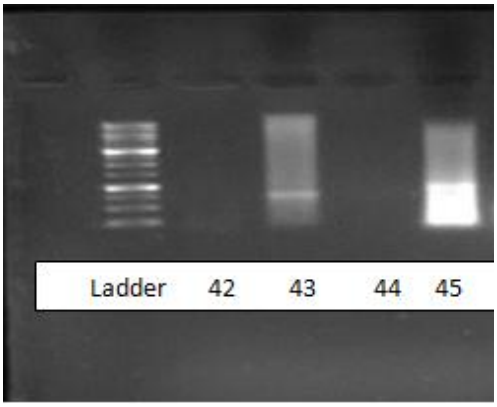


**Fig 8:** Agarose gel electrophoresis of DNA extracted from cutaneous Leishmaniasis culture .Lane 42: L. major, Lanes 43 and 44: unnamed Leishmania, Lane 45: L.tropica.

### 3.2. PCR products

PCR products are needed in order to carry out sequencing, and as was mentioned before. The results of some PCR products that had good resolution are presented in Figures 9, 10, 11, and 12. However, not all the images were attached to this paper due to the poor quality of the gel

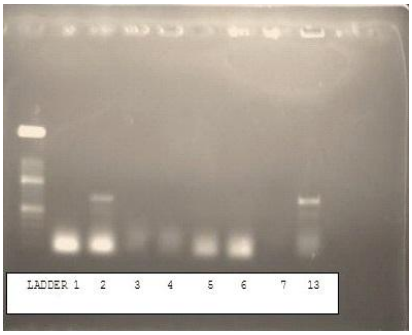


**Fig 9:** Agarose gel electrophoresis of PCR-ITS1 products. Lanes1and6: L.major, lanes2, 3, 4, 5 and 13: crithidia fasciculata, lane7: no PCR product.

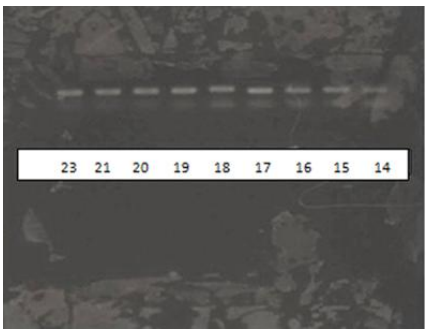


**Fig 10:** Agarose gel electrophoresis of PCR-ITS1 products. Lanes14, 15, 16, and 17: Unnamed Leishmania, lanes 18, 19, 20, 21, and 23: L.major.

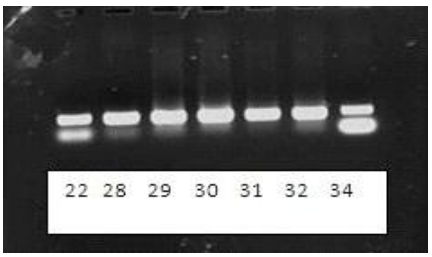


**Fig 11:** Electrophoresis images of PCR ITS1 products. Lanes 22 and 28: L. major, Lanes 29, 30, 31, and 32: Unnamed Leishmania, lane 34: No similar sample was found.

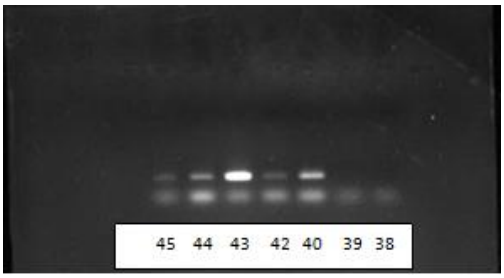


**Fig 12:** Electrophoresis images of PCR ITS1 products. Lanes 38 and 39: no result, Lanes 40 and 42: L. major, lanes 43 and 44: Unnamed Leishmania, lane 45: L.tropica.

After sequencing of ITS1 fragments of all PCR positive samples, they were analyzed in BLASTn Databank and the species are identified (Table 1).

**Table 1:** Phylogenetic results based on the alignment of amplified section of ITS1 region of coetaneous *Leishmania human* isolates.

| Sample No | Species | Accession number |
|---|---|---|
| LP1 | Similar to *L. major* | KF815218 |
| LP2 | Similar to *Crithidia fasciculata* | HM004585, first reported in Yasuj |
| LP3 | Similar to *Crithidia fasciculata* | HM004585, first reported in Yasuj |

| | | |
|---|---|---|
| LP4 | Similar to *Crithidia fasciculata* | HM004585, first reported in Yasuj |
| LP5 | Similar to *Crithidia fasciculata* | HM004585, first reported in Yasuj |
| LP6 | Similar to *L. major* | AJ300482 |
| LP7 | No PCR product | |
| LP8 | Similar to *L.major* MHOM/IR/12/BAM3 | JX289878 |
| LP9 | Similar to *L. major* MHOM/IR/12/BAM3 | JX289847 |
| LP11 | *L. major* MHOM/IR/10/Kashan-CK18 | JX289878, first reported in Kashan |
| LP12 | Similar to *L. major* | KJ425408, first reported in Esfarayen region |
| LP13 | Similar to *Crithidia fasciculata* | HM004585, first reported in Yasuj |
| LP14 | Similar to strain MHOM/CN/80/XJ801 | HQ 830357 |
| LP15 | Similar to strain MHOM/CN/80/XJ801 | HQ 830357 |
| LP16 | Similar to strain MHOM/GS6/CHN/SCgq | HM130599 |
| LP17 | Similar to strain MHOM/CN/80/XJ801 | HQ 830357 |
| LP18 | Similar to *L. major* IRN227 | KJ194181 |
| LP19 | Similar to *L. major* IRN227 | KJ194181 |
| LP20 | Similar to *L. major* | KJ425408, first reported in Esfarayen region |
| LP21 | Similar to *L. major* | KF981809 |
| LP22 | Similar to *L. major* IRN227 | KJ194181 |
| LP23 | Similar to strain MHOM/IR/10/Kashan-CK10 | JX289875 |
| LP24 | Similar to strain MHOM/CN/80/XJ80 | HQ 830357 |
| LP25 | Similar to strain MHOM/CN/80/XJ801 | HQ 830357 |
| LP26 | Similar to strain MHOM/CN/89/GS5 | HQ830360 |
| LP27 | Similar to strain MHOM/GS5/CHN/SC | HM130602 |
| LP28 | Similar to *L. major* IRN227 | KJ194181 |
| LP29 | Similar to strain MHOM/CS11/CHN/S | HM130606 |
| LP30 | Similar to strain MHOM/CN/84/SD1 | HM130604 |
| LP31 | Similar to strain MHOM/GS5/CHN/SC | HM130602 |
| LP32 | Similar to strain MHOM/CN/83/GS2 | HM130603 |
| LP33 | Similar to strain MHOM/CS11/CHN/SCgz | HM130606 |
| LP34 | No similar sample was found. | |
| LP35 | Similar to *L. major* IRN227 | KJ194181 |
| LP36 | Similar to *L. major* | FR796423 |
| LP37 | Similar to strain MHOM/GS6/CHN/SCgq | HM130599 |
| LP38 | No sequence result | |
| LP39 | No sequence result | |
| LP40 | Similar to *L. major* IRN227 | KJ194181 |
| LP42 | Similar to Strain *L. major* MHOM/IR/08/Kermanshah-mk1 | JX289871, first reported in Kermanshah |
| LP43 | Similar to strain MHOM/10Esfahan-8 | JX289862, first reported in Esfahan |
| LP44 | Similar to strain MHOM/CN/80/XJ801 | HQ 830357 |
| LP45 | Similar to strain *Leishmania tropica* MHOM/IR/03/Mash-878 | EU727198, first reported in Mashhad |

## 4. Discussion

In the present study, Leishmania promastigotes isolated from patient samples were typed with DNA extraction, PCR and DNA sequencing using primers specific for the ITS1 region.

All 44 extracted *Leishmania* DNAs were loaded on gel to see whether they produce detectible (DNA) bands or not. This was done and the images were captured (figure 1-8). In figure1, DNA bands for samples number 1-7 can be seen. Figure 2 shows DNA bands for samples 8 and 9. DNA bands for samples 10-13 can be observed in figure 3 (number 10 deleted due to insufficient amount of DNA, needed for PCR reaction). Following this, DNA bands for samples 14-23 except 22 were observed in figure 4. In figure 5, DNA bands for samples 28-32, 22 and 34 were observed. Figure 6 shows that the result of gel electrophoresis for samples 24-27 and 33-37 except 34 were positive and in figure 7 and 8 DNA bands for samples 38-40 and 42-45 were observed, respectively. These 44 Leishmanial DNAs, which produce bands on gel, were amplified by PCR and then, were loaded on gel (figure 9-12). All samples were examined in duplicate. Sample number 1,6 were PCR positive for *Leishmania* Major, number 2,3,4,5,13 were positive for *Crithidia fasciculata* and sample number 7 had no PCR product; therefore, it was not send to be sequenced (figure 9). As we can seen in figure 10, samples number 14-17 were PCR positive for unnamed *Leishmania* and number 18-21, 23 were PCR positive for *Leishmania major*. Figure 11 displays PCR positive samples identified as *Lishmania major* for samples number 22, 28, samples number 29-32 were unnamed *Leishmnia* and for sample 34, although it was PCR positive, there was no similar sample to number 34 in GenBank. Finally, despite of the fact that samples number 38 and 39 had DNA and PCR product, the sequencing results were negative for them; moreover, samples number 40, 42 were positive PCR product, identified as *Leishmania major* and samples number 43, 44 produced detectible PCR band and distinguished as unnamed *Leishmania* species. Also sample 45 was identified as *Leishmania tropica* (figure 12). Other PCR products, loaded on gel, didn't have high quality images to be attached to this article but all of them were sequenced. Species identification was confirmed by sequencing of the ITS1-PCR products for all positive PCR products. All sequence results were opened by CHROMAS software and were typed in BLASTn. Species identified in samples1, 6, 8, 9, 11, 12, 18, 19, 20, 21, 22, 23, 28, 35, 36, 40, 42 were *L. major*. Samples 2, 3, 4, 5, 13 were diagnosed as *Crithidia fasciculata*, and samples 14, 15, 16,

17, 24, 25, 26, 27, 29, 30, 31, 32, 33, 37, 43, 44 were unnamed species of *Leishmania* and sample 45 was *Leishmania tropica*. Moreover, the results found no similar sample-to-sample 34.

By focusing on ITS-rDNA, the present study could differentiate between *Leishmania* and *Crithidia*. The study carried out by Islami et al (2012) was the first genetic report on hybridization between *Leishmania* and *Crithidia*, which showed that these two are far different in phylogenetic and epidemiologic terms [8].

In the following part, different PCR methods are compared in order to target ITS gene to identify *Leishmania* species.

Dabirzadeh et al (2012) have specified PPIP-PCR technique and ITS-RFLP method for patterns of species-species section before use to determine *Leishmania* strains at species level. PCR methods used in this study found a high degree of genetic variability comparable between different strains of *L. major* species. Therefore, PPIP-PCR showed 9 separate bands and ITS-rDNA RFLP indicated 5 different types. The results of this investigation revealed genetic polymorphisms of *L. major* and indicated that strain A is more common than other strains [4]. According to these findings, remarkable heterogeneity was reported with ITS-RFLP of strains of *Leishmania tropica* and *L. major* and other species.

In another study carried out in Iran, 5 genotypes of ITS-rDNA from Kashan, Tehran, Dezful, Dehloran, and Damghan were identified. Similar results to the present study were obtained using 1taq enzyme. PPIP-PCR indicated more strains compared to ITS-rDNA-RFLP, which can be justified through this fact that a change in sequence may remain undiscovered using RFLP analysis because restriction enzymes only examine a subset of all changeable places. However, little attention has been devoted to the exact capacity of changing their sequence. For instance, analysis of DNA fragments through PCR-RFLP and random amplification of polymorphic DNA (PAPD) depends on separating the sections by agarose gel or polyacrylamide gel electrophoresis. Two DNA fragments with similar size but different sequences move same distance trough the gel and it may consider as two same sequences by mistake. In a similar study, PPIP-PCR assessment divided *Leishmania major* into three different species of LmA and two subspecies of 1LmA and 2LMA [4].

According to this information, this is the first application of PPIP-PCR for genetic change of *L. major* in Iran, and its results are summarized as follows:

All of the isolates except for two mixed infections were identified as *L. major* using ITS-RFLP and PPIP-PCR, and the best advantage of the latter was the provision of simple interpretation for the results of PCR products in agarose gel. These products enable us to draw distinction between intra-species differences and similarities. Bands of type A are similar to MHOM/IL/80/Frieldlin except for that instead of a 240bp band, it has bands of 180,220bp. Band C is similar to MHOM/IL/86/Blum, but instead of band 3100bp, it has a 261bp band [4]. Different evolutionary mechanisms including immigration, genetic selection and drift play an important role in density of genes in natural populations; however, reproduction is an essential biological process that affects the genetic structure of the population. Many scholars have reported evidence of hybrid formation in *Leishmania*, which leads to the enhancement if that the possibility that sexual reproduction happens in *Leishmania*. It is obvious that random and rare conflict of sexual recombination can have a deep effect on the extent of genetic diversity (4).

In Iran, *Leishmania* infections are identified either biologically or molecularly, and cases of infection from rodents and sandflies are fewer [14]. In the study conducted by Parvizi et al (2008), 103 out of 120 patient lived in villages and 100 out of those 103 patients had *L. major*. However, none of those sequenced isolates was from Esfahan province, while in the present study, two samples belonged to Esfarayen and Esfahan [16].

In Iran, except for the present study, the experiment carried out by Mohebi et al also amplified species of *Leishmania* through random amplification of DNA-PCR. They identified 105 rodents whose test result was positive and samples that were examined by molecular method identified *L. major* in different rodents [17].

Only by discovering parasite DNA in sandflies, one can conclude that at least 5 common species of fly in Iranian ZCL center and a rat parasite or more by feeding on source hosts that are mostly large rats in the sampling regions have been obtained. The rate of relevant contagion indicates that probably more than one of these flies is the vector of *L. major* (including *P. papatasi*, *P. casasicus*, *P. mongolensis*) and *L. gerbillis* (including *S. sintoni*, *P. papatasi*); however, *P. papatasi* is likely to be the only vector of *L. turanica*. This evidence is more likely to indicate that *P. papatasi* plays the role of key vector in ZCL center, and determine the importance of L. turanica in maintaining *Leishmania* contagion in rats. The importance of *P. papatasi* as a vector of *L. major* in Iran is proposed based on the frequency of flies in and around the heart of rats.

The mechanism of different species of parasites to preserve *L. major* infection was indicated for big rats. Discovering the role of the species of flies transferring any type of *Leishmania* in source hosts and people is as much important (14). Nowadays, available molecular tools can be utilized in ecological-demographic studies in order to respond to such questions about the role of different parasites in rats in maintaining ZCL center and the probability of emergence of contagion and new center. Different molecular methods have been proposed to identify *Leishmania* infection. For instance, PCR aims small laryngeal kDNAcan be very sensitive for tracking *Leishmania* parasites, which is due to the frequency of small laryngeal kDNAs in each kinetoplast [2, 3, 5]

Almeida et al (2011) carried out a study in order to develop a molecular method to distinguishing species levels that only need a pair of PCR primers. They focused on ITS region: rDNA separators are exposed to lower evolutionary pressure and indicate more sequence divergence than coded regions and are proposed as molecular typing aims. Moreover, they determined ITS2 region for their diagnosis aims and designed general PCR primers (LGITSF2/LGITSR2) to strengthen this part of genus *Leishmania* relevant to human infection. Remarkable differences were observed in ITS2 region that is covered with the primers that make general diagnosis of the species possible [1].

As other researchers first banned using ITS2 in identifying source species, Almeida et al considered intra-species variability inmicrosatellite repeats of AT, TC, TG, or GA during analyzing source strains. They also focused on some separate sequences due to changes and trans version changes. In most samples, separate sequences were observed in clones

obtained from those isolates that probably indicate the presence of several different copies of ITS in the same genome. In order to avoid the possibility of improper incorporation of nucleotides, DNAs of some samples were boosted again through DNA of highly accurate polymerase pfu, and the results were the same as those who had conventionally used Taq DNA. During the growth phase of this evaluation, they cloned the amplicons of the reference strain in order to obtain the sequence of the quite diagnostic part, and indicated that partial sequence is enough for identification of species [1].

Similar discrepancies were previously reported for strains of *L. donovani* [13]. However, it seems that a method that was used for several clinical samples (4 out of 6 samples that were classified as *L. donovani*) led to wrong classification of *L. infantum*/*chgasi* as *L. donovani*. In order to determine the capacity of molecular ITS in distinguishing *L. donovani* from *L. infantum/chgasi*, more experiments with bigger samples from specific regions are required [1].

A problem of this method is the need for analysis of DNA sequencing which is not available in most laboratories of these regions. However, ITS2 part has a binding region for enzymes of HinP1I, HhaI (GCGC), and 1Mn I (GAGG), which may be applicable to identify the species that were evaluated in the present study (except for those that belong to *Viannia* family). The binding region for HphI ($GGTGAN_7$) can be used to identify *L. guyanensis*; however, it can be used for *L. brazilian* or *L. panamensis*. These binding regions can be examined as goals for limiting analysis of fragment length polymorphism to distinguish some species by laboratories where DNA sequence analysis is not available [1].

Briefly, the results of the present study confirmed this hypothesis that the ITS-rDNA sequence region can complete the characteristics of *Leishmania* parasites. The method had been developed in the present study can be employed as a diagnostic tool in reference laboratories that have appropriate infrastructures for conducting molecular identification of parasites.

**References**


1. De Almeida ME, Steurer FJ, Koru O, Herwaldt BL, Pieniazek NJ, Da Silva AJ. Identification of Lieshmania spp.by Molecular Amplification and DNA Sequencing Analysis of a Fragment of rRNA Internal Transcribed Spacer 2.*JCM*. 2011; 49(9):3143-3149.
2. Anders G, Eisenberger CL, Jonas F, Greenblatt CHL. Distinguishing *Lieshmaniatropica* and *Lieshmania major* in the Middle East using the polymerase chain reaction with kinetoplast DNA-specific primers. *Trans R Soc Trop Med Hyg*. 2002; 96(Supplement 1):S87-S92.
3. Bensoussan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CHL. Comparison of PCR assays for diagnosis of cutaneous Leishmaniasis. *JCM*. 2006; 44(4):1435-1439.
4. Dabirzadeh M, Mirmohammadsadeghi H, Baghaie M, Hejazi H. Genetic Polymorphism of *Leishmania major* in Two Hyper Endemic Regions of Iran Revealed by PPIP-PCR and ITS- RFLP. Arch Iran Med. 2012; 15(3):151-156.
5. Disch J, Pedras MJ, Orsini M, Primez C, De Oliveira MC, Castro M et al. *Leishmania* (*Viannia*) subgenus kDNA amplification for the diagnosis of mucosal Leishmaniasis. Diagn. Microbiol. Infect. Dis. 2005; 51(3):185-190.
6. Doroodgar M, Doroodgar M, Doroodgar A. Unusual Presentation of Cutaneous Leishmaniasis: Ocular Leishmaniasis. Case Reports in Infectious Diseases, 2017, Article ID 3198547, 2017, 4 pages,doi:10.1155/2017/3198547
7. El Tai NO, Osman OF, el Fari M, Presber W, Schonian G. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania Donovani spotted on filter paper as revealed by single-strand conformation polymorphism and sequencing. *Trans Soc Trop Med Hyg*. 2000; 94:575-579
8. Eslami G, Salehi R, Khosravi SH, Doudi M. Genetic analysis of clinical isolates of *Leishmania major* from Isfahan, Iran. *J Vector Borne Dis*. 2012; 49(3):168-174.
9. Garcia L, Kindt A, Bermudez H, L-Cuentas A, De Doncker S, Arevalo J et al. Culture-independent species typing of neotropical *Leishmania* for clinical validation of a PCR-based assay targeting heat shock protein 70 genes. *JCM*. 2004; 42(5):2294-2297.
10. GrimaldiJr G, McMahon-Pratt D. Monoclonal antibodies for the identification of New World Leishmani species. *J Scielo*. 1996; 91(1):1678-8060
11. Ihalamulla RL, Rajapaksa US, Karunaweera ND. Microculturefor the isolation of Leishmania modified to increase efficacy: a follow-up to a previous study. *Ann Trop Med Parasitol*. 2013; 100(1):87-89.
12. Kar K. Serodiagnosis of Leishmaniasis. Crit Rev Microbiol. 2008; 21(2):123-152.
13. Kuhls KL, Mauricio IL, Pratlong F, Presber W, Schonian G. Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmaniadonovani* complex. Microb Infect. 2005; 7(11):1224-1234.
14. Mohebali M, Javadian E, Yaghoobi Ershadi MR, Akhavan AA, Hajarian H, Abaei MR. Characterization of Leishmania infection in rodents from endemic areas of the Islamic Republic of Iran. *EMHJ*. 2004; 10(4-5):591-599.
15. Parvizi P, Benlarbi M, Ready PD. Mitochondrial and *Wolbachia* markers for the sandfly *Phlebotomuspapatasi*: little population differentiation between peridomestic sites and gerbil burrows in Isfahan province, Iran. *Med Vet Entomol*. 2003; 17(4):351-362.
16. Parvizi P, Moradi GH, Akbari GH, Farahmand M, Ready PD, Piazak N et al. PCR detection and sequencing of parasite ITS-rDNA gene from reservoirs host of zoonotic cutaneous Leishmaniasis in central Iran. *Parasitol Res*. 2008; 103:1273.
17. Parvizi P, Ready PD. Nested PCRs, and sequencing of nuclear ITS-rDNA fragments detect three *Leishmania* species of gerbils in sandflies from Iranian foci of zoonotic cutaneous Leishmaniasis. *TMIH*. 2008; 13(9):1159-1171.
18. Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous Leishmaniasis. *Lancet Infect Dis*. 2007; 7(9):581-596.
19. Reithinger R, Dujardin JC. Molecular diagnosis of Leishmaniasis: current status and future applications. *JCM*. 2007; 45(1):21-25.
20. Tashakori M, Kuhls K, Al-Jawabreh A, Mauricio I, Schonian G, Farajinia S et al. *Leishmania major*: genetic heterogeneity of Iranian isolates by single-strand

conformation polymorphism and sequence analysis of ribosomal DNA internal transcribed spacer. *Acta Tropica.* 2006; 98(1):52-58.