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Full Length Research Paper

Genotyping of isolated cutaneous Leishmaniasis species by polymerase chain reaction- restriction fragment length polymorphism (PCR- RFLP) analysis in endemic foci of Isfahan, Iran

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Genotyping Cutaneous Leishmaniasis (CL) species is important for selecting the appropriate method to control and prevent disease, determine the health effects of drugs, prepare and evaluate the vaccine and finally to determine disease vectors and reservoirs. In order to identify *Leishmania* species, endemic foci of Iran center (Isfahan) was used in this study using polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) method. For this purpose, 305 clinical samples were collected from patients referred to health centers in the city of Isfahan. Two samples were collected from each patient for direct slide and culture. Nucleic acid of samples was separated using deoxyribonucleic acid (DNA) extraction kit and kept in 20°C until the test time. Amplify of ITS1 area using L5.8s and LITSr primers showed 350 bp in a number of isolated cases and 450 bp in some others. Enzyme digestion of bands was compared using the enzyme HaeIII with the digested areas of reference strains. The resulted product of a number of isolates was analyzed after determining the sequence. The results showed that 193 isolated cases had 350 bp bonds and after the enzyme digestion revealed 220 and 140 bp bands that were related to the *Leishmania major* and 7 isolated cases with 350 bp bands that revealed bands 200 and 60 bp after enzymatic digestion that was related to *Leishmania tropica* and 55 isolated cased had 450 bp bond and after enzyme digestion, revealed 300 and 150 bp parts and after determining the sequence with *Crithidia fasciculata* and *Crithidia luciliae* they were matched to about 96%. It seems that by planning new studies and completing current studies, more conclusive results can be accessed and therefore it seems that control, prevention and treatment of the disease in the future may be associated with changed strategies.

Key words: Leishmania, Cutaneous Leishmaniasis, ITS1, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP).

INTRODUCTION

Cutaneous Leishmaniasis is a serious public health problem and a complex disease with a wide range of clinical symptoms being prevalent in more than 88 countries, including Iran and Isfahan is one of the endemic foci of CL (Nadim et al., 1968).

The disease occurs through different species of parasitic protozoa of *Leishmania* type. Cutaneous forms of the disease occur in a range of simple form nodules to active chronic lupoid. Determining *Leishmania* species specifications is very important for determining strategies of control, prevention and treatment (Hajjaran et al., 2004; Kazemi-Rad et al., 2008).

Clinical forms due to different species causing the disease may require different treatment methods. Since the treatments are expensive and drugs have toxic side

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Figure 1. Geographic distribution of Cutaneous Leishmaniasis in endemic cities in Iran.

effects, treatment failures should be reviewed to diagnose the difference between a recurrence of latent infection and re-infection by other species (Kumar et al., 2007). Initial classification of *Leishmania* species has been based on clinical signs, epidemiology features and geographic disperse of parasite especially is endemic regions (Chance et al., 1979).

All these parameters are important in determining parasite species, but they are not sufficient evidence for a complete description of *Leishmania* species due to lack of care and simultaneous presence of several species of *Leishmania* in one region (Eltai et al., 2000; Marfurt et al., 2003).

Classical method of detection and identification of parasite species, including microscopic observation, culturing, biochemical and immunological methods and old methods also are considered not conclusive (McMahon-pratt et al., 1981; Rioux et al., 1990). Currently, molecular methods and the use of parasite DNA by PCR is a gold standard for distinguishing *Leishmania* species (Schonian et al., 2001). Among these methods, RFLP has shown a promising result in the analysis of PCR products from multi-version genes (Baghaei et al., 2005; Kazemi-Rad et al., 2008). This study aimed to use PCR method to identify the species of *Leishmania* and then RFLP method for genotyping of isolated cases in the city of Isfahan.

MATERIALS AND METHODS

Patients and research regions

This study was conducted in 5 suburb of Isfahan (Isfahan, Khorasgan, Segzi, Mohammadabad and Varzaneh) and 4 villages of Borkhar (Habib-abad, Khorzuq, Dastgerd, and Dolat-abad) on a total of 305 samples (Figure 1).

The sampling was from 21st March, 2009 to 20th March, 2010. A variety spectrum of skin ulcers were selected from small nodules to progressive bad form wounds (Figure 2).

Collecting samples and preparation

At first wounds were sterilized with 70% alcohol and saline and then a topical anesthesia (Xylocaine) was applied, and using a sterile surgical knife, surface cuts with length of 2 to 3 mm was created on the wound edges. For each patient, two slides were prepared, one for microscopic observation and the other for PCR. Both slides were air dried first and then were established in pure methanol and both were painted with gymsa.

The prepared surface cuts were entered to Brain Heart Infusion (BHI; Merk, Germany) medium liquid as a transition environment and then were transferred to Novey-Nicol-Mac Neal (NNN) medium

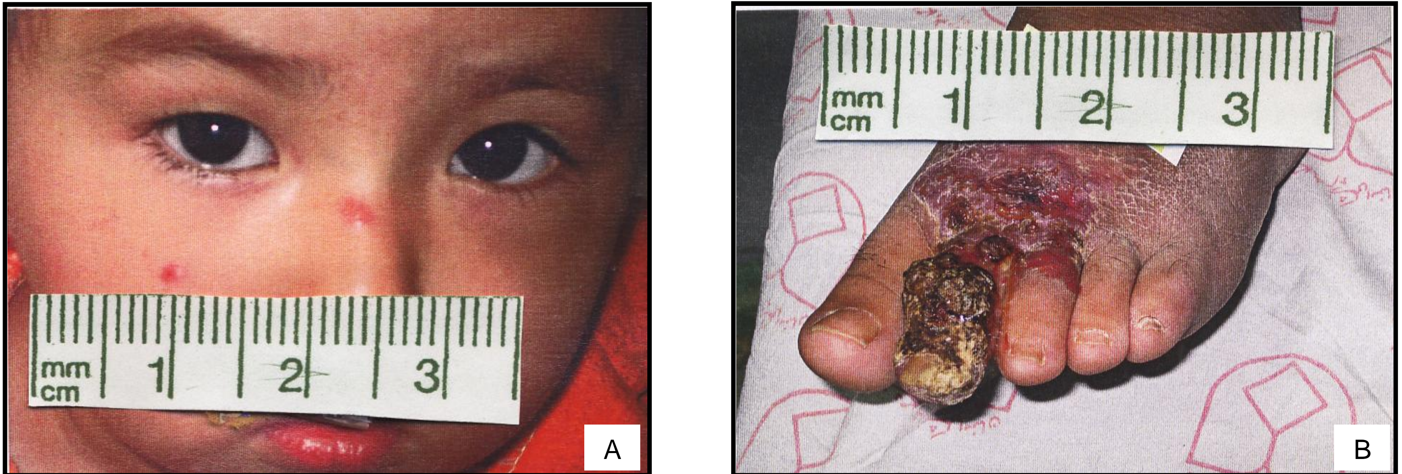

Figure 2. (A) Small nodules of Cutaneus Leishmaniasis with 2 × 2 mm dimensions in Isfahan (B) and Progressive Cutaneus Leishmaniasis wound with 2 × 5 cm dimensions in Borkhar area.

and were placed in incubators with 24±1°C temperature.

Isolation and culture of parasite

Brain heart infusion (BHI) liquids with parasite also were transferred in sterile conditions to NNN medium and were placed in 24 ± 1°C temperature and they were checked once every three days for 6 weeks before they were reported negative. Then multiplied promastigotes were amplified and transmitted to RPMI 1640 medium with 10% FCS (Sigma). Then the parasites in the late logarithmic growth phase (5×10^6, parasites/ml) was harvested and with sterile Phosphate Buffered Salt Solution (PBS) with pH = 7.4 was washed and then was placed in freezer -70°C for next experiments.

Standard species

Three reference species in this study were used: *Leishmania major* (MRHO/IR/75/ER), *Leishmania tropica* (MHOM/IR/99/YAZ1) and *Leishmania infantun* (MCAN/IR/97/LON49).

Deoxyribonucleic (DNA) extraction

All slides were washed with pure ethanol and were placed in 250 µl fertilizer including: (Nacl 50 mM, Tris 50 Mm, EDTA 10 mM, %1 v/v Triton X-100, 200 µg/ml of Proteinase K , pH = 7.4) and were transferred to a pipe of 1.5 ml and were placed in water bath at 72° C for 2- 4 h or in water bath at 56°C for one night (Kazemi-Rad et al., 2008; Schonian et al., 2003). Other stages were done based on the DNA extraction kit (Roch, Germany) guidelines.

For the cultivated parasite, suspension promastigotes of washed Leishmania which contained 5×10^6 parasites were taken out of the freezer at -70°C and 200 µl Binding Buffer and 40 µl proteinase K were added to its sediment and the contents were placed in water bath at 70°C for 10 min, the rest of the steps were done based on the DNA extraction kit guidelines and finally DNA was solved in 200 µl of Elution Buffer. The quality and quantity of extracted DNA were analyzed by spectrophotometers and agarose gel 0.8% in TBE 1X buffer.

Polymerase chain reaction (PCR) amplification of ITS1

To multiply the ITS1 region by PCR method, specific primers of Leishmania species were used with the following sequence:

LITSr (5 ´- CTGGAT CATTTT CCG ATG-3 ´), $L_{5.8S}$ (5ó - TGATAC CACTTA TCG CAC TT- ó).

Using reference strains, PCR conditions were optimized. The reaction was performed in 25 microlitre volume. First, Master Mix including 0.2 µl Taq polymerase, 2 µl $Mgcl_2$ (50 mM), Forward and Reverse primers with a concentration of 10 picomole, 1 µl from each, 2 µl $dNTP_s$ mix, PCR 1X buffer and extracted DNA 1-5 µl and finally, the final volume with sterile distilled water were increased to 50 µl. Samples were placed inside the thermal cycler (Corbet) and the following cycles were scheduled.

The first naturasion stage of 95°C was done for 5 min and following that 30 cycles including denaturasion 95°C for 20 s, annealing 50°C for 30 s, Extention stage equal to 72°C for one min and finally Post extention stage in 72°C for 6 min.

Electrophoresis was done for PCR products with positive and negative control along with molecular marker 50bp on agarose gel 1% containing 0.7 µg/ml ethidium bromide for each mililitre gel in TBE 1X under voltage 80 for 25-45 min and finally PCR products were observed in trans laminator using UV lamps (Kazemi-Rad et al., 2008; Schonian et al., 2003).

Restriction fragment length polymorphism (RFLP) analysis of amplified ITS1

To determine the species, ITS1-PCR products were digested under influence of limiting enzyme HaeIII (Fermentase) and related buffer in 37°C for 2 h and again to determine the species of Leishmania, electrophoresis was done on agarose gel 2% in TBE 1x buffer under voltage 80 for 1 h and were analyzed (Kazemi-Rad et al., 2008; Schonian et al., 2003).

ITS sequencing

To study DNA sequencing, ITS1- PCR products were used. There are three different standard species of *Leishmania* in the gene

Table 1. Frequency distribution of PCR products in two regions of Isfahan and Borkhar in Iran.

Band size		350 bp		450 bp		Total	
Name of the city	**Endemic areas**	**Number**	**Percent**	**Number**	**Percent**	**Number**	**Percent**
Isfahan	Isfahan	49	24.5	20	36.5	69	27
	Khorasgan	13	6.5	5	9	18	7.1
	Segzi	8	4	3	5.4	11	4.3
	Mohammadabad	25	12.5	2	3.6	27	10.6
	Varzaneh	19	9.5	3	5	22	8.6
Borkhar	Habib-abad	25	12.5	6	11	31	12.2
	Dolat-abad	26	13	8	14.5	34	13.3
	Dastgerd	19	9.5	5	9	24	9.4
	Khorzuq	15	8	3	5.5	19	7.5
		200	100	55	100	255	100

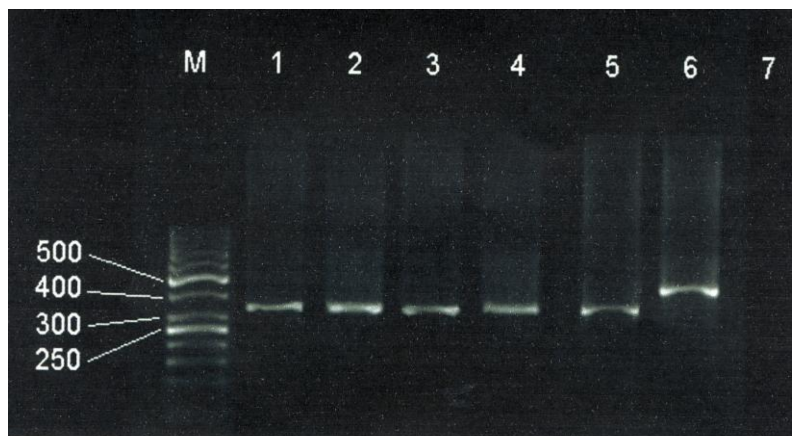

Figure 3. Electrophoresis of PCR products of reference strains, Isfahan and Borkhar M: Molecular marker (50bp) 1: Standard *L. major*, 2: Standard *L. tropica*, 3: Standard *L. infantum*, 4: PCR product of Isfahan based on standard, 5: PCR product of Borkhar based on standard, 6: PCR products of Isfahan and Borkhar different from standard 7: Negative control.

database and the product of species in this study which was different from standard samples was registered in NCBI gene database with Accession Number of GQ331988.

Statistical analysis

To analyze the frequency distribution of *Leishmania* species in two regions of the study, chi square test was used (*P<0.001*).

RESULTS

In this study, among 305 patients suffering from Cutaneus Leishmaniasis, 255 patients were selected from Isfahan and Borkhar and after sampling; slide samples and their cultures were reported positive. Cultures infected with bacterial or fungal agents and negative microscopic slide were excluded from the study. A total of 255 patients in 9 regions of Isfahan and Borkhar were studied using ITS1-PCR technique. In general, from the total 255 isolated cases studied in these two endemic area, 200 isolated (78.4%) 350 bp band and 55 isolated (21.6%) 450 bp band were disclosed (Table 1) (Figure 3).

As it is clear, this difference of 100 bp is seen in about one fourth of isolated. All samples were studied by digesting ITS1-PCR products with HaeIII (Fermentase) enzyme. In 193 isolated (75.7%) of Isfahan and Borkhar pieces of 210 bp and 140 bp were disclosed after enzymic digestion, that was related to *Leishmania major* and in 55 isolated (21.5%) of Isfahan and Borkhar, after enzimic digestion HaeIII 300 and 150 bp were disclosed that was not according to standard pattern and based on the sequence was related to *Crithidia Fasciculta* and *Crithidia lucilia*. In 7 isolated (2.7%) of Isfahan and Borkhar, after enzymic digestion with HaeIII, 60 and 200 bp

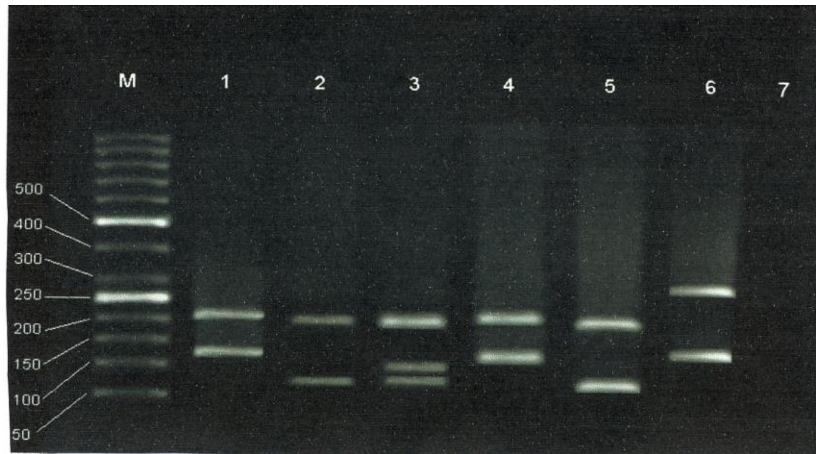

Figure 4. Electrophoresis of HaeIII enzyme digestion products of reference strains, Isfahan and Borkhar. M: Molecular marker (50 bp) 1: Standard *L. major*, 2: standard *L. tropica*, 3: Standard *L. infantum* 4: *L. major* in accordance with standards of Borkhar and Isfahan, 5: *L. tropica* in accordance with standards of Isfahan and Borkhar, 6: Crithidia in accordance with sequence results of Isfahan and Borkhar, 7: Negative control.

Table 2. Frequency distribution of *Leishmania* and *Crithidia* species in Isfahan and Borkhar in Iran.

Name of the city	Endemic areas	*L. major*		*L. tropica*		*Crithidia*			
		Number	Percent	Number	Percent	Number	Percent	Number	Percent
Isfahan	Isfahan	45	23.3	3	43	20	36.4	69	27
	Khorasgan	11	5.6	2	28.6	5	9	18	7
	Segzi	8	4.2	-	-	3	5.5	11	4.4
	Mohammadabad	25	13	-	-	2	3.6	27	10.6
	Varzaneh	19	9.8	1	14.2	3	5.5	22	8.6
Borkhar	Habib-abad	25	13	-	-	6	11	31	12.2
	Dolat-abad	26	13.5	-	-	8	14.2	34	13.3
	Dastgerd	19	9.8	-	-	5	9	24	9.4
	Khorzuq	15	7.8	1	14.2	3	5.5	19	7.5
		193	100	7	100	55	100	255	100

bp pieces were disclosed and based on the standard pattern was related to *Lishmania tropica* (Figure 4) (Table 2). In none of the isolated of Isfahan and Borkhar, after enzymic digestion with HaeIII, three pieces of 60, 80 and 200 bp that belong to *Leishmania infantum* were not disclosed.

Therefore, comparing the above results with the model of enzyme digestion of reference species and PCR-RFLP analysis in ITS1 region, it seems that in both endemic areas of Isfahan and Borkhar, in addition to *Leishmania major* and *Leishmania tropica*, *Crithidia facsiculta* and *Crithidia lucilia* that like *Leishmania* are members of trypanosomathide family and are considered non-pathogenic to humans, have been isolated in relatively large number of patients.

DISCUSSION

In control strategies of Leishmaniasis in an endemic area, a major requirement is to determine specificities of species that cause the disease when different species of *Leishmania* can make similar clinical symptoms. Therefore, identification of *Leishmania* species is very important, because different species may require different treatment and control strategies (WHO). For example, for the treatment of CL caused by *L. guyanensis*, pentamidine and for the treatment of CL due to *L.*

braziliensis, Glucantime is the best choice (Croft et al., 2002).

Furthermore, such information are considered a prerequisite for designing appropriate disease control measures and considered very valuable in epidemiology studies, where there are species *Leishmania* in human and animal hosts and also in insect vectors (El Tai et al., 2000; Schoian et al., 2003). Nowadays, molecular methods has replaced traditional methods as an alternative method in the diagnosis and typing of many microorganisms and are used as the best and most effective diagnostic tool (Singh et al., 2003).

In this study, the molecular PCR-RFLP method was also used. To implement this method, first a specific primer and still a member of *Leishmania* was applied to diagnose the above parasite in the samples by PCR and after that RFLP test was conducted, for positive samples to diagnose *Leishmania* parasite species. Pattern of cutting digestion enzymes is different between various species according to sequence difference of polygraph ITS1 area. Many molecular studies have used various areas of the parasite genome for identification, one of these areas is ITS (Davila and Momen, 2000; Schonian et al., 2000).

According to studies, the reproduced piece of standard strains of different species of *Leishmania* using two primer LITSr and L5.8s has revealed 350 bp (Kazemi-Rad et al., 2008; Schonian et al., 2000; Tashakori et al., 2006), while in the present study, using the same primers and standard conditions, a small number of isolates revealed 450 bp and many of them revealed 350 bp. For better analysis of this 100 difference in the above mentioned groups, the product of some species in each group were selected randomly and was sent for sequence and based on the molecular analysis with Blast software, it was found that gene sequence of a number of the isolates with band 450 bp are 97% similar with *Crithidia fasciculata* and 96% similar with *Crithidia luciliae* and 40% similar to *Leishmania infantum* Strain MCAN / IR / LON49.

This sequence was registered in the world bank of genes NCBI with Accession Number GQ331988.

Thus, ITS1-PCR products were place under the influence of HaeIII enzyme. According to some studies, if PCR - ITS1 products with the enzyme HaeIII Parts would reveal 220 and 140 bp, it was related to *L. major* and in case that two pieces of bp 200 and 60 bp were created, it was *L. tropica* and emergence of three pieces of 200 and 80 and 60 bp would make it *L. infantum* (Kazemi-Rad et al., 2008; Tashakori et al., 2006).

With regard to HaeIII enzyme digestion map, four pieces of 200, 64, 55 and 20 bp must be the result of *L. tropica* enzymatic digestion, but because of the small size of the last piece (20 bp) and the overlap of the two pieces with a molecular weight of about 64 and 55 bp caused only 200 and 60 bp parts to be recognizable (Erans, 1989; Schonian et al., 2003).

To study different species, all samples of the two areas of Isfahan and Borkhar that had 450 and 350 bp were placed under influence of enzyme HaeIII. In 55 (21. 5%) isolates, a different pattern of RFLP compared to the reference strains was observed and after applying the HaeIII enzyme, 300 and 150 bp parts were separated. While in the 200 bp isolate (78.4%) the same RFLP pattern compared with reference strains was observed. It means that in 193 (75.7%) isolates from both regions, after applying the enzymes Hae III, parts 220 and 140 bp were separated that was related to *L. major* and in 7 isolates (2.7%) of both regions, after applying enzymes HaeIII, 200 and 60 bp were separated that was related to *L. tropica.*

This study for the first time reports that *Crithidia* may be pathogenecitically act like *Leishmania* and create similar clinical symptoms as *Leishmania*. Because this report is rare and exceptional considering the previous findings in endemic areas of Isfahan and many parts of Iran (Nadim et al., 1968; Tashakori et al., 2003). It requires very strong justifications and evidences, which can be the basis for designing further studies by researchers who are interested in this field.

In this study, the presence of *Crithidia* cannot be accidental or due to pollution of culture medium with this microorganism, because the studied samples were prepared from all patients in completely sterile conditions and away from any pollution and studied samples included microscopic slides in addition to culture. On the other hand, in accordance with the conducted sequences, a number of isolates were reported, that is, *L. major*, some *L. tropica* and some *Crithidia*.

Another explanation in this regard is that these results could be due to heterozygote species or mixed species (genetic hybrid), because these analyses are the result of studying isolates that were not simulated and were directly isolated from the patients.

About recombination between species, some studies have suggested genetic exchange. Some researchers in the new world have described hybridization of *L. peruviania*, *L. guyanensis* and *L. braziliensis* (Belli et al., 1998; Da-cruz et al., 1992; Dujardin et al., 1995).

In addition, Kreutzer and Christensen (1980) by a quantitative micro-spectrophotometer showed that nuclear integration and sexual reproduction occurs in forms of amastigotes inside the cell. This has also been shown by Youssef et al. (1997) in amastigotes and promastigotes using the nuclear DNA and computer pictogram.

After hybridization, hybrid strains were naturally reproduced in the population. An example of this type of hybrid can be seen between the *L. braziliensis* that creates cutaneous lesions or Cutaneous-Mucous Lesions in humans, which require treatment and *L. peruviana* that creates benign dry cutaneous lesions that can be healed on their own. The hybrid between these two has been seen in patients in Peru that creates cutaneous-epithelial

lesions as well as benign lesions related to infection with *L. peruviana* (Martine-Calvillo et al., 2001).

As it was explained, exchange between genes and hybridizations among the *Leishmania* parasites (Cupolillo et al., 2003) can cause new parasite hybrids and possibly cause different clinical forms of the disease (Baily and Lockwood, 2007). In this regards, one hypothesis is the existence of signaling phenomenon between genes and it is thought that perhaps in the process of this phenomenon, virolancy gene that is a *Leishmania* transfers to *Crithidia* and in this process the non-malicious *Crithidia* turns to a pathogenic organism (Martine-Calvillo et al., 2001).

In general, it can be concluded from the results of the present study that not only *L. major* is the agent of wet Cutaneous Leishmaniasis, but *L. tropica* is the agent of dry Cutaneous Leishmaniasis in Isfahan, but the presence of a new species of the family Trypanosomatidae in these two regions suggests the necessity of rethinking in study of the disease pathogenesis, epidemiology and classification and treatment of parasite. In addition, although clinical signs of Cutaneous Leishmaniasis and review of disease pathogenesis is affected by individual characteristics of the host and genetic characteristics of the parasite, results presented in this study and other researches show that parasite genetic characteristics can play an important role in incidence of clinical symptoms, pathogenesis, epidemiology and classification of parasite (Banuls et al., 1997). However, conclusive commenting about this topic requires extensive studies, especially on samples isolated from spare hosts, vectors and humans in different areas.

On the other hand, wet Cutaneous Leishmaniasis disease exists in a number of Afghan migrants in Isfahan and Borkhar and considering the possibility of person to person transfer by Phlebotominae cannot be ignored (Cupolillo et al., 2003; Delgado et al., 1997), those who are infected will have new source of infection and this issue will provide chances to create new hybrid species between different geographical species and may lead to a new type of wound.

Conclusion

The present study showed that PCR-RFLP is a precise, sensitive and rapid method for diagnosis and genotyping of Cutaneous Leishmaniasis agents and considering the sampling method, which includes direct microscopic slide and culture and both presented similar results, there is no need to culture the parasite that takes time, but by using PCR-RFLP method, within one working day, one can not only diagnose Cutaneous Leishmaniasis with high sensitivity and easy sampling, but can identify the species of parasites and provide patients with proper guidance and tips for treatment. The results of this study are also important in fast diagnosis and epidemiologic studies in Isfahan region.

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