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*Full Length Research Paper*

# Genetic polymorphism analysis of *Leishmania tropica* isolated from three endemic regions (Bam, Kermanshah and Mashhad) in Iran by PCR-RFLP technique and based on ITS1 sequences

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**Anthroponotic Cutaneous Leishmaniasis (ACL) is a parasitic disease caused by a single-celled parasitic protozoan *Leishmania tropica* with an overall prevalence of approximately 12 million cases worldwide. In Iran it is an increasing public health problem with an endemic focus in three regions of Bam, Kermanshah and Mashhad. This study was designed to characterize *Leishmania* spp. by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) technique, regarding to both high incidence rates of the disease in Iran and the variety of different species of *Leishmania* which might affect the prevention strategies. A total of 330 samples were taken directly from skin scars of confirmed cases of leishmaniasis derived from three endemic region of Iran. The samples were used for smear-slide preparations and microbial culture to confirm the infection and then they were subjected to PCR-RFLP detection. DNA from each slide was extracted separately and was examined by means of PCR-RFLP. The information revealed in DNA sequencing was achieved from the amplification of ribosomal internal transcribed spacer 1 (ITS1) which was done using LITSr and L5.8s primers. Six different genotype groups of *L. tropica* were obtained in these three studied regions. In total six genotypic groups LtA, LtB, LtC, LtD, LtE, LtF were identified among *L. tropica* isolates. The most frequent genotype, LtA, belonged to the isolates collected from all three endemic regions of ACL in Iran. Genotypes LtF and LtE were found in the isolate that came from Bam and LtD, LtC and LtB were identified exclusively among isolates of Mashhad. *L. tropica* is the pathogenic agents responsible for ACL in endemic region of Iran. This infectious agent is genetically polymorphism and it was hypothesized that there could be a probable correlation between the prevalence rate of the infection and genotypic variations of the studied stains and also their geographic origin.**

**Key words:** Polymorphism, *Leishmania tropica,* polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP), internal transcribed spacer 1 (ITS1).

## INTRODUCTION

Leishmaniasis is a spectrum of diseases caused by infection with different species of the protozoan parasite *Leishmania*. Cutaneous Leishmaniasis is a serious public health problem with a wide range of clinical symptoms being prevalent in more than 88 countries. *Leishmania tropica* is recognized as one of the causative agent of Anthroponotic Cutaneous Leishmaniasis in the Old World (Peters et al., 1995), causing a broad spectrum of infection in humans with a wide range of clinical symptoms

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from small nodules to widespread cutaneous lesions (Schnur et al. 1995). *L. tropica* is mainly isolated from Visceral Leishmaniasis (VL) and also in some other cases from Post Kala-azar Dermal Leishmaniasis (PKDL) in autochthonous patients (Shahian and Alborzi, 2009). It is generally believed that *L. tropica* causes an anthroponotic disease which is transmissible only between humans without interference of animals. This kind of human infection is transmitted between one person to another by the bite of an infected sand fly *Phlebotomus sergenti.* Leishmaniasis is a zoonotic disease which can be transmitted from animal vectors to people (Kamhawi et al., 1995). In the past decades, *Leishmania* parasites were identified and classified based on the morphological differences found between isolates by means of microscope slide preparation. Other characters such as the their geographical distribution of isolated samples specifically in distinct endemic regions, the level of virulence and infectivity of the isolates for human host and their growth pattern in culture medium were also critical in differentiation procedure of *Leishmania* parasites. But all of these methods lacked the accuracy required for optimal result, for example in some individual region several isolates were found (Marfurt et al., 2003). Molecular techniques for *Leishmania* characterization such as isoenzyme analysis and PCR amplification are currently replaced the old procedures mentioned above (Schonian et al., 2003). Isoenzyme analysis helps us to detect the highest level of genetic diversity in different strains of *L. tropica* but there are some documents that showed according to some evidences, zymodemes were not consistent with geographic distribution and the clinical manifestation of the patient (Mebrahtu et al., 1992). Currently, some PCR technologies such as RAPD-PCR resulted in the precise differentiations of genetic heterogeneities in different *Leishmania* species (Schonian et al., 2003).

Among all of these characterization methods, the use of Restriction Fragment Length Polymorphism analysis (RFLP) in genotype identification has shown promising results. PCR-RFLP analysis of Internal Transcribed Spacer 1 (ITS1) was conducted on RNA gene in different *Leishmania* species of either New World (NW) or Old World (Cupolillo et al., 2003; Alrajhi et al., 2003).

Sequence comparison of the ITS1 gene region is mainly used in the determination of phylogenic relation among species. ITS1 gene region seems to be less conserved even between closely related species. Therefore, the study of this specific region is useful in the determination of intraspecific differences of *Leishmania*. ITS gene region contains different sequences including ITS1 located between 5.8S rRNA and SSURNA and ITS2 situated between 5.8S rRNA and LSURNA.

The importance of ITS1 examination data is the revealing of phylogenic affinity among *Leishmania* species based on the analysis of this highly variable rDNA. In comparison with other rRNA gene region, ITS1 has the highest discriminatory power for determining the closely related *Leishmania* species (Rotureau et al., 2006; Yang et al., 2010).

In this study, PCR-RFLP is reintroduced as a useful and feasible diagnostic tool for identification of *Leishmania* species and for the purpose of highlighting DNA polymorphism observed in *L. tropica* isolates collected from three hyper endemic regions of Iran; Bam, Kermanshah, Mashhad. Moreover new strains were subjected to DNA sequencing.

## MATERIALS AND METHODS

### Patients and research regions

Bam is a city in eastern part of Kerman province of Iran. Area of Bam is 19,424 $km^2$. Kerman is located in the south-east of Iran. It is the second largest province of Iran with an area of 181,714 $km^2$. The city of Bam has a semi-moderate and dry climate and the altitudes of the city from the sea is 1076 m.

The center of Khorasane Razavi province, Mashhad covers an oval measuring 204 $km^2$ and it is situated at 985 m above sea level. The city is located at 36.20º latitude and 59.35º east longitude, in the valley of the Kashaf river near Turkmenistan, between the two mountain ranges of Binalood and Hezar-masjed. Mashhad experiences hot summers and cool winters.

Kermanshah province of Iran covering an area of 24,990 $km^2$ is located in the west of the country.It is bordered by Iraq to the west and located beside two Iraq city of Soleimaniye and Diyali. Kermanshah has a moderate and mountainous climate. City's elevation average about 1335 m above sea level.

In this experimental study, a total of 330 patients positive for Anthroponotic Cutaneous Leishmaniasis were selected. Samples were obtained from patients of 3 endemic regions of Iran; Bam (110), Kermanshah (95) and Mashhad (125) during the period from the beginning of June until the end of March 2010.

### Collecting samples and preparation

First of all the scares were disinfected with 70% alcohol and serum physiology. Lignocaine ointment was applied on the specified areas as a local anesthetic. The biopsy specimens were extracted with the removal of 3 to 4 mm of tissue from the active edge of the lesion by a blade. The samples were then smeared on a microscope slide, and stained with Giemsa. Two smeared glasses were prepared for each patient; one for microscopic observation and the other for molecular analysis. The samples extracted from the internal edges of the skin scares were steriled and transferred into an enrichment medium, Brain Heart Infusion broth (BHI), then into biphasic Novy-Nicole-Macneal (NNN) for culturing and were incubated at 24 ± 1°C. These cultures were examined every 3 days for the period of six weeks before they show negative results.

### Isolation and culture of parasite

After the proliferation of promastigotes, they were transferred to RPMI 1640 medium supplemented with 10% FCS (Sigma) and antibiotics. In their final stage of growth, parasites were taken from the medium with density of $2\times10^6$/ml. They were centrifuged and washed with sterile Phosphate-Buffered Saline (PBS) with pH of 7.4 and then stored at -70°C for further manipulation (Al-Jawabreh et al., 2006).

#### Standard species

*L. tropica* (MHOM/IR/99/YAZ1) and *L. major* (MHOM/IR/75/ER) were used as standard in this study.

#### DNA extraction

All the prepared slides were washed with PBS and then this solution was transferred into a 1.5 ml tube.In the next step, It was disposed to lysis buffer containing; 50 mM Nacl, 50 mM Tris, 10 mM EDTA, 1% V/V Triton X-100, 200 mg/ml proteinase K solution, pH: 7.4 and incubated for 1.5-2 hours at 56°C or for an overnight in a 37°C (Al-Jawabreh et al., 2006). The following procedures were done based on the steps mentioned on the DNA extraction kit (high pure PCR template preparation kit; cat.No.11796828001; Roche, Germany). The treated suspension of washed *Leishmania* promastigotes were taken out from the refrigerator and 200 µl binding buffer and 40 µl proteinase K were added and mixed with the solution. The continent was incubated for 10 min at 70°C in a water bath and resuspended in Elution Buffer. Quality and quantity of extracted DNA was analysed by agarose gel electrophoresis and spectrophotometry, respectively.

#### PCR amplification of ITS1

The ribosomal Internal Transcribed Spacer 1 (ITS1) amplification was subjected to PCR, using the LITSr and L5.8s primers (Table 1). Amplification reaction was performed in a volume of 25 µl containing, 100 mg DNA master 1X PCR buffer, 0.2 mM dNTP, 3.5 mM $Mgcl_2$, 10 pmol for each primer and 1U Taq DNA polymerase. The reaction was performed in a thermocycler (Eppendorf) with the following steps: initial denaturation at 95°C for 5 min followed by 30 cycles containing denaturation at 95°C for 20 s, annealing at 53°C for 30 s and extension at 72°C for 1 min and in the end post extension phase at 72°C for 6 min. PCR products were analyzed using a 1.5% agarose gel. The samples were run at 5V/cm along with a gene molecular marker of 50 bp, a negative control, and positive control. The fragments were visualized with UV light using a UV transilluminator (Schonian et al., 1996).

#### RFLP analysis

For *Leishmania* species discrimination, ITS1-PCR products were assigned to RFLP analysis. For this reason, the restriction enzyme of HaeIII (BUSRI) (fermentase- lithvani) was added to the solution and incubated at 37°C for 2 h. Polymorphism analysis was performed using of the restriction enzymes of Dpn I, Taq I, Hpa II (fermentase- lithvani). The digestion reaction was assessed using electrophoresis in 1.5% agarose and photographed with a documentation device.

#### ITS1 sequencing

The PCR products of isolates representing different profile digestion with Dpn I, Taq I, Hpa II, were purified using (High Pure PCR Product Purification Kit Cat. No. 11732 668001; Roche) and then undergone the sequencing process.

#### Statistical analysis

To assess the distribution frequency rate corresponded to *Leishmania* species and their genotypes in three regions of Bam and Kermanshah and Mashhad, chi-square test was executed.

**Table 1.** LITSr and L5.8s primers.

| | |
|---|---|
| Forward(LITSR) | 5'-CTGGATCATTTTCCGATG-3' |
| Reverse(L5.8S) | 5'-TGATACCACTTATCGCACTT-3' |

## RESULTS

In this study, all the smear samples taken from suspected cases of CL were examined through parasite culture and microscopic tests, amongst them, the positive cases for CL accounted for 103 in Bam, 82 in Kermanshah and 115 in Mashhad whereas other cases in total 30 patients turn out to be affected by bacterial or fungal infections or in some cases they were smear negative.

Of the confirmed cases of CL analyzed via PCR-RFLP in the three mentioned regions, 287 of CL positive cases have displayed single DNA bands 350 bp in the results of PCR whereas 13 have revealed single DNA bands 450 bp.

The isolates that generated 450 bp DNA bands were determined to be similar to *Crithidia* according to sequence analysis and all other isolates that exhibited single DNA bands 350 bp were assesed by restriction enzyme digestion using HaeIII. In the obtained banding pattern, two fragments of 200 and 60 bp were observed in 80(77.66%), 72(87.80%) and 95(82.60%) of the isolates from Bam, Kermanshah and Mashhad respectively which were similar to *L. tropica* following comparative analysis of the PCR product with standard pattern while in banding pattern of 17(16.50%), 8(9.75%) and 15(13.04%) of the isolates from the mentioned regions, two single DNA bands of 150 and 200 bp were detected. According to standard pattern it is similar to *L. major*. These latter isolates were eliminated from the study (Table 2, Figure 1).

After species identification, PCR products of *L. tropica* from the three regions of Bam, Kermanshah and Mashhad were analysid by restriction enzyme digestion using Taq1, DpnI, Hpa II. Digestion with Taq1 showed that 78(97.50%) isolates from Bam had two DNA bands of 80 and 125 bp in comparison with standard pattern, and 2(2.5%) isolates were not digested by Taq1 and displayed the DNA band of 350bp, whereas all of the isolates from Kermanshah had two DNA bands of 80bp and 125 bp in comparison with standard pattern. About 63(66.31%) samples taken from Mashhad have exhibited two DNA bands of 80 and 125 while 20(21.05%) isolates of Mashhad were not digested by Taq1 and displayed the DNA band of 350 bp. The rest of them have shown two DNA fragments of 125 and 50 bp. All of the isolates form the studied areas have revealed the DNA bands of 140 and 185 bp after being affected by Dpn1 and the DNA fragment of 250 and 80 bp after digestion with HpaII according to standard pattern (Figure 2).

According the results declared above, as differences was found between the enzymatic digestion patterns of

**Table 2.** The distribution of frequencies of Tripanosomatidae obtained from microscopic examination and parasite culture in CL positive patients in contaminated regions.

| Region species | Bam | Kermanshah | Mashhad | Total |
|---|---|---|---|---|
| | NO (%) | NO (%) | NO (%) | NO (%) |
| *L. tropica* | 80 (77.66) | 72(87.80) | 95 (82.60) | 247(82.33) |
| *L. major* | 17 (16.50) | 8(9.75) | 15 (13.04) | 40(13.33) |
| *Crithidia* | 6(5/8) | 2(0.01) | 5(4.34) | 13(4.33) |
| Total | 103 | 82 | 115 | 300(100) |

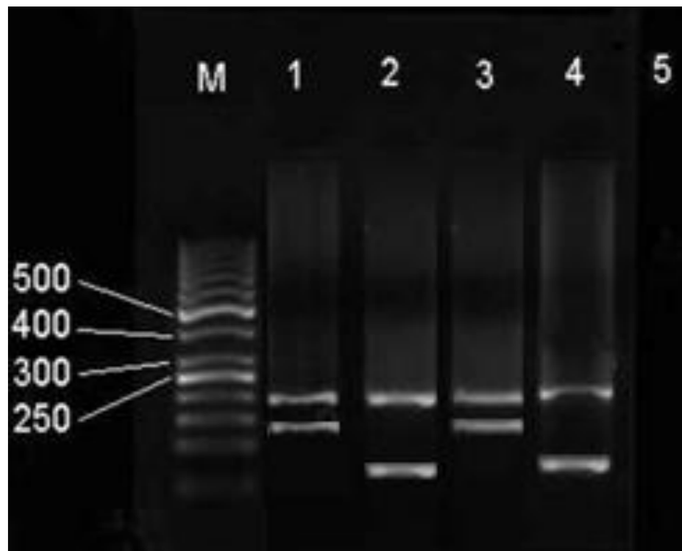


**Figure 1.** The digesting results of amplified ITS1 by HaeIII enzyme from microscopic slides or parasite culture of CL positive patients in three regions of Bam, Kermanshah and Mashhad.
Lane 1: Represents standard *L. major*. Lanes 2: Standard *L. tropica*. Lanes 3: *L. major* related to three geographical. Lane 4: *L. tropica* related to three geographical. Lane 5: Negative control. M. Molecular marker: 50 bp.

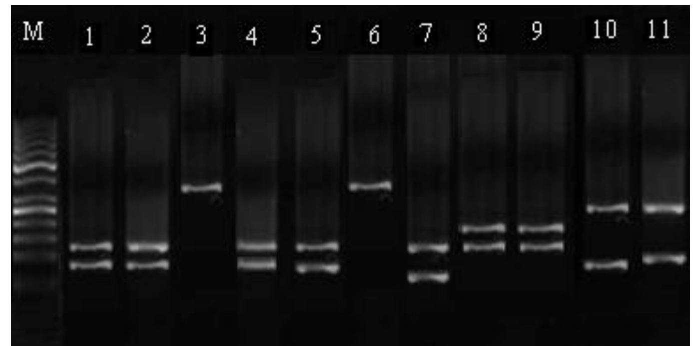


**Figure 2**. The digesting results of amplified ITS1 by the application of Taq1, Dpn I and Hpa II enzymes from microscopic slides or parasite culture of CL positive patients in three regions of Bam, Kermanshah and Mashhad.
Lane 1: Standard *L. tropica* digested by Taq I, Lane 2: L. tropica related to Bam accordance with standard *L. tropica*, Lane 3: *L. tropica* related to Bam no accordance with standard *L. tropica,* Lane 4: *L. tropica* related to Kermanshah accordance with standard *L. tropica*, Lane 5: *L. tropica* related to Mashhad accordance with standard *L. tropica* , Lane 6: *L. tropica* related to Mashhad no accordance with standard *L. tropica*, Lane 7: *L. tropica* related to Mashhad no accordance with standard *L. tropica*, Lane 8: Standard *L. tropica* digested by Dpn I, Lane 9: *L. tropica* related to three geographical regions digested by Dpn I., Lane 10: Standard *L. tropica* digested by Hpa II, Lane 11: *L. tropica* related to three geographical regions digested by Hpa II

the examined species and the enzymatic digestion pattern of reference species in the results of ITS1-PCR, most of the *L. tropica* isolates from three geographical regions of Bam, Kermanshah and Mashhad were transferred for DNA sequencing and finally a group of ITS1-5.85 sequences was reported from GenBank. The sequences were aligned with Clustal X 1.83 and the alignments were checked carefully by eye. Matrix alignment is accessible in one of our reference article (Yang et al., 2010). Based on the results achieved by DNA sequencing, it was possible to characterize 6 Genomic polymorphic profiles for *L. tropica* in association with studied geographical regions summarized in Table 3. Genotypes of LtA and LtB were detected in isolates of *L. tropica* from Bam. Genotypes of LtA, LtC, LtD and LtE were identified in isolates of *L. tropica* from Mashhad and Genotypes of LtA and LtF were found in isolates of *L. tropica* related to Kermanshah.

## DISCUSSION

In the past, common diagnostic tools used for *Leishmania* characterization were mainly the study of its clinical, epidemiological and ecological characteristics (Vega-Lopez F, 2003) but one of the major deficiencies in these conventional criterions is the clinical manifestations of this kind of cutaneous disease which can be easily mistaken with other dermal disorders .This reinforce the need for distinct and meticulous diagnostic laboratorial techniques. Today, to overcome these shortages, a molecular diagnostic assay is used which is a more rapid, accurate and sensitive diagnosis method for *Leishmania* spp. identification and typing. Also it constitutes an appropriate method for phylogenic and epidemiological surveillance (Doudi et al., 2010).

In the present trial, ITS1-PCR approach was designed

**Table 3.** The isolated strains of *Lishmania tropica* used in this study and the results derived from DNA sequencing procedure in consistency the Leishmanial DNA sequences available in the GenBank.

| Sequence type | Sequence length pb | Genbank accession numbers | WHO code | Origin | Reference |
|---|---|---|---|---|---|
| LtA | 320pb | GQ913688 | MHOM/AF/88/KK27 | Bam, Mashhad, Kermanshah, Iran | Mahmoudzadeh-Niknam et al. (2009) |
| LtB | 320pb | HM101131 | MHOM/IR/Bam-163 | Bam, Iran | Doudi et al. (2010) |
| LtC | 350pb | EU727197 | MHOM/IR/02/Mash-2 | Mashhad, Iran | Hajjaran et al. (2008) |
| LtD | 350pb | EF653267 | MHOM/IR/02/Mash-10 | Mashhad, Iran | Kazemi rad et al. (2007) |
| LtE | 350pb | EU727198 | MHOM/IR/03/Mash-878 | Mashhad, Iran | Hajjaran et al. (2008) |
| LtF | 350pb | EU482829 | MHOM/IR/01/Kermanshah | Kermanshah, Iran | Hajjaran et al. (2008) |

with the goal of distinguishing isolated species of *Leishmania* parasites in three different geographic areas of Bam, Mashhad and Kermanshah and PCR-RFLP was applied to discriminate intraspecific and interspecific genetic diversity in *L. tropica* species. The amplified fragment of Leishmanial standard species by using two primers of LITSr and L5.8s is the achievement of a single DNA band with approximate size of 320-350 bp whereas in some cases a 450 bp band was found which after undergoing different sequencing and molecular assessing process with Blast software, it was clarified that this sequence is related to *Crithidia* and was removed from the study. For species characterization of isolated samples, enzymatic digestion were performed using Hae III enzyme which deduces the production of two fragments of 200 and 60 bp from DNA band of 350 bp suggesting the existence of *L. tropica*. In fact banding pattern of *L. tropica* is expected to be dominated by 4 DNA fragments of 20, 55, 64 and 200 bp but due to small molecular weight fragment of 20 bp and segments overlapping of two DNA band with sizes of 55 and 64 bp in the digestion pattern, only two fragment of 200 and 60 bp were able to be detected (Kazemi-Rad et al., 2008). PCR- RFLP analysis of the ITS1 in *L. tropica* as well as sequencing analysis results prove the genetic polymorphism among some infected clinical samples and was used to group the isolates in 6 genetic profiles including LtA and LtB correspond *to L. tropica* in Bam, LtA, LtC, LtD and LtE in Mashhad LtF and LtA in Kermanshah. These results are believed to be correlated with species high degree of genetic heterogeneity or genetic hybridization because the studied samples were derived directly from random patients and they were not disposed to transgenic procedure and replication (Schonian et al., 2001).

On the other hand, genotype sequencing of LtA, LtB, LtC, LtD, LtE and LtF depicted the highest frequency of LtA correspond to MHOM/AF/88/KK27 strain not only in Bam, but also in Kermanshah and Mashhad. Recently Genotype LtB related to MHOM/IR/10/Bam-163 strain was discovered in samples collected from Bam. Our finding based on BLAST molecular analyses software indicated that this new genotype differed from other WHO reference strains of MHOM/IR/02/Mash-2, MHOM/IR/02/Mash-10 and MHOM/IR/03/Mash-878 isolated from Mashhad and MHOM/IR/07/Kermanshah at 3,4,7 and 6 nucleotides respectively within ITS1 gene region (Kazemi-Rad et al., 2008).

The presence of Genotype LtA correspond to MHOM/AF/88/KK27 strain which is significantly dominant in studied endemic regions could be attributed to afghan immigrants who cross the common border between Khorasan province of Iran and Afghanistan frequently. Therefore this genotype has widespread in endemic region of Iran and subsequently observed abundantly in isolates yielded from three regions of research.

From an epidemiological point of view, it has been inferred that there could be a rational relationship between the yielded genotypes and either their geographical origin evidenced their plasticity. For example Genotype LtA localize itself to new endemic focuses proposing the rapid adaptation of *Leishmania* isolates to variable vectors and animal reservoirs in a particular region (Cupolillo et al., 2003).

As addressed in some papers, DNA recombination happened among isolates suggests the genetic exchange in *Leishmania* while in the new world it defines the genetic hybridization among divergent species of *L. braziliensis, L. guyanensis* and *L. peruviania* (Dujardin et al., 1995; Belli et al., 1994; Cupolillo et al., 1998).

It is noteworthy that Kreutzer and his colleagues have demonstrated nucleus fusion and sexual reproduction in the intracellular amastigote form (Kreutzer and Christensen, 1980). The experiment done by Youssef and his colleagues showed the same evidences in amastigotes and promastigotes via nucleus DNA screening and computer analysis (Youssef et al., 1997). After hybridization within DNA species, the new born dihybrid strains are widely spread throughout *Leishmania* population; one of the examples in this case is the hybrid generated between two closely related species of *Leishmania braziliensis*; the causative agent of malignant cutaneous ulcers and visceral lesions and *Leishmania peruviana* which causes leishmanisis characterized by

dry cutaneous lesion with no severe damage and heals spontaneously (Belli et al., 1994).

The generated hybrid from these two mentioned species causes an enhanced degree of severity with combined clinical features of its originated species constitutes a major health problem in Peru.

To elucidate, Hybridization and DNA exchanges among *Leishmania* parasites lead to the generation of new species resulting in heightened symptoms which is indicative of the elevated level of parasite virulence (Kreutzer and Christensen, 1980; Youssef et al., 1997).

## Conclusion

To summarize all the information discussed in this article in brief, it can be concluded that *L. tropica* which causes Anthroponotic Cutaneous Leishmaniasis in three endemic regions of Bam, Kermanshah and Mashhad is highly polymorphic. Although the clinical symptoms of Anthroponotic Cutaneous Leishmaniasis and its pathological assessment is closely related to the genetic traits of *L. tropica* and individual susceptibility of the host as well but the data obtained from this study suggests that genetic properties of *L. tropica* have a great impact on the level of pathogenicity of *L. tropica*, notably its epidemiological and Taxonomic Consequences.

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## REFERENCES

Al-Jawabreh A, Schoenian G, Hamarsheh O, Presber W (2006). Clinical diagnosis of Cutaneous Leishmaniasis: A comparison study between Standaraized graded direct microscopy and ITS1-PCR of Giemsa-Stained Smears, 99: 55-61.

Alrajhi A (2003). Cutaneous Leishmaniasis of The Old World. Skin Therapy, 8: 1-4.

Belli A, Miles MA, Kelly JM (1994). A putative *Leishmania panamensis*/*Leishmania braziliensis* hybrid is a causative agent of human Cutaneous Leishmaniasis in Nicaragua. J. Parasitol., 109: 435-442.

Cupolillo E, Brahim LR, Toaldo CB, De Oliveira-Neto MP, De Brito ME, Falqueto A, De Farias Naiff M, Grimaldi GJ (2003). Genetic polymorphism and molecular epidemiology of *Leishmania (Viannia) braziliensis* from different hosts and geographic areas in Brazil. J. Clin. Microbial., 41: 3126-3132.

Cupolillo E, Momen H, Grimaldi G (1998). Genetic diversity in natural populations of New World *Leishmania*. Memorias do Instituto Oswaldo Cruz., 93: 663-668.

Doudi M, Hejasi SA, Razavi MR, Eslami G (2010). Comparative molecular epidemiology of *Leishmania major* and *Leishmania tropica by* PCR-RFLP technique in hyperendemic cities of Isfahan and Bam, Iran. Med. Sci. Monit., 16: 530-535.

Dujardin JC, Banuls AL, Llanos-Cuentas A, Alvarez E, DeDoncker S, Jacquet D (1995). Putative *Leishmani*a hybrids in eastern Andean valley of Huanuco. Peru. Acta. Trop., 59: 293-307.

Kamhawi S, Abdel-Hafez SK, Arbagi A (1995). A new focus of cutaneous leishmaniasis caused by *Leishmania tropica* in northern Jordan. Transact. R. Soc. Trop. Med. Hyg. 89: 255-257.

Kazemi-Rad E, Mohebali M, Hajjaran H, Rezaei S (2008). Diagnosis and Characterization of *Leishmania* Species in Giemsa- Stained stained slides by PCR-RFLP. Iranian J. Public Health., 37: 54-60.

Kreutzer RD, Christensen HA (1980). Characterization of *Leishmania* spp. By isozyme electrophoresis. Am. J. Trop. Med. Hyg., 29: 199-208.

Marfurt J, Nasereddin A, Niederwieser I (2003). Identification and differention of *Leishmania* species in clinical samples by PCR amplification of mini – exon sequence and subsequent Restriction Fragment Length Polymorphism analysis. J. Clin. Microbiol., 41: 3147-3153.

Mebrahtu YB, Lawyer PG, Pamba H (1992). Biochemical characterization and zymodeme classification of *Leishmania* strains from patients, vectors, and reservoir hosts in Kenya. Am. J. Trop. Med. Hyg. 47: 825-892.

Peters W, killick-Kendrick R (1995). The Leishmaniasis in Biology and Medicine. London and New York: Academic Press, 1(2): 1987.

Rotureau B, Ravel C, Couppie P (2006). Use of PCR-Restriction Fragment Length Polymorphism analysis to identify the main new world *Leishmania* Species and analyze their taxonomic Properties and polymorphism by application of the assay to clinical samples. J. Clin. Microbiol., 44: 459-467.

Schnur LF, Greenblatt CL, *Leishmania (1995)*. In: Parasitic Protozoa, Kreier, J. (editor). San Diego: Academic Press, pp. 1-160

Schonian G, Nasereddin A, Dinse N (2003). PCR diagnosis characterization of *Lesishmania* in local and imported clinical samples. Diagn. Microbiol. Infect. Dis., 47: 349-358.

Schonian G, Schnur L, El Fari M, Oskam L, Kolesnikov AA, Sokolowska-Kohler W, Presber W (2001). Genetic heterogeneity in the species *Leishmania tropica* revealed by different PCR- based methods. Trans. R. Soc. Trop. Med. Hyg., 95: 217-224.

Schonian G, Schweynoch C, Zlateva K, Oskam I (1996). Identification and determination of the relationship of species and strains within the genus *Leishmania* using single primers in the polymerase chain reaction. Mol. Biochem. Parasitol., 77: 19-29.

Shahian M, Alborzi A (2009). Effect of meglumine antimoniate on the pancreas during treatment of visceral Leishmaniasis in children. Med. Sci. Monit., 15: 290-93.

Vega-Lopez F (2003). Diagnosis of Cutaneous Leishmaniasis. Curr. Opin. Infect. Dis., 16: 97-101.

Yang BB, Guo XG, Hu XS (2010). Species discrimination and phylogenetic inference of 17 Chinese *Leishmania* isolates based on internal transcribed spacer 1 (ITS1) sequences. Parasitol. Res. Springer-Verlag., 107: 65-1049.

Youssef MY, Eissa MM, Mansoury ST (1997). Evidence of sexual reproduction in protozoan parasite *Leishmania* of the Old World. J. Egyptian. Soc. Parasitol., 27: 651- 657.