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Molecular identification of *Leishmania* isolates obtained from patients suspected as having cutaneous leishmaniasis referred to reference laboratories from Yazd province in central Iran

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Abstract

Background:

Cutaneous leishmaniasis (CL) continues to be an increasing public health problem in Iran. The dominant etiologic agents of CL in the Old World are *Leishmania major* and *Leishmania tropica*. One of the important endemic foci of CL in Iran is Yazd. Recently, previous studies showed the equal prevalence of *L. major* and *L. tropica* as the agents of cutaneous leishmaniasis in this area. This prompted us to identify the genotype of *L. major* isolates obtained from patients with cutaneous leishmaniasis.

Materials and Methods:

After completing a clinical/epidemiologic data questionnaire for 218 patients with suspected skin lesions, scraping samples were collected, and each specimen was examined using both direct microscopy and molecular assay of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results:

Results showed that of the 218 samples, Leishman body was observed in 77 by direct smear and 104 by PCR assay. Molecular assay indicated 50 cases as *L. major*, 52 cases as *L. tropica*, and two cases as unknown. Molecular characterization of *L. major* isolates showed four patterns, named LmA1, LmA2, LmA3, and LmA4.

Conclusion:

Our study is the first report for molecular characterization of *L. major* from one of the important central province of Iran that could affect the control strategies in this field.

Keywords: Cutaneous leishmaniasis, identification, internal transcribed spacers1, *leishmania major*

INTRODUCTION

Leishmaniasis is a broad spectrum of clinical forms including cutaneous, mucocutaneous, visceral, and post-kala-azar leishmaniasis forms caused by the genus *Leishmania* transmitted by the bite of the infected female sand flies.[1] Leishmaniases are considered endemic in more than 80 countries with the prevalence

of 12 million cases, 400,000-600,000 new cases per year for visceral forms and 1-1.5 million for the cutaneous forms.[2] Cutaneous leishmaniasis is endemic in different European, African, and Asian countries as well as regions in Central Asia and the Middle East.[3] It is prevalent in many areas of Iran such as in Tehran, Mashhad, Yazd, Tabriz, Kerman, Isfahan, Neyshabour, and Bam.[4] *Leishmania* organisms have been classified as different species primarily on the basis of clinical, biological, and epidemiological criteria,[5] but clinical signs and symptoms cannot really be used for identification of *Leishmania* species because a broad differential diagnosis makes for diverse clinical manifestations.[6] Advanced polymerase chain reaction (PCR) based methods with various targets including microsatellite, kinetoplast DNA, telomeric sequences, gp63, hsp70, mini-exon, b-tubulin, or rRNA have been used for identification of *Leishmania* isolates.[7,8,9,10] Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified sequences of multicopy genes such as internal transcribed spacers (ITS) has been proven to be a crucial method for either Old and New World *Leishmania* species.[11,12,13,14,15] As mentioned, one of the endemic province of Iran is Yazd. Recently, our previous study showed that the prevalence of *L. major* in this area is as the same as *L. tropica*. This prompted us to assay the genetic diversity of the parasite *L. major* in the area. In this study, we applied ITS1-PCR-RFLP as a sensitive tool for identification of genotype pattern of *L. major* in this endemic.

MATERIALS AND METHODS

Patients and samples

During September 2011 to December 2012, 218 smears were obtained from skin lesions of the suspicious patients with cutaneous leishmaniasis referred to reference laboratories of Yazd, Iran. Each patient clinical data were collected. Two microscopic smears were taken from each patient by scraping the internal border of skin lesions with a surgical blade; one sample was used for molecular study and the other was fixed with methanol and stained with Giemsa for microscopy examination.

DNA extraction

DNA extraction from smear's scrapings was conducted using the FlexiGene DNA extraction kit (Cat. No. 3032; Bioneer, Korea) as recommended by the manufacturer.

Detection of *Leishmania*

Detection of *Leishmania* isolates was done using ITS1-PCR-RFLP by the primers LITSR (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'- TGATACCACTTATCGCACTT-3'). Amplification reactions were performed in 25 µl containing the end concentration of 1x PCR buffer (CinnaGen), 1.5 mM $MgCl_2$ (50 mM; CinnaGen), 0.2 mM dNTP (10 mM; CinnaGen), 10 pmole of each primer, and 0.5 units of Taq DNA polymerase (CinnaGen). Amplification was started with one cycle at 94°C for 5 min, followed by 35 cycles, 1 min at 94°C, 30 sec at 53°C, and 1 min at 72°C, and then finalized with one cycle at 72°C for 5 min using the thermocycler (ABI-Veriti, American). The PCR products were digested with restriction enzyme of HaeIII (Fermentas, #ER0011) under condition of 37°C for 2 hours. This step will only detect *Leishmania* spp. *Leishmania major* were selected for further analysis.[16]

Identification of *L. major* subtypes

For molecular identification of *L. major* strains, restriction enzymes of *TaqI*, *Dpn1*, and *HpaII* were used. The reaction for all enzymes were done in a final concentration of 1x Tango buffer, 10 U enzyme and 10 µg PCR product in a volume of 20 µl at 37°C for 16 hours. The digested products were analyzed using 2% gel agarose electrophoresis with the pattern of three Iranian *Leishmania* stock species including: *Leishmania tropica* (MHOM/IR/99/YAZ1), *Leishmania major* (MRHO/IR/75/ER), and *Leishmania infantum* (MCAN/IR/97/LON49).[16]

RESULTS

In the present study, among 218 the samples collected during 16 months of sampling, the Leishman body was detected in 77 (35%) and 104 (48%) samples using microscopic evaluation of stained slides identified

and molecular assay, respectively. It means that 27 (25.96%) samples were not detected by microscopic examination. From the positive sample collection by PCR, 102 (98.08%) and 2 (1.92%) samples exhibited the fragments of 350 bp and 450 bp, respectively [Figure 1].

The results of amplicon digestion with *HaeIII* showed the fragments of 210 bp and 140 bp in 50 of 104 (48.08%) samples such as the pattern seen in the standard strain of *L. major* (MRHO/IR/75/ER) and the fragments of 200 bp and 60 bp in 52 of 104 (50%) samples such as the pattern seen in the standard strain of *L. tropica* (MHOM/IR/99/YAZ1) and the fragments of 153 bp and 296 bp, which was not similar to the pattern of all standards [Figure 2]. Data from patients who were infected with *Leishmania major* were studied. After data analysis, it was found that age was between 3 months and 75 years (average age: 26 years), 33 (66%) males, and 17 (34%) females. More information about type of ZCL, duration of its development, number of lesion, and location is shown in Table 1.

Molecular identification using digestion with restriction enzymes of *TaqI* showed four patterns, named LmA1, LmA2, LmA3, and LmA4 with the digested fragments of 200 and 130 bp in 43 of 50 (86%), 210 bp and 150 bp in 1 of 50 (2%), 210 bp and 120 bp in 2 of 50 (4%); and 200 bp and 145 bp in 4 of 50 (8%), respectively [Figure 3]. All the *L. major* isolates were digested with the restriction enzymes of *HpaII* and *DpnI* the same pattern as the standard *L. major* (MRHO/IR/75/ER) with the fragments of160 bp and 90 bp and 205 bp and 125 bp, respectively [Figure 4].

DISCUSSION

The gold standards for the diagnosis of CL are the parasitological methods. The sensitivity of these methods is variable from 27 to 85% for the diagnosis of leishmaniasis.[17,18,19,20,21] The present study could confirm this range (74.04%). This low sensitivity of these methods depends on clinical presentation with a high dependence on the number of parasites in samples and requires technical skills for sampling, parasite species, technical expertise, and other factors, and it is the main disadvantage of these methods.

Techniques based on PCR have improved for the identification of *Leishmania* parasites at species and strain levels in the past decade.[17,18,19] The ability to the differential diagnosis of *Leishmania* species and strains can be used to design appropriate strategies for therapy, ecologic, and epidemiologic studies, especially in regions where more than one type of *Leishmania* species are seen. Also, study of species distribution can help us to design prophylactic strategies for disease control and providing an effective vaccine.[20,21] PCR-RFLP is one of the most sensitive and powerful molecular techniques that have been used for this purpose. This method detects mini variations inside genomic DNA where single base displacements have either created or obsolete a site capable of being digested by a specific restriction endonuclease. PCR has been used in a variety of areas such as gp63,[22] kDNA,[23,24,25] miniexon sequences,[26] ITS1,[27] heat-shock protein 70 (Hsp 70) genes.[11] ITS1 region relative to rRNA genes between species is less protected; therefore, it is useful to examine the phylogenetic relationship of organisms.[28] Also, this region has some conservative area that is completely useful for designing the PCR primers to amplify.[24,25,26,27,28,29] In the present study, we applied ITS1-RFLP as a tool for identification of *Leishmania*. Based on the results, among the 218 suspected cases of leishmaniasis, 104 cases were PCR positive and 114 samples were negative. It may be possible that the negative samples were some other form of ulcers such as Buruli ulcer, Pyoderma streptococcal, actinomycetoma, cutaneous tuberculosis, impetigo, etc.[30,31]

PCR analysis showed that among the positive samples, all isolates produced fragments of about 350 similar to standard strains, but two samples with size of 450 bp, which may be due to contamination or mutation. [31,32,33]

In the last decade, there is some document that showed *L. tropica* to be the dominant species in Yazd.[33] But the recent study showed the same incidence of *L. tropica* and *L. major* among patients with cutaneous leishmaniasis referred to references laboratories of Yazd. Our study could confirm the last study with the rate of 50% and 48.08% for *L. tropica* and *L. major*, respectively.

For incidence of *L. major* in this important endemic region, we decided to genotype all *L. major* isolates. PCR-RFLP analysis with the restriction enzyme of *TaqI* showed four patterns including LmA1-4.

This study could confirm *L. major* heterogeneity that has been proved by Doudi *et al*.[16] Profile of LmA1 subtype is similar to pattern LmA, which was reported by Doudi in Isfahan region.[16] This profile not only had the highest frequency in the Isfahan zone, but was also based on the reports by Tashakori and colleagues, which was found in other geographic regions of Iran, such as Kashan, Tehran, Dezfool, and Dehloran[28] and also in our study. In another study, Dabirzadeh *et al*., showed nine distinct banding profiles in *L. major* with more frequency for LmA (LmA1 in our study) in Isfahan.[34] These heterogeneity may affect the viability, pathogen city, and resistant therapy in strains that could happen with either gene transfer or hybridization between *Leishmania* parasites that could lead to generate new strains with different forms of clinical diseases in different geographical areas.[29,30,31,32,33,35] Also, Calvopina *et al*., that studied KDNA in *L. Mexicana* isolates obtained from three patients with lesions very unusual showed the same genetic heterogeneity that was related to clinical differences and geographic area.[36] In addition, Baghaei *et al*., studied on different clinical forms of cutaneous leishmaniasis by *L. major*. They showed different band profiles that were related with drug susceptibility.[37]

It seems necessary to study on heterogeneity and their effects on clinical managements, treatments, and prognosis.

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Footnotes

Source of Support: Nil

Conflict of Interest: None declared

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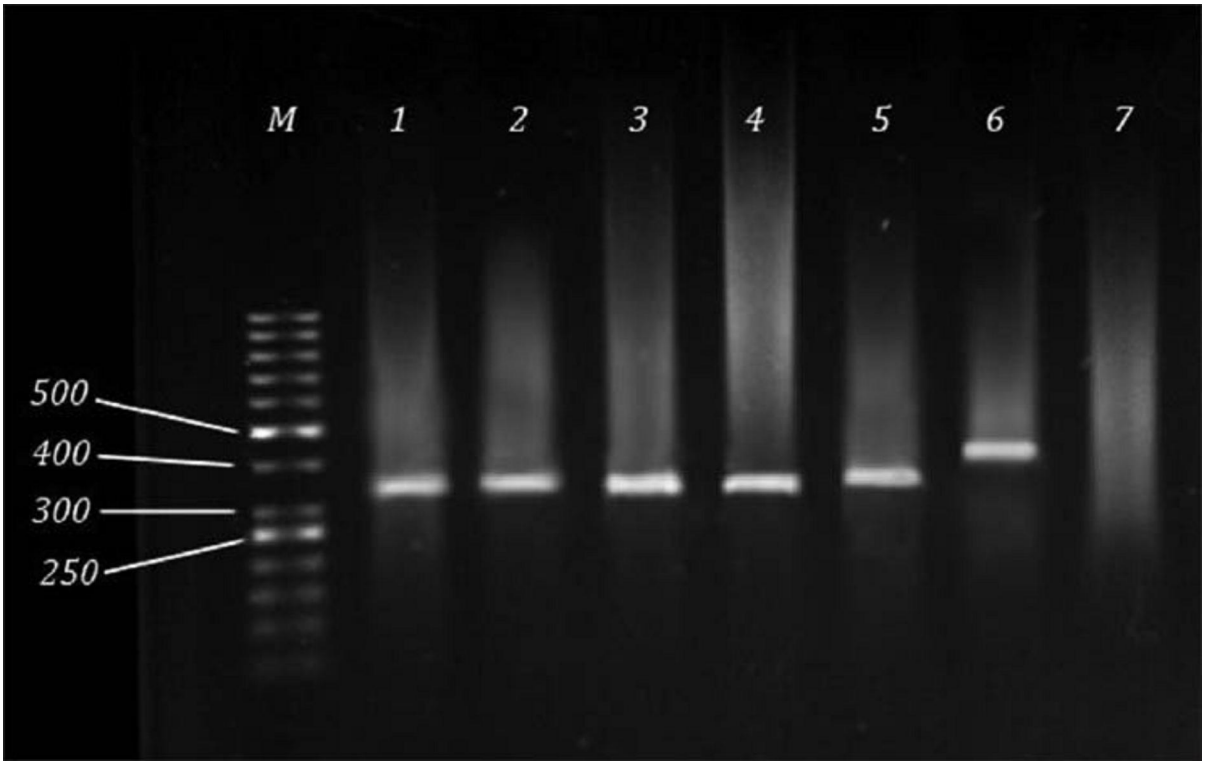
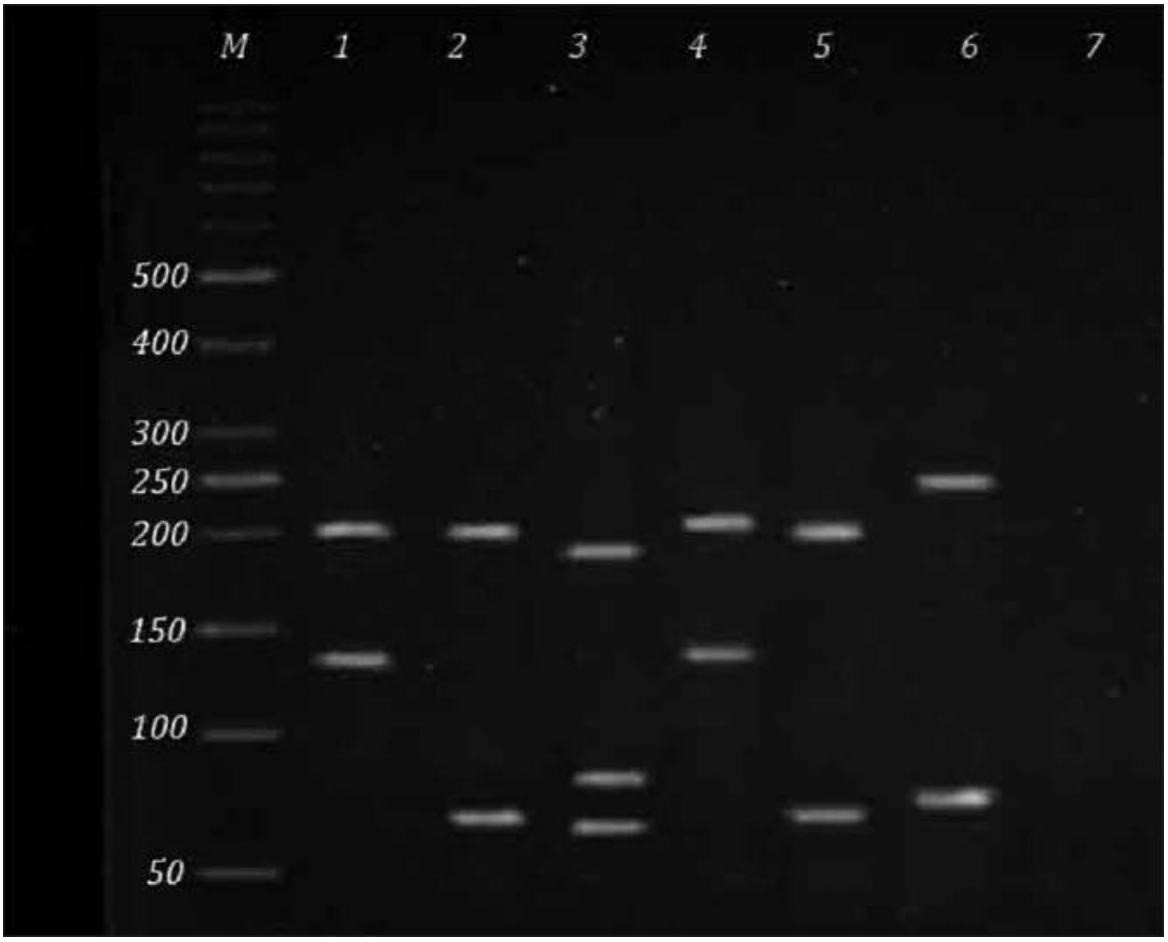
Figures and Tables

Figure 1



Electrophoresis results of ITS1-PCR from smears. M: Molecular marker (50 bp); Lane 1: Standard *Leishmania major* (MRHO/IR/75/ER); Lane 2: Standard *Leishmania tropica* (MHOM/IR/99/YAZ1); Lane 3: *Leishmania infantum* (MCAN/IR/97/LON49); Lane 4, 5: PCR products sample; Lane 6: PCR product s sample; Lane 7: Negative control

Figure 2



Restriction enzyme digestion profile of amplified ITS1 region with the restriction enzyme of *HaeIII*. M: Molecular marker (50 bp); Lane 1: Standard *Leishmania major*; Lane 2: Standard *Leishmania tropica*; Lane 3: Standard *Leishmania infantum*; Lane 4: *Leishmania major*; Lane 5: *Leishmania tropica*; Lane 6: The one sample different from the normal samples and standard patterns; Lane 7: Negative control

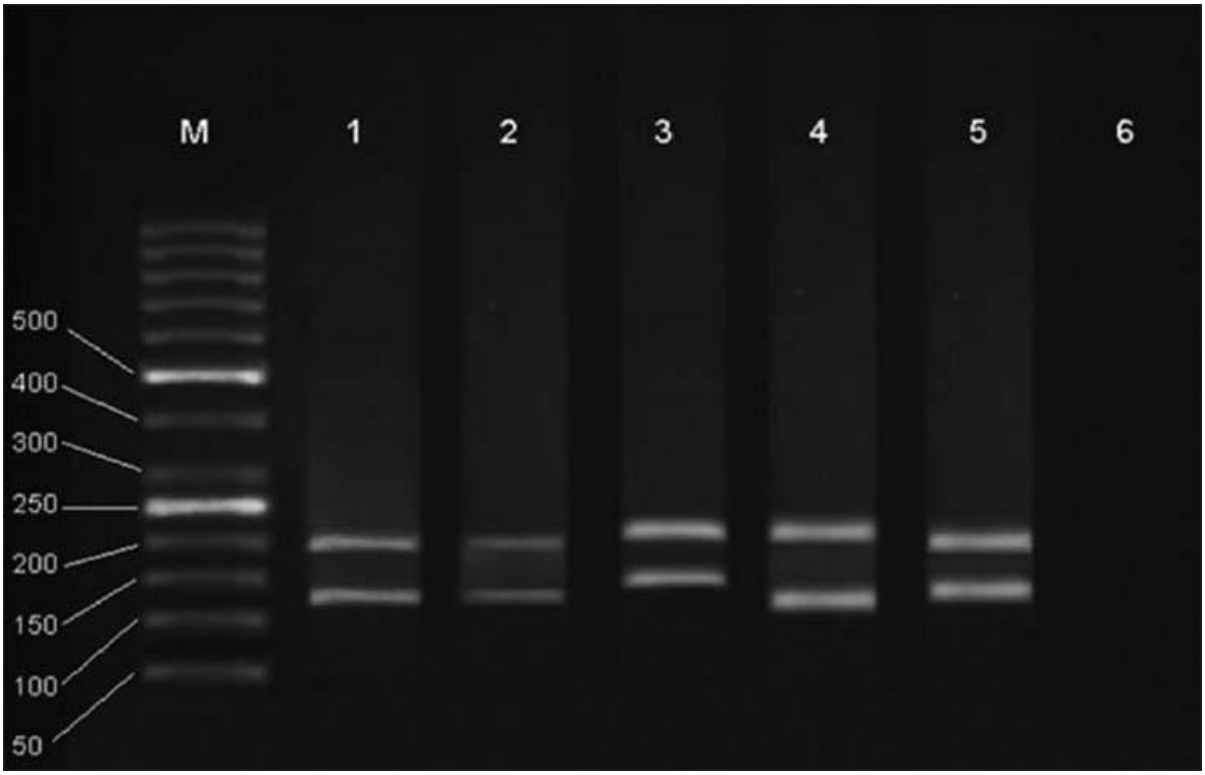
Table 1

Type of ZCL	Number of lesions	Location	Number of patients	Duration (weeks)	Genotype
Crateri form	1-2	Face, hands, feet, trunk, neck, etc.	31	3-9	LmA1, LmA3, LmA4
Papule form	1-5	Face, hands, feet, neck, trunk	16	5-9	LmA1, LmA4
Nodular form	3	Hand	1	26	LmA1
Sporotrichoid form	15	Hands, feet, ear, trunk, neck	1	3	LmA1
Lupoid form	2	Face	1	3 years	LmA2

ZCL: Zoonotic cutaneous leishmaniasis

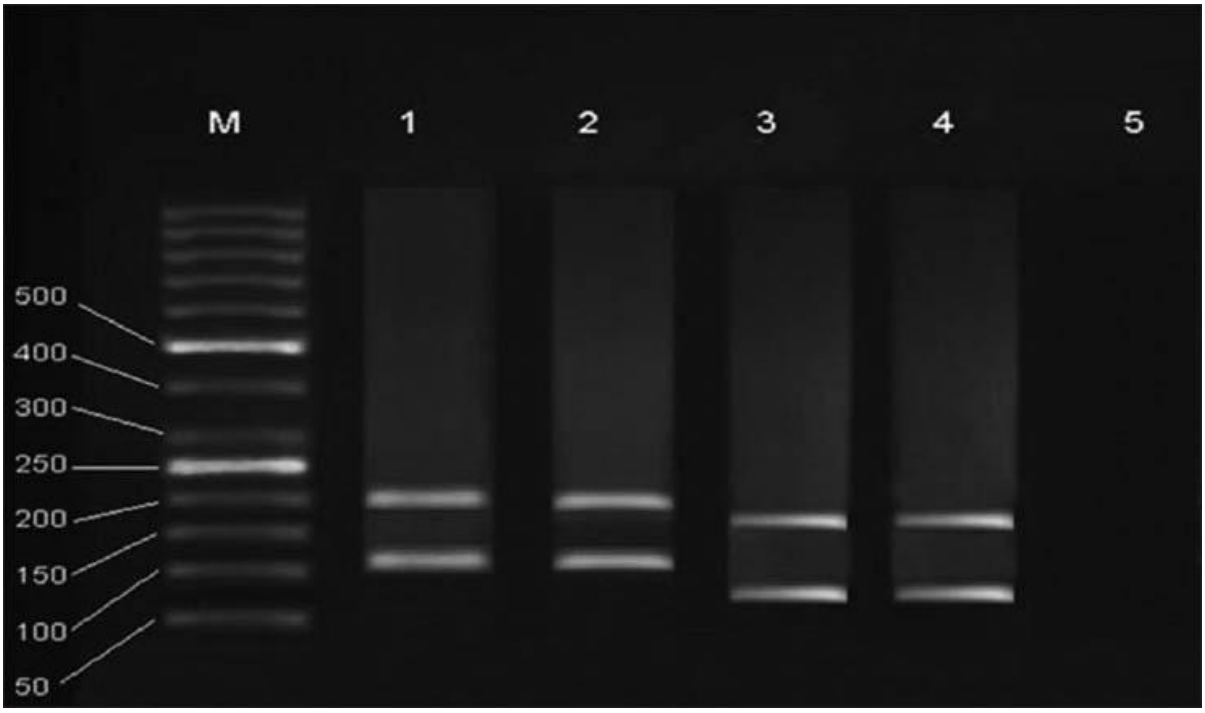
The clinical shapes of different forms of ZCL present in Yazd

Figure 3



Restriction enzyme digestion profile of amplified ITS1 region from *Leishmania major*. Giemsa-stained smears with the enzyme *TaqI*. M: Molecular marker (50 bp); Lane 1: Standard *Leishmania major* with *TaqI*; Lane 2: LmA1 (130 and 200 bp) *Leishmania major*; Lane 3: LmA2 (210 bp and 150 bp); Lane 4: LmA3 (210 bp and 120 bp); Lane 5: LmA4 (200 bp and 145 bp); Lane 6: Negative control

Figure 4



Restriction enzyme digestion profile of amplified ITS1