



Molecular identification of *Leishmania* species in Torbat-e Heydarieh, Khorasan Razavi province, Iran

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ABSTRACT

Cutaneous leishmaniasis is considered as health problem in many parts of Iran such as Torbat-e Heydarieh in Khorasan Razavi province. Identification of the *Leishmaniaspecies* is useful for the control of this disease. Microscopic examination and clinical findings aren't sufficient for the differentiation of the parasites. kDNA - PCR technique is a very reliable method to detect *Leishmania* species. The objective of this study the identification of leishmania species using PCR and compared to the routine microscopic examination in Torbat-e-Heydarieh. Slide smears obtained from skin lesions of 70 patients suspected to the leishmaniasis. Direct microscopy and an optimized PCR method were performed using specific kDNA primers. Data were analyzed with SPSS ver.16 software. Among 70 subjects with skin ulcers suspected to CL, 57(81%) were positive in direct microscopic smear examination. However, specific *Leishmania* PCR band were observed in 60(86%), in which 53 subjects had *L. tropica* and 7 has *L. major*. Although, gender and age distribution does not show any statistically significant differences, the seasonal occurrence of the infection in autumn was significant ($P < 0.05$). The most common site of lesions was the face (37%) ($P < 0.05$). Sensitivity of kPCR for diagnosis *Lashmaniaspp* was calculated 95% in this study. Optimized PCR method revealed that both genus of *Lashmania* are prevalent in the of Torbat-e Heydarieh in which *L. tropica* is the dominant causative species for cutaneous *Leishmaniasis*.

Keywords: Cutaneous leishmaniasis, *L. tropica*, *L. major*, Torbat-e Heydarieh, PCR

INTRODUCTION

Leishmaniasis is endemic in many parts of the world and occurs in different part of Iran [1, 2]. *Leishmaniatropica* and *Leishmania major* are, respectively, the main causative agents of anthroponotic cutaneous leishmaniasis (ACL) and zoonotic cutaneous leishmaniasis (ZCL). CL is endemic in Khorasan Razavi province and the surrounding cities [3- 5]. During 1995-2014, 68958 cases with CL were identified only at 5 health centers of Mashhad [6]. It seems that ACL has become the most important endemic disease and has been considered as a health priority. Torbat-e Heydarieh with a population of 131,150 (2011) in Khorasan Razavi province is near its capital city, Mashhad, that is the main pilgrimage city for Shi-et Muslim in Iran.

The decisive diagnosis of disease factor species is essential to select the proper and effective treatment of various forms of infection as well as control the disease in a region [7]. Direct method is the most technique used for

patients who engaged with leishmania lesions. Direct smear has less sensitivity compared with PCR molecular techniques on leishmanial diagnosis [8, 9]. Many different PCR targets, including the coding and intergenic noncoding regions of the gp63 gene locus, splice leader mini-exon (SLME), and the SSU rRNA gene, have been used for the identification of parasites from cultures and for their direct detection in various animal, sand fly, and human tissues [10, 11, 12]. Sensitivity is correlated with the copy number of the amplified region. The kDNA PCR is considered to be the most sensitive method for diagnosing leishmaniasis since there are ~10,000 minicircles per parasite [13, 14].

In this study, the parasitological techniques (microscopy and PCR methods) were used for CL diagnosis in Torbat-e Heydarieh, Khorasan Razavi province to compare and a differential diagnosis of different genus.

MATERIALS AND METHODS

Sampling

A cross-sectional study was performed in 70 subjects who had at least one skin ulcer suspected to CL. Participants signed informed consent and were referred to the Public Health laboratories for direct examination. Genus specific PCR method then carried out for differential diagnosis of *L.major* and *L.tropica*

Direct examination

To prepare direct Giemsa stained smears, samples were obtained by scraping from the center and edge of each skin lesion using a sterile scalpel. After air drying, the slides were fixed in methanol and stained with Giemsa. Each smear was examined by an expert microscopist and then kept for DNA extraction.

PCR for Leishmaniak DNA

DNA extraction from tissue lesion samples was carried out using the Ge Net Bio Kit (Korea) according to the manufacturer's instructions. The quantification and quality control of the DNA extraction procedures were performed using a nano spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific). All reactions were performed in appropriated places, following the good practice of laboratories to avoid sample contamination. A conventional PCR for detection of Leishmania spices was performed. Specific primers for kDNA were derived as forward (TCGCAGAACGCCCTACC) and reverse (AGGGGTGGTGTAATAATAGG), (5). The optimum amplification conditions were 95°C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 45s, and extension at 72 °C for 1min, with a final extension step at 72 °C for 5 min. Two standard samples of parasites (*L. tropica* and *L.major*) and a negative control sample were used to monitor the reaction. PCR products were visualized by EthBr staining on 2% agarose gel electrophoresis. The results were analyzed by SPSS ver.13. The differences were considered significant if p was ≤ 0.05 .

RESULTS

Optimizations

The kinetoplast DNA of the parasite has been targeted for amplification, using the optimized pair of primers. The PCR products were a 615 bp band for *L.major* and a 744bp band for *L.tropica* (Fig 1)Shows the PCR test for the patient samples.

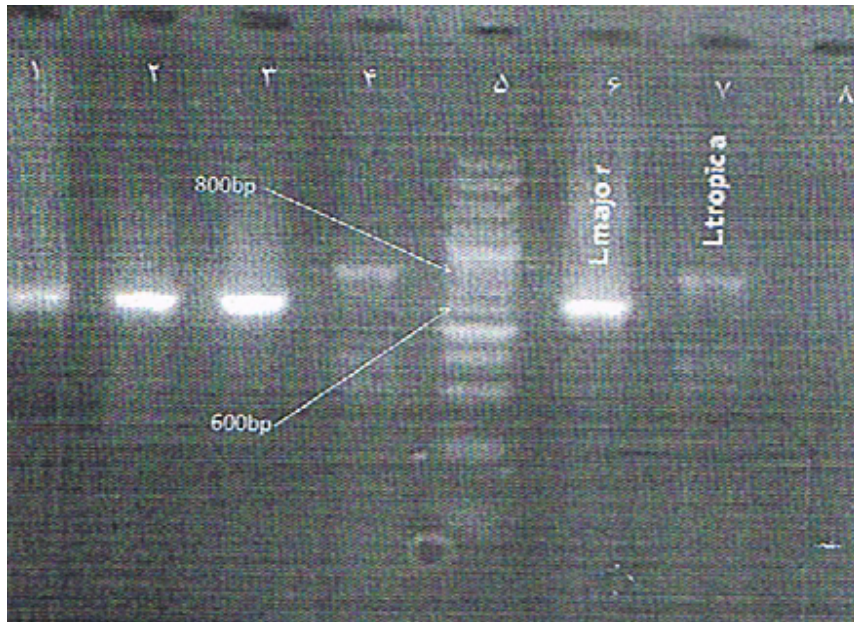


Fig.1: Lanes 1: *L. major* (positive control); lane4: *L. tropica* (positive control); lane 2, 3, 6: *L. tropica*; lane 7: *L. major*; lanes5: ladder; lane 8: negative control.

Patient’s findings

Fifty seven patients out of seventy subjects who enrolled for direct smear examinations were positive for leishmaniasis. However, using PCR results, Sixty 60 (85%) patients were positive and among them 53 (88%) were positive for *L. tropica* and 7(12%) for *L. major*. From this sixty patients 34 individuals (57%) were male and 26 (43%) were female.

Among 60 persons with leishmaniasis, among infected patients, 20 individuals (34%) came from rural areas and 40 individuals (66%) were from urban regions. Most of the lesions of *L. tropica* were presented on the face (38%), and *L. major* related lesions were on the feet (43%). The most of the CL patients (23 cases) had age between 11-20 years. The highest prevalence of CL was observed in autumn ($P = 0.03$) (Fig 2), and the most common site of lesions was in the face (37%) ($P < 0.05$). The findings indicated that two patients (29%) with ZCL had not history of traveling to endemic regions of the disease.

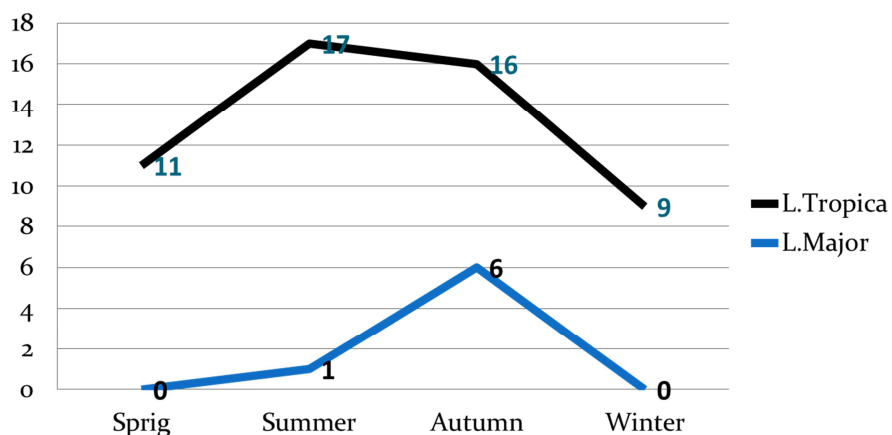


Fig 2: Frequency of Cutaneous Leishmania species by PCR method in Different Seasons of Torbate-haydariyeh in 2014 (P Value= 0.03).

DISCUSSION

Leishmaniasis is widely spread in Iran and is considered to be one of the major health problems for the population [15]. *L. major* is the dominant strain leishmania in Isfahan, Kashan, Ilam, Khuzestan and Semnan provinces [16]. Previous researches identified *L. tropica* in Khorasan province [17- 19]. The optimized PCR test showed more reliable results for detection and differential diagnosis of Leishmania species.

The CL was found to affect a wide range of age, in both male and female subjects. However, in some studies there were no significant difference for these factors [20, 21]. In the longitudinal research by Rafati et al. and Mohammadi Azani, it was discovered that, during the year's 1999 to 2005 in Damghan, 76.3% of patients were bitten during the autumn. Also, 82.5% of patients suffered sand fly bites in the autumn of 2008 [22, 23]. In Kermanshah, the largest proportion (35%) of patients was infected in the autumn [24]. Taken together, our results and the findings of other studies show that LC is more frequent in the autumn in the most parts of Iran.

Several investigations have been performed with different laboratory methods to identify leishmaniasis species [25, 26]. Sensitivity of microscopic techniques, i.e., histopathology and tissue smears, touch preparations, and exudates, has been reported to range from 17 to 83% for CL [27, 28, 29], depending on clinical presentation, parasite species, technical expertise, and other factors. To our results, using kinetoplast DNA (kDNA) and PCR method, produce more reliable molecular results on Leishmaniasis that can differentiate different species. The sensitivity reported in this study for diagnosis of CL by (kDNA) PCR was (95%). This conserved regions are highly proper to design primers for the detection of different Leishmania species [17] and seems to be a useful tool for diagnosis of all kinds of leishmanial species and even for diagnosis of co-infections related to the other diseases [30]. This method has shown the highest sensitivity (98.7%) for the diagnosis of CL in other studies too [13, 14, 31].

Torbat-e Heydarieh is 150 km. far from Mashhad (The capital city of Khorasan province) and its population is about 119,390 people. This is the first report on prevalence of leishmania species by molecular method in Torbat-e Heydarieh. In the present study, 60 individuals (86%) from 70 positive samples were detected to have *L. tropica* by kDNA-PCR assay. Previous studies demonstrated that *L. tropica* is a dominant species in the cities of the Khorasan provinces [3, 5, 32, 33]. However, this study reveals that both species of Leishmania are prevalent in Torbat-e Heydarieh and they are more frequent than estimated previously by direct smear methods.

CONCLUSION

The etiology of leishmaniasis has been unknown in Torbat-e Heydarieh, a city in way of pilgrims to capital of the Khorasan. Using kPCR it has been indicate that both *L. tropica* and *L. major* are the causative agents of cutaneous leishmaniasis in Torbat-e Heydarieh city. Moreover, *L. tropica* is the dominant species in Torbat-e Heydarieh but this city was introduced as a ZCL foci. Therefore, in order to control the disease, the health authorities should focus more on this infection as it has been spread more in this region where it is on the way of pilgrims and tourists.

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