

Original article**Molecular identification of *Leishmania* species causing cutaneous leishmaniasis in Mashhad, Iran**

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How to cite this article:

Mahmoodi MR, Mohajery M, Tavakkol Afshari J, Shakeri MT, Yazdan panah MJ, Berenji F, Fata A. Molecular identification of *Leishmania* species causing cutaneous leishmaniasis in Mashhad, Iran. Jundishapur J Microbiol. 2010; 3(4): 195-200.

Received: May 2010

Accepted: June 2010

Abstract

Introduction and objective: Cutaneous leishmaniasis (CL) is considered as an important health problem in many parts of Iran especially in Mashhad, north-eastern part of Iran. Various species of *Leishmania* cause the disease. Identification of *Leishmania* parasites is useful for control and preventive plans. Although epidemiological and clinical findings are necessary but they are not sufficient for identification of causative agents of CL. In order to identify *Leishmania* spp. a definite molecular technique, Polymerase Chain Reaction (PCR) method was used over a 12 months period.

Materials and methods: A total of twenty-one patients participated. Direct smear and culture in modified NNN medium followed by sub-culture in RPMI-1640 were performed for each case. Genomic DNA was extracted by using proteinase k and amplified by specific primers of kDNA. The PCR product was analysed by gel electrophoresis using 2% agarose. Gel staining was performed by ethidium bromide. The presence of 620bp fragment indicated *Leishmania major* and 800bp indicated *L. tropica*.

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Jundishapur Journal of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, Tel: +98611 3330074; Fax: +98611 3332036; URL: <http://jjm.ajums.ac.ir>; E-mail: editorial office: jjm@ajums.ac.ir

JJM. (2010); 3(4): 195-200.

Results: Of 21 positive cultures out of 53 positive samples, nineteen isolates were identified as *L. tropica* and two others were identified as *L. major*. However, by previous investigations, Mashhad was known, as an endemic focus for Anthroponotic Cutaneous Leishmaniasis (ACL), but it is now concluded that both ACL and Zoonotic Cutaneous Leishmaniasis (ZCL) are present in Mashhad and *L. tropica* is the dominant species.

Conclusion: Both *L. tropica* and *L. major* are the causative agents of cutaneous leishmaniasis in Mashhad. *L. tropica* is the dominant *Leishmania* species in Mashhad. However PCR technique is a very reliable method to detect *Leishmania* DNA, but it is not easy to obtain *Leishmania* culture samples.

Keywords: *Leishmania major*, *Leishmania tropica*, PCR, Anthroponotic cutaneous leishmaniasis (ACL), Zoonotic cutaneous leishmaniasis (ZCL)

Introduction

Cutaneous leishmaniasis (CL) is considered as an important health problem in many parts of Iran especially in Mashhad (north-eastern of Iran) [1,2]. In Iran, two species of *Leishmania* are responsible for CL. Isolation and identification of *Leishmania* species are useful for the control and prevention of the disease. However, epidemiological and clinical findings are helpful in the diagnosis of CL; but they are not sufficient to identify the cause of the disease [3].

Precise identification of these species is important clinically and epidemiologically. *Leishmania* parasites have similar morphology and sometimes cause similar clinical manifestations; therefore, differentiation among species requires molecular techniques such as isoenzyme electrophoresis, DNA probe hybridization, and polymerase chain reaction (PCR) [4]. In order to identify *Leishmania* spp. causing CL in patients living in Mashhad, a study was undertaken over a 12 months period using PCR method.

Materials and methods

This is a descriptive, cross-sectional, prospective study. The population under study was selected among the individuals

clinically suspected to CL who were referred by dermatologists to the Parasitology Lab of University Hospitals, Mashhad University of Medical Sciences, Mashhad, Iran. Considering inclusion and exclusion criteria, all individuals who were living in Mashhad since one year before appearance of the lesion and had at least one ulcer suspected to CL were included in this study. Those individuals whose direct smears showed negative result and had any journey six months before appearance of the lesion were excluded from the study.

Finally, seventy eight patients were selected for isolation of *Leishmania* species from their cultures. A consent form was obtained from each volunteer. Slit-skin technique was used for obtaining the sample, which was then spread on a slide. In order to diagnose the *Leishmania* amastigotes, the prepared smear was stained by Giemsa, and then carefully studied by low and high power microscopy. Then, of all the positive cases (53 individuals), the parasites were isolated by needle aspirates from the edge of the lesions.

Aspirates were inoculated into a tube of modified NNN and incubated at 22°C and checked for growth of *Leishmania* promastigotes [5]. After initial growth in culture tubes, the parasites in 21 culture

tubes multiplied and were mass cultivated for PCR in RPMI 1640 (Sigma, USA) with 17% heat inactivated fetal bovine serum (JRH, Biosciences), 2mM glutamine, 100 IU/ml penicillin G-potassium, and 100-mg/ml streptomycin sulfate (RPMI1640-FBS) at 25°C [5].

After multiplication of the parasite, at least 1000 promastigote/ml of culture supernatant, the PCR assay was performed to identify the parasites using a *Leishmania* species-specific detection kit (Cinna-Gen, Iran) according to the manufacturer's instruction. The specific primer pair in the kit was previously designed [6]. PCR reaction was provided for each sample, using 20µl PCR mix (containing reagents and *Leishmania* kDNA primers), 1 unit (0.5µl) Taq polymerase and 5µl (containing 1µg) DNA templates.

DNA extraction

DNA was extracted from promastigotes multiplied in mass cultivated tubes (1000/ml) in order to be used in molecular diagnosis of *Leishmania* species. The promastigotes were added to 150µl lysis buffer [50mM Tris-Hcl (pH=7.6), 1mM EDTA (pH=8), 1% Tween 20, proteinase k solution (19mg/ml)]. The lysate was extracted twice by phenol-chloroform and followed by addition of sodium acetate (3 M) to supernatant precipitated by ethanol. The precipitate was re-suspended in 50µl dd H₂O stored at -20°C [7].

Molecular diagnosis

PCR amplification was performed by the commercial Kit (Cinnagen-Iran), according to manufacturer's recommendation. All recommended precautions were taken to avoid PCR artifacts, including negative and positive controls in each reaction. Amplification reaction mixture (25λ) contained 5λ of DNA extracted from culture

medium, 20λ of 1x PCR mix (dNTP's, MgCL₂, primers) and 0.3λ Taq-DNA polymerase.

Amplification was performed for 36 cycles that comprised successively a 180 second denaturation step at 93°C, a 30 second annealing step at 63°C, and a 60 second extension at 72°C, in one initial cycle and 40 second at 93°C, 40 second at 63°C, 40 second at 72°C for 35 cycles (by Thermal cycler of Crbet Research, Australia). PCR products were analyzed by 2% agarose gel electrophoresis and visualized by UV trans-illuminator (UVP/Upland, USA) after staining with ethidium bromide. Interpretation of the patterns was based on the size and on the presence or absence of amplified DNA bands. According to manufacturer's recommendation, presence of 620bp fragment indicated *L. major* and 800bp indicated *L. tropica* (Fig. 1).

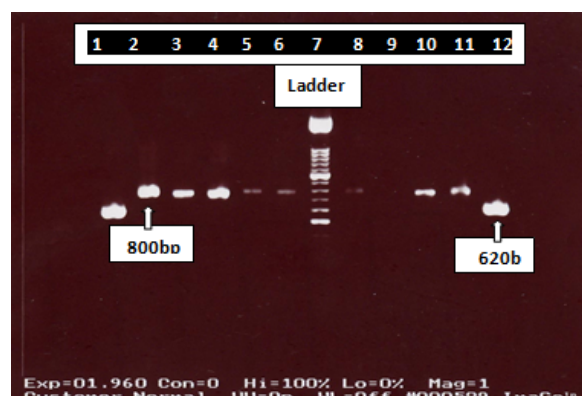


Fig. 1: Visualization of the amplification PCR products on agarose gel stained with ethidium bromide. lane 7; 100bp ladder, lane 9; negative control, lane 6; positive control, lanes 2, 3, 4, 5, 8, 10, 11, 12; patient samples

Results

Among 53 smear positive patients for CL, 22 individuals were female and 31 were male. More than 90% of them had dry (ordinary or lupoid) lesions (Table 1). PCR

technique was performed for 21 mass cultivated cultures. The results showed that nineteen isolates (90.5%) were *L. tropica* and two others were identified as *L. major* (9.5%).

Discussion

World health organization (WHO) has ranked leishmaniasis as one of the six important infectious diseases of the world. Consequently a concentrated research on the different aspects of the disease has been recommended and emphasized [8]. Population growth, immigration of non immune persons to endemic areas, activity of infected sand flies, the presence of rodent burrows and people suffering from active lesions or chronic sores, have provided a suitable environment for the maintenance of the disease [1,9].

Table 1: Clinical appearance of lesions in 21 cases suffering from cutaneous leishmaniasis

Clinical form of the lesion	Male	Female	Total No	%
Dry (Ordinary form)	11	7	18	85.7
Dry (Lupoid form)	0	1	1	4.8
Moist	0	2	2	9.5
Total	11	10	21	100

Leishmaniasis is widely spread in Iran and considered to be one of the major health problems for the population [1]. Several investigators have performed different laboratory methods to identify leishmaniasis species [10-12]. PCR method has been used for identification of *Leishmania* species in other areas of Iran [13-17].

In this study, nineteen isolates were identified as *L. tropica* and two others were sorted as *L. major* by PCR method. Since 1966, Mashhad has been considered as the

focus of ACL [1]. Recent investigations using isoenzyme electrophoresis and monoclonal antibody showed that *L. major*, the cause of ZCL, is also present in Mashhad [2,10]. The disease has been observed in different sites with more occurrences in the western and southwestern parts.

Surrounding villages of Mashhad are also known as infected foci for ZCL [2]. In this study, the results of PCR approved that both parasites (*L. major*, *L. tropica*) are present in this city and revealed that Mashhad is not only one of the important foci of ACL, but also *L. major* is present in this city; however both two latter cases came from Mashhad suburbs. In two previous studies, using isoenzyme electrophoresis and ELISA using monoclonal antibody, it was proved that *L. major* is also present in Mashhad. It was also indicated that *L. tropica* is the dominant species [2,11].

With regard to previous and present investigations, it is clear that both ACL and ZCL foci are present in Mashhad district and ACL is the dominant form. Since *L. tropica* (the dominant species.) is an anthroponotic parasite, therefore in order to control the disease, it is recommended to emphasize on chemotherapy of patients. Since *L. major* is also present in the city, we have to pay more attention on immigrants from rural areas and rodents eradication program. In this regard, PCR technique is a very reliable method to identify leishmaniasis species. However, the samples used in this study for PCR technique were promastigotes isolated from NNN culture, which indicated 21 positive cultures obtained out of 53 positive samples. Comparing the samples isolated from Whatman paper and direct smear in another study [18], it is recommended to

use the samples obtained from direct smear and/or Whatman paper rather than those obtained from the cultures. To renew the epidemiological data of CL in Mashhad district, further studies on larger size populations is also recommended.

Conclusion

Both *L. tropica* and *L. major* are the causative agents of cutaneous leishmaniasis in Mashhad. *L. tropica* is the dominant *Leishmania* species in Mashhad. However PCR technique is a very reliable method to detect *Leishmania* DNA, but it is not easy to obtain *Leishmania* culture samples.

Acknowledgements

This paper is the result of an MS student thesis which is supported by deputy of research, Mashhad University of Medical Sciences. The authors wish to thank Vice Chancellor for education and the research committee of University for their support. The authors would also thank Mr. Mehdi Akbari, Mr. Majid Ganjbakhsh and Mr. Samiei for their assistance in laboratory works.

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