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Differentiation of three species of *Leishmania major, L. tropica* and *L. infantum* by PCR-RFLP technique

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Abstract

Background: The Leishmaniasis is a zoonosis disease with a global spread that occurs in three, cutaneous, mucocutaneous and visceral forms. In Iran, three species of *Leishmania tropica*, *Leishmania major*, and *Leishmania infantum* have been reported from different regions of the country. Nowadays, molecular methods are usually used for the diagnosis and identification of parasite species. Amplification of the ITS1 gene can also be used to differentiate symptomatic and asymptomatic visceral leishmaniasis as well as types of cutaneous leishmaniasis.
Objectives: The main aim of our research was to diagnose and identify the common *Leishmania* species in Iran through ITS1 gene amplification and using the PCR-RFLP method.
Methods: First, *L. major, L. tropica*, and *L. infantum* parasites were proliferated in an RPMI culture medium, and DNA was extracted from these species separately then ITS1 gene of the parasite was amplified using the PCR-RFLP method.
Results: The result of PCR-RFLP after enzymatic cutting indicated, *L. tropica* produced 4 fragments of 139, 76, 56, 20 bp bands; *L. major* showed two fragments of 165, 139 bp bands and *L. infantum* produced three fragments of 141, 91, 54 bp bands.
Conclusion: The results of the present study showed that the use of ITS1 gene and HaeIII enzyme in the PCR-RFLP method is efficient for identifying *L. tropica, L. major*, and *L. infantum.*

Keywords: Leishmania major, L. tropica, L. infantum, RFLP-PCR, ITS1-gene

1. Background

Leishmaniasis is an infectious parasitic disease that can affect the skin, mucous membranes, and internal organs (1). Approximately 20 species of *Leishmania* cause cutaneous, cutaneous-mucosal, and visceral illnesses in humans and manifest as four different clinical symptoms, cutaneous leishmaniasis, visceral leishmaniasis, diffuse cutaneous leishmaniasis, and mucocutaneous leishmaniasis (2). The clinical manifestations of leishmaniasis, in three forms, depend on the involvement of the reticuloendothelial system, the severity of systemic manifestations as well as the host's immunological conditions (3). Except for active visceral leishmaniasis, which may lead to death in most cases if not diagnosed and treated on time, there is no risk of death in other forms. Leishmaniasis may cause unpleasant lesions, disfigurement, and disability, as well as economic and psychological effects (4).

Leishmaniasis is considered an important health problem in the world, which occurs as a result of living in infected areas or traveling to endemic areas. Identifying the parasite species in infected patients is important for proper treatment and favorable prognosis. Because the infecting species are known as the main determinant of clinical manifestations and may affect leishmaniasis treatment administration.

As an endemic country, Iran has three species of *Leishmania major*, *L. tropica* and *L. infantum*. *L. tropica*, the causative agent of anthroponous cutaneous leishmaniasis (ACL) and L.

major, causes zoonotic cutaneous leishmaniasis (ZCL). It is not possible to identify *Leishmania* species microscopically. Today, all *Leishmania* species, both in the old world and in the new world, can be distinguished from each other by molecular methods, including PCR-RFLP (5-9). Usually, genetic analysis targeting kinetoplast and nuclear DNA (kDNA and rDNA, respectively) is widely used for this purpose. In many cases, epidemiological studies of leishmaniasis conducted in endemic countries, common species have been identified using PCR-RFLP (1).

2. Objectives

Considering the importance of leishmaniasis in Iran and the necessity of accurate diagnosis of the causative species of this disease to properly combat and treat and prevent its occurrence. Thus, we aimed to evaluate the RFLP technique to differentiate circulating *Leishmania* specie in Iran.

3. Methods

3.1. Culture of *Leishmania*

Three standard strains of *L. tropica* (MHOM/IR/02/Mash10), *L. infantum* (MHOM/TN/80/IPT1), L. major (MRHO/IR/75/ER), and an unknown sample were used. Two culture mediums were used, modified NNN biphasic medium for survival and enriched RPMI1640 monophasic medium for mass multiplication of the parasite.

3.2. DNA extraction

DNA extraction from the parasite was carried out according to the instructions of the manufacturer of the extraction kit (Sinaclon - Iran). First, DNA concentration was accurately measured by spectrophotometry with ultraviolet light absorption. The electrophoresis method was used to confirm DNA extraction. Results from bands were observed following electrophoresis on agarose gel and by staining with Safe stain and using a U.V Transilluminator device.

3.3. DNA amplification

he positive samples were subjected to a PCR with ITS1(internal transcribed spacers) as a target gene. The *Leishmania* ITS1-specific primers used consisted of Forward: 5'-TCCGCCCGAAAGTTCACCGATA-3'and Reverse: 5'-CCAAGTCATCCATCGCGACACG-3' (6). The reaction mixtures were adjusted to a final volume of 25 µL and consisted of Taq Master Mix 12.5 µL, 10 pmol of each primer (forward primer 1 µM, reverse primer 1 µM), template DNA 1 µL, sterile deionized water 2.5 µL. PCR amplification steps were started with initial denaturation for 5 minutes at 95°C and continued with 52 cycles including denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C continues for 30 seconds and finally, the final extension continues at 72°C for 10 minutes (6, 10). The desired bands of the PCR product were electrophoresed on agarose gel and stained with Safe stain using a UV Transilluminator device.

3.4. RFLP

The enzyme used in this research was the HaeIII enzyme, which was purchased from Sinaclon company (Iran). The NEB cutter program was used to predict bands after enzymatic digestion and compared with the bands obtained from enzymatic digestion.

4. Results

4.1. DNA amplification

The PCR result of all cases was to be positive. It showed different species of cutaneous and visceral leishmaniasis and bands between 378-435 bp (Figure 1).

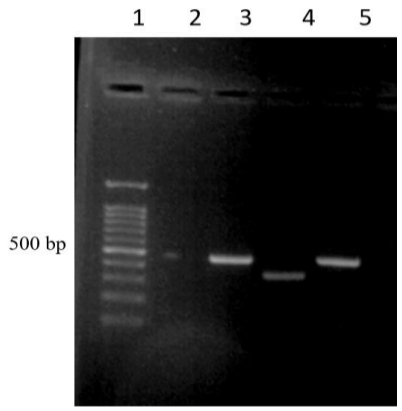

Figure 1. 1% gel electrophoresis of PCR products of different species of cutaneous and visceral leishmaniasis. From left to right: 1- Ladder with a molecular weight of 100bp, 2- Unknown sample, 3- L. major, 4- *L. infantum*, 5- *L. tropica*.

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4.2. RFLP

Based on the PCR-RFLP method, *L. tropica* showed four bands 139, 76, 56, and 20; L. major produced two bands 165, 139, and *L. infantum* three bands 141, 91, 54 bp (Figure 2).

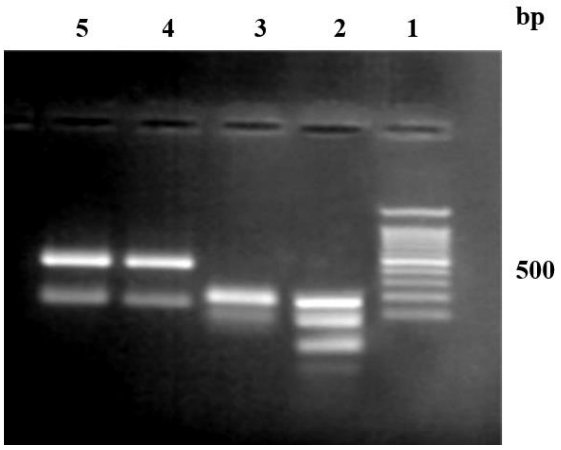

Figure 2. RFLP results following cutting with HaeIII enzyme. From the right to left, 1 - Ladder with molecular weight 100bp; 2 - a digested fragment of *L. tropica*; 3 - a digested fragment of *L. infantum* - 4 - a digested fragment of L. major - 5 - a digested fragment of an unknown sample.

5. Discussion

Leishmaniasis is a broad-spectrum parasitic disease that is reported more or less all over the world. So far no suitable vaccine or medicine has been provided to control the parasite, and no suitable chemical method has been provided to decisively fight against its vector. An estimated 0·7-1 million new cases of leishmaniasis per year are reported from nearly 100 endemic countries.
And a total of 200 million people are at risk and 300 thousand people are infected every year. Leishmaniasis shortens the life of every person between 100 and 200 days and is the cause of 5000 deaths per year (10).
Most of the common methods used for the direct diagnosis of parasites include microscopic tests (Giemsa staining and culture in laboratory conditions), which have low sensitivity because in some species the number of parasites is low and the medium cultures may be contaminated by some microorganism. Indirect methods, including serological methods, have also low sensitivity. These methods are not able to differentiate the infection in the present and that occurred in the past. Characterization of *Leishmania* spp. is important, considering that different species may require distinct treatment regimens and may also have very different prognoses. Moreover, such information is also valuable in epidemiological studies, for which knowledge of the distribution of *Leishmania* spp. in human and animal hosts, and in insect vectors, is a prerequisite for designing appropriate control measures.
Given the importance of leishmaniasis in our country, identifying species is very important for diagnosis, treatment, control, and prevention measures. In recent years, the use of molecular and biochemical tools for classification and phylogenic studies on the diversity of *Leishmania* species around the world, including Iran, has increased. Leishmaniasis classifications are usually based on geographical distribution, vector species, and disease manifestations (11).
To set up a control program in endemic areas like Iran, the correct identification of *Leishmania* species, the prognosis of the disease, the evaluation of the specific chemotherapy regimen, the effective control of the disease, and the prevention of disease transmission are necessary. Therefore, accurate identification of the parasite species(s) and analysis of their genetic diversity should be considered (12, 13).

Different techniques, which include PCR assays, with genetic targets and different post-PCR techniques, enable researchers to carry out a wide range of research with different genes worldwide (14-16). *Leishmania* cells contain hundreds of tandemly repeated nuclear ribosomal (rDNA) genes, which provide species-specific sequence markers and are often identified by restriction fragment length polymorphism (RFLP) analysis of one-step PCR products. In recent years, various studies have been conducted to reveal the phylogenetic relationship between Iranian isolates with different methods in different geographical regions (17, 18).
In the present study, by using the ITS1 gene and HealII enzyme in the PCR-RFLP test on samples of standard and unknown species, different bands were obtained for L. major, *L. tropica*, and L. infatum. This shows the success of this test in differentiating common species of *Leishmania* in Iran. Although infection with these three species manifests different clinical symptoms. But sometimes the symptoms of infection cannot be distinguished, so it is always difficult to diagnose the species of *Leishmania*. Of course, by correct and timely diagnosis of the infection, a different and appropriate treatment protocol can be considered for each species. The application of the results of this research can help clinicians in making the right decision.

In similar studies conducted in this regard, Spanakos et al. (2008) in Greece, using ITS1 gene and HealII enzyme, failed to distinguish between L. major and *L. tropica* and obtained similar bands for both species (19). In Marfurt et al. (2003) study, in Switzerland different *Leishmania* species have been differentiated from each other by using PCR-RFLP method and a mini exon fragment of kinetoplast DNA. They differentiated L. major, *L. infantum*, *L. tropica*, *L. donovani*, and *L. mexicana* from cultured samples (20).

Vaeznia et al. 2009, identified different species of *Leishmania* using the PCR-RFLP technique in Mashhad (21). In this research, it is

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suggested that the use of primers designed for the ITS1 gene and the RFLP PCR method is very suitable for species identification. Beheshti et al. 2018 conducted a cross-sectional study to identify *Leishmania* species in patients with cutaneous leishmaniasis referred to Mashhad health centers during 2012-2013 using the ITS-PCR-RFLP technique (6). In another study, conducted by Mohammadi et al. 2017, by examining the characteristics of *Leishmania* isolates collected from different regions of Razavi Khorasan Province, it was shown that ITS-PCR-RFLP based on designed primers is a suitable method for determining the characteristics of the species (22).

Compared to other studies, the results of the study by Beheshti et al showed that after digesting the amplified fragment by TaqI restriction enzyme, L.major showed two bands of 296 and 141bp, *L. tropica* produced four bands of 193, 129, 115, 68bp, and *L. infantum* five bands of 277, 142, 70 and 33bp (6). In the present study, *L. tropica* showed four bands 139, 76, 56, and 20bp; L. major produced two bands 165, 139bp, and *L. infantum* three bands 141, 91, and 54 bp.

Conclusion

The result of the present study showed that the PCR-RFLP technique, using the ITS1 gene and HaeIII enzyme, is a suitable method for detecting *Leishmania* species, especially *L. tropica*, L. major, and *L. infantum*. According to the findings of the present study, it is suggested that the primers and digesting enzyme of this study be used for the diagnosis of clinical samples.

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