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Detection of *Leishmania major* using PCR-ELISA

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ABSTRACT

Background and objectives: Cutaneous leishmaniasis is endemic in most areas of Iran, and the diagnosis of its species is essential for controlling the disease. *Leishmania major* is the causative agent for cutaneous leishmaniasis in humans. Molecular methods are generally more sensitive than microscopic methods. The present study aimed to use a polymerase chain reaction-enzyme linked immunosorbent assay (PCR-ELISA) technique for detecting live *L. major* from wounds of patients with cutaneous leishmaniasis.

Methods: In the present study, a standard strain of *L. major* promastigotes was used as the positive control for purification of DNA. The Novy–MacNeal–Nicolle and RPMI-1640 media were used for reproduction of parasites. DNA was isolated from specimens taken from 35 patients with suspected cutaneous leishmaniasis whose disease was confirmed by direct smear method. The PCR-ELISA technique was later applied by using the standard strain, patient specimens, and primers specific for the 18s rRNA.

Results: Out of 35 patients, 17 (48.6%) were male and 18 (51.4%) were female. In addition, 8.6% of the patients lived in the Gonbad-e Kavus County, while all patients had been infected in villages around Gonbad-e Kavus. Of 35 patients with confirmed cutaneous leishmaniasis according to the direct smear method, 31 patients (86.31%) had leishmaniasis based on the PCR method and the PCR-ELISA methods.

Conclusion: Based on the results, the PCR-ELISA method is more sensitive and accurate for detecting *L. major*.

Keywords: Leishmania major, PCR-ELISA, Patients.

INTRODUCTION

Leishmaniasis is an important health problem in the world that mainly occurs due to travel to endemic areas. It is caused by the intracellular protozoan *Leishmania* from the kinetoplast phylum that is transmitted by the female sandflies of the genus *Phlebotomus* in the Near East and *Lutzomyia* in South America (1). In Iran, both cutaneous and visceral forms of leishmaniasis are important parasitic diseases. Cutaneous leishmaniasis can be seen in two types, namely anthroponotic (dry) and zoonotic (wet) leishmaniasis. In Iran, the main cause of zoonotic cutaneous leishmaniasis is *Leishmania major*, and the anthroponotic type is mainly caused by *Leishmania tropica*, each of which has several foci (2, 3).

About 1.5 million individuals around the world are annually diagnosed with cutaneous leishmaniasis, and it is estimated that there are about 12 million cases of cutaneous leishmaniasis in different regions of the world. In Iran, about 15,000 individuals are diagnosed with leishmaniasis every year. The incidence of the disease in Iran is estimated at 28 cases per 1000 population, most of which are reported from the Isfahan and Shiraz provinces with 1.66 cases per 1000 population and the least cases are reported from the Mazandaran Province with 0.22 cases per 1000 population (4, 5). The Gonbad-e Kavus County is a center of zoonotic cutaneous leishmaniasis in the Golestan Province, and many cases of leishmaniasis are found in people of villages bordering Turkmenistan. Due to the geographical location of these villages and the proximity of residential areas with numerous rodent nests, as well as the type of materials used in the construction of houses, stables, and poultry farms, this region is a suitable place for reproduction of sandflies. According to the statistics of Gonbad-e Kavus health centers, 172 individuals were diagnosed with cutaneous leishmaniasis in 2007 (6). The use of more sensitive and accurate molecular methods such as polymerase chain reaction (PCR) is conventional for diagnosis of leishmaniasis (7). The PCR- enzyme linked immunosorbent assay (ELISA) technique is a suitable alternative to the PCR method because it is quicker and more sensitive for detection of small amounts of specific gene sequences of pathogens (8). In the present study, the PCR-ELISA technique was applied to accurately determine the species of *L. major* in the Gonbad-e Kavus County, Golestan Province, Iran. The method utilizes probes specific for a region of the target gene, labeled with biotin in the 5' end and digoxigenin-dUTP nucleotides for the synthesis of a specific region of the target gene, as well as anti-DIG peroxidase antibodies to detect the reaction between the probe and the PCR product using the ELISA method.

MATERIALS AND METHODS

The present descriptive-analytical study was performed in the Molecular Cell Department of Golestan University of Medical Sciences during 2016-2017. Patients whose wounds were infected with zoonotic cutaneous leishmaniasis were selected via convenience sampling. After cleaning the wounds with 70% ethanol, samples were taken from the wound margin using a sterile scalpel. Some wound specimens were used for DNA extraction and spread on the chamber. Diagnosis of suspected patients was made by smear preparation of the wound, spread on the chamber, and Giemsa staining. For this purpose, a small incision was made at the wound margin, and the Leishman bodies were examined inside and outside of macrophages under a microscope. Detection of *L. major* was based on the gold standard parasitological methods (microscopic examination and culture) due to its high specificity. Furthermore, demographic information and personal characteristics of the patients were collected via a questionnaire. Overall, 35 patients with suspected leishmaniasis were enrolled in the study. *L. major* strain MRHB/IR/15/ER was used as the positive control. After preparing the parasites, the Novy–MacNeal–Nicolle (NNN, Sigma-Aldrich, USA) and RPMI-1640 (Sigma-Aldrich, USA) media were used for reproduction of parasites. Next, growth of the parasite in the NNN medium, at temperature of 25 ℃, was examined after 48-72 hours. After the reproduction of parasites in the NNN medium, the parasites were transferred to enriched *RPMI-1640* medium and incubated at 24-26 ℃. Growth was controlled daily using an invert microscope.

For DNA extraction, 1-2 billion promastigotes in static phase were required. The DNG™-Plus kit (CinnaGen Co., Iran) was used for DNA extraction if specimens of the suspected patients were fixed on the chambers. After

0reading optical density (OD) of specimens by spectrophotometry, DNA extraction was performed using isopropanol, 75% ethanol, and sterile distilled water. For this purpose, the DNG™-Plus solution (400 µl) was mixed with 100 µl of specimen, and vortexed for 15-20 seconds. Then, 300 µl of isopropanol was added to the solution. Due to the low amount of parasites, the specimens were placed at -20 ℃ for 20 minutes after adding isopropanol, and then the specimens were centrifuged at 12,000 rpm for 10 minutes, and the supernatant was aspirated. Next, 1 µl of 75% ethanol was added. After through mixing by vortexing, the solution was centrifuged at 12,000 rpm for 5 minutes, and this step was repeated. After removing the supernatant, the plate was placed at 65℃ for 5 minutes to completely dry the content. Then, 25 µl of distilled water was added to each well of the 96-well plate.

The following primers were used in the PCR method: reverse: 5'-AGGGCCGGTAAAGGCCGAATAG-3', and forward: 5'-DIGCCAAAGTGTGGAGATCGAAG-3'. The probe sequence was as follows: 5'- biotin GCCCGCTTTTACCAACTTACG-3'.

The PCR reaction was carried out in final volume of 25 µl. The reaction solution contained 12.5 µl PCR MasterMix (CinnaGen Co. of Iran), 1 µl of each primer, 5.5 µl deionized water, and 5 µl of DNA template. The PCR cycling conditions were as follows: initial denaturation at 94 ℃ for a minute, denaturation at 98 ℃ for 5 seconds, annealing at 55 ℃ for 5 seconds, extension at 72 ℃ for 5 seconds, and 30 cycles of final extension. Results of the PCR experiment was analyzed by electrophoresis on 1.5% agarose gel and visualized under a UV transilluminator.

For the PCR-ELISA method, 5 µl of each PCR specimens (positive and negative controls as well as patient specimen) were added to a 1.5 ml microtube. Next, 20 µl of the denaturation solution were added, and the microtube was incubated at room temperature for 10 minutes. Then, 175 µl of hybridization buffer were added and the microtube was vortexed. After transferring 200 µl of the solution to well of a 96-well plate, the hybridization reaction was done by placing the plate on a shaking incubator at 55 ℃ for 3 hours. Then, the content of the plates were removed, and the wells were washed 5 times with a wash buffer. Next, 20 µl of peroxidase conjugated anti-D (Bio-Techne Co., USA) were added to each well. After incubation at 37 ℃ for 30 minutes, the washing step was repeated, and 200 µl of chromogenic substrate ABTS were added to each well. The plate was incubated in the dark, at 37 ℃ for 30 minutes. Absorbance at 492 nm was read using an ELISA reader, and a PCR mixture containing distilled water was used as the blank.

Data analysis was done with SPSS software (version 15) at an alpha level of 0.05.

RESULTS

Out of 35 patients, 17 (48.6%) were male and 18 (51.4%) were female. The frequency of leishmaniasis was highest subjects aged 0-9 years (22.9%) and the lower in those aged 30-39 (11.4%), 10-19 years (14.3%), 20-29 years (17.1%), and 49-40 years (17.1%). Moreover, 8.6% of patients lived in the Gonbad-e Kavus County, while all patients had been infected in villages around Gonbad-e Kavus due to the round-trips of people to villages bordering Turkmenistan.

In the study, the frequency of leishmaniasis wounds was highest in the arms and hands (54.3%) (Table 1).

It should be noted that some of the subjects had skin sores or wounds on more than one body part.

Table 1- Frequency distribution of cutaneous leishmaniasis in terms of wound location

Wound location	Number	Percentage
Arms and hands	19	54.3%
Legs	9	25.7%
Face	6	17.1%
Neck	1	2.9%

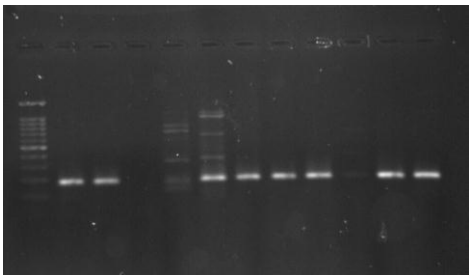

Figure 1-Agarose gel electrophoresis of the PCR products for detection of *L. major* in patients with suspected cutaneous leishmaniasis. Lane 1:100 bp DNA Ladder (Fermentas), Lane 2: Positive control, Lane 3: Smear sample, Lane 4: Negative control, Lane 5-12: Positive specimens. Detection of 200 bp bands confirmed the presence of *L. major*).

Table 2- Mean OD read by ELISA Reader

Positive control	Negative control	Specimen No1	Specimen No2	Specimen No3	Specimen No4	Specimen No5	Specimen No6
0.088	0.007	2.994	2.356	3.018	0.173	2.975	0.111
7	8	9	10	11	12	13	14
0.103	0.109	2.630	0.108	2.552	1.948	1.712	0.140
15	16	17	18	19	20	21	22
3.107	3.105	1.979	2.190	0.853	0.135	0.164	0.181
23	24	25	26	27	28	29	30
2.717	2.526	0.415	0.219	0.332	0.236	0.137	0.598
31	32	33	34	35			
0.232	0.522	0.337	0.404	0.260			

Based on the results, 71.4% of the patients had 1 or 2 wounds, 22.9% had 3 to 6 wounds, and 5.7% had more than 6 wounds.

Of 35 patients with confirmed cutaneous leishmaniasis according to the direct smear method, 31 patients (86.31%) had leishmaniasis using the PCR method and the PCR-ELISA technique (Table 2). Figure 1 shows the results of the PCR experiment for detection of *L. major* in patients suspected of cutaneous leishmaniasis.

DISCUSSION

In this study, the rate of cutaneous leishmaniasis was not significantly different between men and women. This finding is consistent with findings of studies by Dehghan et al. in Larestan, Iran (9) and Karimian in Mashhad, Iran (10) but inconsistent with studies by Silveira et al. in Brazil (11) and Rajabi in Isfahan, Iran (12).

According to the results, the frequency of cutaneous leishmaniais was highest in patients below 9 years of age and lowest in patients aged 30-39 years. This finding is consistent with results of other studies in Iran. In the study by Dehghan et al., the highest rate of the disease was seen in patients below 9 years of age (9). In a study by Tohidi, the frequency of leishmaniasis was highest in patients below 5 years of age (13). Similarly, in two previous studies, the frequency of leishmaniasis was highest in individuals aged 5-9 years (14, 15).

In this study, most wounds were located on the hands and arms (54.3%) as well as on the feet (25.7%). This finding is in line with findings of previous studies (9, 16, 17).

Due to the multiplicity and similarity of the disease agent, the accurate detection of causative parasite species is essential for developing prevention and control programs. On the other hand, the epidemiological patterns of cutaneous leishmaniasis have changed in Iran. Molecular methods have revolutionized the identification of parasites (18).Diagnosis of cutaneous leishmaniasis is mainly based on the parasitological methods and direct observation of the parasite (19). In the direct observation method, it is possible to determine the parasite species by using the PCR method (20).

According to a study by Pagheh, 52.3% of patients with cutaneous leishmaniasis that had

tested negative in the direct microscopic test, were tested positive in the PCR method. Therefore, the PCR method should be used for more accurate diagnosis of the disease and preventing misdiagnosis (false negative) (21). In a study by Fakhar et al. on 62 direct extensions in which Leishman bodies were not found, 35 cases (56.4%) contained the parasite DNA (22).

In the present study, 31 out of 35 specimens, which were positive in the direct smear method, were also found positive in the PCR method. In addition, all specimens were reported as positive in the PCR-ELISA method. Based on the results, the PCR-ELISA method had higher precision and sensitivity for detection of *L. major*. It should be noted that ELISA has overcome many limitations and defects of other methods, including conventional PCR. A prominent feature of ELISA-assisted diagnosis is the possibility of simultaneous analysis of multiple specimens (23, 24).

The PCR-ELISA technique has been previously used for diagnosis of schistosomiasis (25) and *Acinetobacter* (7). In this regard, another study also reported that the PCR-ELISA is more sensitive for detecting *Leishmania* parasites compared to the PCR method (26).

CONCLUSION

The results indicate that the molecular methods have higher precision and sensitivity for the detection of *Leishmania* species. Therefore, the development of molecular methods for the diagnosis of *Leishmania* species should be further exploited**.**

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Ethics approvals and consent to participate

The study was approved by the ethics committee of Golestan University of Medical Sciences (ethical code: 172305937203).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding publication of this article

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