

Comparison of Traditional Methods and PCR for Diagnosis of Cutaneous Leishmaniasis in South-West of Iran

Shahram Khademvatan,¹ Jasem Saki,^{*2} Sharif Maraghi²

1. Department of Medical Parasitology, Cellular and Molecular Research Center, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran
2. Department of Medical Parasitology, Infectious and Tropical Diseases Research Center, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran

Article information	Abstract
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<p>*Corresponding author at: Department of Medical Parasitology, Infectious and Tropical Diseases Research Center, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran E-mail: jasem.saki@gmail.com</p>	<p>Background: The usual method for laboratory diagnosis of cutaneous leishmaniasis was the direct observation of parasites under a light microscope. Although this method has high specificity, it has low sensitivity. The purpose of this study is to compare three methods of direct observation, culture and Mini-exon-PCR to diagnose cutaneous leishmaniasis in Khuzestan province. This study intends to compare sensitivity of PCR approach with sensitivity of the existing traditional methods to diagnose cutaneous leishmaniasis using Mini-exon gene.</p> <p>Materials and Methods: A total 216 skin biopsies prepared from patients with cutaneous leishmaniasis were studied through direct method, culture in NNN, culture in RPMI 1640 and Mini-exon-PCR and the sensitivity of these methods were compared with each other. In this study Mini-exon-PCR was considered as the gold standard method.</p> <p>Results: Results showed that 46.7% with direct method, 35.1% with culture method in RPMI 1640, 57.8% with culture method in NNN and 70.3% with PCR were positive. Sensitivity was obtained 66.4% for microscopic observation, 50% for culture in RPMI1640, and 82.2% for culture in NNN and 100% for PCR.</p> <p>Conclusion: This study showed that PCR on samples stored in normal saline has higher sensitivity and specificity than other traditional methods ($p<0.05$). Thus, Mini-exon-PCR on samples in normal saline is a reliable method to diagnose cutaneous leishmaniasis, especially in cases where the diagnosis is negative with the other methods.</p> <p>Copyright © 2012 Zahedan University of Medical Sciences. All rights reserved.</p>

Introduction

Leishmaniasis is a parasitic infection that is caused by a protozoan called *Leishmania*. Depending on the species type, the clinical symptoms in the host are different. Visceral leishmaniasis, cutaneous leishmaniasis, mucosal and cutaneous leishmaniasis, and diffuse cutaneous leishmaniasis are of important forms of the diseases. The disease is considered of the world's health problems [1]. 350 million people across the world are at risk for this disease. Among 88 countries endemic for the disease, 22 countries were in the New World and 66 countries in the Old World 16 of which were developed and 72 were developing countries [2-4]. In our country, both forms of visceral and cutaneous leishmaniasis are common [5]. Khuzestan province is considered one of the important endemic areas of our country for cutaneous leishmaniasis [6].

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]. It is highly important to select a method with high sensitivity and specificity to diagnose *leishmania* parasites in clinical samples. To do this in laboratories, direct method is often used that has less sensitivity compared with PCR molecular techniques. In the past two

decades, DNA based methods were common to diagnose leishmania species. Leishmania detection using PCR techniques has high sensitivity and specificity [8]. Therefore, in this study, three direct methods, culture and PCR were used to diagnose cutaneous leishmaniasis in Khuzestan province and the results were compared with each other. Give the efficiency of Mini-exon gene in detection of Leishmania species [9], Mini-exon-PCR was used in this study. Mini-exon gene is an intra-nuclear gene with 200 copies on leishmania parasite genome which is composed of three sections of Exon, Intron and nontranscribed spacer. Nontranscribed spacer section is different in various species and makes them distinct from each other [9]. One of the distinctions of this gene to the other target is that all leishmania species of the old and new world can be identified through a pair of primers [9]. So far, PCR methods conducted in our country have used genes and locus other than Mini-exon and this study is the first study in Iran which has used Mini-exon gene to diagnose leishmaniasis.

Materials and Methods

Given the endemic areas of cutaneous leishmaniasis, sampling was performed from different parts of

Khuzestan province from September 2010 to September 2011. Samples were prepared from 216 patients referred to health centers and laboratories. First, for each person, a questionnaire containing personal information was completed and after filling out the consent by the patient or their family, sampling was performed with a scalpel from the inflamed surrounding of lesions and 4 samples were prepared from each individual.

A sample to make the expansion on the slide, a sample for culture in Novy-Nicolle-McNeal NNN medium, a sample for culture in RPMI 1640 medium enriched with 10% of fetal calf serum (FCS) and a sample for culture in normal saline. Mediums were kept in the temperature of 21°C. High initial parasites were transferred to the RPMI1640 medium enriched with 15% fetal calf serum so that parasites would reach mass production. The expansions provided were examined under light microscopy after staining with Giemsa.

DNA Extraction: high promastigotes were centrifuged in large numbers with round 3000 rpm at 4°C, and were washed three times in sterilized PBS with pH=7.2. DNA was extracted using DNeasy blood and tissue kit according to the manufacturer's instruction. Obtained DNA was dissolved in 50 µl sterile distilled water and was kept at -20°C till the time of PCR test.

PCR Test: for amplification of Mini-exon gene, primers: Fme (5' - TATTGGTATGCGAAACTTCCG-3') Rme (5' - ACAGAAACTGATACTTATATAGCG-3') were used [9]. The amounts of 2-7 µl (75-100 ng) of DAN were extracted and 0.5µmol of each primer was used in 20 µl of Taq DNA polymerase Master Mix RED reaction (Bioneer Korea) solution. At this stage, to perform PCR reaction, Dimethyl sulfoxide (DMSO) (78.13 g/mol Cinnagen, Iran) was added to the solution as much as 12% of reaction.

After preparation of overall mixture, the thermal program of Thermocycler (Eppendorf AG 22331, Hamburg, Germany) was set so that first the sample was put at 94°C for 5 min; then, the following steps were repeated for 35 cycles: At 94°C for 30 seconds Denaturation, at 51/5°C for 30 seconds Annealing, at 72°C for 45 seconds extension and at the final step, elongation was performed at 72°C for 10 minutes. Obtained PCR products were loaded on agarose gel 1.5%, and then, they were read after staining with ethidium bromide in Geldoc device (Bio-rad).

Determination of specificity of PCR reaction: To achieve this goal, PCR reaction was performed with DNA purified from rabbit blood, human blood and yeast fungus according to the previous PCR programs. After loading on agarose 1.5%, no band was observed for any of them. For positive control, DNA was extracted from standard samples of *L. major* (MHOM/IR/75/ER), *L. tropica* (MHOM/IR/02/Mash10) and *L. infantum* (49MCAN/IR/97/LON) and PCR test was conducted through provided primers.

To analyze the obtained results, SPSS-19 software, statistical t-test as well as equations of sensitivity and specificity were used. The sensitivity of the applied

diagnostic methods was calculated considering PCR as the standard method.

Results

Samples collected from 216 patients suspected with skin lesions studied using direct diagnostic method, culture and PCR method. The results compared with each other (Table 1).

Out of total 216 skin samples taken, 101 samples (46.7%) with the direct method, 76 samples (35.1%) with culture method in RPMI 1640, 125 samples (57.8%) with culture method in NNN and 152 samples (70.3%) with PCR method were detected to be positive.

Through PCR, all positive samples in the medium RPMI 1640 and NNN became positive (sensitivity 100%). Also, 32 samples of 91 samples (35.1%) which were negative in the medium NNN were shown positive through PCR method. 42 out of 140 samples (30%) which were negative in the medium RPMI 1640 became positive through PCR method. In this study also, from 115 negative slides in the direct method, DNA was extracted and tested through PCR method 39 of which (33.9%) were shown positive. 100 out of 101 positive slides (99%) were shown positive through PCR method.

In all cases of PCR tests, standard strains of *L. major*, *L. tropica* and *L. infantum* were used as positive control. Using PCR method, 4 out of 152 samples (2.6%) were identified as *L. tropica* and 148 samples (97.4%) as *Leishmania major*. 400 bp band was obtained for *Leishmania tropica*, 427 bp band for *Leishmania major* and 434 bp for *Leishmania Infantum*, respectively (Fig. 1), which was performed for final approval using enzymes Eae I and HaeIII and PCR-RFLP test.

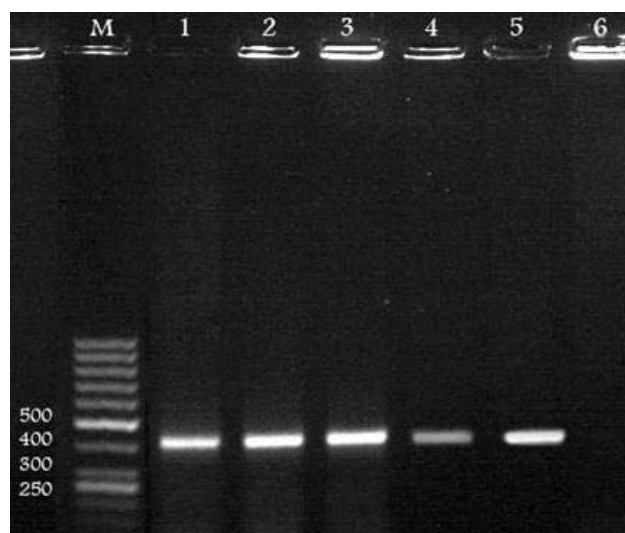


Figure 1. PCR products obtained from amplification of Mini exon gene of leishmania on agarose gel 1.5%. lane 1 and 2 of leishmania major, lane 3 of leishmania major of positive controls, lane 4 of leishmania tropica, lane 5 of leishmania tropica of positive control, and lane 6 of negative control

Table 1. Comparison of sensitivity of three methods of direct microscopy, culture and PCR to diagnose cutaneous leishmaniasis in South West Iran

Method	Number	Positive N (%)	Negative N (%)	Sensitivity
Direct microscopy	66.4%	115(53.3%)	101(46.7%)	216
Culture in RPMI1640	50%	140(64.9%)	76(35/1%)	216
Culture in NNN	82.2%	(42.2) 91	125(57.85)	216
*PCR of normal saline	100%	64(29.7%)	152(70.3%)	216
PCR on Positive NNN culture	100%	0	125(100%)	125
PCR on negative NNN culture	52.4%	59(64.9%)	32(35.1%)	91
PCR on negative RPMI1640 culture	55.2%	98(70%)	42(30%)	140
PCR on negative smear	76.4%	76(66.1%)	39(33.9%)	115
PCR on positive smear	99%	1(1%)	100(99%)	101

Keys: *Gold standard method, PCR; Polymerase chain reaction, NNN; Novy-MacNeal-Nicolle, RPMI1640; Roswell Park Memorial Institute 1640

Discussion

In this study, the highest sensitivity was determined using PCR method, from the samples provided in saline. Also with PCR method, a significant percentage of negative samples became positive through the methods used in this study.

Iran is considered one of the important endemic areas of cutaneous leishmaniasis in the world; so that the incidence of this infection in various provinces has been reported between 1.8% and 37.9% [10, 11]. More than 90% of cases of cutaneous leishmaniasis reported across the world are related to 6 countries of Iran, Afghanistan, Syria, Brazil Saudi Arabia and Peru [12].

Correct diagnosis of cutaneous leishmaniasis infection and its differentiation from other fungal and bacterial diseases is important and essential for proper treatment and control and prevention of disease in a region [13]. Although the direct method of diagnosis of cutaneous leishmaniasis has 100% specificity, this method has less sensitivity [14]. The study of isoenzymes of *Leishmania* is considered a reliable and standard method with high sensitivity and specificity to determine species. However, this method is time-consuming, expensive and is laborious [15]. Therefore, researchers welcomed PCR molecular methods which have high sensitivity and specificity [16]. Comparison of conventional diagnostic methods with PCR molecular methods to identify leishmania parasite in clinical samples is of particular importance. In this study, direct methods, culture in RPMI 1640, culture in NNN and PCR were compared with each other and culture in RPMI 1640 and direct test method showed the least sensitivity.

The results of this research were higher (46.7%), than the results obtained in the study of Al-Jawabreh et al. [14], and Aviles et al. [17] which have reported positive cases with microscopic method to be 37% and 42% respectively. In a study conducted by Meryem et al. [18]. On 3361 patients, 69.2% of the samples were identified by microscopic methods that have reported a higher sensitivity compared to this study. Also, this researcher had obtained sensitivity of culture method with the direct method to be the same which showed a higher sensitivity compared to the results of both culture methods in NNN and RPMI1640 in the present study. However, the sensitivity of PCR method in the study is mentioned 84.6% that showed less sensitivity than our study. It may be due to the difference in the amplified segment on these

two studies which was SSUrRNA in the mentioned study. Marfurt et al. have reported the sensitivity of Mini-exon-PCR method to be more than SSU-ITS-PCR method [9]. Results obtained in this study are consistent with results of the study of Bariio et al. on cases of mucosal leishmaniasis with PCR method in which sensitivity of this method was reported to be 100% [19].

In their study, Marques et al. concluded that KDNA-PCR method is able to identify American leishmaniasis agent species and to be a substitute for microscopic method and Montenegro skin test, especially in cases where both methods fail to detect the infection [20]. Our research findings are consistent with the research of Bensoussan et al. which examined the sensitivity of three culture, microscopic and PCR methods in detection of leishmania parasites in patients with cutaneous leishmaniasis which reported sensitivity of PCR to be 98.8% which was higher than other methods [21].

In the study of Pour-Mohammadi et al. on 219 patients with cutaneous leishmaniasis, sensitivity of microscopic method, culture in NNN and PCR were respectively, 79.71%, 50.68% and 93.61% [22]. The sensitivity of microscopic method in the study was higher than the sensitivity of microscopic method in this study, but sensitivity of its culture method was lower than sensitivity of culture method in our study. Sensitivity of PCR in the study of this researcher, conducted on stained slides as well as medium, is shown higher than sensitivity obtained in our study conducted on normal saline samples. However, is shown less than sensitivity of PCR method in the present study conducted on promastigotes of NNN medium. In the present study, to investigate the results of microscopic methods and culture, PCR was performed from slides and negative test samples. 33.9% of the slides, 30% of negative samples of RPMI 1640 culture and 35.1% of negative samples of NNN became positive. In the study of Venazzi et al., to diagnose American cutaneous leishmaniasis through PCR, 27.3% of negative people with direct method, but positive with Montenegro test, were shown positive [23].

The study of Brustoloni et al. in Brazil, to compare the direct method, culture and PCR in the diagnosis of visceral leishmaniasis, sensitivity of direct method, culture and PCR was respectively reported 79.1%, 59% and 92.3% [24]. In this research, PCR method performed on slides prepared from the bone marrow of patients. The study of Shahbazi et al. [25], conducted in Mashhad

reported sensitivity of direct method, culture and PCR respectively 79.3%, 86.2% and 100% that is consistent with the results of this study in terms of sensitivity of PCR method, but it shows a higher value of sensitivity for direct method and culture. One of the limitations of this study is that due to time and budget constraints, the samples were collected from limited areas. It is recommended that more areas should be examined in future studies.

According to the type of the segment used in the PCR method, parasite type, tester's experience, accuracy of instruments and the size of the sample used in the experiments are of factors that can affect the results. The difference between the results of this study with other studies conducted inside and outside the country is expected. Given the higher sensitivity of PCR method than microscopic methods and culture.

This method can be used to diagnose leishmania parasites, especially in cases where results are obtained negative through other methods. If there is no possibility

of using PCR, two combined methods of microscopic and culture in NNN can be used for diagnosis of cutaneous leishmaniasis.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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