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Original Article

Comparison of Three Methods for Diagnosis of Cutaneous Leishmaniasis

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ABSTRACT

Background: Leishmaniasis is one of the infectious parasitic diseases of highest incidence in the world. Cutaneous Leishmaniasis (CL) has long been reported in Shiraz, Southern Iran. There is a need to find a sensitive and specific method for treatment and control of the disease.
Methods: We have compared the sensitivity of the conventional methods microscopy and cultivation of lesion scrapes against PCR amplification of parasite kinetoplast DNA from these samples. The samples (n=219) were obtained from the patients clinically suspected of CL. The smears were stained with Giemsa for microscopy and cultured in Novy-Nicolle-McNeal (NNN) blood agar for promastigote growth. For PCR, the dry smears were scraped off the slides and DNA was extracted.
Results: The positive rates from 219 specimens were 76.71%, 50.68%, and 93.61% for microscopy, cultivation, and PCR, respectively. The highest correlation was found between PCR and microscopy method (*P*= 0.014). In PCR assay, 95.61%, 3.9%, and 0.49% of the samples were identified as *Leishmania major*, *L. tropica*, and dermatropic *L. infantum*, respectively.
Conclusion: The PCR method appears to be the most sensitive for the diagnosis of CL and is valuable for identifying the other species of *Leishmania* with confusing dermatropic signs.

Keyword(s): *PCR, Diagnosis, Cultivation, Cutaneous Leishmaniasis*

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Introduction

Leishmaniasis is a vector- born parasitic disease caused by a protozoan, *Leishmania* spp. (1). The disease is endemic in many tropical and subtropical regions, at least 88 countries of the world. The annually estimated incidence of the disease is 1-1.5 million cases of the disfiguring cutaneous leishmaniasis, the most common form of the disease, and 500,000 cases of the visceral form (2, 3). Both forms of the disease with diverse clinical manifestations are prevalent in Iran and remain a severe main public health problem (4). There are several foci of zoonotic cutaneous leishmaniasis (ZCL) in the north, east and south of Iran. Fars, a province in South Iran, is one of the endemic foci of CL (5, 6).

Diagnosis of CL is difficult because of the varied symptoms and the different species involved (7, 8). There are many reliable laboratory diagnostic methods such as direct smear examination, culture, immunologic and molecular techniques. The routine diagnosis of CL patients depends on examination of skin lesions using smears and cultures of dermal scrapings or examination of sections obtained from a skin biopsy (2). The investigations available have a wide range of reported sensitivities. The sensitivity of direct microscopy is not high, and tissue culture is not uniformly available and successful. Polymerase Chain Reaction (PCR) is a sensitive test for the detection of low amounts of DNA in tissues (9).

On the other hand, due to the wide clinical spectrum of leishmaniasis and the variety of response to treatment according to the parasite species, there is a need to find a highly sensitive method for both diagnosing and identifying the causative agents of the disease especially in the endemic regions.

The present study was carried out to compare the sensitivity of a molecular method, PCR, not only for diagnosis but also for species discrimination with traditional methods, microscopy and cultivation in an endemic area, Shiraz, Southern Iran, during October, 2007 to January, 2008.

Materials and Methods

Patients and Samples

A total of 219 patients referred to the Microbiology and Parasitology unit of Valfajr Health Center in Shiraz during Oct. 2007 to Jan. 2008, and were clinically suspected to cutaneous leishmaniasis, were investigated to diagnose and identify the causative agent of the disease. The following diagnostic investigations were performed for each case:

1-Microscopic examination of Geimsa-stained smears from the ulcer border;

2-Culture from skin lesion scrapes;

3-PCR on dried and/or fixed and even stained specimens.

The patients were given the diagnosis of proven CL if at least 1 of the 3 techniques (smear, culture, or PCR) produced positive findings.

Parasitology

For making stained smears, tissues were taken using a disposable lancet. A small incision was made in the cleaned margin of the nodules and lesions with the point of the blade. The blade was turned 90 degrees and scraped along the cut edge of the incision to remove and pick up the skin tissue, which was smeared on a clean glass microscope slide. After the smears dried completely, they were fixed with 100% methanol, allowed to dry again, and stained with Geimsa stain for microscopic examination (10). At

least two specimens were prepared for each case. One was stained and the other stored to be applied in the next appropriate time if necessary.

Culturing

The lesions and the adjacent normal-looking skin around them were cleaned, sterilized with 70% ethanol, and allowed to dry. Similar to the preparation of the slide smears, a small amount of the scraped tissue was inoculated on the liquid phase of Novy-McNeal-Nicolle (NNN) medium (10% of rabbit blood). The cultures were incubated at 25ºC and examined for parasite growth by the inverted microscope and also light microscope every 4 days until promastigotes were seen or up to one month before being discarded as negative. The cultures were made at least in duplicates for each case.

DNA extraction

Each fresh or dried smear was scraped off the slide with a sterile scalpel and the scrapings were added to 200 µl lysis buffer [50 mM Tris- HCl (pH 7.6), 1 mM EDTA, 1% (v/v) Tween 20] containing 8.5 µl of a proteinase K solution (19 µg/ml), in a 1.5-ml tube (11). The tube was incubated for 2 h at 56°C before 200 µl of a phenol: chloroform: isoamyl-alcohol mixture (25:24:1, by volume) was added. After being shaken vigorously, the tube was centrifuged at 6000g for 10 min and then the DNA in the supernatant solution was precipitated with 400 µl cold absolute ethanol, resuspended in 50 µl double distilled water and then stored at -20 °C, until it could be tested for leishmania kDNA.

PCR amplification

The PCR was performed on all 219 methanol fixed and/or Giemsa-stained samples.

The PCR used to amplify the variable area of the minicircle kinetoplastic DNA of any *Leishmania* in the smears was a slight modification (12) of that described by Aransay et al. (2000). The forward LINR4 (5′-GGG GTT GGT GTA AAA TAG GG-3′) and reverse LIN17 (5′- TTT GAA CGG GAT TTC TG-3′) primers were used have been designed within the conserved area of the minicircle kinetoplastic contained the conserved sequence blocks 3 and 2, respectively (11). Each 25- µl reaction mixture contained 250 µM of each deoxynucleoside triphosphate, 1.5 mM MgCl2, 1 U Taq polymerase (CinnaGen, Tehran, Iran), 1 µM LINR4, 1 µM LIN17 Primers, 100pg DNA extract, and 2.5 µl PCR buffer. Each reaction mixture was overlaid with mineral oil before being transferred to a CG1-96 thermo cycler (Corbett Research, Sydney, Australia) set to give 5 min at 94°C, followed by 30 cycles, each of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C, and then a final extension at 72°C for 5 min. A 5- µl sample of each PCR product was subjected to electrophoresis in 1.5%-agarose gel. The bands were then stained with ethidium bromide and visualized under ultra-violet trans-illumination. The parasites were identified by comparing the size of the band produced from a test sample with those produced from reference strains of *L. infantum* (MCAN/IR/96/LON49), *L. tropica* (MHOM/IR/89/ARA2) and *L. major* (MHOM/IR/54/LV39). A band of 720 bp, for example, would have indicated that *L. infantum* (or, at least, *L. infantum* kDNA) was present in the tested smear.

Statistical analysis

The results of the various diagnostic techniques were analyzed using McNamara test. If P was<0.05, the difference was considered significant.

Results

A total of 219 patients with a clinical suggestion of CL were investigated in a routine setting. Direct microscopy, culture, and

PCR were the diagnostic techniques performed.

All of the 219 examined people were identified as CL patients with a positive result in at least 1 of the 3 performed techniques. All the cases showed obvious clinical symptoms for CL and were found to be positive with combination of three performed techniques. Of the 219 cases, 165 (76.7%), 111(50.7%) and 205(93.6%) were positive by direct microscopy, cultivation and PCR, respectively. All the three diagnostic tests were positive for only 89 of the 219 cases. PCR and microscopy showed more correlation (*P*= 0.014). One dermatropic *L. infantum* case was identified by two PCR methods.

Ninety six percent, 3.9% and 0.49% of isolates were identified as *L. major*, *L. tropica* and *L. infantum*, respectively (Fig. 1). Eighty-five (38.8%), 43(19.63%) and 91(41.55%) of cases had 1, 2 and 3 or more than 3 lesions respectively. The most frequently affected sites were the upper extremities (63%). The lesions were more often in wet form.

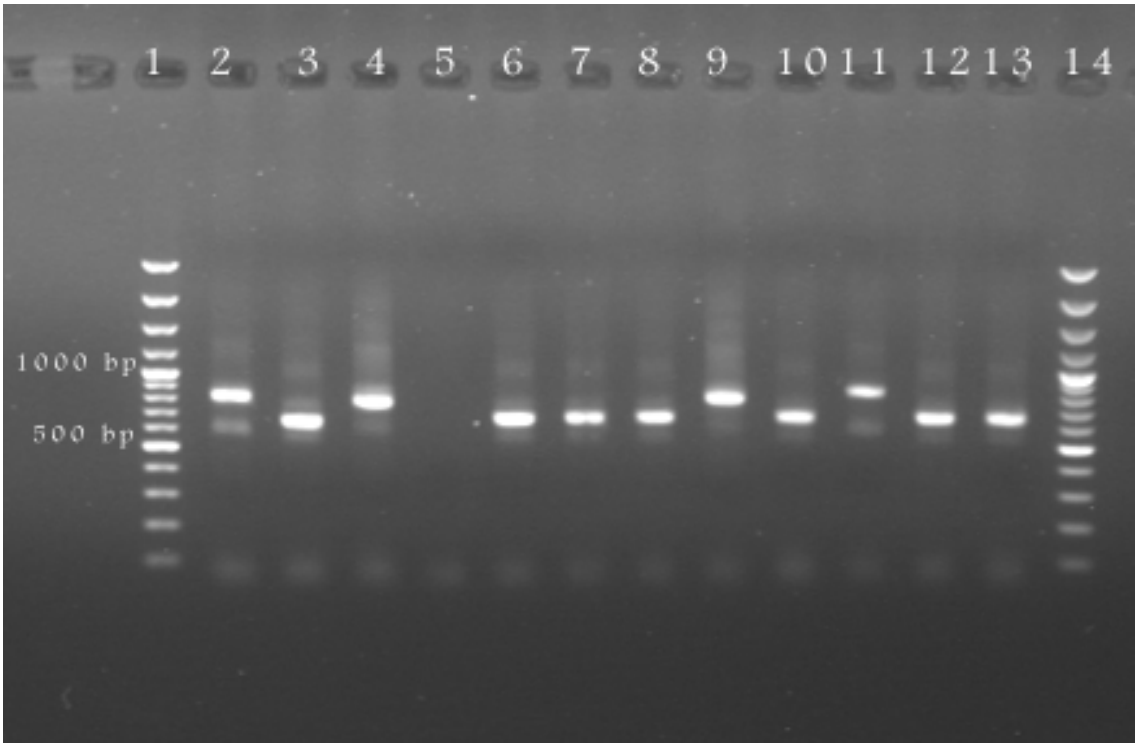

Fig. 1: The result of the PCR-based amplification of kinetoplast DNA extracted from skin lesions of the patients. The bands shown on 1.5% agarose gel stained with ethidium bromide correspond to molecular weight markers. Lanes 1 and 14: ladder markers; lanes 2, 3 and 4: positive controls of *L. tropica* (760bp), *L. major* (650bp) and *L.infantum* (720bp) respectively; lane 5: negative control; lanes 6, 7, 8, 10, 12 and 13: *L. major*; lane 9: *L.infantum* and lane 11: *L. tropica* of patients samples

Discussion

Several provinces in Iran are endemic foci for both forms of CL. Zoonotic Cutaneous Leishmaniasis (ZCL), due to *L. major*, is found in many rural foci of Isfahan, Khuzestan and Khorasan provinces, while Anthroponotic Cutaneous Leishmaniasis (ACL), due to *L. tropica*, is endemic in many large and small cities including Bam and Kerman in the southeast, and Mashed in northeastern Iran. Shiraz and other cities in Fars Province were reported as ACL (15, 16). Recently different studies showed the spread of ZCL in this province especially in Shiraz City. According to the result of this study, more parasites were identified as *L. major*. The reason was discussed elsewhere (6, 11). In this study, *L. infantum* was identified as causative agents of cutaneous lesion (dermatropic) in a patent. This species also was confirmed by specific primers for *L. donovani* as *L. infantum*. A few dermatropic *L. infantum* cases also have been reported previously from Iran (14).

Appropriate diagnosis and characterization of the particular parasite is important for evaluating prognosis and prescribing appropriate treatment (17). Until recently, diagnosis of CL was based primarily on clinical symptoms, microscopic observation of the parasites in stained tissue smears, and/or culture of promastigotes from tissue (17). Even today, microscopic identification and parasite cultivation are still primary diagnostic tools employed in many regions where leishmaniasis is endemic. Culture of promastigotes from the infected tissues and/or direct identification of amastigotes in microscope smears have long been considered as the standard for diagnosis. While these techniques are highly specific for diagnosing leishmaniasis, they are not sensitive (10). The use of PCRs has slowly become the preferred way for diagnosing leishmaniasis since conventional parasitological methods are not sufficiently sensitive (10).

At present, no single laboratory technique is accepted as the gold standard for diagnosing *Leishmania* infection (18). Diagnosis of cutaneous leishmaniasis by PCR seems to be approaching a 'gold standard' status as novel techniques offer considerable advantages in the collection and transport of specimens and DNA extraction procedures that are more efficient in individual and field-based protocols. Many researchers have reported consistent 100% specificity with increasing sensitivity which in overall between 92 and 98 % (17).

The PCR appears to be the most sensitive single diagnostic test for each form of leishmaniasis (10, 19). In the present study, PCR presented 93.61% sensitivity, significantly higher than that of direct microscopy (76.71%) and culture (50.9%) methods alone (P=0.001) for diagnosis of cutaneous leishmaniasis. However, microscopy and culture in combination improved the sensitivity totally to 84.93%. These are consistent with other reports in different endemic areas of the world including Iran (10, 19-22). It has been indicated that the PCR technique has a higher sensitivity as compared to other microscopical techniques (19, 23).

Culha et al. and Belli et al. have reported 100% sensitivity for PCR method (19, 26). Aviles et al. and safaei et al. both have reported that PCR was 92% sensitive (25, 9). The values reported by Bensussan et al. for diagnosis of CL by microscopy and parasite culture were 74.4% and 62.8%, respectively (10).

It is worth noting that in our study, PCR was positive in 43 out of 219 cases (19.63%) which were negative for direct microscopy

and also it was positive in 96 out of 219 specimens (43.83%) which were negative for culture and finally was positive in 27 out of 219 patients (12.33%) who were negative for direct and culture methods combined.

Our culture result is lower than usual and is not consistent with other researchers' findings. It may be affected by some technical problems such as the type of materials applied in the media and fungal or bacterial contaminations that sometimes occurred in this study.

Traditional methods (direct, culture) can be time consuming, are limited by access to specialized laboratories and microscopic expertise, and have a reported sensitivity of 50-70 % (20, 24). When the PCR results were compared with the combination of direct microscopy and culture methods, its sensitivity was higher than that of the two other techniques (93.61% vs. 84.93%). The results revealed that *L. major* species was dominant (95.61%) in the studied areas. When PCR was compared with the combined results from the two traditional tests, a significant difference in sensitivity was found. The direct microscopic examination and culture, when associated, are not sufficient to diagnose all the CL cases.

In conclusion, the PCR assay indicated a high sensitivity for diagnosis and identification of CL caused by three identified species of *Leishmania* parasites including *L. major*, *L. tropica* and dermotropic *L. infantum* in the study area. Identification of the parasite in addition to diagnosis of the infection is necessary. In these situations, using a sensitive molecular method such as PCR will be helpful.

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