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A comparative study of the diagnosis of Old World cutaneous leishmaniasis in Iraq by polymerase chain reaction and microbiologic and histopathologic methods

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Abstract

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Background The diagnosis of cutaneous leishmaniasis (CL) largely depends on the clinical appearance, especially in endemic areas. A diagnostic challenge arises when the lesions appear in nonendemic areas, when the clinical picture is distorted, or when an atypical variant is seen, even in endemic regions.

Aim To assess the correlation of microbiologic and histopathologic diagnosis with polymerase chain reaction (PCR) findings in clinically diagnosed cases of CL.

Methods This was an observational and descriptive study. The patients were seen at outpatient departments of dermatology in the middle Euphrates region of Iraq. Sixty-five patients with clinically suspicious CL lesions were screened. Fifty-seven clinically diagnosed cases were then subjected to slit-skin smear and skin biopsy. PCR examination was conducted in 40 cases.

Results Direct slit-skin smear examination was positive in 38 patients (66.7%), and 48 specimens (84.2%) were positive by the slide-touch skin biopsy method. Histopathologic examination showed features suggestive of CL in 34 specimens (59.6%). The results of PCR examination were positive in 37 cases (92.5%).

Conclusion The PCR technique is highly specific (100%) and sensitive (92.5%) for the diagnosis of CL. In addition to the confirmation of the diagnosis, it may be useful in identifying a relationship between the type of microorganism and the clinical presentation of the disease.

Introduction

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Cutaneous leishmaniasis (CL) is a growing public health problem in several parts of the world, including Iraq.¹ It is an anthroponotic disease caused by protozoans of the genus *Leishmania*, which infect the vertebrate host after a bite by infected female phlebotomus sand flies.^{2,3} There are many species of *Leishmania* that cause human disease, leading to a spectrum of clinical presentations dependent on several factors. Only a few studies have been performed in Iraq to evaluate the species responsible for this disease using culture characteristics.⁴ Most cases of Old World CL in Iraq, as in neighboring countries, are found to be caused by *Leishmania tropica* and *Leishmania major*.⁴

The diagnostic methods available at present are based on clinical and epidemiologic features, parasitologic detection (stained smears, culture, and histopathology), and immunologic methods.⁵⁻⁷ The histologic picture in CL varies according to the stage of infection and the clinical type. It is important to search for amastigotes, which are diagnostic.^{6,8,9}

All of these traditional laboratory methods have limitations, including low sensitivity, the requirement for culture facilities, and their time-consuming nature.^{5,10} In order to overcome such difficulties, several DNA-based molecular techniques have been developed.¹¹ DNA amplification through polymerase chain reaction (PCR) has several advantages compared with traditional techniques, such as the ability to detect infectious agents present at very low copy numbers and the ability to be performed with a broad range of clinical specimens.¹²⁻¹⁶ PCR utilizes amplification of circular DNA molecules of the kinetoplast, an organelle unique to kinetoplastids.¹¹ The abundance and other characteristics of these molecules have made them the target for a number of PCR-based techniques.

The aim of this study was to compare the routine laboratory methods used for the identification of the *Leishmania* parasite, histopathologic examination, and PCR for the diagnosis of Old World CL in Iraq.

Materials and Methods

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Sixty-five patients with lesions clinically suggestive of CL were evaluated in the outpatient dermatology clinic in a teaching hospital in Najaf, Iraq. The laboratory work was performed in the Department of Microbiology, College of Medicine, University of Kufa, Kufa, Iraq. The study was performed between November 2003 and February 2005.

The definition of a confirmed case of CL was based on the following: (i) the presence of typical lesions, a compatible epidemiologic history, and a clinical response to specific treatment; and (ii) positivity by microscopic smear examination, histopathologic examination, or both.¹¹ The clinical diagnosis was dependent on familiarity with the condition in daily practice in this endemic area. It was considered, in particular, in the presence of one or more discrete, relatively painless, skin lesions (nodules, plaques, ulcers, or noduloulcerative lesions), mostly on exposed parts of the body.⁴⁻⁷ There is a general rule in this part of the world that any boil that is present for several weeks and does not respond to ordinary therapy should be considered as CL unless proven otherwise.⁴

After a detailed clinical and epidemiologic history had been obtained, a slit-skin smear from the margin of the lesion was taken for direct microbiologic examination using a #15 sterile knife in a bloodless field. The specimen was fixed with methanol and stained with Giemsa stain to look for Leishman–Donovan (LD) bodies. A skin biopsy from the peripheral part of the skin lesion was obtained and each specimen was divided into three samples. The first sample was used for direct smear examination by slide-touch preparation. This was performed by touching the biopsy with a glass microscope slide, followed by staining with Giemsa. Subsequently, 50 high-power fields (magnification, $\times 100$) were searched for *Leishmania* organisms with a light microscope.¹⁷ The second skin biopsy sample was stored in 10% formalin at 4 °C and then processed and stained with hematoxylin and eosin and Giemsa stains for histopathologic examination. The third sample was stored in absolute ethanol at –20 °C until use for PCR.

PCR was performed in only 40 patients because of the difficult circumstances in Iraq. The standard PCR technique was followed:

1 DNA extraction from the saved part of the incisional biopsy and DNA purification.^{18,19}

- 2 PCR amplification using the following pair of primers: 5'-TCGAGAACGCCCCTACC-3' and 5'-AGGGGTTGGTGAATAGG-3'.20-22

- 3 Analysis of amplification products: PCR products were analyzed by 2% agarose gel electrophoresis with 8 mL of the reaction mixture, and the bands on an ethidium bromide-stained gel were visualized and photographed under UV light with a polaroid MP4+ system.^{23,24}

Several negative controls (no DNA added) and positive controls (DNA added) were included for each PCR to detect false-positive results caused by contamination or variation in sensitivity. The number of cases of Old World CL identified by the reference standard provided the denominator of the sensitivity calculation. A case of CL considered to be test positive for the test being compared.

Statistical methods

McNemar's test was used to obtain paired chi-squared and two-tailed P values to assess the level of significance of the difference of the other tests in relation to PCR. The odds ratio with a 95% confidence interval was also assessed.

Results

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The study included 57 patients with lesions consistent with a diagnosis of Old World CL. There were 26 males (45.6%) and 31 females (54.4%). Their ages ranged from 6 to 53 years (28.3 years). The duration of illness varied from 4 to 18 weeks (6.5 weeks). The total number of lesions in all patients was 137, including both ulcerative (79, 57.7%) and nodular or dry (58, 42.3%) lesions.

Amastigotes were detected by direct examination in slit-skin smear in 38 patients (66.7%); this compared with 48 positive specimens (84.2%) by the slide-touch skin biopsy method. The morphology of amastigotes varied from rounded forms to spindle-shaped or umbrella-like structures. On histopathologic examination, features consistent with the diagnosis of CL were found in 34 specimens (59.6%), and negative results were present in 23 specimens (40.4%). LD bodies were detected in 10 positive biopsies (29.4%), mostly in those with early ulcerative lesions.

PCR examination showed positive results in 37 cases (92.5%) and negative results in three cases (7.5%). Of the 37 positive cases, 21 (56.7%) involved *L. major* and 16 (43.3%) involved *L. tropica* (Figs 1–3). The positive results of the conventional methods in these patients were as follows: 26 patients (65%) by slit-skin smear, 32 patients (80%) by slide-touch method, and 25 specimens (62.5%) by histopathologic examination. The results of statistical analysis using McNemar's test to obtain paired chi-squared and two-tailed P values, using PCR as the control, were as follows. In slit-skin smear, the two-tailed P value was 0.0055, and this difference was considered to be very statistically significant. The odds ratio was 12.000, with a 95% confidence interval extending from 1.775 to 512.972. Statistical comparison with the slide-touch method showed a two-tailed P value of 0.1306, which was not considered to be statistically significant. The odds ratio was 6.000, with a 95% confidence interval extending from 0.728 to 275.986. For histopathologic examination, the difference was also statistically very significant with a P value of 0.0033. The odds ratio was 13.000, with a 95% confidence interval extending from 1.953 to 552.471. The microbiologic and PCR results were evaluated by the same authors. The histopathologic findings were assessed blind with regard to the results of the other tests.

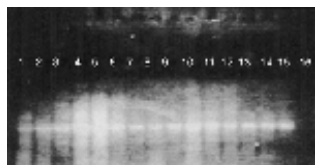


Figure 1. Representative agarose gel of polymerase chain reaction (PCR) products obtained from biopsy samples with primers specific for *Leishmania major*. Lanes 1–10, biopsy samples from patients with cutaneous leishmaniasis. Lanes 11–15, positive controls. Lane 16, negative control

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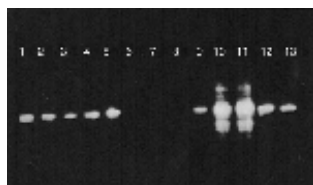


Figure 2. Representative agarose gel of polymerase chain reaction (PCR) products obtained from biopsy samples with primers specific for *Leishmania tropica*. Lanes 1–5 and 9–11, biopsy samples from patients with cutaneous leishmaniasis. Lanes 6–8, negative controls. Lanes 12 and 13, positive controls

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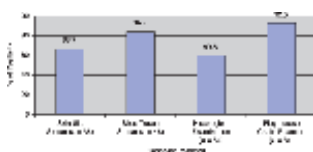


Figure 3. Comparison of the results of different techniques used to detect *Leishmania* parasites. They were detected in 66.7% of cases in direct smear examination, in 84.2% of cases by the slide-touch technique, in 59.6% of cases by histopathologic examination, and in 92.5% of cases by polymerase chain reaction

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Discussion

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CL is a known endemic disease in Iraq with the local name of “Baghdad boil.”⁴ The diagnosis of CL can be based on the clinical presentation of patients in geographic regions in which the infection typically occurs.^{1,2} It may be misdiagnosed as other common causes of slowly growing lesions and ulcers, such as tuberculosis, syphilis, and leprosy. Parasitologic confirmation of the diagnosis is critical because of the high cost and significant toxicity of current treatment regimens for leishmaniasis.²² It is also important to identify the species of *Leishmania* for both clinical and epidemiologic reasons.

In this study, we have attempted to use PCR to confirm the clinical diagnosis of this disease, and have compared it with the most commonly used conventional methods, such as direct smear, slide-touch biopsy method, and histopathologic diagnosis.

These traditional diagnostic methods are easily employed, but have limitations of low sensitivity, the requirement for a large sample of tissue, the need for specially trained personnel to perform all three methods, and their time-consuming nature.⁵ When an attempt was made to detect parasites using these methods, comparable results were obtained with those described in other reports.^{4,6,11,18,25} Parasites were detected in 66.7% of patients by direct slit-

skin smear examination and in 84.2% of patients by the slide-touch skin biopsy method. The result for the slide-touch skin biopsy method was higher than that reported previously,^{11,17,21,26} except for the study by Andersen *et al.*²⁷ This may be attributed to the deeper structures reached by biopsy and the larger surface area examined by slide-touch.

Histopathologic findings may be suggestive and occasionally diagnostic when LD bodies are present. This study revealed that 59.6% of patients showed positive findings on histopathologic examination. The low number of positive results by this test may be attributed to secondary infection of ulcerative lesions by bacteria, such that the histologic changes may be nonspecific. In addition, biopsy specimens from old (more than 6 months) and partially treated lesions, or those with low infection burden, are frequently nondiagnostic histopathologically. Great experience is also required because of the scarcity of the parasites and their small size. In spite of all of these limitations, histopathology allows other diseases that show clinical features similar to those of leishmaniasis to be identified.^{6,8,9}

PCR is increasingly being used to improve the diagnostic sensitivity for CL by detecting nucleic acids unique to the parasite. Many studies have used PCR examination to diagnose CL in the Old and New World.^{10–16,18–24,26,27} These studies have shown conflicting results from different groups. In some reports, the technique has not been found to be very sensitive (63–80%) or specific (60%) with biopsy specimens from patients with CL.^{10,12–18} In other studies, however, this test has shown detection rates of 97% with samples from patients from the New World.^{11,19} In this study, an attempt has been made to show the pattern of test results in Iraq for the first time. In spite of the relatively small number of cases examined in this work, it has been shown that PCR is a good method for the diagnosis of CL in Iraq. In this study, PCR showed detection rates of 92.5%. Possible explanations for the discrepancies in various studies include differences in the quality of the DNA samples and in the DNA extraction protocols used for PCR (the samples may contain different amounts of DNA polymerase inhibitors).

The sensitivity of PCR (92.5%) in our work was significantly higher than that of direct smear (66.7%) and histopathologic examination (59.6%). This is in line with the results of other studies, showing that PCR is consistently more sensitive than conventional methods.^{13,16,21} Although PCR examination was comparable with the slide-touch skin biopsy method in this study, PCR can be used for both the diagnosis of leishmaniasis and the identification of *Leishmania* species. This aids the investigation of the relationships between causative agents and the clinical manifestations and epidemiology of the disease. In addition, PCR has the potential to provide specific results in less than 1 day. The possibility of PCR automation, the simplification of sample collection and processing (for example, using exudative materials collected by cotton swab),^{22–24} and the in-house preparation of reagents may make this technique economically attractive for the processing of large numbers of samples in endemic regions.^{22–24}

With regard to species identification by PCR, the results were not surprising and were consistent with the few studies performed in Iraq using culture and isoenzyme identification of *Leishmania* species.⁴ The results were also consistent with the findings of other studies of Old World CL, where most cases were caused by *L. major* or *L. tropica*.^{3,4,14}

In conclusion, this study shows that PCR is a useful test for the diagnosis of CL in Iraq. In spite of the small amount of experience with this test in Iraq, this study provides interesting results on the features of the disease in this area, and opens up the possibility of performing PCR using different sampling techniques.

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