A Comparative Study Of Diagnosis By Polymerase Chain Reaction (PCR), Microbiological, And Histopathological Methods Of Old World Cutaneous Leishmaniasis In Iraq.

Sundus Nsaif AL- Hucheimi* MSc
Baqur A. Sultan* PhD
Muhsin A. Al-Dhalimi** MD

*Department of Microbiology, College of Medicine, University of Kufa-Iraq.
**Department of Dermatology, College of Medicine, University of Kufa-Iraq.

Abstract:
Background: The diagnosis of cutaneous leishmaniasis (CL) is largely depends on its clinical appearance especially in endemic areas. Diagnostic challenge arises when the lesions appear in nonendemic area, when clinical picture is distorted, or any atypical variant is seen even in endemic regions.
Aim: The study was designed to assess the correlation of microbiological and histopathological diagnosis with the Polymerase chain reaction (PCR) findings in clinically diagnosed cases of old world CL.
Methods: It was an observational and descriptive study. The patients were collected from the outpatient departments of dermatology in the middle Euphrates region of Iraq, Al-Najaf. Sixty-five patients with clinically suspicious lesions of CL 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: The direct smear examination was positive in slit skin smear in 38 patients (66.7%) compared with 48 specimens (84.2%) of slide-touch method of skin biopsy samples. The histopathological examination showed features suggestive of cutaneous leishmaniasis in 34 specimens (59.6%). The results of polymerase chain reaction examination were positive in 37 cases (92.5%).
Conclusion: PCR technique is a highly specific and sensitive test in the diagnosis of CL. In addition to confirmation of the diagnosis, it may be useful to find a relationship between the type of microorganism and the clinical presentation of the disease.
Introduction:
Cutaneous leishmaniasis (CL) is a growing public health problem in several parts of the world including Iraq (1). It is an anthropozoonotic disease caused by protozoan of the genus Leishmania, which infect the vertebrate host after a bite by infected female of phlebotomus sand flies.(2,3) There are many species of leishmania causing human disease leading to a spectrum of clinical presentations depending on many factors. There are only few studies done in Iraq to evaluate the species responsible for this disease by culture characteristics.(4) Most of the cases of old world CL in Iraq, like other neighboring countries found to be caused by Leishmania tropica and Leishmania major.(4)

The diagnosis of leishmaniasis has traditionally been made by direct identification of leishmania amastigotes in tissue samples or smears.(5-7) The histological picture in CL differs according to the stage of infection and the clinical type.(6,8,9) Different immunological tests also used for the diagnosis of CL, but they are not useful for leishmania species discrimination. All these traditional laboratory methods have several
limitations due to one reason or another; prominent among them are low sensitivity, they require culture facilities and they are time-consuming.\(^{(5,10)}\)

In order to overcome such difficulties, several DNA-based molecular techniques have been developed.\(^{(11)}\) The polymerase chain reaction (PCR) was the most prominent among them. It 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. PCR has several advantages compared to 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.\(^{(12-16)}\)

The aim of this study is to compare between routine laboratory methods that used for identification of leishmania parasite, histopathological examination with polymerase chain reaction (PCR) for diagnosis of old world CL in Iraq.

Materials and Methods:

A total of 65 patients with lesions clinically suggestive of CL were evaluated in the outpatient dermatology clinic in Alsadr teaching hospital in Najaf, Iraq. The laboratory work was done in the department of microbiology, college of medicine, university of Kufa. The study was done between November, 2003 and February, 2005.

The definition of a confirmed case of CL was based upon (a) the presence of typical lesions, a compatible epidemiological history, and a clinical response to specific treatment and (b) positivity by at least one of the following tests: microscopic smear, biopsy- slide touch and histopathological examination.\(^{(11)}\) The clinical diagnosis was depending on familiarity with the diagnosis of the condition in the daily practice as an endemic area. It is considered especially in 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 country: any boil that stays for few weeks and does not respond to ordinary therapy should be considered to be CL unless proved otherwise.\(^{(4)}\)

After a detailed clinical and epidemiological history, a slit-skin smear from the margins of the lesions for direct microbiological examination was taken, fixed with methanol and stained with Giemsa stain looking for Lieshman Donovan (LD) bodies. A skin biopsy was done from the peripheral part of skin lesions and each specimen was divided into three samples. The first sample was used for direct smear examination by slide- touch preparation.\(^{(17)}\) The second part of skin biopsy was stored in 10% formalin at 4°C and then processed and stained with Haematoxylin-Eosin and Giemsa stain for histopathological examination. The third sample was stored in an absolute ethanol at - 20°C until used for PCR.

PCR test was done in 40 patients only due difficult circumstances in Iraq. It is done by standard PCR technique including the following steps:
1. DNA extraction from the saved part of incisional biopsy and DNA purification.\(^{(18,19)}\)
2. PCR amplification using a pair of primers of 5' TCGAGAACGCCCCTACC 3' and 5'AGGGGTTGTTGAAATTAGG 3' DNA.\(^{(20-22)}\)
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 ethidium bromide – stained gel were visualized and photographed over UV Light with the Polaroid MP4+system.(23-24)

Several negative controls (no DNA was added) and positive controls (DNA was added) were included each time that PCR was undertaken to detect false –positive results due to contamination or variations in sensitivity. The number of cases of old world leishmaniasis identified by the reference standard provides the denominators of the sensitivity calculation. A case of CL considered to be test positive for the test being compared. 

Statistical Methods: Wilcoxonrank sum test and chi-square were applied to find out the significant difference between the data by (SPSS v.10) under (Windows).

Results:
The number of patients who fulfill the required criteria and regarded as a confirmed case of CL was 57 patients. 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 most common site of the lesions was the arms (46%), followed by the legs (34.4%), and face (19.6%). Many patients have multiple lesions and the number varied between one and 9 with a mean of 2.13. The total number of the lesions in all patients was 137 of both ulcerative (79, 57.7%) and nodular or dry (58, 42.3%).

The amastigotes can be detected by direct examination in slit skin smear in 38 patients (66.7%) compared with 48 specimens (84.2%) of slide-touch method of skin biopsy samples. The morphology of amastigotes varied from rounded forms to spindle shaped or umbrella like structures. On histopathological examination, features goes with the diagnosis of CL were found in 34 specimens (59.6%), while negative results were present in 23 specimens (40.4%). LD bodies were detected in 19 (55.8%) biopsies of the positive cases, mostly in those with early ulcerative lesions.

The PCR examination showed positive results in 37 cases (92.5%) and negative results in 3 cases (7.5%). Out of these 37 cases, 21 (56.7%) were Leishmania major (Figure1) and 16 (43.3%) were Leishmania tropica (Figure 2). The positive results of the conventional methods in these patients were as the following: 26 patients (65%) in slit skin smear, 32 patients (80%) slide-touch method and 25 specimens (62.5%) on histopathological examination. The sensitivities of the PCR based methods were significantly higher than those of direct smear examination (P<0.05), while the difference was statistically insignificant when compared with the slide-touch method of skin biopsy (P > 0.05) (Figure 3). Statistical relationship between the results of PCR and histopathological examination showed a high significant difference. The value of chi square for PCR was more than the value of chi-square for histopathology examination. This means that the PCR was much better (Table 1).
Figure (1): Representative agarose gel of PCR products obtained from biopsy samples with primers specific for *leishmania major*. Lanes 1 to 10 (from left to right): biopsy samples from patients with CL; lane 11 to 15: positive control; lane 16: negative control.

Figure (2): Representative agarose gel of PCR products obtained from biopsy samples with primers specific for *leishmania tropica*. Lanes 1 to 5 and 9 to 11 (from left to right): biopsy samples from patients with CL; lane 6 to 8: negative control; lane 12 and 13: positive control.
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, 84.2% by slide- touch technique, 59.6% on histopathological examination, and 92.5% by PCR.

Table (1): The Correlation between the results of PCR and histopathological examination in CL.

<table>
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<tr>
<th>Tests</th>
<th>Result of test</th>
<th>Total</th>
<th>Calculative value of $x^2$</th>
<th>Tabling value of $x^2$ (1.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR examination</td>
<td>37 (92.5%)</td>
<td>3 (7.5%)</td>
<td>40 (100%)</td>
<td>3.8095</td>
</tr>
<tr>
<td>Histopathological</td>
<td>34 (59.6%)</td>
<td>23 (40.4%)</td>
<td>57 (100%)</td>
<td>2.0699</td>
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<td></td>
<td></td>
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<tr>
<td>Total</td>
<td>70</td>
<td>27</td>
<td>97</td>
<td>5.8788</td>
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</table>

Discussion:

Cutaneous leishmaniasis is a known endemic disease in Iraq with a local name of Baghdad Boil. The diagnosis of CL can be based on the clinical presentation of patients in geographical regions where the infection typically occurs. It may be misdiagnosed with other common causes of slowly growing lesions and ulcers like tuberculosis, syphilis, and leprosy. Parasitologic confirmation of 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.

We try in this study to use PCR technique to confirm the clinical diagnosis of this disease and compare it with the most commonly used conventional methods like direct smear, biopsy slide-touch method and histopathological diagnosis.

These traditional diagnostic methods are easily employed but with the limitations of low sensitivity the requirement for a large sample of tissue, the need for specially trained personnel to perform all the three methods for each patient and are time consuming\(^{(5)}\). When we attempted to detect parasites by these methods, we obtained results comparable to those described in other reports\(^{(4,6,11,18,25)}\). This study showed that parasites were detected in 66.7\% from direct slit smear examination and in (84.2\%) of slide-touch method of skin biopsy samples. The result of slide-touch method of skin biopsy samples is relatively higher than those reported previously\(^{(11,17,21,26)}\), except Andersen study that showed a similar observation.\(^{(27)}\) This high figure may be attributed to deeper structures reached by biopsy and the larger surface area searched by touch with the slide.

Histopathological findings may be suggestive and occasionally diagnostic when the LD bodies are present. This study revealed that 59.6\% of patient showed positive findings in histopathological examination. The relatively low positive results by this test may be attributed to secondary infection of ulcerative lesions by bacteria, in which case, histological changes may be nonspecific. In addition, biopsy specimens from old, more than six months, partially treated lesion, or low –burdens infections frequently are non diagnostic histopathologically. High experience also required because of the scarcity of the parasites and their small size. In spite of all the above limitations, histopathology allows one to identify other diseases that show clinical features similar to those of leishmaniasis\(^{(6,8,9)}\).

PCR is one of the increasingly used methods to improve the diagnostic sensitivity of CL by detecting nucleic acids unique to the parasite. There are many works done to use PCR examination for the diagnosis of CL in old and new worlds.\(^{(10-16,18-24,26,27)}\) These studies showed conflicting results from different groups. The technique was found to be not very sensitive (63 to 80\%) or specific (60\%) with biopsy specimens from patients with CL.\(^{(10,12-18)}\) While, in other studies, this test showed detection rates of 97\% with samples from patients in the New World.\(^{(11,19)}\) We try in this study to show the pattern of the results of this test in Iraq for the first time. In spite of the relatively low number of cases examined in our work, it showed that PCR well diagnoses CL in Iraq. In this study, PCR showed detection rates of 92.5\%. A possible explanation for the discrepancies of various studies is a difference in the quality of the DNA samples and DNA extraction protocol used for PCR (the samples may contain different amounts of DNA polymerase inhibitors).

The sensitivity of PCR in our work was significantly higher than those of the direct smear and histopathological examination. 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 slide-touch method of skin biopsy in this study, it is obvious that PCR has the attractive value of not only making the diagnosis of leishmaniasis, but also specifying the Leishmania species. This help in investigation of
relationships between causative agents and the clinical manifestations and epidemiology of the disease and. In addition, it has the potential to provide specific results on smaller sample in less than 1 day. The possibility of PCR automation, the simplification of sample collection and processing like using exudative materials collection by cotton swab,(22-24), as well as the in-house preparation of reagents can make this technique economically attractive for the processing of large numbers of samples in regions of endemcity.(22-24)

Regarding the species identification by PCR, the results were not surprising and were consistent with the few studies done in Iraq using culture and isoenzyme identification of Leishmania species.(4) The results were also goes with the findings of other studies dealing with old world CL where most of the cases were caused by Leishmania major or Leishmania tropica.(3,4,14)

In conclusion, this study shows that PCR is a useful test for diagnosing cutaneous leishmaniasis in Iraq. In spite of low experience with this test in this country, this first time study gives us promising results about the features of this disease in our country and opens the way to do PCR using different sampling techniques.

References:

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