

THE COMPARISON OF HISTOPATHOLOGICAL FINDINGS AND POLYMERASE CHAIN REACTION IN LESIONS WITH PRIMARY CLINICAL DIAGNOSIS OF CUTANEOUS LEISHMANIASIS WITH NEGATIVE SMEAR

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ABSTRACT

Objective: Cutaneous leishmaniasis (CL) is a common skin disease in Middle East region which involves all ranges of ages and can affects both sexes. With regard to this fact that on time diagnosis and treatment would lead to reduction in its permanent complications and because it resembles other skin diseases, various techniques have been introduced to diagnose its agent in affected tissue.

Methodology: The reliability of pathological examination and PCR technique for diagnosis of CL was investigated in present study. In total 30 patients with clinically CL lesions having negative direct smear, were investigated. Tissue biopsies were taken from the patients, histopathologic sections were prepared and the sections were examined to confirm or rule out the disease. DNA was extracted from biopsy samples by using standard DNA extraction kit and PCR assay was performed according to standard protocol using *Leishmania tropica* species-specific primers.

Results: Among 30 biopsies from patients with clinical diagnosis of CL under investigation, five were positive on PCR (16.7%) and 4 were positive on histopathologic examination (13.3%), There was not any meaningful statistical difference between PCR assay and pathological examination ($P= 0.87$) for diagnosis of CL cases with a negative smear.

Conclusion: The present study indicated that PCR is a reliable technique for detection of some cases of suspected patients to CL with negative direct smear, but did not find any priority of PCR compared to histopathology in respect to similarity and economics.

KEYWORDS: Leishmania, Cutaneous leishmaniasis, PCR, Smear, Histopathology.

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INTRODUCTION

Leishmaniasis represents a major public health problem and is endemic in more than 88 countries and threatens more than 350 million people.^{1,2} At least 21 species and subspecies of *Leishmania* have been recorded as being infective to humans, many of which cause extensive morbidity and are responsible for a wide spectrum of clinical symptoms.³ Cutaneous leishmaniasis (CL) is a common skin disease in middle east region which involves all ranges of ages and can affects both sexes.⁴ Correct and early diagnosis of the parasite is important for appropriate treatment, which could leads to reduce its permanent complications.⁵ Since CL is similar to other skin diseases,

various techniques have been introduced to diagnose its agent in affected tissue.

Cutaneous leishmaniasis (CL) caused by *L. tropica*, is an important health problem in some regions of Iran including Khuzestan province in the Southwestern of Iran. A definitive diagnosis of CL is based primarily on clinical symptoms and is routinely made by the demonstration of parasites microscopically in tissue smears or sections.⁶ Another conventional method is parasite cultivation from lesions samples, which together with microscopic examination are still primary diagnostic tools in many regions where leishmaniasis is endemic, despite that the cultivation requires long period of incubation.^{7,8} Current diagnostic techniques based on parasite detection (stained smears, culture) and immunological methods (direct agglutination test, Enzyme-linked immunosorbent assay [ELISA], etc.) have several limitations, including low sensitivity and specificity.⁹ In recent years, PCR-based diagnostic methods have been described for leishmaniasis, with a wide range of sensitivity and specificity. These techniques have been developed and optimized over time.^{10,11} An excellent target for a sensitive and rapid detection method is the kinetoplast mini-circle DNA, which is present at thousand copies per cell. The mini-circles have been used as targets for selective amplification of parasite DNA in various studies.^{12,13} In the present study the reliability of pathological examination and PCR technique for detection of leishman body (LB) in patients with clinical diagnosis of CL and a negative direct smear was investigated.

METHODOLOGY

Sampling: Samples were taken from 30 patients with clinical CL (diagnosed by 2 dermatologists) referred to Outpatient Clinic of Dermatology, Imam Khomeini Hospital, Jondishapour University of Medical Sciences, Ahwaz, Iran at the pretreatment stage. They had negative direct smear for LB. Tissue for pathological examination and PCR was taken using a disposable scalpel blade or punch. Direct smear was prepared by aspiration of

fluid from beneath the ulcer bed. After smear had dried completely, it was fixed with 100% methanol, allowed to dry again, and stained with Wright's Giemsa for microscopic examination. Informed consent was taken from the patients in all cases and permission was obtained from human ethics committee at the university and the relevant authorities prior to study.

DNA extraction and PCR analysis: The second part of the skin biopsy was subjected to DNA extraction. Tissue sections were incubated overnight at 37°C in lysis buffer containing proteinase K. DNA was extracted by using standard extraction kit (Cinnagen Co., Tehran, Iran) according to manufacturer's instructions. As a negative extraction control, negative sample was processed in exactly the same manner as the positive controls (parasite culture positive and pathological positive section) and test samples.

Amplification was performed using assay based on a 116-bp fragment in the constant region of the *Leishmania* spp. kinetoplast DNA. The two oligonucleotide primers used were 13a (5'- GTG GGG GAG GGG CGT TCT-3' and 13b (5'- ATT TTC CAC CAA CCC CCA GTT-3') according to that described by Rodgers et al.¹³ and the amplification was carried out according to instructions of PCR kit provider (Cinnagen Co., Tehran, Iran). In brief, the reaction mixture (25ul) was composed of 50mmol KCl, 10mmol Tris-HCl (pH 8.3), 1.5mmol MgCl₂, 0.2mmol of each deoxynucleotide triphosphate, 0.5μmol of each primer, 1.25 units of Taq polymerase and 5ul of processed sample. Amplification was performed in a thermal cycler (Techgene, UK) using the program described by Reale et al,¹⁴ as below: 30 cycles of denaturation at 94°C for one minute, annealing at 60°C for one minute, extension at 70°C for one minute and final extension at 72°C for 7 minutes. For analysis of the amplified products, they were loaded on a 2% agarose gel containing 0.5mg/ml ethidium bromide in electrophoresis and the products were visualized by UV transillumination.

The result of both techniques were then analysed and compared using T-test and SPSS 11.5 for windows software.

RESULTS

The objective of this study was to assess the ability of PCR to detect leishmanial parasites in clinical biopsies and to compare PCR with histopathology staining. The samples were obtained from patients that were clinically diagnosed as CL. The patients consisted of 18 males (60%) and 12 females (40%), age ranging from 2-65 years with the mean of 29.5.

The samples were taken from different sites of suspected lesions. The majority of samples were from forearm lesions twelve (40%) the other main sites of lesion location were face and wrist five each (16.7%). Table-I represents the rate of positivity of PCR and pathology examination in correlation with lesion sites. We could not find any significant correlation between the site of lesions to positivity of PCR or histopathology staining.

From 30 samples with a negative direct smear tested by both histopathology examination and PCR technique, 4 (13.3%) were positive by pathology and five (16.7%) were positive by PCR. There was no statistically meaningful difference between PCR technique and pathological test (P= 0.87) in detecting leishmania from negative smears.

DISCUSSION

PCR is at present the most widely used molecular technique for the study of clinical and epidemiological aspects of infectious diseases, due to its high sensitivity. The use of PCR method has slowly become the preferred way for diagnosis leishmaniasis since conventional parasitological methods are not sufficiently sensitive.³

The PCR method appears to be very sensitive for diagnosis of CL and several workers have reported the high sensitivity of the technique in detecting leishmaniasis in lesions compared microscopy and culture.¹⁻⁴ Similar studies were undertaken by Rodriguez et al. who reported 98% positivity of PCR among patients

Table-I: The value of PCR and histopathology examination in relation to lesion site

<i>Lesion site</i>	<i>No.(%)</i>	<i>PCR assay</i>	<i>Histopathology examination</i>
Forearm	12(40%)	1	1
Face	5(16.7%)	3	2
Wrist	5(16.7%)	-	-
Leg	3(10%)	-	-
Elbow	2(6.7%)	-	-
Thigh	1(3.3%)	-	-
Nose	1(3.3%)	1	1
Forehead	1(3.3%)	-	-

clinically diagnosed as having CL, compared to 64% positive pathology staining¹⁵ and Weigle et al. which in their study the PCR technique was evaluated for diagnosis of acute and chronic CL and as they concluded in both cases, PCR showed priority to conventional methods including pathology staining.¹⁶ Anderson et al. compared PCR and conventional microscopic diagnosis technique using frozen sections from skin lesions of 28 CL cases. They described a positive rate of 86% for PCR alone and 93% for a combination of PCR and southern Blot analysis.¹⁷ Despite that most of the PCR assays were evaluated and the sensitivity of the method in suspected lesions smear compared to conventional methods, however there are a few reports on the application of PCR for direct smear negative cases. Safaei et al., used PCR for both cases of positive and negative direct smears and they reported 92% sensitivity for PCR in detecting negative smears (24 out of their 29 negative samples were positive by PCR).¹⁸

Bensoussan et al. used three different PCR assays for diagnosis of CL and as they concluded, the kinetoplast DNA PCR showed the highest sensitivity,³ so we decided to apply this PCR assay to the samples in present study. Our results showed that there was no significant difference between PCR technique which detected 5 out of 30 negative samples and histopathology examination with detection rate of 4 out of total samples (P= 0.87). We still do need to examine more negative samples by using PCR method to make a clearer

conclusion on the value of PCR in detecting cases with clinically diagnosed CL with a negative direct smear or culture. However based on present findings, we believe that still detection of granulomatous infiltration is highly a reliable histopathologic findings in suspected cases, and PCR alone, is not sensitive enough to make correct diagnosis of CL cases with a negative smear.

In conclusion, although PCR is rapid technique without any complication for diagnosis of suspected cases of CL, but its ability to discover leishmania parasite in the lesions with primary clinical diagnosis of CL and negative direct smear is limited. Our study showed despite the fact that PCR had ability to detect some cases of the patients suspected to CL with a negative direct smear, but it may not be more effective than pathological examination because granulomatous infiltration is important for diagnosis.

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