

Detection of Leishmania DNA in paraffin embedded specimens of chronic lupoid leishmaniasis using polymerase chain reaction

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Background: Chronic lupoid leishmaniasis (CLL) is a chronic form of cutaneous leishmaniasis that is usually resistant to anti leishmania agents and leishmania is not seen in the histologic sections. We used polymerase chain reaction (PCR) to detect leishmania DNA in CLL specimens.

Method: This descriptive cross sectional study was done on 20 paraffin embedded specimens of CLL cases referred to the dermatology clinic of Imam Reza Hospital, Mashhad, Iran. Patients' information including age, sex, duration and location of the lesion was obtained and then skin specimens were reviewed histopathologically and assessed for leishmania DNA using PCR.

Result: Eleven male and 9 female patients with a mean age of 17.95 years were included in our study. The mean duration of lesions was 3.69 years. Most of the lesions (18 of 20 cases) were located on the face and others were on the limbs. Histologic study showed tuberculoid granulomatous inflammation with a variable ratio of histiocytes and lymphocytes without detection of leishman body. PCR was positive in 12 cases (60% of cases). We could not find any correlation between the results of PCR and sex, age, duration and location of the lesions. Histiocyte- prominent lesions were more prone to be positive on PCR examination but that was not statistically significant.

Conclusion: Despite the very high sensitivity of PCR for the diagnosis of usual forms of cutaneous leishmaniasis, it fails to detect leishmania in a remarkable proportion of CLL specimens.

Key words: cutaneous leishmaniasis, chronic lupoid leishmaniasis, polymerase chain reaction

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INTRODUCTION

The annual incidence of cutaneous leishmaniasis (CL) is about 1.8 million cases with 350 million people at risk of infection. About 90% of these cases are in 7 countries including Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. Lupoid Leishmaniasis is the chronic form of Old World Cutaneous Leishmaniasis (OWCL) that follows

4-10% of *L.tropica* infections^{1,2}. On histological examination, leishman bodies are not seen and the lesions are usually resistant to antileishmania treatments in the clinical practice. Practitioners in our region sometimes use intralesional steroids with antileishmania drugs to subside lesions. This practical value tends to remind that the leishmania parasite may not be the key element in lesion persistence, as it is rare to find leishmania by

routine parasitological methods like direct smear or cultures.

In this study, paraffin embedded specimens of chronic lupoid leishmaniasis (CLL) were examined by polymerase chain reaction (PCR) for the detection of *Leishmania* DNA.

PATIENTS AND METHODS

Twenty paraffin embedded skin biopsies of CLL cases obtained from the archives of the dermatology department of Imam Reza Hospital, Mashhad, Iran, were enrolled to study. Inclusion criteria were as follows:

- 1) Clinically typical features of chronic or lupoid leishmaniasis
- 2) Positive results of the direct smear before lesions became chronic
- 3) Disease duration more than 1 year

Paraffin embedded specimens were sent for laboratory investigation. Hematoxylin and eosin (H&E) stained sections were studied for histopathologic features and unstained sections of each specimen were processed for PCR according to the following procedure:

- 1) Deparaffinization and DNA extraction: First, 5- μ m sections from paraffin-embedded blocks were cut and after octane deparaffinization, the solution was centrifuged and then incubated in 100 μ l of proteinase K and detergent in 37°C for 24 hours until digestion was complete. Then, proteinase was deactivated by heating for 10 minutes in boiling temperature, and the solution was taken by a sampler after centrifugation at 8000rpm for one minute.
- 2) Amplification of DNA was performed by a leishmania genus specific primer. The primer was obtained from Pasteur Institute, Tehran, Iran. These genus-specific primers include 13 kA and 13kB (forward and reverses) that amplify the 120-bp band and are able to detect 1 pg of promastigote genomic DNA, they also work well on archived specimens^{3,4}. We used Genet Bio PCR kit for DNA amplification.
- 3) Gel electrophoresis of 5 μ l of PCR product on the 2.5% agarose gel.

We found 62°C as the best annealing temperature and to avoid the amplification of nonspecific sequences, we used touchdown style PCR by

decreasing annealing temperature from 68°C to 62°C.

Slides of H&E stained sections were studied and histological features were evaluated according to their inflammatory reaction patterns in the dermis and the presence or absence of leishmania parasites.

RESULTS

Twenty cases of CLL (11 male and 9 female) with a mean age of 17.95 years were studied. They had the lesions for a mean duration of 3.69 years. Eighteen lesions (90%) were located on the face and two lesions were on sun-exposed areas of the upper limbs.

Histological examination showed granulomatous inflammation including epithelioid histiocytes, giant cells (Langerhans or foreign body type), lymphocytes and plasma cells often surrounding histiocytic aggregations. Distribution of inflammation in the dermis was variable. In some cases, it had a band like pattern in the upper dermis while in others, it was diffuse through the deep dermis. The spectrum of the granulomatous reaction was from histiocyte predominant infiltrates with well formed histiocytic aggregations surrounded by lymphocytes (but the histiocyte were predominant infiltrates) and a lot of giant cells in the granuloma, to a predominantly lymphocytic infiltrate with less apparent foci of histiocytes.

PCR for the leishmania genus specific primer of 120bp was positive in 12 cases (7 male and 5 female). The mean age of the PCR positive cases was 20.08 years and the mean duration of the lesions was 3.75 years (range: 1-12 years). No correlation was found between PCR results and the lesion duration or location.

According to the pathogenesis of the disease, the lesions begin with histiocytic inflammation and the lymphocytic infiltration increases gradually as the histiocytes decrease or make well-formed granulomas with variable giant cells⁵. The results of the histopathologic evaluation were classified in the three groups and the PCR results compared with each other.

- 1) The first group had granulomas consisting of histiocytic aggregations surrounded by sparse lymphocytic infiltration. The histiocytes were predominant cells in the infiltrate. Of the 9 cases with these features, 7 had positive PCR

results.

- 2) The second group had lymphohistiocytic granulomas consisting of histiocytes surrounded by remarkable lymphocytic or lymphoplasmocytic infiltrates. The number of histiocytes and lymphocytes were almost equal. Of five patients that had these features, 2 were positive for leishmania by PCR.
- 3) The third group had relatively dense lymphocytic infiltrations with a variable number of plasma cells in the dermis. Foci of histiocytes are included between them but the lymphocytes and plasma cells are clearly predominant. There were 6 cases in this group including 3 cases with positive PCR results for the parasite.

Comparison of the results of PCR showed that the parasite was more likely to be found in cases with predominantly histiocytic infiltrations, but the difference was not statistically significant.

DISCUSSION

In our study, leishmania DNA was detected by PCR in 12 out of 20 paraffin embedded specimens of CLL cases. Despite the granulomatous reaction through the dermis compatible with the diagnosis of leishmaniasis, as well as a history of positive direct smear in the acute stage, 8 cases showed neither leishmania in the histologic sections nor DNA of parasite using PCR. We would like to discuss the following questions:

- 1) What is the sensitivity of PCR for the detection of leishmania DNA in chronic or lupoid forms of cutaneous leishmaniasis? The importance of this question remains in the fact that it is hard or even impossible to find leishmania parasites in CLL and routine parasitological methods (direct smear) are usually negative in lesions lasting more than 6-12 months⁴.
- 2) Are there factors other than parasite persistence that may influence the chronicity of the disease in some cases of CL?

Safaie et al, studied 62 paraffin-embedded skin biopsies of CL in comparison with 20 specimens of non leishmaniasis specimens to assess the validity of PCR in the diagnosis of CL. They found the parasite in all 33 cases whose skin biopsies had

shown the leishmania parasite by light microscopy. PCR results were also positive in 24 of 29 specimens which had clinical diagnosis of CL but amastigotes were not visible in histologic sections. They reported 92% sensitivity for PCR in the diagnosis of CL and none of the non leishmaniasis cases in their study showed positive results (specificity 100%)⁶. It is notable that almost all their cases had leishmaniasis for less than one year whereas all our cases had leishmaniasis for more than one year.

Shahbazi et al, evaluated PCR sensitivity and specificity and compared it with culture and microscopic examination as standard methods of Leishmania diagnosis. They examined 155 specimens of patients suspicious of CL; 69 (44.5%) specimens were positive on microscopic examination and 75 (48.4%) specimens were culture positive. PCR correctly identified 75 of the 75 culture positive specimens (100% sensitivity). Also, PCR was positive in 11 of the 80 negative cultured specimens. No PCR band was observed in leishmania negative control specimens included from other infectious diseases (100% specificity)⁷.

In another study, Venkataram et al showed that 65% of lesions (including acute, subacute and chronic ones) demonstrated leishmania parasites in sections⁸. They attribute their results variability to a previous study by Cannizares that emphasized parasites would disappear from the lesion after 5-7 years⁹. Although all of our cases had leishmaniasis for more than a year, but we did not find any relationship between duration of lesion and PCR results.

Ergin et al, determined the validity of PCR for the diagnosis of CL on paraffin-embedded specimens. PCR was positive in all 20 cases in whom the leishmania parasite was detectable by light microscopy, as well as in 27 of 34 cases that were microscopically negative, with an overall sensitivity of 87%. They claimed a specificity of 100% for PCR by obtaining negative PCR results in all of their non leishmaniasis control cases¹⁰.

Although standard methods are frequently unable to show the etiologic agent in CLL, few studies have been conducted to detect leishmania parasites by PCR in these cases. Momeni et al, used PCR to detect leishmania amastigote DNA in tissue samples obtained from 65 CLL patients. The results were confirmed by Southern blot analysis. Using histologic examination and cultures in the NNN

medium. They found only occasional macrophages containing a few amastigotes in 12 histologic specimens and *Leishmania* promastigotes grew only in 20 cultures. Polymerase chain reaction identified *Leishmania* DNA in 30 of 63 cases (47.6%)¹¹. These results are comparable with our findings, as we had positive PCR in 60% of chronic leishmaniasis.

Weigle et al, also evaluated PCR in the diagnosis of acute (25 cases) and chronic forms (44 cases) of cutaneous leishmaniasis in comparison with conventional methods (microscopic examination and culture). They found PCR was more sensitive than each conventional method alone or even combined together for both types of CL. They found that PCR sensitivity for the diagnosis of acute CL was slightly higher than the conventional methods combined (75.7% versus 70.2%) while for chronic cases, the sensitivity of PCR was much higher than the conventional methods (45.5% versus 4.5 to 27.3 %)¹².

According to these data, sensitivity and specificity of PCR in diagnosis of cutaneous leishmaniasis is documented, but our results and those of previous studies^{9,10} suggest that a remarkable percentage of chronic lupoid leishmaniasis are negative even with this most sensitive method. One possibility is that unknown subtypes of the leishmania parasite contribute to the development of CLL that could not be detected by the 120 bp general primer for leishmania parasite.

Another possibility is that other factors instead of parasite persistence contribute to the chronicity of lesions in CLL. Many studies have been conducted on the immunopathogenesis of the non-healing types of cutaneous leishmaniasis which have mostly shown a predominantly Th2 lymphocyte response to *Leishmania* major antigen and this ineffective type of immune response has been considered as the most probable cause of chronic and non-healing CL. The Th1/Th2 paradigm of resistance/susceptibility to intracellular infection is largely based on investigations using animal models of cutaneous and visceral leishmaniasis induced by *L. major*; however, there is no animal model for chronic lupoid leishmaniasis. This non-healing type of CL has its own unique clinical and immunological characters, namely the presence of a positive skin reaction to the Leishanin test and restriction of the lesion to the primary site of inoculation, which is contradictory to animal models

of non healing leishmaniasis that disseminate to the viscera and follow a lethal course in case of no treatment. Another remarkable feature of lupoid leishmaniasis is predilection to sun exposed areas of the body, as 18 cases in our study had lesions on the face and 2 had lesions on exposed parts of the upper extremities. Moreover, unlike mice models of non healing CL, the lesions did not extend to other sites of the skin or other organs. In a recent study, Meymandi et al showed that the inflammatory infiltrate in lupoid leishmaniasis is predominantly Th1 type and is not similar to mice models of non-healing leishmaniasis which is related to Th2 type immune response¹³. Then, according to our results (negative PCR in 40% of CLL specimens) and what Meymandi et al reported recently, the question whether factors other than parasite persistence influence the disease chronicity in some cases of CL remains unanswered.

We found that the leishmania DNA was not detectable in a noticeable proportion of cases with CLL even using PCR, which is the most sensitive diagnostic method in cutaneous leishmaniasis.

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