Original Article

Molecular Pathology and Histopathological Findings in Localized *Leishmania* Lymphadenitis

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Abstract

Background: A rare variant of Leishmaniasis is Localized *Leishmania* Lymphadenitis which has been occasionally reported from south-eastern parts of Iran. So far, no molecular assay has been performed for diagnosing this variety of Leishmaniasis.

Methods: Nineteen lymph node paraffin blocks were collected from 1994 to 2007. Parasite load count and histopathological patterns reported on Hematoxylin-Eosin and Giemsa stained slides.DNA extraction was carried out just on the remaining available 7 lymph node paraffin blocks according to QIAamp DNA FFPE kit instructions. A pair of primers and a probe were designed for rRNA ITS region with Allele ID 6.0 software, followed by real time PCR amplification.

Result: The most common histopathological pattern was necrotizing granuloma with few Leishman bodies. Parasite load was the highest in submental lymph node (3 ± 1.41 per oil field) which was significantly higher compared to cervical and inguinal nodes ($P < 0.05$). Absolute load of parasite DNA was detectable in all 7 cases. The positive cases revealed a 201 bpamplicon after electrophoresis of end product which was confirmative for *Leishmania tropica*.

Conclusion: Real time PCR revealed *Leishmania tropica* as the etiologic agent of Localized *Leishmania* Lymphadenitis. Although this molecular method is a sensitive diagnostic tool, histopathological findings are still important.

Key words: Histopathology, Localized *Leishmania* Lymphadenitis, *Leishmania tropica*, real time PCR

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Introduction

Leishmaniasis refers to a group of diseases caused by the protozoan genus *Leishmania* in about 98 countries. *Leishmania* includes more than 20 species which can cause a spectrum of manifestations. They involve different body organ, from skin to the reticuloendothelial system.[1–3] The most common clinical forms of Leishmaniasis are cutaneous, visceral and mucosal involvements.[4]

A rare clinical form of Leishmaniasis is Localized *Leishmania* Lymphadenitis (LLL) which is defined by isolated involvement of lymph node without systemic manifestations and excellent prognosis.[5] Although this clinicopathologic entity is not prevalent, it has been reported several times from south-eastern provinces of Iran, including Kerman and Fars.[5–9] Immunologic studies have revealed its relationship with dry-type cutaneous Leishmaniasis agent, *Leishmania tropica*.[10,11] This kind of leishmaniasis might be a self-limiting condition for which no treatment is required and it also responds to antimonial derivatives properly.[5,12]

PCR-based diagnostic methods are highly sensitive and specific for detection of *Leishmania* parasites in cultures or clinical samples.[1]On the other hand, identification of *Leishmania* species is important for correct diagnosis, prevention and proper treatment regimen.[13–15] Therefore, this study was designed for molecular diagnosis, species identification and parasite burden determination. Moreover, possible correlation with histopathological findings was investigated.

Materials and Methods

Sampling

Nineteen patients with clinicopathologic diagnosis of LLL from Kerman province were selected from 1994 to 2007. Demographic data were gathered from their medical records and kept confidential.

Microscopic examination

Paraffin blocks of lymph node biopsies were cut by microtome and two series of slides were prepared by Hematoxylin-Eosin and Giemsa stainings. The slides were reviewed double blindly by pathologists. Histopathological patterns based on Azadeh classification (Azadeh,1985) fell into four groups: group 1) Anergic histiocytes with many Leishman bodies; group 2) Necrotizing granuloma with many plasma cells and few Leishman bodies; group 3) Organized epithelioid granuloma with few plasma cells and very occasional necrosis or Leishman bodies; and group 4) Necrotizing granuloma with stellate microabscess. In addition,

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the number of Leishman bodies in oil power field was counted and the mean value was reported.

DNA extraction

This step was performed by QIAamp DNA FFPE kit for DNA extraction in accordance with manufacturer's instructions on the only remaining seven paraffin blocks due to lack of enough paraffin embedded tissue in old samples. Each paraffin block was cut to 8 sections of 10 µm thickness with microtome. Then, they underwent deparaffinization with 1cc xylene. Subsequently, xylene was removed with 1cc ethanol. The remaining pellet was resuspended with 180 µL buffer ATL and proteinase K. It was incubated at 56°C until the sample was completely lysed, and incubated again at 90°C for 1 hour. Subsequently, 200 µL buffers AL and 200 µL ethanol were added. After centrifuging the lysate in QIAamp MinElute column, it was washed with 500 µL buffer AW1 and 500 µL buffer AW2.Ultimately, 50 µL buffer ATE was applied into tubes which were incubated at room temperature for 5 minutes.

Primer design

With focus on rRNA Internal Transcribed Spacer region (gene bank accession number: FJ948464.1), a pair of primers (forward: L.ITS.F: 5'-CAAATACACGCATGCACTCTC-3' and reverse: L.ITS.R: 5'-TTTAATAATCCTGGTCACAGCC-3') which had been designed previously by bioinformatics softwares, AlleleID 6.0, were used. These primers were specific for *Leishmania tropica* and led to a 201 bp product according to NCBI BLAST. A flurogenic Taq man probe was also synthesized and marked with 6-carboxy-fluorscein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) as reporter and quencher, respectively. Its sequence was as follows: L.ITS.P: 5'-AGCGTCGAAACTCCTCTCTG-GTGC-3'.Beta actin was also selected as the housekeeping gene for normalization of PCR reactions (NCBI reference sequence NM 001101.3). Its primers and probe were as follows: Forward primer: BAF: 5'- ACCACCTTCAACTCCATCATG-3', Reverse primer: BAR: 5'-CTCCTTCTGCATCCTGTCG-3', Probe: BAP: 5'- JOE- ACATCCGCAAAGACCTGTACGCC- TAMRA -3'.[16]

Real time PCR assay

PCR amplification reaction was fulfilled in a 25 µL of reaction mixture containing 12.5 µL of mastermix, 2 µL of forward primers for Beta actin and rRNAITS regions, 2 µL of reverse primers of both mentioned genes, 1.5 µL of probes, 2 µL of H2O and 5 µL of extracted DNA.

Thermal cycling conditions started at 95°C for 2 minute followed by 95°C for 20 seconds (denaturation) and 60°C for 30 seconds (annealing and extension) which were programmed for 45 cycles.

Quantification of parasite DNA load

For absolute quantification, the standard strain (MHOM/Sudan/58/OD) of *Leishmania tropica* was cultured in Novy-MacNeal-Nicolle medium[16] and six dilutions (rate, 1 to 10) were prepared by counting parasites on neubar cell chambers. Subsequently, the standard curve was drawn by usage of serial dilutions.

Statistical analysis

SPSS software version 15.5 was used for descriptive and statistical evaluation. Analysis of variance (ANOVA) and Pearson correlation were performed to compare and depict correlation of variables. Parasite loads were shown as mean ± standard error. *P* value < 0.05 was considered significant.

Results

Histopathological and molecular findings of 19 patients (14 men and 5 women) with LLL were described with ages ranging from 7 to 65 years. Only 3 of the 19 patients had previous or concomitant skin lesions. The most common site of involvement was inguinal lymph node (42.1%) followed by cervical (26.3%) submandibular (15.8%) and submental (15.8%) lymph nodes. Based on Azadeh classification, the most common histopathological pattern was group 2 (8 cases) followed by combined pattern of group 2 and 3 (7 cases), group 3 (3 cases) and finally, the least prevalent group 1 (1 case)(Figure 1). The most common region of involvement in the lymph node was paracortex and cortex (14 cases) followed by paracortex alone (5 cases). Parasite count, by anatomic site of the lymph node, was 3±1.41 (mean±standard error) in submental area followed by 1.26 ± 0.37 in submandibular, 0.46 ± 0.15 in cervical and ultimately 0.33 ± 0.05 in inguinal region. There was a significant relationship between anatomical site and higher parasite load count in submental region compared to cervical and inguinal area ($P < 0.05$). The highest mean of parasite load count per oil field was found in histopathological pattern of the first group defined as "anergic histiocytes with Leishman bodies and necrotizing granuloma" in Table 1.

In order to show the linearity of real-time PCR reactions, we plotted results of cycling threshold (Ct) values of extracted DNA against different parasite dilutions prepared by serial addition of negative sample (rate, 1 to 10). The results suggest a linear relationship between quantification and parasite concentration with a constant slope of -3.711 and a correlation coefficient which was greater than 0.977. Absolute quantification of *Leishmania tropica* DNA was determined from the appropriate standard curves and demonstrated detectable DNA copies of *Leishmania* in all 7 tested samples (Table 2). Non template controls were applied by use of ultra-pure water which yielded no amplicons. All of the positive cases had detectable Leishman bodies on microscopy, except one which showed only epithelioid granuloma with a history of local cutaneous Leishmaniasis. Ultimately, electrophoresis of PCR end product in positive cases revealed a 201 bp amplicon compared to a 100 bp ladder marker which was confirmative for *Leishmania tropica* (Figure 2).

Discussion

LLL is a relatively rare presentation of Leishmaniasis and its correct diagnosis requires clinicopathologic correlations and ruling out other granulomatous lymphadenitis, such as toxoplasmosis, tuberculosis and cat scratch fever.[5] Previously, some investigators used inoculation of animals or culture media to confirm LLL. Ardehali et al., used culture media and isoenzyme determination to reveal that its causative agent was *Leishmania tropica*. However, *Leishmania major* and *Leishmania braziliensis* have also been reported as underlying causes of necrotizing lymphadenitis in other parts of the world.[17,18]

Histopathological findings in the present study indicated that necrotizing granuloma with few Leishman bodies was the most common pattern in LLL and there was a reverse relationship between the number of parasites and granulomatous reaction; i.e.,

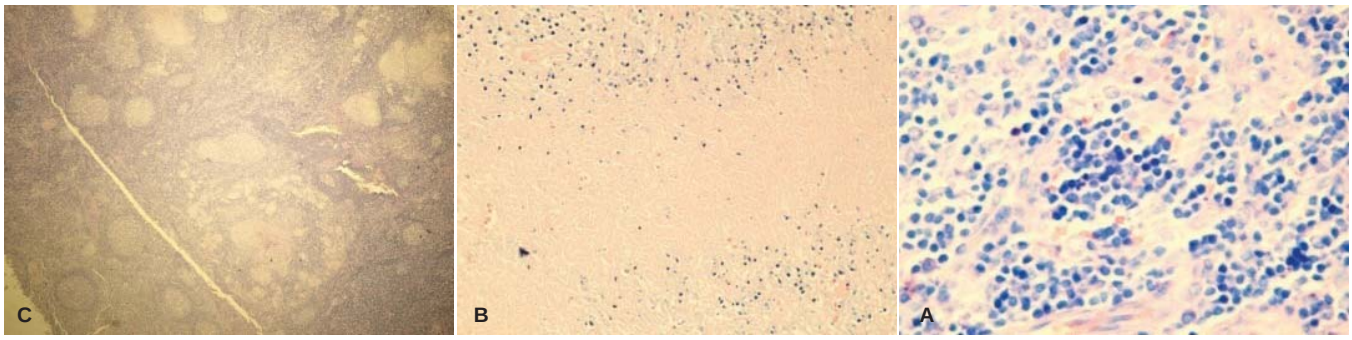

Figure 1. Histopathologic patterns. **(A)** Anergichistiocytes containing numerous Leishman bodies, Giemsa stain magnification x1000. **(B)** Necrotizing granuloma, showing central necrosis surrounded by palisaded histiocytes and mononuclear cells, few Leishman bodies were detectable, Hematoxylin-Eosin stain, magnification x400. **(C)** Epithelioid granuloma with very occasional leishman bodies located in cortical and paracortical region, Hematoxylin-Eosin stain, magnification x40.

Table 1. Mean count of parasite load per oil field on the basis of histopathological pattern.

Histopathologic pattern	Mean count of parasite load per oil field
Anergic histiocytes with Leishman bodies and necrotizing granuloma	4.8
Necrotizing granuloma with few Leishman bodies and epithelioid granuloma	0.94±0.52
Necrotizing granuloma with few Leishman bodies	0.61±0.20
Epithelioid granuloma with occasional Leishman bodies	0.5±0.25

Table 2. Absolute load of parasite DNA obtained by real time PCR.

Number	CT Beta actin gene	CT ITS gene	*Leishmania* DNA copies / reaction
1	32	33	29.5
2	30	31	102.3
3	27	29	346.7
4	23	28	645.6
5	24	30	190.5
6	25	28	645.6
7	29	35	8.51

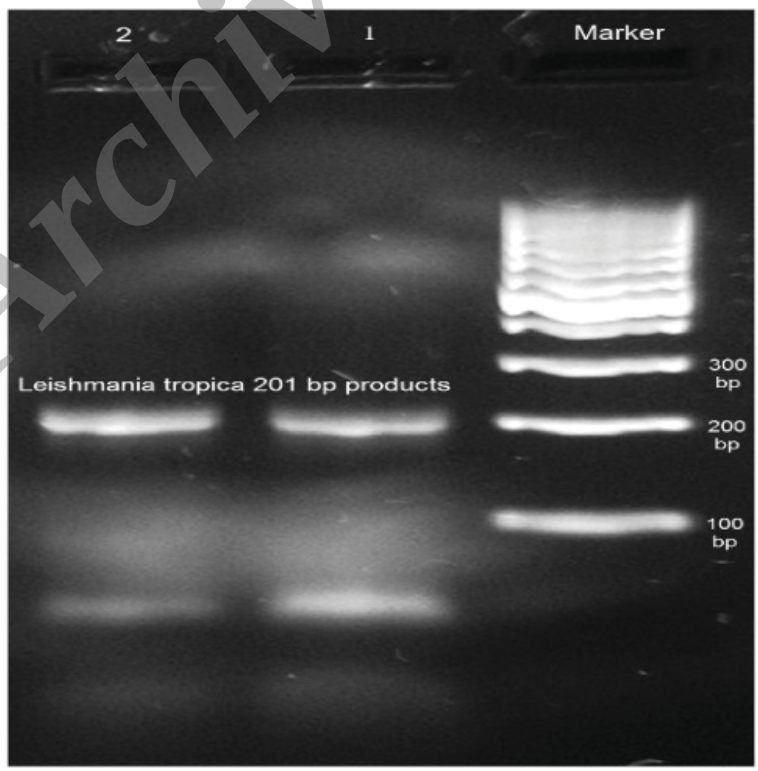

Figure 2. End product electrophoresis on 2% agarose gel. The right column demonstrated a 100 bp ladder marker. Column 1 and 2 pertained to real-time end product electrophoresis of standard strain and the subject strain in our study, respectively. Both demonstrated a 201 bp amplicon confirmative for *Leishmania tropica*.

the more the granulomas, the less the parasite load. In a similar study on mucosal Leishmaniasis, a reverse relationship was reported between parasite load and granulomatous reaction, and PCR-based methods were recommended for such conditions.[19] As the detection of parasite is necessary for diagnosis of Leishmaniasis, this pathologic entity may mandate the application of more sensitive methods such as molecular assays for confirmation of diagnosis.

Paracortex and cortex were the most prevalent regions of lymph node involved in LLL. This finding could be justified by the prominent role of T cell immunity in histogenesis of parasite eradication.[5,20]

The load of parasite was significantly higher in submental area compared to the inguinal and cervical areas. This phenomenon might be due to the denser lymphatic network and shorter lymphatic pathway from site of primary inoculation to regional lymph node because of more superficial position of this node. Unlike *Leishmania donovani, Leishmania tropica* is sensitive to temperature and grows well at 35°C, less well at 37°C and poorly at 39°C.[20] This is a probable explanation for the tendency of this parasite for more superficial locations such as the submental area.

Finally in this study, real time PCR exhibited detectable load of parasite DNA in all 7 tested samples which was acceptable for a sensitive method. Although Leishman bodies were not detectable on microscopy in one case with high clinicopathologic suspicion and previous cutaneous leishmaniasis, real time PCR yielded detectable DNA copies of *Leishmania tropica*. This case demonstrated epithelioid granulomas without Leishman bodies. Therefore, presence of granulomatous reaction with clinicopathological suspicion of LLL, even in the absence of Leishman bodies, might obligate us to perform molecular confirmation with real time PCR. However, high sensitivity of molecular methods compared to conventional ones, such as microscopic evaluation of stained slides of tissue or multilocus enzyme electrophoresis for diagnosis of leishmaniasis might support the use of PCR-based methods as confirmative in suspicious patients.[14,21–24] Real time PCR is also a highly sensitive method for detecting *Leishmania* parasite in lymph nodes of patients with visceral Leishmaniasis, although the load of parasite DNA is markedly higher when compared to this entity.[23–25]A reasonable justification for this finding is disseminated parasitemia in visceral Leishmaniasis. The other Leishmaniasis entity caused by *Leishmania braziliansis* had low parasite burden and therefore the PCR method could be helpful for detection of parasite.[26,27] In this study we focused on the rRNA internal transcribed spacers, which were less prone to evolutionary pressure and demonstrated more divergence of sequence compared to the coding regions for molecular typing.[27,28] This study was the first one to confirm *Leishmania tropica* as the etiologic agent of LLL with molecular assays.

Based on these findings, real time PCR is a precise method for the diagnosis of LLL and especially helpful in clinicopathologically suspected cases without evidently detectable Leishman bodies. The PCR-based methods can also be applied for species identification of the parasite. The causative agent was *Leishmania tropica* in LLL. Despite their high sensitivity, PCR-based methods should be considered as the second line diagnostic tool due to their high cost. Thus, histopathological evaluation still remains the first diagnostic step.

Leishmania tropica has unique presentations which differ from other species manifestations such as Lupoid Leishmaniasis and LLL. The latter, with its excellent clinical outcome, raises this question, "what happens if the parasite is presented to immune effector cells properly?" Future investigations may yield vaccines via lymph node inoculation.

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