# Ability of Real-time PCR in diagnose Differentiation Various Forms of Cutaneous Leishmaniasis: A Comparative Study with Histopathology

**CURRENT STATUS:** ACCEPTED

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**SUBJECT AREAS**
- Infectious Diseases

**KEYWORDS**
- Leishmaniasis, Real-time PCR, Ridley Scoring System
Abstract

Objective: Histopathological studies suggest that parasite load is different between acute and chronic forms of cutaneous leishmaniasis (CL). However, highly sensitive detection methods are still needed to distinguish different forms of leishmaniasis. In the present study, we developed a quantitative real-time polymerase chain reaction (PCR) to detect and quantify Leishmania tropica parasites in paraffin-embedded tissue samples. Results: The ability of real-time PCR for leishmania detection was higher than histopathological evaluation. The parasite loads were quantified by qPCR assay and microscopic evaluation were highly correlated (r = 0.598; P < 0.001). Among patients, the parasite load was inversely correlated with disease duration (acute CL lesions had very higher parasite loads than chronic CL lesions), but there was no difference in parasite load according to the patients’ age and sex as well as location of the lesions. In contrast to Ridley scoring system (P < 0.001), there were no statistically significant differences in the relative number of parasites among the lupoid and non-lupoid forms of chronic lesions in real-time PCR (P = 0.549), which indicates the superiority of histopathological evaluation in CL forms differentiation.

Introduction

Dry cutaneous leishmaniasis (CL) caused by Leishmania tropica is a significant parasitic disease in Iran (1). The clinical phenotype, histopathology, and the number of organisms are diverse among acute, chronic lupoid, and chronic non-lupoid forms of this infectious disease (2). In histopathology of acute CL, plasma cells, histiocytes, epithelioid cells, and occasionally eosinophils and giant cells, and dense dermal infiltrate of lymphocytes are seen. Also, numerous intracytoplasmic Leishman bodies parasitized macrophages and sometimes neutrophils are seen throughout the reticular dermis. A small number of
infected macrophages and multifocal small tuberculoid granulomas composed of epithelioid cells, histiocytes, and occasional giant cells are seen more in chronic form. Mild to moderate mononuclear infiltrates (lymphocytes and plasma cells) adjacent to the granuloma along with fibrosis and telangiectasia are present. Low numbers of organisms, erythematous papules at the periphery of a scar of a healed acute lesion, and granulomas consisting of tubercles surrounded by lymphocytes, histiocytes, and giant cells are the most pathological findings in the lupoid forms of the disease; although, for scanty organisms in cutaneous lesions specifically in chronic leishmaniasis, microscopic studies has less sensitivity (2-9).

Laboratory diagnosis of CL relies on either the microscopic detection of Leishman bodies in cutaneous tissue or the culture and isolation of parasites from lesions biopsy samples (10, 11). Apart from high specificity, inadequate sensitivity, difficulty, and time consuming nature are among disadvantages of these methods (12). Nowadays, PCR-based testing of skin lesion biopsies is known as a sensitive and specific test for diagnosis and quantification of leishmaniasis (13-16). The analysis of the load of leishmania parasites within the skin lesions would be important not only for diagnostic purposes, but also for an eventual follow-up of a patient’s response to treatment (17). Accordingly, in the present study we applied a standardized qPCR assay to detect Leishmania. tropica load in paraffin blocks of various CL forms. The differentiation ability of this quantitative method was compared with semi quantitative pathological scoring system.

Materials And Methods

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Patients and Sampling

Forty patients presenting with acute (n=10), chronic lupoid (n=14), and chronic non-lupoid (n=16) forms of CL who attended the Dermatopathology Department of Afzalipour
Hospital (2010-2013) were selected to participate in our study. It should be noted that the study design, consent form, and sampling procedure were approved by the Ethics Committee of Afzalipour Hospital and patient selection was performed after evaluation of inclusion/exclusion criteria. The patients were considered to be included in the study if they presented with parasitologically confirmed CL with long-term illness (≥3 years), had received at least 3 times glucantime treatment, and were able to give contact information for the follow-up. We excluded patients with other skin diseases or with small biopsy samples. Informed consent was obtained from all the participants prior to enrolment.

**Histopathology:**

After making diagnosis for three different clinical forms based on Azadeh classification (18):

I- Anergic macrophage reaction

II- Focalized histiocytic reaction

III- Diffuse necrotizing reaction

IV- Diffuse lympho-histiocytic reaction

V- Lupoid granulomatous reaction

We also did Ridley scoring for parasite loads as follows from 0 to +4: (19)

+1: one or more amastigotes

+2: 10 or more amastigotes

+3: 100 or more amastigotes

+4: 1000 or more amastigotes

**DNA extraction**

For DNA extraction, 5 μm sections from paraffin-embedded blocks were cut using disposable blades and deparaffinized by hot xylene and then, they were hydrated (descending grades of alcohol) and incubated in proteinase K (20 μg/μL, at 60°C). After
digestion completed (3 days), the DNA was isolated using a QIAamp® DNA Mini Kit (QIAGEN, 51304), according to the manufacturer’s protocol.

**Real-time PCR assay**

We applied a probe-based assay targeting rRNAITS region to detect and quantify parasites in the samples. PCR amplification reaction was fulfilled using ABI StepOne system (Applied Biosystems, USA) and in a 25 μL of reaction mixture, containing 12.5 μL of master mix, 2 μL of forward and reverse primers for beta-actin and rRNAITS regions, 1.5 μL probe, 2 μL of H₂O, and 5 μL of extracted DNA. Thermal cycling conditions started at 95°C for 2 minutes followed by 95°C for 20 seconds (denaturation), and 60°C for 30 seconds (annealing and extension), which were programmed for 45 cycles. A cycle threshold (Ct) for each sample was determined based on the required cycles for the fluorescent signal to cross the background level.

| Primers   | Sequences (5’-3’)                                             |
|-----------|---------------------------------------------------------------|
| L.ITS.F   | 5’-CAAATACACGCATGCACTCTC-3’                                   |
| L.ITS.R   | 5’-TTTAATAATCCTGGTCACAGCC-3’                                  |
| L.ITS.P   | FAM-5’AGCGTGCAGAGTCTCTCTGAGCTC3’-TAMRA                       |
| Actin.F   | 5’-ACCACCTTCAACTCCATCATG-3’                                   |
| Actin.R   | 5’-CTCCTTCTTGATCTGCGTAC3’                                     |
| Actin.P   | JOE-5’ ACATCCGCAAAGACCTGTACGCC 3’-TAMRA                      |

F=Forward, R=Reverse, P=Probe, L.ITS=Leishmania ITS (internal transcribed spacer) gene

**Quantification of parasite DNA load**

For absolute quantification, the standard strain (MHOM/Sudan/58/OD) of *L. tropica* was cultured in RPMI1640 medium and serial dilutions (10 to 10⁷) were prepared. Subsequently, a standard curve was set by plotting the Ct values against each standard of known concentration of the parasite’s DNA.
**Statistical analysis**

The differences between experimental groups were analyzed using the ANOVA (Tukey test) and Spearman's rank correlation coefficient was used for evaluation of the relationship between real-time PCR and histopathological results. SPSS software (version 22) was used in this study.

**Results**

Histopathology and real-time PCR results in studied patients with different forms of CL are summarized in table 2. 40 patients with confirmed CL were enrolled: 25 (62.5%) men and 15 (37.5%) women, with mean age of 32 years (range 6-73 years). To evaluate the correlation between the qPCR assay and histopathological evaluation, collected samples were analyzed in parallel by both methods. The linearity of qPCR results was approved (diagram slope of -3.23 and correlation coefficient (r^2) of ≥0.997) (20) and this assay allowed the quantification of the parasite load in all samples, while the microscopic evaluation allowed this in 32 samples (80%, 8 negative samples corresponded to lupoid patients), which is indicating that the former method is more sensitive than the latter.

As presented in table 2 (see Supplementary Files), acute form has higher parasite load than chronic ones (P<0.001) by real-time PCR. The mean parasite load in chronic lesions (n=30) was 0.08×10^3 parasites, compared with 13.064×10^3 in acute lesions (n=10) (P<0.001). Interestingly, there was no significant difference in parasite load between chronic- lupoid and non-lupoid lesions by real-time PCR (P=0.549). According to histopathological analysis, there were statistically significant differences in the relative number of parasites among the acute and chronic (P<0.01) and chronic -lupoid and non-lupoid forms (P<0.001). These results indicate the superiority of histopathological evaluation (Ridley scoring system) for differentiation of various forms of CL.
Discussion

In order to accurately and confidently quantify parasites in paraffin-embedded biopsy samples, we evaluated the parasitic load in acute and chronic forms using real-time PCR and histopathological scoring system. The focus of the present study was to compare the diagnostic ability of two common methods in a relatively large number of patients with CL. The power of the used qPCR assay (21) has allowed the quantification of a broad range of parasite load levels in tissue lesions. In terms of diagnostic sensitivity, our results confirmed that the sensitivity of real-time PCR is indeed higher than histopathological scoring system. Our findings are also consistent with the findings of previous studies that focused on parasite abundance in various forms of CL, pointing to inversely correlation of parasite load with the disease duration. Namely, in both methods of this study, acute form has higher parasite load than chronic ones. Interestingly, in contrast to Ridley scoring system (P<0.001), there were no statistically significant differences in the relative number of parasites among the lupoid and non-lupoid forms of chronic lesions in real-time PCR (P=0.549), which indicates the superiority of histopathological evaluation in differentiation of various forms of CL.

It should be noted that the analysis performed here revealed no significant differences in parasite load with regard to the age, sex, and location of skin lesions. These findings were consistent with other studies (22-25). For example, Mashayekhi and colleagues in a study on 11 male and 9 female patients with a mean age of 17.5 years showed that PCR was positive in 60% of the samples and no correlation was found between the results of PCR and age, sex, duration, and location of the lesions (26). Venkataram and co-workers indicated that 65% of acute, subacute, and chronic lesions manifested leishmania parasites in tissues. But they could not find the relationship between the duration of lesions and PCR results (25).
Weigle and others showed that PCR sensitivity was higher than the conventional assays for the diagnosis of acute lesions while for chronic samples, the sensitivity of PCR was much higher than the conventional assays (27).

Sandeep Verma and colleagues conducted real-time assay to estimate parasite burden in clinical samples of visceral leishmaniasis and patients with post kala-azar dermal leishmaniasis. The study for diagnosis as well as prognosis of both visceral leishmaniasis and post kala-azar dermal leishmaniasis, provided a simple molecular instrument to show the efficacy of anti-leishmanial drugs or vaccines (28).

Another study done by Dabiri and others compared the effect of different treatments on parasite (leishmania) DNA load following treatments using real-time PCR method (29). Jara and colleagues improved a quantitative real-time PCR (qPCR) method targeting mini-circle kinetoplast DNA (kDNA) to find and quantify Leishmania (Viannia) parasites. According to the parasite species, the patients’ age, and number or area of lesions, there was no difference in parasite load (30). Sirian and co-workers conducted a comparison between conventional, molecular, and immunohistochemical methods for CL detection and reported that immunohistochemical and molecular techniques were more sensitive (31-33). Our observations support the validity of using real-time PCR to simultaneously detect and quantify the leishmania load in tissues from human lesions, particularly in chronic lesions. This highly sensitive quantitative technique (10, 20, 21) can be employed also for monitoring the parasite load during treatment and follow-up as a way to assess the outcome of treatment.

**Limitations**

Our observations support the validity of using real-time PCR to simultaneously detect and quantify the leishmania load in tissues from human lesions, particularly in chronic lesions. This highly sensitive quantitative technique (10, 20, 21) can be employed also for
monitoring the parasite load during treatment and follow-up as a way to assess the outcome of treatment.

Declarations

Ethics approval and consent to participate
The study approved in Kamran university of medical science ethical committee and The Ethic Approval Cod is IR.KMU.REC.1397.813.

Consent for publication
All authors consent for publication in Parasites & Vectors journal.

Availability of data and material
Please contact author for data requests.

Competing interests
The authors declare that they have no conflict of interests.

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Authors' contributions
And it was designed on Kerman university of medical science, all authors read and approve the final manuscript.

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Table 2

Due to technical limitations, table 2 is only available as a download in the supplemental files section.

Supplementary Files

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Table 2.jpg