

Molecular diagnosis of Old World leishmaniasis: Real-time PCR based on trypanothione peroxidase gene for the detection and identification of *Leishmania* spp

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ABSTRACT

Background & objectives: Rapid and accurate diagnosis and identification of *Leishmania* sp causing cutaneous leishmaniasis is crucial in control and therapeutic programs. The problem of diagnosis with traditional methods is that they have a low sensitivity or time consuming but molecular techniques would be an alternative method for rapid and accurate diagnosis. In this work, trypanothione peroxidase gene-based real-time PCR was used for accurate identification of *Leishmania* spp causing Old-World cutaneous leishmaniasis.

Methods: In this study, biopsies of specimens were taken from the ulcerative sites in 100 patients and used for direct microscopy, culture in NNN or fixed in alcohol for identification of *Leishmania* spp using trypanothione peroxidase gene-based realtime PCR (qPCR).

Results: Using direct microscopy and culture method, *Leishmania* parasites were isolated from 68 out of 100 patient samples. However, 13 patients with negative finding on traditional tests, had positive results on RT-PCR test. After melting curve analysis of PCR product, *Leishmania major* in 75 and *L. tropica* in 4 cases were identified. The sensitivity and specificity of RT-PCR for diagnosis of cutaneous leishmaniasis was 98.7 and 59.8%, respectively.

Conclusion: Results of this study showed that RT-PCR was the most sensitive diagnostic test for cutaneous leishmaniasis and represents a tool for rapid species identification.

Key words Cutaneous leishmaniasis; diagnosis; Iran; peroxidase; RT-PCR; trypanothione

INTRODUCTION

Leishmaniasis comprises a group of diseases caused by the genus *Leishmania* widely distributed in tropical and subtropical regions throughout the world. These diseases transmitted by various species of *Phlebotomus* sandfly in Old World. *Leishmania* genus can be divided into several species complexes and some of them including *L. major*, *L. tropica* and sometimes *L. infantum* are the causative agents of cutaneous leishmaniasis in Old World¹. Cutaneous leishmaniasis has been increased in endemic and non-endemic regions due to geographical expansion of the disease; changing patterns of international travel; and population migrations^{2,3}.

Identification of *Leishmania* is crucial to select the most appropriate therapeutic regimen to be administered to each individual, to determine possible control measures in epidemiological studies and to predict the risk of dissemination in immunocompromised patients^{4,5}.

Frequently, geographical distribution and clinical

manifestations⁶ are applied for *Leishmania* species identification. However, geographical distribution is not a suitable index in non-endemic areas, as well as endemic areas where multiple species of *Leishmania* may co-exist; and clinical manifestations could not be useful since several species cause cutaneous leishmaniasis and these clinical presentations are not specific enough to allow species identification⁷. Due to similar morphology, it is not possible to identify *Leishmania* species by parasitological methods. Moreover, direct microscopy is not sensitive enough and culture method is time consuming^{8,9}. The gold standard for species identification is multilocus enzyme electrophoresis (MLEE)¹⁰ which requires expertise, isolation of the parasites and cultivation *in vitro*. Therefore, MLEE would not be rapid enough to guide first-line therapeutic decisions. PCR-based methods, as a powerful tool to detect *Leishmania* directly in clinical samples as well as for parasite characterization, have proven to be highly sensitive and specific compared with traditional methods^{8,11} providing results in one or two working days⁷. Peroxidase

gene with antioxidant activity is an important gene in parasite validity. Three copies of this gene in the form of tandem repeat are located in chromosome 15. With these properties it is easy to detect the parasite even in low quality in patient samples. In the present work, the tryparedoxin peroxidase gene-based qPCR method including a diagnostic step of cutaneous leishmaniasis followed by species identification has been developed.

MATERIAL & METHODS

Clinical patients

In this experimental investigation, study population consisted of patients with cutaneous sore suspected to leishmaniasis referred to Sedighe Tahereh Clinic in Isfahan, Iran from 2007–08. Sample size was 100 patients and biopsy specimens were taken with informed and free consent from the ulcerative sites of every individual and used for direct microscopy, culture in NNN or fixed in alcohol for molecular investigations. Results of each method were recorded and for determination of sensitivity and specificity, combination results of direct microscope slide and culture was considered as golden standard. Sensitivity was calculated by dividing the number of true positive cases to total number of positive cases and false negative cases. Specificity was also calculated by dividing the number of true negative cases to total number of negative cases and false positive cases.

Parasites

Two standard strains, *L. major* (MRHO/IR/75/ER) and *L. tropica* (MHOM/IR/NADIM₃) were used as positive controls. Promastigotes were cultured on NNN medium supplemented by BHI (4%), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 24°C.

DNA extraction

DNA was extracted using QiAampDNA mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The incubation time with proteinase K was increased to 1 h at 56°C and DNA was eluted from the column with 100 µl of PCR-grade H₂O. The extracted DNA was analyzed by spectrophotometric determination of A₂₆₀.

Real-time PCR

The identification of the parasites was done by qPCR of the tryparedoxin peroxidase gene with accession number of EU251502. All RT-PCR assays were performed on the Corbett Rotor gene 3000 as per the manufacturer's instructions. Amplification was done in reactions using

Quantifast SYBR green (Qiagen) with the following primers: TRYP-F(5' GGAATTCATATGTCCTGCGG-TAACGCCAAG 3') and TRYP-R (5'CGCGGATCCT-TACTTGTGTGGTCGACCTTCATGC 3'). The amplification and detection program of (95°C for 3 min, 35 cycles: 7s at 95°C, 30s at 60°C, 30s at 72°C) was used. Melting program consists of one cycle of 95°C for 0s, 65°C for 20s and heating at 98°C. The PCR products of interested gene in *L. major* and *L. tropica* presented amplicons with TM of 91.3 ± 0.7 and 89.5 ± 0.2. Therefore, they were distinguishable by two distinct peaks in the melting curves obtained from the real-time apparatus.

Analysis

All the reactions were analyzed by the software provided with the instrument. The generation of amplification plots, standard curves, and dissociation stage analysis were as per the manufacturer's protocol. The calculation of the melting temperature of each amplicon (T_m) was done directly by the software provided. Each of the assays was repeated at least thrice to check the reproducibility and reliability.

RESULTS

Leishmania parasite was detected in lesion of 59 patients (59%) in microscopic examination and in lesion of 36 patients (36%) according to the culture method (Table 1). DNA of patient's biopsy samples was extracted and subjected to qPCR and melting curve analysis. Considering the species-specific difference in melting temperature of reference strains (*L. major* and *L. tropica*) and comparing melting temperature of samples with standard strains, 79 positive cases in qPCR were detected, of which 75 were *L. major* and 4 were *L. tropica*. As an example qPCR melting curves of two patient's samples, standard strain of *L. major* and negative control have been demonstrated in Fig. 1.

Overall, 68 out of 100 samples were positive in parasitological diagnostic methods (culture and/or direct microscopy) and 81 out of 100 samples were positive by at

Table 1. Results of various diagnostic techniques applied for the diagnosis of cutaneous leishmaniasis in skin biopsy samples of 100 patients referred to Sedighe Tahereh Clinic, Isfahan, Iran

Diagnostic technique	No. (+)ve samples	No. (-)ve samples
Smear	59	41
Culture	36	64
RT-PCR	79	21

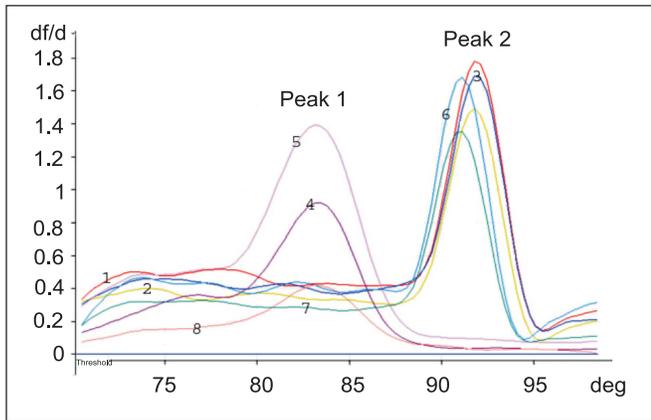


Fig. 1: Melting curve analysis of amplification products of samples from two patients (4, 5, 6, 7), standard strain of *L. major* as positive control (1, 2, 3) and negative control (8). Detection was done using the double-stranded DNA-binding dye SYBER Green.

Table 2. Sensitivity and specificity of the different techniques applied for the diagnosis of Cutaneous leishmaniasis in skin biopsy samples of 100 patients referred to Sedighe Tahere Clinic in Isfahan, Iran. Combination positive results of direct microscopy and culture were considered as gold standard

Diagnostic technique	Sensitivity (%)	Specificity (%)
Smear	86.8	100
Culture	52.9	100
RT-PCR	98.7	59.8

least one of three methods (microscopic examination or culture or qPCR) and 13 out of 100 samples were positive only in qPCR.

Sensitivity and specificity of qPCR in comparison to other methods considering culture method as golden standard have been shown in Table 2. Statistical analysis showed a significant difference in the sensitivity of qPCR, microscopy and culture.

DISCUSSION

Results of this study showed that qPCR is more sensitive than parasitological methods in detection of leishmania parasite in skin biopsy samples. Furthermore, it is possible to determine rapidly the leishmania species by this method. Other investigations also achieved the same results about the high sensitivity of qPCR in diagnosis of Leishmaniasis^{9, 13-20}. RT-PCR simply refers to amplification of DNA (by PCR) that is monitored while the amplification is occurring. The benefit of this RT-PCR is that it allows the researcher to better determine the amount of starting DNA in the sample before the amplification by PCR. Also qPCR can distinguish specific sequences from

a complex mixture of DNA. However, its application requires the availability of primers and probes that must be selected according to very rigid conditions, which cannot always be easily applied.

Wortmann *et al*²⁰ investigated diagnosis of leishmaniasis using qPCR. They showed a sensitivity of 96% for this test while that of direct microscopy and culture was 30 and 33% respectively^{7,9,18-20}. Moreover, they showed that comparing with other methods qPCR was a rapid test^{18,21}. qPCR has also been used in diagnosis of *Leishmania* species in animals such as dogs and mice, and demonstrated that it is a rapid and sensitive test¹²⁻¹⁷. It has also been shown that qPCR is more sensitive than conventional PCR in diagnosis of *Leishmania* species²².

In this study, 13 samples which were negative for leishmania parasite, converted positive in qPCR. This result is in agreement with what Schulz *et al*¹³ found in clinical samples of patients. Results of this study together with finding of other investigations emphasize on high sensitivity of qPCR in detection of leishmania infection and the ability of test in species discrimination. Therefore, this test can be used in reference laboratory for rapid diagnosis and species determination of leishmaniasis.

Also it is recommended to design convenient primers and probes for diagnosis of different species of leishmania using Syber Green and taqman reagents. Finally, further work is recommended for diagnosis of other forms of leishmaniasis by this technique.

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REFERENCES

1. Talmi-Frank D, Nasereddin A, Schnur L, Schönián G, Töz S, Jaffe C, *et al*. Detection and identification of old world *Leishmania* by high resolution melt analysis. *PLoS Negl Trop Dis* 2010; Jan 12; 4(1): e581.
2. Desjeux P. The increase in risk factors for leishmaniasis worldwide. *Trans R Soc Trop Med Hyg* 2001; 95(3): 239-43.
3. Rodriguez-Gonzalez I, Marin C, Vargas F, Cordova O, Barrera M, Gutierrez-Sanchez R, *et al*. Identification and biochemical characterization of *Leishmania* strains isolated in Peru, Mexico, and Spain. *Exp Parasitol* 2006; 112(1): 44-51.
4. Foulet F, Cosnes A, Dellion S, Blum L, Bachmeyer C, Raguin G, *et al*. *Leishmania major* cutaneous leishmaniasis in HIV-positive patients does not spread to extralesional sites. *Arch Dermatol* 2006; 142(10): 1368-9.
5. Guiguemde RT, Sawadogo OS, Bories C, Traore KL, Nezien D, Nikiema L, *et al*. *Leishmania major* and HIV co-infection in Burkina Faso. *Trans R Soc Trop Med Hyg* 2003; 97(2): 168-9.
6. Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP,

- Felger I. Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the minixon sequence and subsequent restriction fragment length polymorphism analysis. *J Clin Microbiol* 2003; 41(7): 3147–53.
7. Foulet F, Botterel F, Buffet P, Morizot G, Rivollet D, Deniau M, *et al.* Detection and identification of *Leishmania* species from clinical specimens by using a real-time PCR assay and sequencing of the cytochrome B gene. *J Clin Microbiol* 2007; 45(7): 2110–5.
 8. Bensoussan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL. Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. *J Clin Microbiol* 2006; 44(4): 1435–9.
 9. Wortmann G, Hochberg LP, Arana BA, Rizzo NR, Arana F, Ryan JR. Diagnosis of cutaneous leishmaniasis in Guatemala using a real-time polymerase chain reaction assay and the Smartcycler. *Am J Trop Med Hyg* 2007; 76(5): 906–8.
 10. Spanakos G, Piperaki ET, Menounos PG, Tegos N, Flemetakis A, Vakalis NC. Detection and species identification of Old World *Leishmania* in clinical samples using a PCR-based method. *Trans R Soc Trop Med Hyg* 2008; 102(1): 46–53.
 11. Chargui N, Bastien P, Kallel K, Haouas N, Akrouf FM, Masmoudi A, *et al.* Usefulness of PCR in the diagnosis of cutaneous leishmaniasis in Tunisia. *Trans R Soc Trop Med Hyg* 2005; 99: 762–8.
 12. Shahbazi F, Shahabi S, Kazemi B, Mohebbali M, Abadi AR, Zare Z. Evaluation of PCR assay in diagnosis and identification of cutaneous leishmaniasis: a comparison with the parasitological methods. *Parasitol Res* 2008; 103(5): 1159–62.
 13. Schulz A, Mellenthin K, Schonian G, Fleischer B, Drosten C. Detection, differentiation, and quantitation of pathogenic leishmania organisms by a fluorescence resonance energy transfer-based real-time PCR assay. *J Clin Microbiol* 2003; 41(4): 1529–35.
 14. de Paiva Cavalcanti M, Felinto de Brito ME, de Souza WV, de Miranda Gomes Y, Abath FG. The development of a real-time PCR assay for the quantification of *Leishmania infantum* DNA in canine blood. *Vet J* 2009; 182(2): 356–8.
 15. Francino O, Altet L, Sanchez-Robert E, Rodriguez A, Solano-Gallego L, Alberola J, *et al.* Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniasis. *Vet Parasitol* 2006; 137(3–4): 214–21.
 16. Nicolas L, Prina E, Lang T, Milon G. Real-time PCR for detection and quantitation of leishmania in mouse tissues. *J Clin Microbiol* 2002; 40(5): 1666–9.
 17. Solano-Gallego L, Rodriguez-Cortes A, Trotta M, Zampieron C, Razia L, Furlanello T, *et al.* Detection of *Leishmania infantum* DNA by fret-based real-time PCR in urine from dogs with natural clinical leishmaniasis. *Vet Parasitol* 2007; 147: 315–9.
 18. Wortmann G, Hochberg L, Houg HH, Sweeney C, Zapor M, Aronson N, *et al.* Rapid identification of *Leishmania* complexes by a real-time PCR assay. *Am J Trop Med Hyg* 2005; 73(6): 999–1004.
 19. Wortmann GW, Sweeney C, Houg HS, Aronson N, Stiteler J, Jackson J, *et al.* Rapid diagnosis of leishmaniasis by fluorogenic polymerase chain reaction. *Am J Trop Med Hyg* 2001; 65(5): 583–7.
 20. Wortmann GW, Romero LI, Paz HM, Ortega-Barria E, Bayard V, Hochberg LP, *et al.* Real-time polymerase chain reaction diagnosis of leishmaniasis in Panama from both fresh and frozen tissue. *Trans R Soc Trop Med Hyg* 2004; 98(3): 148–51.
 21. Van der Meide W, Guerra J, Schoone G, Farenhorst M, Coelho L, Faber W, *et al.* Comparison between quantitative nucleic acid sequence-based amplification, real-time reverse transcriptase PCR, and real-time PCR for quantification of *Leishmania* parasites. *J Clin Microbiol* 2008; 46(1): 73–8.
 22. Mary C, Faraut F, Lascombe L, Dumon H. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. *J Clin Microbiol* 2004; 42(11): 5249–55.

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