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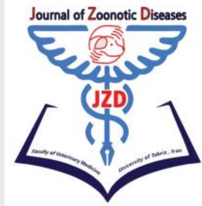

A simple and innovative quantitative PCR analysis for diagnosis and therapy management of human cutaneous leishmaniasis

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Abstract

Leishmania infection manifests across various clinical syndromes, predominantly affecting humans in less developed regions globally. Cutaneous leishmaniasis (CL), caused by *Leishmania major* and transmitted by infected female sand flies, is a crucial neglected disease prevalent in tropical and subtropical areas. Diagnosis of leishmaniasis is performed mostly through staining of blood or skin samples, which can accompany some problems such as appropriate slide preparation avoiding artifacts, low amount of parasites, and expertise of the technicians. Therefore, it is important to develop an easy, sensitive, and specific method that harbors a quantitative aspect like a semi-quantitative method. We present a new, easy-to-handle, innovative, and specific quantitative method to detect *L. major* in skin lesions of suspicious leishmaniasis patients. For this aim, DNA was extracted from the promastigote form of *L. major*, human cells, and the slide smears prepared from the skin lesion. The quantitative PCR was performed with the primer pair derived from 18S rRNA genes of *L. major* and human gene, which can amplify the DNA of both origins simultaneously. As expected, the PCR product originating from human cells had a PCR product of 255 bp in length, whereas the PCR product originating from the parasite had a PCR product of 360 bp in length, which can be used for quantitative analysis. Since this method is innovative, sensitive, specific, and convenient, we believe that it can also be used for the management of successful therapy.

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Introduction

Leishmaniasis, a vector-transmitted disease, is caused by infection with intracellular parasite protozoans from the *Leishmania* genus. This infection is a substantial public health issue, especially in low and middle-income countries (LMICs), with high mortality and morbidity (1). *Leishmania* parasites transmitted by the bite of an

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infected female sandfly (Diptera: Psychodidae), Phlebotomus in the Old World, and Lutzomyia in the New World (2).
During a blood meal, an infected female sandfly injects the parasite's metacyclic promastigote stage into the host. Then the parasite develops into asexually replicating amastigotes in the macrophages, plus the fact that this parasite can also affect various organs and tissues based on some factors such as parasite species, host species, and immunological status of the host (3). *Leishmania* species that infect humans can also be found in mammalian reservoir hosts, including canids and rodents. Zoonotic cutaneous leishmaniasis, caused by *L. major*, is emerging as a major health issue in several Middle Eastern countries such as Iran, Saudi Arabia, and Iraq (4).
In cutaneous leishmaniasis (CL), it is believed that parasites remain localized, leading to non-healing ulcers on exposed body regions. Lesions, starting as red papules 1 week to 3 months post-infection, progress into larger plaques or nodules in subsequent weeks (5). Within 1 to 6 months, an ulcer with a darker border and crusted base forms, occasionally accompanied by painless, rubbery nodules, papules, or hardened masses around the ulcer site, will occur. Enlarged surrounding lymph nodes may itch, but discomfort is usually minor or absent. In cutaneous leishmaniasis, lesions can heal spontaneously over a period of 1 to 36 months, leaving patients with pigmented scars often with social and psychological consequences. Secondary infections may worsen specific cases (6).
The causative agent of mild cutaneous infection, *L. major* (CL), tends to go unnoticed and neglected, which boosts the risk of interpersonal transmission. A major challenge in controlling leishmaniasis originates from the wide range of disease states and the lack of proper ability to link clinical symptoms efficiently. Relying only on clinical diagnosis through physical examination and patient travel history is unsatisfactory (7). For the diagnosis of leishmaniasis, the investigators have tried to introduce different methods based on DNA analysis. Chaouch et al. (2019) evaluated loop-mediated isothermal amplification (LAMP) assays that target the cysteine protease B (cpb) gene for the specific detection of *Leishmania major* and *L. tropica* in skin lesions (8). They believed that the LAMP assay for *L. major* was extremely sensitive, with a detection limit of 20 fg (femtogram) DNA, and it was completely specific, with no cross-reactions with other species of *Leishmania* (8). Owino et al. (2021) reported that *Leishmania major*, *Leishmania donovani*, and *Trypanosoma* species were found in *Sergentomyia squamipleuris* sand flies from a visceral leishmaniasis hotspot in Merti Sub-County, eastern Kenya. The detection was accomplished by PCR amplification and sequencing of the internal transcribed spacer 1 (ITS1) region, which indicated positive samples, including bands that match *L. major* and other Leishmania species (9). Talmi-Frank et al. (2010) used real-time PCR with high-resolution melting analysis to detect and quantify Old World *Leishmania* species, including *L. major*. They believe that unique melt patterns were detected for *L. major*, allowing for its sensitive identification and quantification to identify as few as 2-4 ITS1 gene copies per reaction (10). A quantitative real-time PCR (qPCR) assay has been developed by Wu et al. (2020) to detect pan-*Leishmania* by targeting the minicircle kinetoplast DNA (mkDNA). While it is not tailored specifically for *L. major*, this qPCR method exhibits high sensitivity and specificity in identifying and quantifying various *Leishmania* parasites, including *L. major* (11).
Aqeele et al. (2021) have examined the advantages of PCR over alternative methods for parasite screening and treatment. It has been proven that curcumin is highly effective against both forms *L. major* promastigotes and amastigotes, which implies its probability as a safer treatment for leishmaniasis than hazardous prescription drugs (12).
Due to the absence of an effective vaccination, conventional diagnostic methods are critical for minimizing post-infection effects such as scarring.

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Based on data from WHO and the Centers for Disease Control and Prevention, molecular approaches such as PCR are the most trusted for diagnosing and differentiating various leishmaniasis types (13).
Serological and microscopic tests are simple to perform, but they are accompanied by some problems like cross-reactivity, preparation of suitable smears, quality of staining, and the need for high experience (14).
Diagnosing leishmaniasis requires the accurate and precise detection and identification methods of *Leishmania* infection. A quantitative method, which is easy to perform, not only supports the early and improved treatment approaches but also allows for the deployment of effective control measures, which can improve patient outcomes and reduce persistent reservoirs in a cycle of transmission. Optimized diagnostic technologies would also improve the One Health approach to leishmaniasis control (15, 16).
The present study evaluates the advantages and drawbacks of current diagnostic techniques, with a special focus on PCR-based methods using a specifically designed primer pair amplifying simultaneously 18S rRNA gene of both host cell and parasite with easily distinguishable PCR products on agarose gel, which can be used for management of therapy process.

Material and methods

Sample

Promastigote form of *Leishmania major* (MRHO / IR / 75 / ER) and the definitive diagnosis and suspicious slides for Cutaneous Leishmaniasis (CL) were acquired from the Department of Parasitology at the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.

Preparation of promastigote

Promastigote form of *Leishmania major* (MRHO / IR / 75 / ER) was cultured at 25°C in RPMI-1640 medium (CAISSON company) supplemented with penicillin-streptomycin 100 µg /mL (gibco company) and 10% fetal calf serum (PAA company) as described by Aqeele et al. (2021) and used for DNA extraction (12).

Preparation of human cell line

Human colon carcinoma cell line (HCT116) was purchased from Pastor Institute (Tehran, Iran) and cultured in RPMI, 10% FCS, and pen/strep, and the collected cells were used for a mixture composed of promastigote form of *L. major* and cells in different concentrations.

DNA extraction

Genomic DNA was isolated from lesion smear slides using a DNA Extraction Kit (MBST, Tehran, Iran), following the manufacturer's protocol. DNA was also isolated from the Human colon carcinoma cell line (HCT116) and promastigote in different concentrations. The concentration and quality of the extracted DNA were assessed using a Nanodrop machine (Bio-Rad, Germany). Cell DNA's OD (260/280) and OD (260/230) were measured.
After calculation of the concentration of DNA extracted from the promastigote sample (70 µg/mL), the DNA was prepared in different dilutions (7, 0.7, 0.07, 0.007, 0.70007 and 0.00007 ng). Furthermore, for detection of the ratio of competitive inhibition in PCR, mixtures of DNA extracted from the cell line (3 ng, as a constant part in all mixtures) were prepared with different amounts of DNA extracted from promastigote (70, 7, 0.7, 0.07, and 0.007 ng) and used in PCR analysis.

Polymerase chain reaction (PCR)

For the performance of PCR analysis, primer pairs were designed from the 18S rRNA gene of *L. major* (under accession no. XR_002460811.1), and the 18S rRNA of the human (under accession number XR_007084308.1). The forward primer (F-primer) has the sequence 5`-AGAGGTGAAATTCTTGGACCG-3`, and the reverse primer (R-primer) has the sequence 5`-TTCCGTCAATTCCTTTAAGTTTCA-3`. These primers can simultaneously amplify the corresponding region in the genomic DNA from *L. major* and human cells. The amplification of the genomic DNA from both probes (human and *L.*

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major) resulted in a PCR product of 255 bp in length for human cells and 360 bp for promastigotes. Since some fungal skin infections can mimic *Leishmania* skin lesion, the nucleotide sequence of primer pair was checked by https://www.ncbi.nlm.nih.gov/tools/primer-blast/ and looked for input PCR template for *Dermatophytes* (taxid:34384), *Trichophyton* (taxid:5550), *Microsporidians* (taxid:6029), *Epidermophyton* (taxid:34390) and *Arthroderma* (taxid:63399). No targeted PCR templates were found for the mentioned agents. Furthermore, we checked the nucleotide sequence of primer pair using https://www.ncbi.nlm.nih.gov/nucleotide/ program for *Trichophyton rubrum* (accession no: JX431933), *Trichophyton verrucosum* (accession no: EF631623.1), *Trichophyton mentagrophytes* EF631618.1), *Microsporum canis* (accession no: EF631606.1), *Microsporum distortum* (accession no: EF631608.1), *Teratosphaeria microspore* (accession no: EU343066.1), *Nannizzia lorica* (accession no: EF631613.1), *Nannizzia gypsea* (*Microsporum gypseum*) (accession no: EF631611.1), *Lophophyton gallinae* (accession no: EF631610.1), *Epidermophyton floccosum* (accession no: EF631604.1), *Arthroderma uncinatum* (accession no: EF631614.1), *Paraphyton cookie* (accession no: EF631607.1), *Aspergillus niger* (accession no: XR_009468004.1), *Aspergillus flavus* (accession no: D63696.1) and *Candida albicans* (accession no.: NG_070791.1), *Candida sp*. (accession no.: AY242210.1) and due to three thymidines (TTT) instead (CCG) at 3` end of the forward primer no PCR target template could be formed. Only by *Aspergillus flavus* strain VCG1 chromosome (accession no.: CP051097.1), *Aspergillus flavus* strain AF36 chromosome 7 (accession no.: 7CP051025.1) and *Aspergillus flavus* strain A1 chromosome 7 (accession no.: CP051065.1), the nucleotide sequence differed from the corresponding *Aspergillus flavus* nucleotide sequence at the position mentioned above. Apart from this, the PCR product from mentioned fungi DNA would then be ca. 255 bp like that from human. Taken together, it seems that the designed primer pair is specific for the detection of *L. major* in the skin lesion.

The PCR reaction mixture consisted of 20 µM of each primer, 1 µl of extracted DNA, 9.5 µL of double distilled water, and 12.5 µL of Taq PCR Master Mix (SinaClon), for a final volume of 25 µL. The reaction was conducted in an automatic thermocycler (Bio-Rad, Germany) with the following thermal cycling conditions: initial denaturation at 95°C for 5 minutes, followed by 38 cycles of 95°C for 45 seconds (denaturation), 54°C for 45 seconds (annealing), and 72°C for 45 seconds (extension), and a final extension step at 72°C for 5 minutes. The PCR products were analyzed on 2% agarose gel under UV light with a safe stain (Red-safe SinaClon). A 100 bp DNA ladder (SinaClon) was used as a DNA molecular weight marker. To ensure the accuracy of the PCR results, an external positive control was included to assess the PCR's performance, and a negative process control checked for contamination during the DNA extraction process.

Results

DNA was extracted from promastigotes, human cell line, and Giemsa stained lesion smears and amplified using primer pairs, resulting in PCR products of 250 bp for human genomic DNA and 360 bp for L. major genomic DNA. Figure 1 shows the PCR analysis of DNA extracted from cells infected with L. major. The above-mentioned PCR products were easily distinguishable on the 2% agarose gel electrophoresis. In the next step, the DNA extracted from the promastigote was used to calculate the sensitivity of the tests. The concentration of the DNA extracted from promastigote was 70 ng/µL. The amplification was performed with different dilutions, namely 0.1 (7 ng), 0.01 (0.7 ng), 0.001 (0.07 ng) and 0.0001 (0.007 ng), 0.00001(0.0007) and 0.00001 (0.00007). The PCR analysis showed that it is possible to detect the DNA of *L. major* in 0.007 ng

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(7 pg) genomic DNA. In the next step, the extracted DNA from promastigote, in different dilutions, was added to the constant amount of extracted DNA from human cells. The PCR analysis showed that in the dilution of 1/1 (70 ng DNA of promastigote/3 ng DNA of human cells), a prominent PCR product band for promastigote was detected. Interestingly, no visible (or very weak) PCR band was detectable for human DNA by this ratio. From the dilution of 0/1 promastigote DNA to 0.1 human DNA, it was possible to detect PCR products for both promastigote and humans. In this dilution, the PCR product derived from promastigote remained prominent and the PCR product of humans was visible. Our experiments showed that the smaller the ratio of DNA amount of promastigote to DNA from humans became, the more prominent the PCR product of humans became. The results showed that in a mixture of promastigote and human DNA, it is possible to detect *L. major* in at least 0.007 ng (7 pg) DNA of promastigote to 3 ng DNA of humans. What bothered us was why the PCR product from humans was not visible in the first mixture. It is known if there is more than one amplicon in the sample, like in the lesion region consisting of uninfected and infected human cells (also genomic DNA from the host cells and genomic DNA from the amastigote of *L. major*), the higher concentration of amplicon can lead to competitive inhibition of amplification of the DNA with lower concentration of parasite (17).

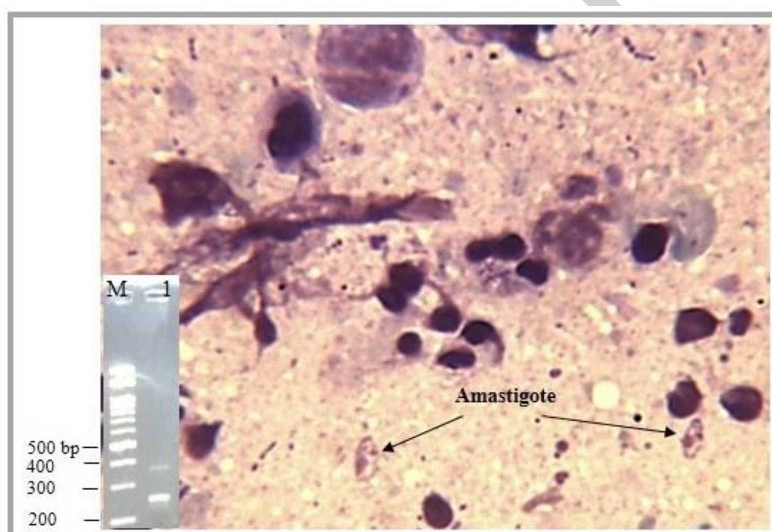

Fig.1. The *leishmania* suspected lesion smear slide was stained with Giemsa and the DNA was extracted and amplified using mentioned primer pair in the text. The amastigote form of *Leishmania* are maked with the arrows and Insert shows amplification of the extracted DNA resulting in two PCR products (human (255 bp) and *Leishmania* (360 bp).

Taken together, our results showed that the PCR with the mentioned primer pair can be used for the detection of *L. major* in the lesion region in a quantitative manner, because the amplification from both DNA from parasite and host cell can be performed under the same conditions in the same tube. Comparing the density ratio of the PCR product band of the parasite to the host cell PCR product can give sufficient information about the quantitative analysis, especially in the case of management of the treatment. To show that the extraction of DNA from previous stained lesion smear slides was also suitable for molecular analysis, the DNA was extracted from Giemsa

stained skin lesion smear slides, which were kindly provided by the *Leishmania* reference laboratory (Department of Parasitology at the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran). For this aim, the deep part of the smears (which was not suitable for microscopic analysis) was collected and lysed in lysis buffer and the DNA was extracted from the so prepared samples. Interestingly, PCR analysis confirmed 6 positive diagnosed slides from 7 positive diagnosed slides by Giemsa staining, and the other positive slide by Giesma staining was negative in PCR. Furthermore, 4 of 4 negative slides in Giemsa staining were also negative in PCR analysis.

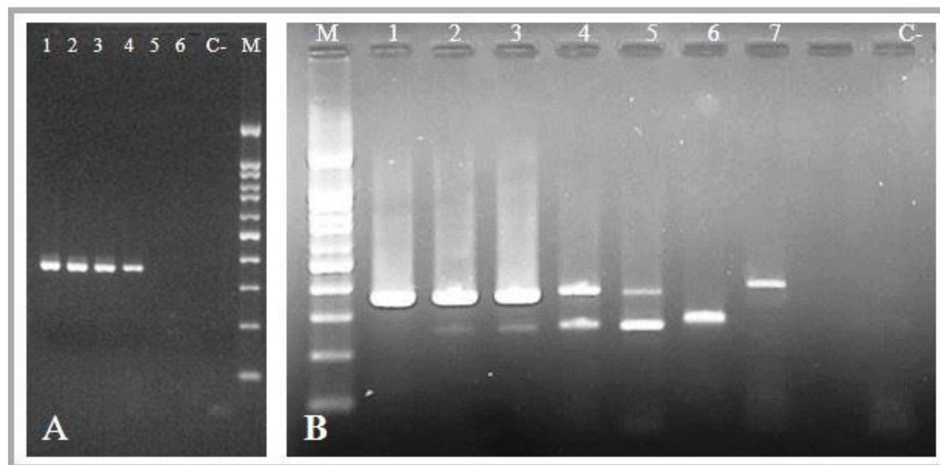

Fig. 2. DNA was extracted from promastigote (approximately 70 ng/µl) and cell line (approximately 30 ng/µl). **A:** Subsequently, dilutions of the extracted DNA from promastigote of *L. major*. was prepared (lane 1= 0.1 µl, lane 2= 0.01 µl , lane 3= 0.001 µl, lane 4= 0.0001 µl, lane 5= 0.00001 µl, lane 6= 0.000001 µl) and amplified. **B:** The extracted DNA from promastigote of *L. major* in different ratios was added to the constant amount of DNA of cell line and amplified. Lane 1= 1/0.1, lane 2= 01/ 0.1, lane 3= 0.01/0.1, lane 4= 0.001/0.1, lane 5= 0.0001/0.1, lane 6= human cells, lane 7= Promatigote. M was 100 bp DNA marker.

Discussion

Leishmaniasis is an important public health problem that occurs in Southeast Asia and West Asia, Nord Africa, and East Africa causing severe diseases (18). For public health management, early diagnosis of *Leishmania* infection is of great importance. Technological advancements have considerably increased the speed and accuracy of these approaches. While each technique for detecting and identifying Leishmaniasis has unique strengths and limitations, an ideal test should have high sensitivity, specificity, rapid turnaround times, and affordability. Accurate diagnosis of Leishmaniasis is essential for disease prognosis and suitable patient treatment strategy. Microscopic detection of parasites typically demands direct aspiration smears, which are frequently stained with chemical substances like Giemsa and Trypan blue to aid with cell clarity. After staining, *Leishmania* amastigotes tend to occur inside macrophages, with pale blue cytoplasm, red nucleus, and a nearby purple-pink-stained kinetoplast. As a result, the data at hand show that detection accuracy depends on variables such as primary sample selection, staining reagent quality, and technical expertise. Sensitivity is significantly influenced by the infection stage, as demonstrated in CL, where amastigote quantities decrease with progression. To counteract the disadvantages of skin lesion smear staining, molecular methods can represent a safe era in the clinical *Leishmania* diagnosis, surpassing the limits of prior detection methods concerning sensitivity,

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specificity, speed, and ease of use. Many investigators used conventional PCR, nested PCR, or real-time PCR techniques to diagnose leishmaniasis. They used different targets for their analysis. For example, Mauricio et al. (1999) used gp63 genes (19). The G6PD gene was targeted by Castilho et al. (2003), HSP70-I-3`- non-translated region was used by Jariyapan et al. (2021) which empowered them to detect and differentiate simultaneously, 18S rRNA gene and genomic ITS-1 region were used by Chargui et al. 2005 and Spanakos et al. (2008) and de Lima et al. (2011), respectively (20-24). Ceccarelli et al. (2014) ran a PCR assay in which the Kinetoplast microcircle DNA was targeted (25). In the case of *L. infantum*, Mary et al. (2004) also used kinetoplast minicircles as targets for real-time PCR. They suggested that it could be used not only for the diagnosis but also for monitoring the therapy and epidemiological proposes (26). It is known that the quantitative real-time PCR is not only more convenient but also it is more sensitive than the other common methods as it can detect the infection in the lowest dosage of parasitic load, which can be considered as a limitation of old methods such as staining. The only disadvantage in such methods compared to the presented method is that in quantitative real-time PCR methods the use of expensive equipment and a housekeeping gene are essential. In the present method, since the primer pair can amplify simultaneously both the genomic DNA from *L. major* and as well as from humans, the amplification of a housekeeping gene is not necessary.

For quantitative analysis, the intensity of PCR product on the agarose gel deriving from the parasite can be divided through the intensity of PCR product deriving from the human genome, and the result can be used as a marker for parasite burden in the lesion and for monitoring of the course of therapy.

Since the primer pair can simultaneously amplify the corresponding region of the DNA from humans and parasites, competitive inhibition can occur for the DNA amplification of the DNA source with a very low amount in the sample. This can be considered as a disadvantage for the presented method. Despite it, we believe that the present quantitative PCR technique can be used as a suitable method for the diagnosis of the cutaneous *Leishmanina* infection and also for the management of the treatment. We surely accept that the number of slides used in the present study was not enough and in the future, the mentioned method can be checked with more lesion smear slides with different grades of infection.

Conclusion

The primer pair used in the present study can amplify both the DNA extracted from human host cells and *Leishmania* spp. Therefore, this method can be used as a quantitative PCR method without the need for expensive equipment for real-time PCR and a housekeeping gene as it is needed for real-time PCR and is easy to perform in any molecular laboratory worldwide.

Acknowledgments

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Ethical approval

Since, the experiments were performed with the probes acquired from the reference diagnostic laboratory of the Department of Parasitology at the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, we hereby declare all ethical standards have been respected in the preparation for the submitted article.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Corrected Proof