Is Real-Time Polymerase Chain Reaction (PCR) More Useful Than a Conventional PCR for the Clinical Management of Leishmaniasis?

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Abstract. It is currently unknown if the use of a real-time polymerase chain reaction (PCR) adds value to the diagnosis and follow-up prognosis of patients affected by leishmaniasis. We performed a study using a real-time PCR directed against the α-polymerase gene and a semiquantitative PCR that target the SSU ribosomal RNA (rRNA) gene as control for the diagnosis and quantification of parasites in patients with visceral (VL) and cutaneous (CL) leishmaniasis. Our single copy real-time PCR missed one diagnosis of VL compared with the conventional PCR, whereas both PCR methods were able to detect Leishmania parasites in CL. Under anti-leishmania treatment the kinetics of parasitemia were comparable with the two methods. The real-time PCR directed against α-polymerase of Leishmania despite being able to make a more accurate quantification of parasites does not add to the decision-making management compared with a semiquantitative PCR, and it is comparatively expensive.

INTRODUCTION

Polymerase chain reaction (PCR)-based methods have been used to detect Leishmania spp. in different clinical samples using several targets, such as ribosomal RNA (rRNA) genes, miniexon genes, kinetoplast DNA (kDNA), and repetitive nuclear sequences.1–3 Despite the fact that the sensitivity of such methods depends on the choice of the target sequence, several works have shown that PCR is actually the most sensitive and specific technique for the diagnosis of visceral leishmaniasis especially in the immunocompromised patients.4,5

Real-time PCR is considered an emerging technology for the detection, genetic characterization, and quantification of, among all, protozoan parasites. Taqman probes have been the most widely used methods for real-time quantitative PCR of parasite DNA, including Cryptosporidium parvum, Leishmania infantum, Plasmodium falciparum.6–8 In the field of Leishmania, the use of real-time PCR has been initially proposed in animal models and was thought especially useful to assess the efficacy of antileishmanial drugs.9,10

The application of real-time PCR to human leishmaniasis has been limited so far to a few studies conducted on stored skin biopsies of culture confirmed and suspected cutaneous leishmaniasis (CL)11–13; a mixed case study of nine patients with cutaneous (CL) and visceral (VL) leishmaniasis14; a study regarding 10 patients affected by AIDS, and finally a population of 10 patients with imported CL and 2 acquired immunodeficiency syndrome (AIDS) patients with skin disease (1 post-Kala-azar dermal leishmaniasis [PKDL], 1 disseminated disease).

DNA extraction. The DNA was extracted from 350 μL of whole peripheral blood and bone marrow aspirates collected in EDTA by using the commercial Easy Kit DNA (Invitrogen, San Diego, CA), according to the manufacturer’s instructions. Cutaneous biopsy was digested and DNA was extracted by phenol-chloroform, according to Sambrook and others19 and 1 μg was used to perform PCR. Scraping from skin lesions was subject to direct lysis with proteinase K (120 μg/mL) and specific buffer (Tris HCL 10 nM, KCL 50 nM, Tween 20 0.5%, NP40 0.5%) in a final volume of 350 μL and then 10 μL was carried out in PCR. The DNA concentration was assessed by UV spectrophotometry.

Qualitative PCR. The DNA target for PCR amplification was the gene coding the 18S rRNA and the primers used were R223 (5′-TCCCATGCAACCTCGGTT-3′) and R333 (5′-AAAGCGGGGCGGTGCTG-3′), which amplify a 359-bp fragment of the Leishmania DNA. The presence and integrity of human DNA in the extracted samples were assessed by amplifying a 252-bp fragment of the β-globin gene with the following primers: hβ3if (5′-CGGCTGTCACTAGCCTAGACCT-3′) and hβ4ir (5′-TTTCATCCACCTTGCACACAC-3′). The semiquantitative results for the positive samples were determined by the serial 10-fold dilutions of the extracted DNA ranging from 1 μg to 0.1 pg and were arbitrarily expressed as the number of Leishmania parasites per 5 × 106 peripheral blood leukocytes, assuming that each parasite harbors 160 copies of the SSU rRNA gene. Negative samples were considered to have less than 1 copy genome per 150,000 leukocytes (i.e., 1 μg of DNA extracted from peripheral blood).

Real time PCR. For the Leishmania spp. Taqman system, the target DNA was the DNA polymerase of L. (L.) infantum, which is a single-copy-number gene.9,10 The PCR primers and probe were designed on the consensus sequence created by the alignment of different sequences derived from different species of Leishmania. For the alignment the Macvector program (version 7.1.1, Accelyers, Inc., Cambridge, UK) was used to align different sequences from the NCBI database. The primers and probe were designed on the consensus sequence created by the Macvector program (version 7.1.1, Accelyers, Inc., Cambridge, UK).

PATIENTS AND METHODS

Patients. All samples assayed came from patients with a confirmed diagnosis of either VL or CL leishmaniasis: 22 patients with a diagnosis of VL; 7 immunocompetent human immunodeficiency virus (HIV)-negative children, and 15 adults (9 HIV-positive and 6 HIV-negative). Samples came from 19 cases of primary diagnosis of VL and three relapses. Regarding CL, all samples were collected from 5 adult patients with imported CL and 2 acquired immunodeficiency syndrome (AIDS) patients with skin disease (1 post-Kala-azar dermal leishmaniasis [PKDL], 1 disseminated disease).

The aim of this study is to evaluate detection and quantification of Leishmania parasites in blood, bone marrow, and skin samples by using both a real-time PCR using specific Taqman probes and a conventional semiquantitative PCR that amplify the small subunit ribosomal 18S sequence.

<table>
<thead>
<tr>
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<th>* Address correspondence to Spinello Antinori, Department of Clinical Sciences L Sacco, section of Infectious Diseases and Immunopathology, University of Milan, Via GB Grassi 74, 20157 Milano, Italy.</th>
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used. The accession numbers of all *Leishmania* α-polymerase sequences were L. (L.) *aethiopica* AF009235; L. (V.) *amazonensis* AF009136; L. (L.) *arabica* AF009137; L. (V.) *braziliensis* AF009138; L. (L.) *chagasi* AF009139; L. (L.) *donovani* AF009141; L. (L.) *infantum* AF009147; L. (L.) *major* AF009148; L. (V.) *mexicana* AF009149; L. (V.) *panamensis* AF009150; L. (L.) *tropica* AF009152. The real time assay was designed using Primer Express Software (PE Biosystems, Foster City, CA) and the generated PCR product was 123 bp long. A fluorogenic probe was synthesized by PE Biosystems with a 6-carboxylfluorescein (FAM) reporter molecule at the 5′ end and a 6-carboxytetramethylrhodamine (TAMRA) quencher at the 3′ end. The Lpol probe sequence was 5′-CCCATCCTGCGT-TAMRA-3′. Taqman PCR mix contained 2× Taqman Universal Master Mix, 45 pmol of forward and reverse primer, 100 nM of the Taqman probe, 1× Exogenous Internal positive Control Mix (containing primer and probe VIC labeled), 1× IPC DNA and 10 µL of template (equal to 1 µg) in a total volume of 50 µL. To normalize the parasite load for cell equivalents a 294 bp fragment of the human β-Actin gene was amplified with the forward primer 5′-TCACCCACACTGGTCCCATCTTACGA-3′ and reverse primer 5′-CACGGGAAACGCTCATTGCCATGGG-3′, and quantified using a fluorogenic probe 5′-FAM-ATGCCCTCC CGTCAATTTCTTTAACGTTC-3′. The PCR Mix was the same as that used for *Leishmania* amplification except for the primers and probe concentrations (100 nM primer forward, 600 nM primer reverse, and 200 nM probe). In addition, for the Human β-Actin gene each sample was analyzed in triplicate.

**Standard preparation.** To prepare the standards for the α-polymerase of *Leishmania* and the Human β-Actin gene a culture of *Leishmania infantum* zymodeme MON1 a pellet of Ramos cell line were digested, respectively, in a lysis buffer containing proteinase K 120 µg/mL, KCL 50 mM, Tris-HCL ph 8 10 mM, NaCl 0.1 M, EDTA 25 mM, Tween 20 0.5%, Nonidet containing proteinase K 120 µL of Ramos cell line were digested, respectively, in a lysis buffer containing at least one amplificable template by PCR; the SGU value was determined in a Poisson distribution test using nested-PCR positive and negative results. The SGU value allowed calculating the real concentration of the undiluted standards. In our experiments, we generated a standard curve of α-polymerase *Leishmania* of six 10-fold dilutions ranging from 150,000 to 15 copies and a human β-actin gene standard curve from 300,000 to 3 copies; to reproduce the sample conditions, all the standard dilution were diluted in 1 µg of exogenous DNA and tested in triplicate.

**Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).** Molecular Old World *Leishmania* identification was carried out by PCR- restriction fragment length polymorphism (RFLP) analysis, according to Minodier and others.20 PCR-RFLP allowed identification of parasites at the complex level: L. (L.) *infantum* (type A) 250 bp; L. (L.) *infantum* (type C) 250 bp, 180 bp, and 70 bp; L. (L.) *donovani* 180 bp and 70 bp, L. (L.) *major* 215 bp, 155 bp, 95 bp, and 35 bp; L. (L.) *tropica/aethiopica* 215 bp and 35 bp. Molecular typing of the New World *Leishmania* was carried out by amplification and sequencing of a larger fragment of 882 bp of the SSU rRNA gene with the primers R200 (5′-CCGGGTAATTCACGCTTCCA-3′) and R300 (5′-CGTCAATTCTTAAACGTTCC-3′), originally described by Van Eys.1

**Costs, time-use.** For each PCR assay (qualitative, semi-quantitative, and absolute quantitative) the total cost for each reaction was calculated; we also calculated time spent (total time for each reaction necessary to perform each assay).

**RESULTS**

We have chosen archived DNA of 22 patients (7 children and 15 adults) with a confirmed diagnosis of VL (19 primary VL, 3 relapses) and 5 with imported CL to make a comparative evaluation of *Leishmania* parasite detection and quantification using two different PCR methods: a newly developed real-time PCR compared with our conventional PCR. A total of 162 clinical samples (135 peripheral blood specimens, 17 bone marrow aspirates, and 10 skin scrapings or biopsies) were tested and the comparative results are summarized in Table 1. All of the children had culture-confirmed VL caused by L. (L.) *infantum* MON1. Among HIV-negative adults 3 out of 5 had culture-confirmed VL: 1 L. (L.) *donovani*, 2 L. (L.) *infantum*. Among HIV-positive adults VL was culture-confirmed in 4; 3 L. (L.) *infantum*, 1 L. (L.) *donovani* MON37, with positive microcopy in 6 patients.

At the time of primary diagnosis of VL, the single-copy gene real-time PCR was found positive in the peripheral blood in all but 1 of the 19 patients (94.7%) that had been tested positive by the conventional PCR.

At the time of primary diagnosis of VL, the median value of parasites/5,000,000 cells was 2856.4 (range 6–124,887) on peripheral blood and 18,710 (range 780–158,334) on bone marrow, with the real-time PCR and 100 parasites (range 1–10,000) and 10,000 (1,000–100,000), respectively, for the conventional PCR. The median value of parasites detected on peripheral blood were lower using real-time PCR among HIV-negative children (430, range 6–3,531) compared with either HIV-positive (6,879, range 170–12,171), and HIV-negative adults (6,954, range 2,767–17,884).

In patients coinfected with HIV and *Leishmania*, the median value of parasites at primary diagnosis (5,030, range 170–12,335) was equal to those observed at the time of relapse (5,490, range 2,525–9,781). Table 2 summarizes mean and median values of *Leishmania* PCR in the different settings.

**Kinetics of parasitemia after anti-Leishmania treatment.** The comparative kinetic of parasitemia was evaluated under treatment of 6 HIV-positive adults (4 primary diagnosis and
2 relapses), 5 HIV-negative adults (all primary diagnosis), and 4 HIV-negative children (3 primary diagnosis and 1 relapse). At the end of treatment all patients but one (the AIDS patient) had undetectable *Leishmania* DNA on peripheral blood.

The kinetics of parasitemia of 6 illustrative patients (4 adults of whom 3 HIV-infected and 2 HIV-negative children) that were treated with different regimens is shown in Figure 1. As expected, the real-time PCR was more accurate in the measurement of parasites, the curves using the two methods were comparable; excluding the HIV-infected patients without negative results, the median time of days from the start of treatment and the first negative result was 8.5 days (13 days for HIV-infected patients; 10 days for HIV-uninfected adults;
5.5 days for children). Among HIV-uninfected patients, the longest intervals before achieving negative results were observed in a child (19 days) treated with meglumine antimoniate (Glucantime; Aventis Pharma, Milano, Italy) and in a women (17 days) initially treated with amphotericin B lipid complex (Abelcet; Elan Pharma, Ireland) before switching to liposomal amphotericin B (Ambisome; Gilead, Foster City, CA).

### Table 2

Mean and median value of *Leishmania* DNA parasites detected by means of real-time polymerase chain reaction (PCR) in different groups of patients at the time of primary diagnosis of visceral (VL) and cutaneous (CL) leishmaniasis*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Children</th>
<th>Adults HIV-negative</th>
<th>Adults HIV-positive</th>
<th>HIV-pos</th>
<th>HIV-neg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PB</td>
<td>BM</td>
<td>PB</td>
<td>BM</td>
<td>PB (relapse)</td>
</tr>
<tr>
<td>Median</td>
<td>430</td>
<td>23951</td>
<td>4905,2</td>
<td>88431,35</td>
<td>5000</td>
</tr>
<tr>
<td>Mean</td>
<td>866,43</td>
<td>26878</td>
<td>6558</td>
<td>88431,5</td>
<td>21408,47</td>
</tr>
</tbody>
</table>

*HIV = human immunodeficiency virus; PB = peripheral blood; BM = bone marrow.

**Diagnosis and level of parasitemia for cutaneous leishmaniasis.** The 5 patients with imported leishmaniasis had the diagnosis confirmed either by microscopy and culture (2 patients) or microscopy alone (3 patients). In all cases PCR was positive either with conventional PCR or real-time PCR; *Leishmania* were identified by means of culture as *L. (L.) major* MON25 and *L. (V.) panamensis* (one case each) and by PCR-RFLP as

![Figure 1](image_url)

**Figure 1.** Kinetics of parasitemia in six representative patients affected by visceral leishmaniasis undergoing different drug regimens. (A) Human immunodeficiency virus (HIV)-negative patient affected by Wegener’s granulomatosis with a rapid response to liposomal amphotericin B therapy without any relapse; (B) AIDS patient with a multitrated (antimonials and liposomal amphotericin B) visceral leishmaniasis at the time of his sixth relapse when he underwent a new treatment with miltefosine for 2 months. Besides controlling the clinical symptoms the treatment never achieved an undetectable parasitemia and this was observed with both polymerase chain reaction (PCR) methods. (C) HIV-negative children who had a clinical and parasitologic relapse after 30 days treatment with meglumine antimoniate. Interestingly, the patient had negative PCR on peripheral blood at Day 30, whereas PCR done on bone marrow aspirate was positive with both methods. The patient was considered apparently cured (because microscopy on bone marrow aspirate was negative and the PCR assayed subsequently) but 2 months later he presented again fever, hepatosplenomegaly, and pancytopenia. A new course with liposomal amphotericin B achieved clinical and parasitologic cure. (D) HIV-positive patient treated with liposomal amphotericin B showing a relapse 1 year after first diagnosis; (E) HIV-positive patient treated with liposomal amphotericin B showing a relapse 1 year after first diagnosis; (F) AIDS patient with an early relapse after treatment with liposomal amphotericin B; despite a new cycle of liposomal amphotericin B the patient had a slow response and was subsequently switched to pentamidine with rapid response. PB = peripheral blood; BM = bone marrow; qPCR = quantitative PCR; cPCR = conventional PCR.
L. major (two cases); in patient 24 we were unable to make identification at the species level. Semiquantitative parasite load was determined with conventional PCR for 3 patients with values ranging from 1,000 to 1,000,000 parasites; the median value obtained with real-time PCR was 90,973 (range 3,208–1,059,268). Two HIV-infected patients showed cutaneous lesions (one PKDL) and underwent biopsy showing positive microscopy and the highest value recorded by quantification with real-time PCR: 2,607,321 and 6,426,951, respectively. Both cases were the result of L. (L.) infantum. It is worth noting that patient 15 was hospitalized again 1 year later for a relapse of VL. At this time no skin lesions were observed. Nevertheless, a skin biopsy was randomly done on intact skin and was positive either on histology or PCR with a high parasitic burden (i.e., 10,454,093).

**Costs and time-use.** The cost for each reaction for the conventional PCR was 0.63€ and 2.72€ for the real-time PCR; the total cost for a single patient was 5.04€ for the qualitative conventional PCR (4 housekeeping tubes + 4 target tubes), 12.6€ for the semiquantitative (10 housekeeping tubes + 10 target tubes), and 54.4€ for the real-time quantitative PCR (10 housekeeping tubes + 10 target tubes). Semiquantitative PCR required the longest assay time (4.5 hours), whereas real-time PCR required 2.5 hours.

**DISCUSSION**

A real-time PCR that quantifies DNA of *Leishmania* has been used initially in the mice-infected models, using as a target a single-copy gene for *L. (L.) infantum* and the kinetoplast for *L. (L.) major*; subsequently, the real-time PCR has been studied in humans using the kinetoplast DNA or the SSU rRNA gene. 

For the microbiologic diagnosis of leishmaniasis, the PCR assay based on kinetoplast amplification is probably the most sensitive because this molecular target is present in about 10,000 copies per parasite. However, the heterogeneity of kinetoplast minicircles could be a problem for accurate quantification. Moreover, the high sensitivity of the kinetoplast target might be a double-edged sword when used as a tool to verify the therapeutic response, because it is able to detect also the asymptomatic carriage of *Leishmania*. For that reason, we have developed a real-time PCR for the detection and quantification of *Leishmania* DNA loads in blood, bone marrow, and skin, choosing as target the DNA polymerase of *L. (L.) infantum*, which is a single-copy gene. In each sample tested the parasite count is normalized for cell equivalents on the basis of its DNA content thus allowing a result that is independent of the quantity of the DNA tested and is comparable in the single patient and between different patients. The results of our study that compares this quantitative real-time PCR (qPCR) with a conventional PCR (cPCR) directed against the SSU-rRNA shows that for the impact of VL, our real-time was unable to identify one HIV-uninfected adult patient with a confirmed diagnosis; however, this result was not unexpected because the target chosen for our real-time PCR was a single-copy gene. From a practical point of view, this means that a single copy real-time PCR is less useful with respect to a kinetoplast DNA-based method for the diagnosis of the disease and for epidemiologic purposes. However, Mary and others, using a real-time PCR with kinetoplast as a target sequence, showed that qPCR does not offer an advantage for initial diagnosis of VL because of the high parasite loads.

Because it has been claimed that an accurate quantification of parasite burden is particularly useful in the monitoring of drug treatment, especially among HIV-infected patients, we aimed to compare the results obtained with the two assays for 15 patients. Although our experience confirms previous studies showing that real-time PCR is the best method to quantify as accurately as possible the *Leishmania* parasite load, the kinetic of *Leishmania* DNA observed using a semiquantitative PCR was substantially similar to those obtained with real-time PCR. In other words, no difference in terms of parasitologic cure or relapse were observed compared with the results obtained with the semiquantitative PCR, meaning that the clinical decision was largely unaffected by a more precise quantification. In fact, we have previously shown with our semiquantitative PCR to make possible a result in the six orders of magnitude that is in the dynamic range of six-eight log10 copies observed with real-time PCR.

At variance with the results of Mary and others who observed a lower parasite load in 3 HIV-infected patients at the time of relapse in comparison with the initial diagnosis of VL, our experience in 4 HIV-positive patients show higher parasite load in two patients, equal value in one patient, and a lower value in the remaining patient. However, with the low number of patients studied in both cases the different primers used and the possible differences in terms of biologic variables (CD4 cell count, HIV viral load) and treatment make any definitive conclusion unfeasible.

Regarding cutaneous leishmaniasis, it is now well accepted that PCR-based diagnosis is superior to histology and culture in our experience regarding imported and autochthonous CL all samples tested positive by either conventional or real-time PCR using in all but one case sample collected by skin scrapings. The PCR was able to detect either Old World *Leishmania* (e.g., *L. major* and *L. infantum*) or New World *Leishmania* (*L. braziliensis* panamensis), as previously shown by Schultz and others. However, in one case we were unsuccessful in the identification of the parasite at the species level by using PCR-RFLP. In two AIDS patients showing different cutaneous manifestations (PKDL and disseminated disease) the real-time PCR assay found a high value of parasites quantification. Worth noting was the fact that the patient with disseminated disease relapsed 1 year later and at this time the previously observed cutaneous lesions have completely regressed. However, a randomly done skin biopsy on intact skin was positive by either histology or PCR and quantitative PCR showed a very high parasite burden. This case raised the possibility that in the Mediterranean basin patients co-infected by HIV/Leishmania may act as reservoir for anthroponotic transmission of *Leishmania*. When interpreting the results of the present study, several limitations should be considered. First, as Bastien and others correctly highlights that PCR is not one technique but a method encompassing a number of techniques depending on a variety of factors. Therefore, a direct comparison between different PCR should not be made.

Second, the choice of different PCR targets should be guided by the aim to which it is directed. In fact, PCR-based assays using kinetoplast DNA targets are the most sensitive either for the diagnosis of visceral or cutaneous leishmaniasis, but they identify leishmanial parasites only to the generic or subgeneric level, whereas other PCR targeting intergenic regions in nuclear DNA are better when a rapid and reliable species identification is needed. Finally, although real-time qPCR
will become the reference technique in the future, because it is more rapid and less prone to contamination (no post-PCR handling is required) compared with conventional PCR, in our experience the cost is 3-fold higher ($€ 54 versus 17). The choice of a specific PCR assay will therefore depend on different criteria: a central reference laboratory would probably require using a real-time PCR, whereas single test applications could be done with conventional PCR. Furthermore, when sensitivity must be maximized (i.e., detection and quantification) high-copy number targets (e.g., rRNA genes, kinetoplast DNA minicircles) should be chosen.

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REFERENCES


