Protozoa of the genus *Leishmania* are the causative agents for leishmaniasis, a zoonotic and anthropontic disease that is endemic throughout tropical and subtropical regions (18). Twelve million people are affected worldwide, and 350 million are at risk. The incidence of new cases is estimated to be 1.5 to 2 million per year (2). Coinfection with *Leishmania* organisms and human immunodeficiency virus type 1 (HIV-1) has recently evolved as an additional major public health issue (1, 7, 13).

The clinical picture of leishmaniasis is heterogeneous and can be roughly classified into three major forms of increasing severity: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (12). CL involves ulcerative lesions of the skin, which in general are self-limiting but functionally impeding and cosmetically unsightly. It can be caused by any pathogenic *Leishmania* strain. MCL causes destructive lesions, especially of the nasopharyngeal mucosa and cartilages. In the vast majority of cases it is associated with parasites that belong to the *Leishmania brasiliensis* complex (*L. brasiliensis brasiliensis*, *L. brasiliensis panamensis*, *L. brasiliensis guanensis*), which are endemic or enzootic in the New World only (12). VL is a severe systemic disease characterized by destructive infiltration of organs and high rates of fatality in symptomatic patients. It is generally caused by parasites belonging to the *Leishmania donovani* complex, which are endemic in both the Old World (*L. donovani* and *L. infantum*) and the New World (*L. chagasi*) (12). Rare exceptions to these rules of etiological association do exist (3, 17, 30, 32).

Leishmaniasis exhibits symptoms that are very similar to those seen in several other diseases, and thus laboratory confirmation is mandatory when the diagnosis is suspected. Serological tests show a high degree of cross-reactivity and cannot discriminate between past and current infections. Their sensitivity is low for CL and MCL (16, 33). The serological diagnosis will furthermore yield false-negative results for about half of those patients coinfected with HIV-1 due to lack of detectable seroconversion (7, 14, 27). Therefore, detection of parasites is required in order to reliably confirm the diagnosis (6, 27). Parasites can be detected in clinical samples by histology or immunohistochemistry and parasite culture.

Furthermore, PCR has been applied successfully in recent years to detect *Leishmania* spp. in cases with any of the clinical manifestations of leishmaniasis. Some PCR methods also allow differentiation between parasite strains, which can facilitate more-efficient treatment (6). Several PCR protocols for combined detection and differentiation of parasites exist, including multiplex PCR (15), PCR plus sequencing (39), and restriction fragment length polymorphism (RFLP) analysis (24, 34). However, the multiple steps of post-PCR manipulation in these procedures require time and pose the risk of DNA contamination. Very recently, real-time PCR for *Leishmania* has been applied to circumvent these steps (4, 28, 41). Nevertheless, differentiation of parasites by real-time PCR has not been possible so far.

We have developed a real-time PCR, based on the fluorescence resonance energy transfer (FRET) technology (21, 35), that facilitates discrimination between three clinically relevant *Leishmania* groups (the *L. donovani* complex, the *L. brasiliensis* complex, and species other than these). It should be noted that the differentiation of parasites by real-time PCR has not been applied in other real-time PCRs, the variability in the quantification of DNA was small (coefficient of variation [CV], <2%). However, human samples containing low levels of parasites (100 parasites per ml of blood) showed higher variation (CV, 60.89%). Therefore, despite its superior analytical performance, care must be taken when real-time PCR is utilized for therapy monitoring.
complex, and others), as well as quantification of parasites, directly from clinical samples. The assay has been evaluated focusing on analytical sensitivity, discriminatory power, and reliability of quantification. Moreover, the applicability of the assay is demonstrated for the detection and simultaneous differentiation of *Leishmania* organisms directly from blood, bone marrow, and skin or live biopsy specimens.

### MATERIALS AND METHODS

**Parasites.** Cultured *Leishmania* reference strains were obtained from the strain collections of the Bernhard Nocht Institute, Hamburg, Germany; the Institute of Biochemistry, Charité, Berlin, Germany; and the Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Ga. (Table 1). The identities of all strains had been verified by zymodeme analysis and RFLP-PCR (34) or direct sequencing of ribosomal DNA (rDNA) internal transcribed spacer regions by using published primers (11). The strains were routinely cultivated at 25°C in supplemented Medium 199 (Sigma-Aldrich, Munich, Germany). After 3 to 5 days, promastigotes (11) were harvested, washed, and resuspended in phosphate-buffered saline.

**Clinical specimens.** EDTA-supplemented blood and bone marrow aspirates, as well as tissue biopsy specimens contained in 0.9% sodium chloride solution, were obtained from nine patients with confirmed leishmaniasis. The maximum time from sampling to processing was 2 days. The diagnosis in all cases had been established by previously described PCR (29), culture and microscopy, indirect immunofluorescence assay, enzyme immunoassay, or complement fixation test (Table 2).

**DNA extraction.** The Puregene DNA blood kit (Gentra Systems, Indianapolis, Ind.) was used for DNA extraction with minor modifications depending on the material tested.

For blood and bone marrow, 300 μl of the EDTA-supplemented sample was incubated for 15 min in buffer RBC at room temperature. White cells were pelleted by centrifugation at 10,000 × g for 2 min. The cell pellet was resuspended in 300 μl of buffer CLS (cell lysis solution; a component of the kit) and incubated with proteinase K (final concentration, 300 μg/ml) at 55°C for 2 h. Subsequent steps were carried out according to the manufacturer’s instructions, with the exception that DNA pellets were resuspended in 100 μl of DNA hydration solution. RNase A treatment was omitted from the procedure.

For skin and organ biopsy specimens, 2-mm-thick pieces were incubated overnight in 300 μl of buffer CLS containing the same concentration of proteinase K as above. The procedure described above was then followed.

For cultured parasites, about 10,000 promastigotes were inoculated in 300 μl of buffer CLS, incubated with proteinase K for 2 h, and processed as described above.

**Seeded blood samples.** Cultured promastigotes of *L. donovani* strain LO8 were counted cytocentrifugally and spiked in EDTA-supplemented blood from a healthy volunteer in concentrations of 50,000, 5,000, 1,000, 500, 100, 50, 10, and 1 parasite per ml. For each concentration, two different promastigote solutions were spiked into two different negative blood samples each, resulting in four independently prepared blood samples of each concentration.

**Selection of oligonucleotides and optimization of PCR.** An alignment of leishmanial 18S rDNA sequences was generated with the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST; query sequence M81429) (nucleotide positions are numbered accordingly). Oligonucleotides were chosen in suitable regions of the alignment by using the Primer Express software package, version 1.0 (Perkin-Elmer, Weiterstadt, Germany). A region spanning nucleotide positions 366 to 392 was chosen as a binding site for a probe (termed CDLP3) (Fig. 1). At this site, three different patterns of point mutations were present in three distinct *Leishmania* groups (*L. donovani* complex, *L. braziliensis* complex, and other *Leishmania* species). The calculated melting points of the template-probe hybrid were different for each of the three patterns. A second probe was designed in close proximity to the first probe (Fig. 1, probe CDLP5) to obtain a pair of FRET probes (fluorescent labeling; see below). Two different sense primers and two different antisense primers were selected upstream and downstream of the probes. All four possible combinations of primers were tested in a standard PCR containing *Leishmania* DNA. The most sensitive pair of primers (Fig. 1) was chosen for experimental optimization of PCR. In order to most accurately determine the optimal reaction conditions, various concentrations (n = 4) of one reaction component were tested simultaneously versus various concentrations (n = 4) of another component in the manner of a checkerboard titration (4 × 4 = 16 reactions). To implement this optimization strategy, real-time detection of PCR products was done with the pair of probes chosen above, which enabled exact measurement of yield and sensitivity. Criteria for optimum reaction efficiency were both the intensity of fluorescence at the end of the reaction (as high as possible) and the number of cycles needed before a signal became detectable (as low as possible). The determined optimum concentrations of these four parameters are given below.

**Real-time PCR.** PCR amplification was carried out in a reaction volume of 20 μl, including 8 μl of DNA solution, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 3.5 mM MgCl2, 400 μM each deoxynucleotide triphosphate, 350 nM primer CDLS (5′-GCT CCA AAA GCG TAT ATT AGT GCT GT-3′), 500 nM primer CDLA (5′-TCC TTC ATT CCT AGA GGC CGT GAG T-3′), 200 nM probe CDLP5 (5′-GGT TTG AAA GGT CTA TGG GAG ATT ATG GAG CGT TCG C-3′, label, 6-carboxyfluorescein), 200 nM probe CDLP3 (5′-CAA GGC CCT TTC CAT CC GAC CTC GGT-5′, label, LC red 640, 3′ phosphorylated), 0.8 μg of

### TABLE 1. Results for *Leishmania* strains

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference no.</th>
<th>Origin</th>
<th>Melting point (°C)</th>
<th>Melting point group</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. donovani</td>
<td>MHOM/IN/80/DD8</td>
<td>India</td>
<td>71.4</td>
<td>C</td>
</tr>
<tr>
<td>L. donovani</td>
<td>MHOM/CN/Wangie1</td>
<td>China</td>
<td>71.5</td>
<td>C</td>
</tr>
<tr>
<td>L. infantum</td>
<td>MHOM/TN/80/TTP1</td>
<td>Tunisia</td>
<td>71.7</td>
<td>C</td>
</tr>
<tr>
<td>L. chagasi</td>
<td>MHOM/BR/74/PP75</td>
<td>Brazil</td>
<td>71.5</td>
<td>C</td>
</tr>
<tr>
<td>L. donovani</td>
<td>LO8</td>
<td>Sudan</td>
<td>71.6</td>
<td>C</td>
</tr>
<tr>
<td>L. aethiopica</td>
<td>MHOM/ET/94/Abuaye</td>
<td>Ethiopia</td>
<td>64.9</td>
<td>A</td>
</tr>
<tr>
<td>L. aethiopica</td>
<td>MHOM/ET/94/1769</td>
<td>Ethiopia</td>
<td>64.9</td>
<td>A</td>
</tr>
<tr>
<td>L. aethiopica</td>
<td>MHOM/ET/94/1470</td>
<td>Ethiopia</td>
<td>64.9</td>
<td>A</td>
</tr>
<tr>
<td>L. tropica</td>
<td>MHOM/SU/58/OD</td>
<td>Soviet Union</td>
<td>65.0</td>
<td>A</td>
</tr>
<tr>
<td>L. tropica</td>
<td>MHOM/SU/74/k27</td>
<td>Soviet Union</td>
<td>65.0</td>
<td>A</td>
</tr>
<tr>
<td>L. tropica</td>
<td>MHOM/IQ/66/L75</td>
<td>Iraq</td>
<td>65.0</td>
<td>A</td>
</tr>
<tr>
<td>L. tropica</td>
<td>MHOM/IL/80/Singer</td>
<td>Israel</td>
<td>65.0</td>
<td>A</td>
</tr>
<tr>
<td>L. tropica</td>
<td>TRO-33</td>
<td>Turkey</td>
<td>64.9</td>
<td>A</td>
</tr>
<tr>
<td>L. major</td>
<td>MHOM/SU/73/5-ASKH</td>
<td>Soviet Union</td>
<td>65.0</td>
<td>A</td>
</tr>
<tr>
<td>L. mexicana amazonensis</td>
<td>LV9</td>
<td>Brazil</td>
<td>64.9</td>
<td>A</td>
</tr>
<tr>
<td>L. tropica</td>
<td>MHOM/IR/72/NADIM3</td>
<td>Iran</td>
<td>64.7</td>
<td>A</td>
</tr>
<tr>
<td>L. aethiopica</td>
<td>BHI</td>
<td>Ethiopia</td>
<td>65.0</td>
<td>A</td>
</tr>
<tr>
<td>L. mexicana mexicana</td>
<td>L.MEX.MEX.</td>
<td>Mexico</td>
<td>64.9</td>
<td>A</td>
</tr>
<tr>
<td>L. brasiliensis brasilensis</td>
<td>MHOM/BR/75/M2903</td>
<td>Brazil</td>
<td>66.9</td>
<td>B</td>
</tr>
<tr>
<td>L. brasiliensis brasilensis</td>
<td>L.BRAS.</td>
<td>Brazil</td>
<td>67.0</td>
<td>B</td>
</tr>
</tbody>
</table>

* C. *L. donovani* complex; B. *L. brasiliensis* complex; A. other strains.
bovine serum albumin (Sigma Aldrich, Munich, Germany), and 1 U of AmpliTaq Gold DNA polymerase (PCR reagents were from Applied Biosystems, Weiterstadt, Germany; oligonucleotides were from Tib-Molbiol, Berlin, Germany). Precautions to avoid laboratory contamination, as suggested by Kwok and Higuchi (19), were strictly complied with. Amplification was conducted in sealed 20-μL LightCycler glass capillaries. Thermal cycling in a Roche LightCycler, version 3.5, comprised an initial denaturation at 95 °C for 15 min, followed by 45 cycles of 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 40 s. A final melting curve analysis was performed by initial denaturation at 95 °C for 10 s, followed by 50 °C for 15 s and continuous heating at 0.1 °C/s to 95 °C. A reaction was considered positive when the normalized fluorescence signal at the end of PCR (ratio of the signal from detection channel F2 [640 nm] to the signal from detection channel F1 [495 nm], read at 55 °C) showed an exponential increase in fluorescence.

The melting temperatures of probe-template hybrids were automatically determined

<table>
<thead>
<tr>
<th>Case no.</th>
<th>History</th>
<th>Result(s) a by:</th>
<th>Melting point (°C) b by FRET-PCR (group c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acute case; unknown travel route</td>
<td>IIFA – BM+ BM+ BM+ BM+</td>
<td>BM, 71.3 (C)</td>
</tr>
<tr>
<td>2</td>
<td>Trip to Morocco; HIV positive</td>
<td>IIFA – BM+ BM+ BM+</td>
<td>L. donovani, 71.3 (C)</td>
</tr>
<tr>
<td>3</td>
<td>Trips to Saudi Arabia and Turkey; nodule on hand</td>
<td>IIFA – SB+ SB+ SB+</td>
<td>L. donovani, 71.9 (C)</td>
</tr>
<tr>
<td>4</td>
<td>Trip to Spain; 5-week history of fever; splenectomized patient</td>
<td>IIFA, 1:320 ND BM+ BM+</td>
<td>BM, 72.2 (C)</td>
</tr>
<tr>
<td>5</td>
<td>Trip to Italy; ascites, fever, elevated liver enzymes; immunosuppressive therapy</td>
<td>EIA – BM+ BM+ BM+</td>
<td>L. donovani, 71.8 (C)</td>
</tr>
<tr>
<td>6</td>
<td>Trip to Costa Rica; ulcer on arm</td>
<td>IIFA – SB+ SB+ SB+</td>
<td>L. brasiliensis panamensis, 66.9 (B)</td>
</tr>
<tr>
<td>7</td>
<td>Trip to Guatemala; solid pretilial skin ulcer</td>
<td>IIFA, 1:20 ND SB– SB+</td>
<td>L. brasiliensis panamensis, 67.6 (B)</td>
</tr>
<tr>
<td>8</td>
<td>Trip to Niger; skin ulcer and enlarged lymph node on neck</td>
<td>EIA, 25 CFA, 1:4 SB– SB+</td>
<td>L. major, 65.5 (A)</td>
</tr>
<tr>
<td>9</td>
<td>Trip to Southeast Asia; nodules and erythema on nose</td>
<td>ND ND SB– SB+</td>
<td>L. tropica, 65.3 (A)</td>
</tr>
</tbody>
</table>

a IIFA, indirect immunofluorescent assay (titers of ≥1:80 are considered positive); EIA, enzyme immunoassay (a numerical result of ≥30 antibody units is considered positive); CFA, complement fixation assay (titers of ≥1:16 are considered positive). BM, bone marrow aspirate; PB, peripheral blood sample; SB, skin biopsy specimen; LB, liver biopsy specimen. ND, not done; i, inhibited.

b C, L. donovani complex; B, L. brasiliensis complex; A, other strains.

c See reference 28.
RESULTS

Analytical sensitivity. The analytical sensitivity of a PCR test is determined by prerequisite steps such as preparation of DNA and the efficacy of the amplification reaction itself. To test the efficacy of the reaction, i.e., formulation and thermal cycling profile, the target region of strain M2903 (L. brasilensis) was cloned into Escherichia coli. Ten replicates of a limiting dilution series of plasmids were then amplified. An average of 10 plasmids per reaction could be detected in all attempts (10 of 10). Reactions containing an average of one plasmid were occasionally positive, indicating that the reaction conditions were optimized to the possible maximum (note that only some reactions could be positive due to random distribution of plasmids among samples). The same sensitivity was achieved with cloned DNA from L. donovani strain DD8 and L. tropica strain K27.

Next, the efficacy of the combined procedure of PCR, including the extraction of DNA, was tested. To this end, blood samples spiked with defined amounts of Leishmania parasites (1,000, 500, 100, 50, 10, and 1 promastigote per ml) were prepared. Four samples of each concentration were extracted and amplified in triplicate (n = 12 reactions). At concentrations of 1,000 and 500 parasites per ml, 12 of 12 reactions turned out positive. Detection rates at 100, 50, 10, and 1 parasite per ml were 11 of 12, 8 of 12, 8 of 12, and 1 of 12, respectively.

Finally, the probability of achieving a positive PCR result, depending on the Leishmania input concentration, was determined by probit regression analysis (Fig. 2). The calculated minimum concentration at which 95% of results could be expected to be positive was 94.1 parasites/ml (95% confidence interval [95% CI], 7.0 to 145.3 parasites/ml).

Detection of Leishmania organisms in clinical samples. A total of 12 clinical samples from nine patients with imported leishmaniasis, including 4 bone marrow aspirates, 2 peripheral blood samples, 5 skin biopsy specimens, and 1 liver biopsy specimen, were tested in the new assay (Table 2). Parasite cultures yielded positive results in 4 of 6 (66.7%) blood or bone marrow samples as well as in 2 of 6 (33.4%) skin or liver biopsy samples. Only two of eight serologically tested patients yielded a positive result by at least one of three methods (immunofluorescence, enzyme immunoassay, and complement fixation test). The new real-time PCR, as well as conventional PCR (29), detected parasites in all samples.

Genotyping. Plasmids containing the real-time PCR target region from L. brasilensis brasilensis strain M2903, L. donovani strain DD8, and L. tropica strain K27 were amplified in parallel with whole-parasite DNA of the corresponding strains. In agreement with the calculation of melting points, each strain exhibited a distinct melting curve (Fig. 3). The melting curves for the plasmids and the corresponding reference strains were congruent, indicating that the melting temperature depended solely on the 18s rDNA sequence and not on the sequence of the rest of the genome.

To test the melting point interassay variability, each plasmid was included in 11 different test runs. The means of the automatically determined melting points for L. donovani DD8, L. brasilensis M2903, and L. tropica K27 were 72.14, 67.58, and 65.27°C, respectively. Coefficients of variation (CV) were extremely small (0.68, 0.2, and 0.28%, respectively).

Next we assessed whether melting curve analysis would allow genotyping in cultured and clinical samples. To this end, another 17 characterized cultured Leishmania strains, as well as 12 characterized clinical samples, were tested together with the reference strains mentioned above (Tables 1 and 2). The 95% CIs of the three resulting groups of melting points did not overlap; mean melting points were 71.7°C for L. donovani complex (95% CI, 71.5 to 71.8°C), 67.1°C for L. brasilensis complex (95% CI, 66.5 to 67.6°C), and 65.0°C for other Leishmania species (95% CI, 64.9 to 65.1°C). All culture samples and all clinical samples containing parasites of the L. donovani complex could be grouped together with L. donovani donovani reference strain DD8; those containing parasites of the L. brasilensis complex could be grouped together with L. brasilensis brasilensis strain M2903; and samples containing parasites belonging to neither of these complexes were all grouped together with reference strain L. tropica strain K27.
Quantification of parasite DNA. Because the rDNA binding regions of the detection probes were not identical for different Leishmania genotypes, it should be clarified whether this might interfere with the results of quantification. In order to ensure that parasites of all three groups could be quantified with the same accuracy and within the same concentration range, the first detectability of a PCR signal (crossing point) and the input DNA concentration was determined for six, three, and six samples containing 100, 500, and 1,000 parasites per ml of blood (x axis), respectively. Single samples containing 5,000 and 50,000 parasites per ml were included to allow linear regression of the data. In order to ensure that parasites of all three groups could be quantified with the same accuracy and within the same concentration range, the first detectability of a PCR signal (crossing point) and the input DNA concentration was determined for six, three, and six samples containing 100, 500, and 1,000 parasites per ml of blood (x axis), respectively. Single samples containing 5,000 and 50,000 parasites per ml were included to allow linear regression of the data.

**DISCUSSION**

In this report we describe the establishment of a real-time PCR assay for detection of Leishmania in clinical samples. The test is based on the application of FRET probes that provide a rough genotypic characterization of the parasite. Three clinically relevant groups (L. donovani complex, L. braziliensis complex, other Leishmania spp.) can be discriminated reliably. The utilization of an inexpensive DNA preparation and the use of basic PCR reagents instead of a preformulated master mix promote the application of the assay in resource-poor settings. Test costs for DNA preparation, PCR reagents, and consumables in our laboratory are less than U.S. $3 per reaction (without license fees).

Our primers target a portion of the 18S rDNA of Leishmania. This sequence has been found to be highly sensitive for the confirmation of leishmaniasis from peripheral blood (20, 23, 40) as well as tissue biopsy specimens (26, 41). To standardize and compare diagnostic PCR procedures between laboratories, the analytical performance of an assay must be accurately characterized. We therefore evaluated two analytical levels of our procedure separately: first, the reaction sensitivity of the PCR reagents and thermal cycling profile, and second, the analytical sensitivity of the complete procedure including extraction of DNA. For testing of the reaction sensitivity, purified plasmid DNA was directly spiked into the PCR, avoiding interfering effects of low DNA quality or inhibiting substances present in samples. The detection frequency achieved under these conditions (occasional detection of 1 copy, reliable detection of 10 copies)
shows that the reaction components work at maximum efficiency.

When blood samples seeded with *L. donovani* LO8 promastigotes were tested, 95% of tests turned out positive with at least 94.1 parasites present per ml. While our seeded samples are slightly different from the blood of infected humans, in which *Leishmania* organisms are present within macrophages in amastigote form, we believe that the sensitivity under these circumstances would be similar, since leukocyte DNA and parasite DNA are coprocessed in our procedure. A sensitivity in this range has been determined by Lachaud et al. in a huge patient cohort to be clinically sufficient to diagnose VL and VL-AIDS coinfection solely from peripheral blood samples (20).

Confirmation of CL and MCL by PCR requires testing of tissue biopsy specimens. However, we did not evaluate our test with seeded tissue samples because the analytical sensitivity limit that is clinically required in PCR diagnosis of CL and MCL is not known. Nevertheless, the fact that all six tissue biopsy specimens available tested positive in our assay, while only two tested positive by culture and three by histology, suggests that the sensitivity of PCR may be superior. This is consistent with other findings concerning PCR-based diagnosis of CL (31). The comparably low sensitivity of culture (23, 31) is attributable to the fact that several samples were sent to our laboratory over long distances, probably leaving no parasites alive. Consequently, it can be considered an additional benefit of PCR that DNA can be detected even from dead parasites or old samples.

In a comparison of the serological patient data to the results of PCR, only one of four PCR-positive patients yielded positive results in any of three serologic tests. Though these results are in concordance with earlier findings (13, 33), the clinical sensitivity figures obtained here are of a preliminary nature only, and larger groups of patients will have to be studied.

To discriminate relevant groups of *Leishmania* spp., FRET probe binding sites were selected that generate distinct melting complexes other than these. Since these groups are associated with distinct clinical courses, our test may allow prediction of the clinical manifestations that a patient will develop. Furthermore, in selected situations, the combination of a certain melting point group with an unusual clinical course can allow identification of highly virulent strains (e.g., recurrent VL caused by *L. tropica* in India [37]). Early clinical decisions can be based on these data.

Several reports exist on drug resistance in VL (36, 38). When leishmaniasis is treated, the response to treatment can be judged by the disappearance of parasites in lesions, blood, or organs such as bone marrow or the spleen. The concentration of parasite DNA in such a body compartment, as well as its tendency to rise or fall under therapy, may become a valuable tool for identifying resistant strains or noncompliant patients.

Three studies involving real-time quantitative *Leishmania* PCR have been conducted so far, suggesting that the method may allow monitoring of the parasite concentration. In two studies, the intra-assay variability of quantification has been determined to be 0.43 to 1.25% (28) and 1 to 1.6% (4), respectively. Our results were in the same range (<2%), which is concordant with results generally seen in testing of water-diluted DNA by real-time PCR (5, 8–10, 22; our unpublished data). However, in none of the studies conducted so far have human samples containing defined amounts of parasites been tested. Moreover, the variation in the low concentration range, which is obviously most relevant for monitoring the therapeutic outcome, has not been determined. When we tested blood samples seeded with small amounts of parasites, we observed a considerable degree of variation (up to 60.89% at 100 parasites/ml). Most probably, this resulted from an unequal distribution of low-level parasitemia in different aliquots of test samples (Poisson distribution). Consequently, for determining parasitemia in patients, replicate testing and averaging of results must be recommended so that reliable results can be obtained. Further studies will be required to confirm these results, and serial samples from various patients under treatment will be necessary to clinically evaluate the usefulness of real-time PCR for monitoring the success of antileishmanial therapy.

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