Diagnostic accuracy of a new *Leishmania* PCR for clinical visceral leishmaniasis in Nepal and its role in diagnosis of disease

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Summary

OBJECTIVE To develop a new PCR for *Leishmania* detection and to estimate its diagnostic accuracy in a visceral leishmaniasis (VL) endemic area.

METHODS After providing the proof-of-concept, the diagnostic accuracy was estimated on blood from 247 non-endemic control persons and on blood and bone marrow from 173 confirmed VL, 39 probable VL and 87 non-VL patients from south-eastern Nepal.

RESULTS The PCR showed a specificity of 99.64% [95% confidence interval (CI): 98.93–100%) on non-endemic controls and a sensitivity of 92.1% (95% CI: 87.6–96.6%) on blood and 92.9% (95% CI: 89–96.8%) on bone marrow from the confirmed VL patients. *Leishmania* DNA was detected in blood and bone marrow of 67.6% (95% CI: 50.8–80.9%) and 71.8% (95% CI: 56.2–83.5%) of the probable VL patients, respectively, and of 38.2% (95% CI: 28–49.4%) and 29.9% (95% CI: 21.3–40.2%) of the non-VL patients, respectively. The PCR showed 97% concordance with a positive DAT status while for a negative DAT status this was only 41.3% (kappa-index 0.416, 95% CI: 0.30–0.53).

CONCLUSIONS Our findings indicate that PCR alone rather provides a marker for infection than a marker for disease and its role in VL diagnosis in endemic regions is discussed.

keywords visceral leishmaniasis, diagnosis, PCR, sensitivity and specificity, diagnostic accuracy, asymptomatic infection

Introduction

Visceral leishmaniasis (VL) is a potentially fatal vector-borne parasitic disease due to infection by *Leishmania donovani* in the Indian subcontinent and eastern Africa and by *L. infantum* in the Mediterranean basin, Central Asia and South America. The Indian subcontinent is one of the most affected regions and Nepal together with India and Bangladesh account for 70% of all reported deaths (Unicef 2006). Diagnosis is currently based on antibody detection in blood with the direct agglutionation test (DAT) or rk39 dipstick, while antigen detection with the KATEX test in urine is still under evaluation (Boelaert *et al.* 2008). The disease is generally confirmed by microscopic demonstration of the parasite in bone marrow or spleen aspirates, requiring invasive sampling techniques. Sensitivity may be increased by prior *in vitro* cultivation of the parasite from these specimens but this is cumbersome and time-consuming.

Amplification of the parasite's DNA by the polymerase chain reaction (PCR) has evolved as one of the most specific and sensitive surrogate methods for parasite detection. Over the last two decades many PCR applications for detecting *Leishmania* have been reported (Antinori *et al.* 2007). Attractive *Leishmania* genome target regions for PCR are high-copy sequences such as the small subunit ribosomal RNA (rRNA) gene (van Eys *et al.* 1992), the kinetoplast DNA (Maurya *et al.* 2005), or the spliced leader mini-exon gene (Reale *et al.* 1999). Despite the wealth of existing PCR assays, very few have been sufficiently validated on a large number of clinical samples and none of them has become a reference tool in leishmaniasis diagnosis.

We here present a new PCR for *Leishmania* detection, targeting a short sequence within the 18S rRNA gene. After providing the proof-of-concept, we evaluated the PCR on experimental samples and on a large series of non-endemic controls. Finally, we estimated the diagnostic accuracy of the test on a consecutive series of blood and bone marrow samples from 299 patients suspected of VL recruited in Nepal between 2000 and 2002.

Materials and methods

Parasite DNA

DNA from *L. donovani* (MHOM/SD/00/1S), *L. infantum* (MHOM/67/MA/ITMAP263), *L. braziliensis* (MHOM/PE/93/LC2177), *L. peruviana* (MHOM/PE/90/HB44), *L. guyanensis* (MHOM/BR/75/M5378), *L. amazonensis* (MHOM/BR/73/M2269), *L. panamensis* (MCHO/PA/00/M4039), *L. lainsoni* (MHOM/PE/92/LC1581), *L. major* (MHOM/SU/73/5-ASKH), *L. aethiopica* (MHOM/ET/72/L100) and *L. tropica* (MHOM/SU/74/K27) and from *Trypanosoma brucei gambiense* (MBA strain) and *Trypanosoma cruzi* (Y strain) was obtained from the DNA reference bank at the Institute of Tropical Medicine Antwerp (ITMA). DNA from *Mycobacterium tuberculosis*, *Plasmodium falciparum* and *Schistosoma mansoni* was obtained from other research groups.

Samples

The human experimentation guidelines of the ITMA were followed. Ethical clearance for the study was obtained from the institutional review boards in Nepal and Belgium.

Spiked blood

Leishmania donovani promastigotes were grown in GLSH medium (Le Ray 1975) with 10% foetal calf serum at 26 °C. At day 3, post-inoculation, parasites were counted in a Bürker counting chamber (Marienfeld, Germany) and 10-fold dilution series were made in human blood ranging from 10 000 to 1 parasite/180 μl blood. Non-spiked blood was used as a negative control.

VL patients and endemic patient controls

Blood and bone marrow samples from clinically suspected VL cases were collected in the framework of a study conducted between 2000 and 2002 at the B.P. Koirala Institute of Health Sciences in Dharan, Nepal (Boelaert *et al.* 2004). This study compared the diagnostic accuracy of 4 serological tests on 310 clinically suspected VL cases. Clinical suspicion for VL was defined as a history of fever for 2 weeks or more and splenomegaly. Bone marrow aspirates were analysed by microscopy and positive smears were scored from 1 to 6 according to the WHO classification (WHO 1990). If the bone marrow aspirate was negative and malaria had been ruled out, a spleen aspirate was proposed. The DAT and rK39 dipstick were performed on the patient's serum in 2000–2002 as previously described. PCR analysis was performed in June 2007. The patient cohort

was divided into three classes as follows: (i) *confirmed VL patient* if positive in parasite detection on bone marrow and/or spleen aspirate, (ii) *probable VL patient* if negative in parasite detection but seropositive in the rk39 dipstick and/or if DAT titre was 1:3 200 or more or when the patient showed positive response to sodium stibogluconate treatment and (iii) *non-VL patient* if negative in parasite detection; negative in rk39 dipstick and DAT titre was below 1:3 200 and therapeutic response was observed when other treatment than VL treatment was given. The HIV status was tested during the study between 2000 and 2002 for 303 patients who gave consent. Sera were screened with the HIV 1&2 Double Check (Orgenics Ltd., Israel) where after seropositive samples were confirmed using the INNO-LIA HIV I/II Score assay (Innogenetics, Ghent, Belgium).

Non-endemic controls

We defined VL non-endemic regions as regions where no active transmission of VL is reported. However, we cannot exclude sporadic autochthonous or imported cases in those areas. DNA extracts from blood of 19 healthy human Belgian blood donors, 25 confirmed *T.b. gambiense* infected persons from the Democratic Republic of the Congo and 230 confirmed *P. falciparum* infected persons from Benin (70), Burkina Faso (100) and Thailand (60) were obtained from the collection of the ITMA. All samples were anonymous. *T.b. gambiense* and *P. falciparum* infections were confirmed by direct parasite detection on blood.

DNA extraction from bone marrow and blood samples

Bone marrow aspirates were sent frozen to ITMA and DNA was extracted for further PCR analysis using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). The 180 μl blood samples were stored in AS1 buffer (Qiagen), shipped to ITMA at ambient temperature and DNA was extracted with the QIAamp DNA blood mini kit. DNA extracts were stored at −20 °C until PCR analysis.

Primers

Sequences of the 18S rRNA gene of the *Trypanosomatidae* parasites *L. donovani* (GenBank accession number X07773), *T.b. gambiense* (AJ009141) and *T. cruzi* (AF303660) were aligned and *Leishmania* primers were designed using the 'DNAman' software (Lynnon Corporation, Quebec, Canada). The sense primer 18S-L-F 5′-CGTAGTTGAACTGTGGGCTGTGC-3′ and the anti-sense primer 18S-L-R 5′-ACTCCCGTGTTTCTT-

GTTTCTTTGAA-3′ amplify a 115 bp sequence within the 18S rRNA gene of *Leishmania*.

PCR amplification

The 50 μl reaction mixture contained 1 × Qiagen PCR buffer, 2.5 mM MgCl$_2$ (Qiagen), 200 μM of each dNTP (Roche, Mannheim, Germany), 0.8 μM of each primer (Sigma, Bornem, Belgium), 0.1 mg/ml acetylated bovine serum albumin (Promega, Madison, WS, USA), 1 U of HotStar *Taq* polymerase (Qiagen) and 5 μl of sample DNA. An initial denaturation step at 94 °C for 15 min to activate the Hot Star *Taq* polymerase was followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s with a final extension at 72 °C for 5 min. Amplification was conducted in 200 μl thin-wall PCR tubes (Abgene, Epsom, UK) in a T3 thermocycler 48 (Biometra, Göttingen, Germany). Amplified products were analysed by electrophoresis in a 2% agarose gel (Eurogentec, Seraing, Belgium) and U.V. illuminated (Syngene, Cambridge, UK) after ethidium bromide staining (Sigma, Bornem, Belgium).

Agreement PCR and DAT

The concordance between the PCR status and the DAT status was estimated using the kappa index. The PCR status was considered positive when a positive PCR result was observed on blood and/or bone marrow and negative when both blood and bone marrow were PCR negative. The DAT status was considered positive when the DAT titre was 1:3 200 or more.

Results

Analytical sensitivity

The detection limit of the PCR was evaluated on a tenfold serial dilution of *L. donovani* DNA in water containing 0.1 mg/ml acetylated BSA and on blood sample series spiked with decreasing numbers of living *L. donovani* promastigotes. The lower detection limits of the PCR were 10 fg DNA and 1 parasite/180 μl blood (Figure 1).

Analytical specificity

The analytical specificity of the PCR was assessed with purified DNA from target *Leishmania* species and from relevant non-target parasites at a concentration of 1 ng per assay. The PCR amplified DNA from all *Leishmania* species tested, while no amplification was observed with DNA from *P. falciparum*, *M. tuberculosis* and *S. mansoni*. To verify the broad applicability of the PCR in other regions than Nepal we tested DNA from the closely related parasites *T.b. gambiense* and *T. cruzi* and no cross-reaction was observed.

Specificity on non-endemic control blood samples

The specificity of the PCR was evaluated on the 274 non-endemic control blood samples. One sample from Thailand was positive in the PCR thus yielding a specificity of 99.64% with a 95% confidence interval (CI) scored by Wilson's method (Wilson 1927) of 98.93–100%.

Diagnostic accuracy in VL patients and endemic patient controls

Confirmed VL patients (n = 184)
DNA extracts were available from 173 patients (140 blood and 170 bone marrow samples). The PCR showed a positive test result in 129 of the 140 blood and in 158 of the 170 bone marrow samples indicating a sensitivity of 92.1% (95% CI: 87.6–96.6%) and 92.9% (95% CI: 89–96.8%), respectively. From the 127 patients with PCR positive bone marrow and for whom PCR data on blood
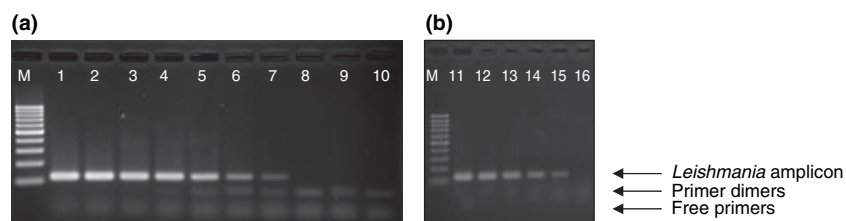
Figure 1 Analytical sensitivity of the 18S PCR on (a) a serial dilution of *L. donovani* DNA in water and (b) on a serial dilution of *L. donovani* promastigotes in naïve human blood. Lanes 1–10: 10 ng; 1 ng; 100 pg; 10 pg; 1 pg; 100 fg; 10 fg; 1 fg DNA per PCR, negative control for dilution, negative control for PCR. Lanes 11–16: 10 000; 1 000; 100; 10; 1; 0 parasites per 180 μl human blood. M: 100 bp DNA marker (Fermentas, St.Leon-Rot, Germany).

were available, 120 were also PCR positive on blood. Out of the 12 PCR negative bone marrow samples, 2 were negative in microscopy (but positive in microscopy on spleen, grade 1), and the 10 others showed low to moderate parasite loads (4 with grade 1, 3 with grade 2 and 3 with grade 3).

Probable VL patients (n = 39)
DNA extracts were available from 34 blood and 39 bone marrow samples. Twenty-three blood samples (67.6%, 95% CI: 50.8–80.9%) and 28 bone marrow samples (71.8%, 95% CI: 56.2–83.5%) were positive in PCR.

Non-VL patients (n = 87)
DNA extracts were available from 76 blood and 87 bone marrow samples. *Leishmania* DNA could be detected in 29 of the 76 blood samples (38.2%, 95% CI: 28–49.4%) and 26 of the 87 bone marrow samples (29.9%, 95% CI: 21.3–40.2%). Thus the specificity for acute VL disease of the PCR was 61.8% (95% CI: 50.6–71.9%) and 70.1% (95% CI: 59.8–78.7%) on blood and bone marrow, respectively.

If we consider the confirmed VL group and the probable VL group as one group of VL cases, the sensitivity of conventional microscopy on 223 patients is 82.5% (95% CI: 77–86.9%) while the PCR shows a sensitivity of 87.4% (95% CI: 81.6–91.5%) and 89% (95% CI: 84–92.6%) on the 174 and 209 available DNA extracts from blood and bone marrow, respectively.

Fourteen of the 303 patients tested for HIV infection showed a positive test result with the HIV 1&2 Double Check, of which four were confirmed with the INNO-LIA HIV I/II Score assay (one patient belonging to the probable VL group and three to the non-VL group). Three patients showed a doubtful positive test result with the HIV 1&2 Double Check but none were confirmed with the INNO-LIA HIV I/II Score assay.

Agreement between PCR and DAT

The PCR status of blood and bone marrow within each DAT titre group up to 1:204 800 is shown in Figure 2a. Of 131 patients with a positive DAT (titre ≥1:3 200) and for which PCR data on blood and/or bone marrow were available, 127 were positive in PCR while 38 of the 92 patients with a negative DAT were also negative in PCR (Figure 2b). Thus we observed a concordance on positive status in 97% and on negative status in 41.3% with a kappa index of 0.416 (95% CI: 0.30–0.53) indicating moderate agreement. The same trend was observed when analysing the PCR results on blood and bone marrow separately (data not shown).
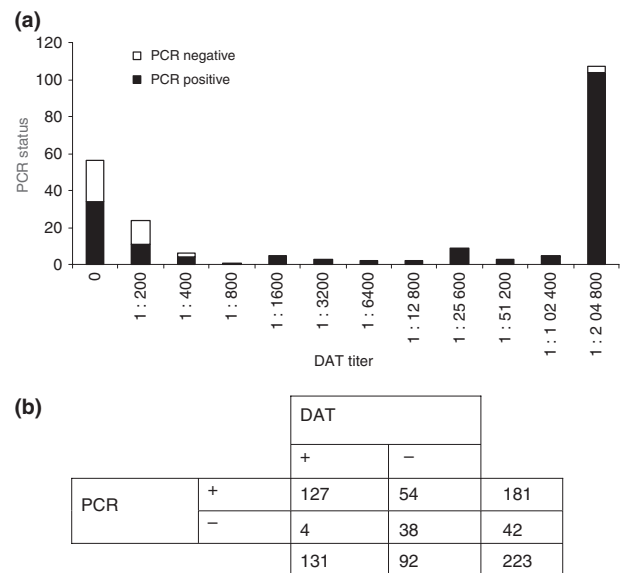
Figure 2 (a) PCR status by DAT titre on 223 patients with febrile splenomegaly from Nepal. PCR positive: positive in blood and/or in bone marrow, PCR negative: negative in both blood and bone marrow. (b) Two by two table of the PCR status and DAT status of 223 patients with febrile splenomegaly from Nepal.

Discussion

We here report on the phases I and II evaluation of a new *Leishmania* PCR targeting a short sequence in the parasite's 18S rRNA gene. A shorter DNA fragment was amplified compared to the existing rRNA gene PCRs (van Eys *et al.* 1992) in order to (i) obtain higher PCR yield, (ii) shorten the elongation time during PCR and (iii) be able to transform the PCR into a simplified dipstick format, the *Leishmania* OligoC-TesT (Deborggraeve *et al.* in press). The lower detection limits of the PCR are 10 fg of purified *Leishmania* DNA and one single parasite in 180 μl human blood. No cross reaction with DNA from non-target parasites was observed while all target species can be detected. Because of the high similarity of the primer target sequences, our PCR might give positive results in *Endotrypanum* and the monoxenous trypanosomatids *Crithidia*, *Wallaceina* and *Leptomonas*. While the chances of finding such protozoa in immune competent patients are negligible, they may present as opportunistic infections in immune compromised patients (Chicharro & Alvar 2003). Furthermore, these trypanosomatids should be taken into account when testing wild animals and vectors (Arias *et al.* 1985; Dereure *et al.* 2001). If needed, confirmation would be possible by direct sequencing of the PCR product since *Leishmania* possess a series of specific point mutations within the targeted region (data not shown).

The sensitivity of our PCR was first evaluated on 173 confirmed VL patients, revealing a sensitivity of 92.1% on blood and 92.9% on bone marrow. The high sensitivity of the PCR on blood together with the observation that 94.5% of the patients with a positive PCR on bone marrow were also PCR positive on blood is encouraging. Indeed, this means that the molecular diagnosis on blood may be as sensitive as on invasive bone marrow aspirates. *Leishmania donovani* might be naturally more abundant in blood than *L. infantum*, thus favouring its anthroponotic transmission. The lack of a positive PCR result in some of the microscopically confirmed bone marrow aspirates can be due to inhibition during PCR, low parasite load given the low grades scored during microscopy and the delay between sample collection and PCR testing (up to 7 years). Furthermore, although usually deemed as 100% specific, false positive microscopic results due to staining artefacts cannot be excluded. Misclassification is most likely to occur in those specimens read as 'low parasite load'. Moreover, several cases in the probable VL group might be true VL cases, because of the suboptimal sensitivity of the reference test used. If all the probable VL are considered as true VL and added to the confirmed VL cases, we observed a significantly higher sensitivity of PCR on bone marrow compared to conventional microscopy (89% *vs.* 82.5%; Mc Nemar's test: *P* < 0.05).

The specificity of the PCR was first tested on 274 non-endemic control blood samples and 99.64% specificity was observed. The positive PCR result on one *P. falciparum* infected patient from Thailand might be due to a *Leishmania* infection since sporadic autochthonous VL cases have been reported in this country (Kongkaew *et al.* 2007) and since imported VL cases cannot be excluded. Secondly, the specificity was evaluated on Nepalese patients showing longstanding fever and splenomegaly but negative VL serology and microscopy and whose symptoms were attributed to another etiology. In this group of non-VL patients, *Leishmania* DNA was amplified in 38.2% of the blood samples and in 29.9% of the bone marrow samples. The high number of positive PCR results in the non-VL group is in fact not surprising, given the known high number of asymptomatic *L. donovani* carriers in endemic areas, which might develop febrile splenomegaly for other reasons. Though our PCR is not able to discriminate between infection and disease, quantitative real-time PCR might be helpful. Mary *et al.* (2004) observed a correlation between the parasite load and the clinical status of *L. infantum* infected persons by quantitative real-time PCR. A technological less complex alternative could be the combination of a very and a moderately sensitive PCR, as described by Lachaud *et al.* (2000). Studying the concordance between PCR (DNA detection) and DAT (antibody detection), we observed a high concordance for a positive status and a low concordance for a negative status. In the DAT negative asymptomatic carriers the *Leishmania* infection is controlled mainly by the host's cell-mediated immune response. Four patients showed a positive HIV status of which three belonged to the non-VL group. Hence, negative DAT results for these three patients can be due to HIV co-infection.

Asymptomatic infections are currently not treated in VL endemic regions since the available drugs are very toxic and to avoid the raise and spread of resistant strains. Consequently, PCR provides a good marker for infection but may be of less value as a marker for acute clinical disease in VL endemic regions. However, immunodiagnosis faces the same limitations since a significant proportion of healthy individuals living in VL endemic regions have anti-*Leishmania* antibodies in the blood (Sundar *et al.* 2006). Therefore PCR and antibody detection test results should always be interpreted in combination with a standardized clinical VL case definition. Although its reported high sensitivity and specificity, PCR might currently not be able to compete with the immunodiagnostic tests due to the complexity of the technique and the lack of standardization. Efforts for simplification and standardization of PCR should therefore be strongly encouraged. At present, PCR can offer an added value compared to immunodiagnosis in HIV co-infected VL patients as well as in patient follow-up after treatment. Antibodies remain detectable for years after successful treatment (Hailu 1990) while the parasite's DNA is rapidly degraded following parasite death (Prina *et al.* 2007). Finally, as it is potentially one of the best markers of *Leishmania* infection, further evaluation of the PCR as a tool for estimating prevalence and incidence of infection in epidemiological studies is recommended.

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