Study of parasite kinetics with antileishmanial drugs using real-time quantitative PCR in Indian visceral leishmaniasis

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Objectives: This study describes parasite kinetics in the blood of visceral leishmaniasis patients treated with liposomal amphotericin B (L-AmB) or a preformed fat emulsion of amphotericin B (ApL) using real-time quantitative PCR (qPCR).

Methods: Forty-six patients were treated with a single dose (15 mg/kg of body weight) of either L-AmB ($n=13$) or ApL ($n=33$). qPCR was used to estimate parasite kinetics by detection of *Leishmania donovani* DNA using kinetoplast DNA-specific primers in peripheral blood samples using an absolute quantification method.

Results: The mean parasite load decreased from baseline (day 0) values of 894.07 and 980.48 to 71.72 and 211.52 parasite genomes/mL at day 7 in L-AmB and ApL groups, respectively, and at day 30 these further declined to 8.30 and 133.98 parasite genomes/mL, respectively. At day 30 post-treatment evaluation, the decline in parasite load was significantly greater ($P=0.024$) with L-AmB compared with ApL. Four of 33 patients in the ApL group failed treatment (1 primary failure and 3 relapses) with the presence of parasites, whereas all patients in the L-AmB group were cured at 6 month follow-up.

Conclusions: qPCR can be a tool to measure parasite dynamics accurately and provide a marker to measure the efficacy of various drugs. It can be used as a test of cure, allowing us to do away with invasive and risky methods such as splenic or bone marrow aspiration.

Keywords: efficacy, fat emulsion of amphotericin B, liposomal amphotericin B, *Leishmania donovani*, qPCR, VL

Introduction

Visceral leishmaniasis (VL) has been treated with pentavalent antimony (Sb^v) compounds in India and elsewhere for more than 70 years; however, Sb^v is no longer effective in most endemic areas of India. Various alternatives, such as miltefosine, paromomycin and various lipid formulations of amphotericin, are now used in this region for the treatment of VL.[1–3] Despite the initial high efficacy demonstrated by every antileishmanial drug, there is a lurking danger of the development of increasing unresponsiveness with the passage of time due to poor compliance, the inherent nature of the drugs and inefficient public health systems.[4] Apart from the need to develop new therapeutic drugs that are easy to administer, economical and safe, it is important to effectively monitor the efficacy of drugs in terms of parasite clearance. Determining parasitic kinetics to assess treatment efficacy could be an important tool. Since sterile cure is never achieved in leishmaniasis after treatment,[5,6] a quantitative approach [real-time quantitative PCR (qPCR)] can be employed not only for diagnostic purposes, but also for

monitoring parasitaemia in patients during their treatment.[7–10] In VL, the number of parasites found in blood is low compared with the spleen.[11] However, in most cases it is feasible to diagnose VL by culturing either peripheral blood mononuclear cells or buffy coat/whole blood cells isolated from a small volume of blood,[11] which is helpful in replacing the invasive splenic aspiration. As the *Leishmania* parasite is an intracellular organism, the source of the DNA detected in blood could be either live or dead parasites harboured inside phagocytic cells or DNA released from phagocytic cells.[12] Kinetoplast DNA-specific primers are more suitable to detect the *Leishmania* parasite, as there are ~10000 copies in a single parasite.[13–15] We used a qPCR experiment based on the SYBR Green method and targeting the minicircle kinetoplast DNA. We took a subset of patients randomized to either liposomal amphotericin B (L-AmB; AmBisome, Gilead Sciences, Foster City, CA, USA; $n=13$) or a preformed fat emulsion of amphotericin B (ApL; Bharat Serum and Vaccines Limited, Mumbai, India; $n=33$) to study the parasite kinetics resulting from the use of these two drugs using qPCR as a molecular tool.

Materials and methods

Study site, patients and sample collection

This study was carried out at the Infectious Diseases Research Laboratory of the Department of Medicine, Banaras Hindu University, Varanasi, India, and at its field site Kala-Azar Medical Research Centre, Muzaffurpur, Bihar, India. The experiment was approved by the Ethics Committee of the Institute of Medical Sciences, and all subjects provided written informed consent. Forty-six patients with parasitologically confirmed VL (6–55 years, males and females, and HIV negative) were enrolled in the study. Confirmation of VL was done by demonstration of amastigotes in Giemsa-stained smears of splenic aspirate. Patients were treated with L-AmB or the cheaper ApL. The drugs were administered at 15 mg/kg of body weight in a single infusion. Baseline blood samples were obtained for haematological and biochemical evaluation. For parasite quantification, 500 μL of blood was drawn from all patients on days 0, 3, 5 and 7. Patients were discharged on day 7 and post-treatment follow-up was done on day 30. Patients were followed up for a further 6 months for evaluation of definitive cure. On day 30 and at the 6 month follow-up, blood samples were drawn for parasite quantification by qPCR, as well as for haematological and biochemical evaluation. At the 6 month follow-up, clinical and laboratory evaluation was carried out in order to look for features of VL relapse. If relapse was suspected earlier, blood for parasite quantification and other haematological evaluations was drawn at that time.

DNA isolation from blood

DNA was extracted from 200 μL of blood collected in citrate tubes. Extraction was carried out using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and DNA elution was carried out in 30 μL of MiliQ water. Only DNA that had an optical density (OD) 260/280 ratio of 1.8–2.0 and an OD 260/230 ratio >1.5 by spectrophotometer measurements (ND-2000 spectrophotometer; Thermo Scientific, Waltham, MA, USA) was taken for qPCR experiments.

Standard curve

Parasite load was determined using a standard curve made with serial dilutions of *Leishmania* DNA. A stock solution of *Leishmania donovani*

DNA was obtained by extraction (QIAamp DNA mini kit) from 2×10^8 promastigotes. After eluting in 200 μL of H_2O, the concentration was 10^6 parasites/μL, assuming the extraction was nearly 100% efficient (120 *Leishmania* parasites have 10 pg of DNA[16]). Ten-fold serial dilutions of the DNA stock solution yielded eight points on a curve spanning $0.1–10^6$ parasites (Figure 1).

qPCR experiment

SYBR Green-based qPCR was applied for quantification of the *Leishmania* parasite in patient blood. For accurate sensitivity, *Leishmania*-specific kinetoplast DNA was chosen as the target region. The PCR was performed in a final volume of 20 μL containing 10 μL of SYBR Green master mixture (2×) [Applied Biosystems (ABI), Carlsbad, CA, USA], 3 μL of MiliQ water and 5 μL of DNA template (20 ng/μL)[16] and 1 μL (5 μM) of forward and reverse primers, 5′-CTTTTCTGGTCCTCCGGGTAGG-3′ and 5′-CCACCCGGCCCTATTTTACACCAA-3′ (Integrated DNA Technologies, Coralville, IA, USA). Amplification was conducted using a 7500 real-time PCR system (ABI). The thermal cycling conditions included an initial incubation at 50°C for 2 min, followed by a 10 min denaturation at 95°C and 40 cycles at 95°C for 15 s and 60°C for 1 min each. Each sample was tested in duplicate. Duplication was maintained both at the DNA isolation and PCR level. The generation of amplification plots, mean values and standard curves and dissociation stage analysis were carried out using ABI SDS software. The calculation of the melting temperature of each amplicon was done directly by the software provided. Appropriate negative controls (no template and healthy controls) were included in each plate to deal with contamination issues. A human tumour necrosis factor (TNF)-α primer (unpublished sequence; submitted for patent) targeting the single-copy TNF-α gene was used as a control to check for differences in human DNA amplification from samples taken at different times from the same individual. PCR conditions were the same as for the kinetoplast DNA qPCR.
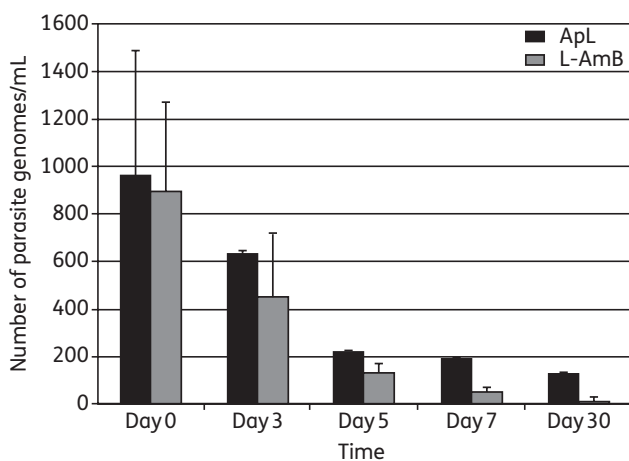
Statistical analysis

Data analysis was carried out using the non-parametric Mann–Whitney test and the one-way ANOVA test using SPSS (IBM, Somers, NY, USA) and Prism (GraphPad Software, La Jolla, CA, USA) software.
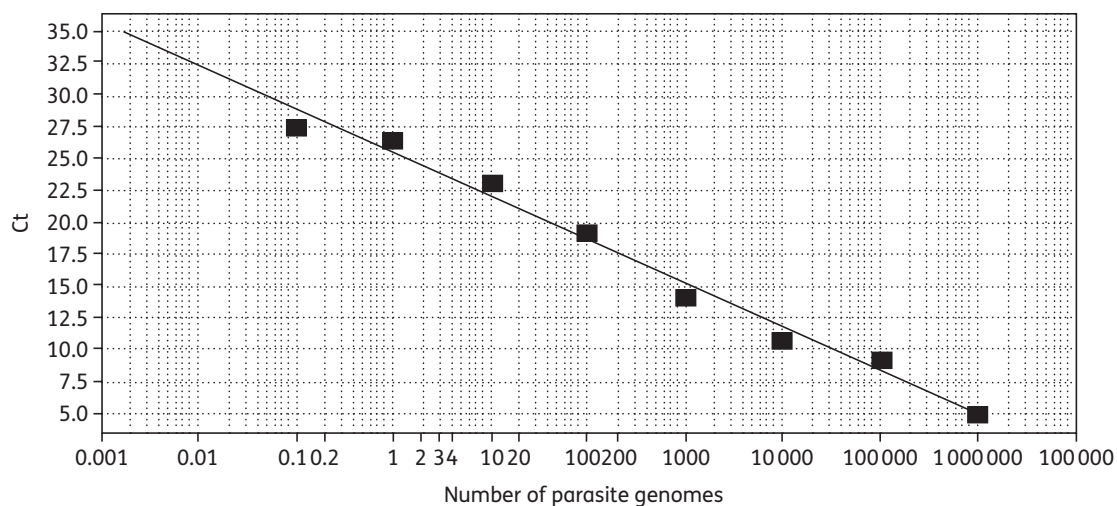
Figure 1. qPCR standard curve for quantification of *Leishmania* parasite. The standard curve was obtained from serial dilutions of *Leishmania* DNA with $0.1–10^6$ parasites. The *y*-axis shows the Ct values at different parasite numbers. Efficiency=96.775%, slope=−3.402 and r^2=0.987.

Results

Results are expressed as the absolute number of parasite genomes/mL of blood from 200 μL of blood, and of the total DNA eluted, 100 ng was taken. Therefore, the number of *Leishmania* genomes found in 100 ng is further calculated in total DNA from 200 μL of blood and then per mL of blood. The difference in cycle threshold (Ct) values between two dilutions is 3.2. At the time of diagnosis, all patients were highly parasitaemic. There was good correlation between the splenic smear score and the blood parasite burden ($r^2 = 0.56$, $P \leq 0.001$). *Leishmania* DNA was found in all confirmed patients at day 0; the mean $\pm$ SEM parasite genomes/mL of blood in the L-AmB and ApL groups was 894.07 ± 376.99 (range $= 23.8–4471.5$) and 980.48 ± 528.5 (range $= 20.16–6453.20$), respectively, and the difference was not significant. The mean parasite load, in parasite genomes/mL, decreased to 71.72 ± 19.82 and 211.52 ± 3.47 in the L-AmB and ApL groups, respectively, at day 7. At day 30, the values were 8.30 ± 2.59 (range $= 0.26–30.9$) parasite genomes/mL and 133.98 ± 0.194 (range $= 1.12–2681.25$) parasite genomes/mL, in the L-AmB and ApL groups, respectively. At day 3, 5, 7 and 30, there was a significant decline in parasitaemia (ApL, $P = 0.01$; L-AmB, $P = 0.045$; Figure 2). However, the decline was greater with L-AmB compared with ApL at day 30 ($P = 0.024$). Parasite clearance in L-AmB group is significantly higher compared with ApL group ($P < 0.0001$) (Figure 3). At the 6 month follow-up, all the L-AmB-treated patients had a negligible parasite number (<1 parasite genomes/mL) and achieved definitive cure, whereas in the ApL group, one patient had a parasite burden of >1000 parasite genomes/mL at day 30, one patient had a parasite burden of 1266 parasite genomes/mL at 4 months and two patients had parasite burdens of 43 and 1204.13 parasite genomes/mL at the 6 month follow-up. All four of these ApL patients (after 1 month of follow-up) who failed treatment were given rescue treatment. The remaining 29 patients in the ApL group showed <2 parasite genomes/mL in their blood, and were definitively cured. Figure S1 (available as Supplementary data at *JAC* Online) shows the parasite load at different times in some patients in both treatment categories.
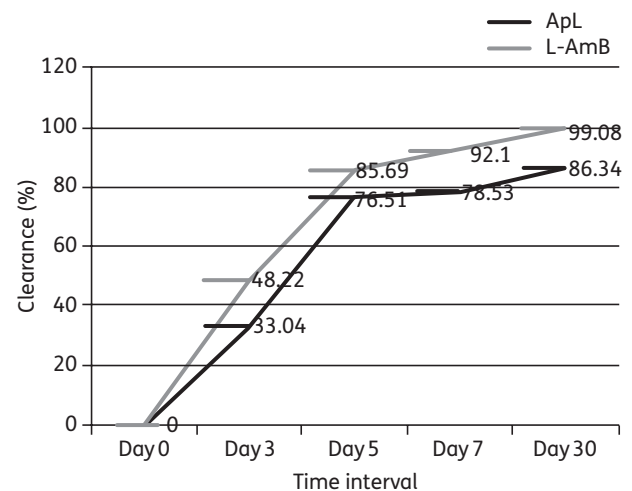
Discussion

This study demonstrates the utility of qPCR in determining an association between parasite load and clinical symptoms in patients undergoing treatment for VL. The benefits of molecular monitoring are already widely accepted in antimalarial drug trials.[17,18] In leishmaniasis, however, microscopy is considered as the gold standard and splenic/bone marrow slide reports are graded as 1+ to 6+, showing different levels of parasite burden at day 0 and discharge of patients,[19] but it deals only with a range of parasite loads. A great disadvantage of microscopy is the requirement for an invasive and risky splenic aspirate. PCR quantification also has the advantage of having the ability to assess the interim parasite load, and can be used as a test of cure. At diagnosis, parasite burden in peripheral blood correlated significantly with splenic score. We used the SYBR Green method for qPCR, which is less expensive than the Taqman assay. Targeting the minicircle kinetoplast DNA maximizes the sensitivity of the assay, and melting curve analysis in the SYBR Green technique confirms the specificity of the kinetoplast DNA primer (melt curve around 79°C) and also reveals the presence of primer dimers. Because of their small size, primer dimers melt at lower temperatures than the desired product. Using the melting peak of a specific product, which in our case is ~79°C, we can be sure that the sequence of interest (amplicon) is amplified. In addition, non-specific amplification may result in PCR products that melt at temperatures above or below that of the desired product. Since readings in triplicate are highly recommended for the interpretation of results at the individual sample level, and since we estimated the parasite load in duplicate, our results should be treated with caution.

We also studied parasite kinetics in terms of burden before treatment and at different time intervals after drug administration in order to observe parasite clearance. This was primarily done to see if there was a difference in the rate of parasite clearance with the two drugs under study. We found a significant difference in the rate of parasite clearance with L-AmB and ApL in most of the patients, with significantly more rapid and

Figure 2. Comparative graph of the *Leishmania* parasite load in blood at different times in VL patients of different treatment groups.

Figure 3. Comparative parasite clearance from the blood of patients of the L-AmB and ApL groups from day 0, i.e. baseline, to day 30.

greater clearance with L-AmB (Figure 3). These results correlated with clinical outcome. The cure rate with L-AmB was 100%, with every patient having <1 parasite genome/mL in peripheral blood at 6 months, which might be due to degraded residual parasite genome in the blood.[20] Thus, monitoring parasite load by qPCR can indicate complete cure or early detection of treatment failure or relapse. In a few patients there was a slight increase in parasitaemia level at day 5 or 7, but it significantly decreased by day 30. The increment at this stage in some patients might be due to the release of parasites from intralesional tissues, such as the spleen, into the blood. Though this study was not designed to measure the efficacy of the two drugs, ApL performed poorly with slower and lower parasite clearance and persistence of higher levels of parasites in four patients, all of whom had to be given rescue treatment due to treatment failure. However, the parasite burden at day 30 in the 3 relapsing patients gave no indication of treatment failure, and was in a range similar to that of the 29 definitively cured patients. The resurgence of parasitaemia and, importantly, the increase in the parasite burden correlated with clinical relapses. Previous studies in human leishmaniasis in immunocompromised patients indicate that a clinical relapse is associated with a certain level of parasitaemia, and it is estimated that an increase above 10 parasites/mL of blood precedes a clinical relapse.[21,22] Here, three relapsing patients in the ApL group also had >10 parasites/mL of blood. Splenic smears showed the presence of amastigotes in these patients and reconfirmed the relapse of VL. The utility of qPCR in comparing drug effectiveness is clearly demonstrated here. As qPCR helps to detect relapse cases and in India post-kala-azar dermal leishmaniasis (PKDL) appears after 3–6 years of VL, it remains to be seen whether the residual parasite burden at the end of treatment is a predictor of PKDL or not. We conclude that with the introduction of new combination therapies as well as new drugs for VL treatment, qPCR could be an important tool for monitoring their efficacy and for helping us choose the best chemotherapeutic strategies.

However, qPCR requires a high initial investment for it to be established in the laboratory and each measurement of parasite load is several times more expensive (~$10/test) than usual parasitological examination. Highly skilled personnel are required, as well as good operating conditions, such as an uninterrupted good quality power supply, air conditioning, etc. In endemic regions of developing countries these are hard to achieve. Nevertheless, such facilities could bring about a paradigm shift in the management of VL, and reference centres equipped with qPCR facilities could guide the region about the parasite kinetics related to a particular drug. Where possible, qPCR facilities should be established, both for diagnosis and prognosis of patients.

Acknowledgements

Funding

Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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