

A Correlative Study of Splenic Parasite Score and Peripheral Blood Parasite Load Estimation by Quantitative PCR in Visceral Leishmaniasis

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Parasitological diagnosis of visceral leishmaniasis (VL) by splenic smear is highly sensitive, but it is associated with the risk of severe hemorrhage. In this study, the diagnosis of VL using quantitative PCR (qPCR) in peripheral blood was evaluated in 100 patients with VL. Blood parasitemia ranged from 5 to 93,688 leishmania parasite genomes/ml of blood and positively correlated with splenic score ($P < 0.0001$; $r^2 = 0.58$). Therefore, quantification of parasite genomes by qPCR can replace invasive procedures for diagnostic and prognostic evaluations.

Visceral leishmaniasis (VL), also known as kala-azar, is a systemic protozoan disease that is caused by the parasite *Leishmania donovani* complex and is transmitted through the bite of an infected phlebotomine sand fly. VL is characterized by prolonged fever, splenomegaly, weight loss, and pancytopenia, and it is complicated by serious infection (1). These clinical features can easily be mistaken for other common febrile illnesses, such as malaria, enteric fever, tuberculosis, etc. If untreated, VL is fatal (2). There are an estimated 200,000 to 400,000 new cases of VL each year worldwide, and over 60% of these occur in the Northern Indian state of Bihar and the bordering regions of Nepal and Bangladesh (3). Therefore, it is necessary to use a diagnostic test that is highly sensitive and specific. At present, diagnosis is either confirmed parasitologically by the demonstration of amastigotes in bone marrow or spleen smears or by the demonstration of antibodies using the rapid immunochromatographic test, the direct agglutination test (DAT), or molecular tests (4–10). The sensitivity of amastigote detection in splenic smear is 96% to 98% (11, 12); however, splenic aspiration is associated with the risk of life-threatening hemorrhage. Additionally, serological tests cannot discriminate between subclinical, current, and past infections (13, 14). Various DNA-based molecular methods (PCR) have been used for the diagnosis of VL using blood, buccal swabs, or urine samples; however, their inability to discriminate between symptomatic or asymptomatic VL is a major drawback (7, 15, 16). Hence, more appropriate tools are urgently needed. Real-time quantitative PCR (qPCR) is a promising tool that can be employed in the diagnosis of VL (17, 18). qPCR distinguishes symptomatic from asymptomatic VL and has the ability to diagnose VL early (19, 20). We recently reported the usefulness of real-time PCR (RT-PCR) for estimation of parasite load during the treatment of VL (21). In the present study, we looked for a correlation between the splenic smear score (5) and the number of circulating parasites in patients with active VL.

The study was carried out at the Infectious Diseases Research Laboratory (Department of Medicine, Banaras Hindu University, Varanasi, India) and at its field site Kala-Azar Medical Research Centre (Muzaffarpur, Bihar, India). The study was approved by the Ethics Committee of the Institute of Medical Sciences, Banaras Hindu University, and all subjects provided written informed consent. One hundred patients with parasitologically confirmed

VL (aged 6 to 55 years, both sexes, and HIV negative) were enrolled in the study. Confirmation of VL was done by demonstration of amastigotes in Giemsa-stained splenic smears. DNA was extracted from 200 μ l of blood collected in citrate tubes. DNA extraction was carried out using a QIAamp DNA minikit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The SYBR green-based qPCR assay was performed in a final volume of 10 μ l containing 5 μ l SYBR green master mixture ($2\times$) (Applied Biosystems [ABI], Carlsbad, CA, USA), 4 μ l of DNA template, and 0.5 μ l (5 μ M) of forward and reverse kinetoplast DNA (kDNA)-specific primers (Integrated DNA Technologies, Coralville, IA, USA). Amplification was conducted in a 7500 real-time PCR system (Applied Biosystems [ABI], Carlsbad, CA, USA). A standard curve-based absolute quantification method for quantification of parasite load, employing kDNA-specific primers (21), was used according to a previously described technique (20). For data interpretation, a correlation study was done by logarithmic transformation of linear regression analysis.

qPCR was positive in all patients and thus had a sensitivity of 100% in the peripheral blood. Peripheral blood parasite load ranged between 5 and 93,688 leishmania parasite genomes/ml of blood. The number of parasite genomes per milliliter of blood varied in patients and correlated well with their respective splenic score ($r = 0.58$; $P < 0.0001$) (Table 1).

Our findings suggest that qPCR is an excellent alternative to the existing modes of diagnosis of VL. Because of the significant positive correlation between splenic score indicating parasite load and the number of parasite genomes present in the peripheral

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TABLE 1 Different splenic score grades with reference to corresponding median values of parasite genomes per milliliter of blood

Slide score (result range) [no. of samples]	No. of parasite genomes per milliliter of blood (mean \pm SD)
1+ (1–10 parasites/1,000 field) [31]	130 (191 \pm 214)
2+ (1–10 parasites/100 field) [24]	427 (666 \pm 860)
3+ (1–10 parasites/10 field) [24]	1,166 (1,397 \pm 1,169)
4+ (1–10 parasites/field) [10]	4,228 (6,361 \pm 6,230)
5+ (10–100 parasites/field) [11]	34,098 (33,130 \pm 29,024)

blood, it was possible to assess the parasite load using peripheral blood, though the range at a particular score was wide with considerable overlap. A good correlation between splenic score and the number of parasites in the blood means that the monitoring of the efficacy of therapy may also be possible, though this needs to be demonstrated. A big advantage is that we need only 200 μ l of blood, and blood collection in citrate tubes is easy and can be performed in the field or in any health center and transported to the lab for qPCR experiments. We have recently reported the usefulness of qPCR for the estimation of parasite load during the treatment of VL patients (21).

In this study, 98% of patients had parasitemia of >10 parasite genomes/ml. The remaining 2% had parasite loads of 5 and 10 parasite genomes/ml, and these 2 patients had low splenic scores of 1+ each. Cultures of splenic aspirate, peripheral blood mononuclear cells (PBMC), and buffy coat are positive in most patients (22); however, parasite culture is labor-intensive and time-consuming, and up to 7 days may be needed for the culture to become positive. Further, in cultures, there is no correlation between splenic score and parasite load (22).

qPCR has the advantage over serological diagnosis that by the absolute number of parasites, it can be determined whether a subject has an active disease. Qualitative (conventional) PCR also suffers from the similar handicap of not being able to differentiate between infected and diseased individuals (23). In one of our studies, this kDNA-specific SYBR green-based qPCR assay clearly distinguishes symptomatic VL from asymptomatic *Leishmania* infection with a threshold value of 5 parasite genomes/ml of blood and helps to achieve early detection in epidemiological studies.

Limitations of qPCR include high initial investment and a relatively higher cost per test compared to serology. The requirement of skilled personnel can be another limiting factor; however, if completely equipped and manned, central laboratories are established at strategic locations to cater to one or several districts, and a reliable diagnosis can be provided to patients in large areas without resorting to either risky or less-reliable diagnostic processes.

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