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Novel PCR primers to diagnose visceral leishmaniasis using peripheral blood, spleen or bone marrow aspirates

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ABSTRACT

Objective: To establish a suitable method of diagnosis of visceral leishmaniasis (VL) using peripheral blood, spleen or bone marrow aspirates.

Methods: Peripheral blood, bone marrow and spleen aspirate samples were collected from clinically suspected VL patients (n = 26). A new PCR primer pair (MK1F/R) was designed targeting kinetoplast mini circle DNA sequences of *Leishmania donovani*, and *Leishmania infantum*, and was used to diagnose VL along with some other established primers for VL in polymerase chain reactions. Test was validated by comparing with several other diagnostic methods.

Results: The designed primer set showed 100% specificity and 98% sensitivity in detecting VL using blood samples, when compared with more invasive samples: bone marrow or spleen aspirates.

Conclusions: The newly designed primer MK1F/R could be a better alternative for PCR based diagnosis of VL using less invasive sample, peripheral blood instead of bone marrow or spleen aspirates.

1. Introduction

Visceral leishmaniasis (VL), or kala-azar is a symptomatic infection of the spleen and bone marrow, caused by the *Leishmania donovani* (*L. donovani*) complex that includes *L. donovani* and *Leishmania infantum* (*L. infantum*) [1]. They are responsible for causing significant health problems in up to 400 000 people and up to 40 000 deaths per year [1]. Of the 200 000–400 000 new cases of VL worldwide, 90% are reported from India, Brazil, Bangladesh, Sudan, South Sudan, and Ethiopia [2].

VL can be diagnosed in laboratory by three ways: 1) demonstration of *Leishmania* parasite in bone marrow or splenic tissue specimens by microscopic observation of the stained sample, culture *in vitro*, or inoculation into animal; 2) detection of *L. donovani* complex DNA in tissue samples [mostly by polymerase chain reaction (PCR)]; or 3) serodiagnosis by detection of antibodies against *L. donovani* complex, or by diagnosis for leishmania-specific cell-mediated humoral immunity, and by detection of *L. donovani* complex antigens in tissue, blood, or urine samples [3,4].

The commonly used method for diagnosing VL has been the direct detection of the parasite on Giemsa stained smears made from aspirates of bone marrow, lymph node or spleen which is considered as the gold standard [5]. The sensitivity of parasitological diagnosis from splenic aspiration, bone marrow aspiration/biopsy, and lymph node aspirations are 93%–99%, 53%–86%, and 53%–65% respectively [5,6]. Although the sensitivity of the bone marrow/splenic smear is high, but the recovery of splenic tissue carries the risk of fatal hemorrhage, and collection of bone marrow aspiration is very painful [3].

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In vitro culture is sensitive but it requires specialized laboratories and requires much time to get the diagnostic result ranging from one to several weeks [4]. *Leishmania* parasite can also be demonstrated after inoculation of test animals (such as hamsters, or mice) with infected sample, but this technique is not usually utilized as a diagnostic techniques, since several months may be needed to get a positive result [3].

Serological tests [rK39 ELISA, rK39 dipstick, IFAT, soluble *Leishmania* antigen ELISA (SLA-ELISA) *etc*.] are also routinely used to diagnose *L. donovani* complex. rK39 immunochromatographic test (ICT) is used generally as the test showed good sensitivity and specificity, some recent report however, showed that it is less accurate in East Africa [7–9]. Serological assays are associated with several intrinsic drawbacks, like cross-reactions with antibodies against other similar pathogens. Further, most of these tests cannot readily distinguish among current, subclinical, or past infections. Besides, as immune response depends on the health status of the patient, false-negative results can be obtained from immune suppressed patients [10].

Several antigen-based systems have been developed to detect VL [11,12]. Monoclonal antibody based detection kit, KAtex was recently reported to have a sensitivity of 48%–87% [5], while the sensitivity of the same kit was initially shown in the range of 68%–100% [3]. In eastern Nepal it is found that KAtex is less sensitive (47.7%) in diagnosing kala-azar [13]. Other antigen based detection using urine samples have also shown promise in diagnosis of VL [11]. Abeijon and Campos-Neto generated antibodies against three *L. infantum* proteins and developed a single capture ELISA for the clinical detection of these antigens in the urine of 20/20 patients with VL while the same antibodies did not react with 62 urine samples obtained from control subjects [11]. According to Jamal *et al.*, detection of *Leishmania* species by circulating antigen have a sensitivity of 90%–100%, however, more study is required for its validation and authentication [12].

PCR is another suitable method for diagnosis, as it can be applied on blood, bone marrow and skin, with good sensitivity and specificity. PCR provides a prompt and sensitive method for the detection of *L. donovani* complex using peripheral blood (70%–100%) [5], the collection of which is less invasive [14]. Sensitivity and specificity of PCR-based detection depend on both the samples and primers to amplify the target sequence. In a background of myriad PCR primers, reported with varying sensitivity and specificity for *Leishmania* detection, here, we evaluated several existing methods of *L. donovani* complex diagnosis, and reported a newly designed PCR primer pair that was found to be equally sensitive and specific to detect VL in both peripheral blood specimen, and bone marrow/spleen aspirates.

2. Material and method

2.1. Sample collection

An Ethical clearance from Bangladesh Medical Research Council (reference number BMRC/NREC/2013-2016/816, dated 8 May 2014) and consents from individuals contributed in this study were duly obtained at the onset of the study. Venous blood, bone marrow and splenic aspirates from clinically-suspected VL patients, showing signs of fever, splenomegaly and hepatomegaly were collected. Patients were recruited, either from Surjo Kanto Hospital (S. K. Hospital) Mymensingh, or Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) Hospital, Dhaka, Bangladesh. Bone marrow (n = 19) and splenic (n = 7) aspirates were collected from the recruited patients, while peripheral (venous) blood was collected from all of them (n = 26). There were 3 mL blood drawn from antecubital vein in a vacuette containing EDTA (K3 EDTA tube) (Thermo Scientific, USA), and they were then processed only to separate the buffy coat by concentration gradient centrifugation using the histopaque solution (Histopaque-1119; Sigma-Aldrich) [15]. For control experiments, 66 venous blood samples were collected, consists of healthy individuals of non-endemic (Dhaka, n = 25) and endemic areas (Mymensingh) [16] (n = 16), and patients with other similar symptomatic diseases (n = 25; 10, 3 and 12 patients diagnosed for tuberculosis, malaria and dengue respectively). All these control samples were collected from BIRDEM hospital.

2.2. Immunochromatography

Antibody was detected by ICT by using the CTK Biotech Leishmania IgG/IgM Combo Rapid test kit (CTK Biotech, SanDiego, USA), as per manufacturer's instructions. All malaria suspected patients, used for control experiments were tested for ICT dipstick test in order to detect the presence of antigen of *Plasmodium vivax* and *Plasmodium falciparum* in patient's blood by using Excel Quick test device (Malaria P.f./P.v. rapid test cassette, TUN, USA) as per manufacturer's instructions. All suspected dengue patients were tested for ICT dipstick test in order to detect the presence of antibody in patient's plasma against dengue by using the dengue IgG/IgM Combo rapid test kit (Standard Diagnostics, INC, Korea).

2.3. Microscopy and culture

The smears of the collected specimens (bone marrow, splenic aspiration or blood) taken on a clean glass slide were dried completely, fixed with 100% methanol, and then stained with leishman stain for examination under oil immersion objectives [17]. At least 1 000 fields per slide preferably around the edges of the preparation were examined to detect amastigote form of *Leishmania*. For culture, one drop each from bone marrow, splenic aspiration or blood samples were inoculated into the liquid phase of Novy-McNeal-Nicolle (NNN) medium (1908), incubated at 22 °C and were observed in every week by microscopy until the presence of the parasite is recorded before they were discarded eight weeks post-inoculation. For control experiments, the sputum specimens of suspected TB samples were diagnosed by microscopy after staining with Zeihl Neelsen (ZN) stain.

2.4. Designing of primer pair and PCR

For PCR-based detection, three sets of primer pairs were used: LD1-F/R [18], BHUL18SF/R [19] and a newly designed MK1F/R (this study). The latter was designed by aligning kinetoplast minicircle DNA sequences of two *Leishmania* species: *L. donovani*, and *L. infantum* (GenBank accessions: AF169137.1, AF103739.1, AF190476.1, Y11401.1, X84844.1, and Z35273.1) using ClustalW [20]. An almost conserved region of about 150 bp that is found to be very unique for

L. donovani complex was selected for primer designing. The Primer 3 program [21] was then used to confirm their suitability as primers. The MK1F/R primers were designed to amplify a 102 bp fragment of leishmanial kinetoplast DNA (Figure 1 and Table 1). Figure 1 also describes the relative positions of other primers in *Leishmania* kinetoplast mini circle DNA used in this study.

DNAs from bone marrow, splenic aspiration and buffy coat were extracted by using EZ -10 Spin Column Genomic DNA Miniprep Kit, Blood (Bio Basic Inc, Canada, Cat No. 84) according to the manufacturer's protocol. The purified DNAs were used as template for PCR reactions for 3 sets of primers following the conditions outlined in Table 1. For PCR involving MK1F/R, each reaction was set with a Mastermix (Thermo scientific, California, USA) added with 10 pmoles of each primer and 50 ng of template DNA isolated from different specimens in a final volume of 25 μL. The annealing temperature (°C) was determined by setting up a temperature gradient PCR reactions in ABI Prism 3130 Genetic Analyzer PCR system (Veriti, ABI, Foster City, USA) in increments of 1 °C starting from 43 °C. The amplification conditions was set as follows: heating at 95 °C for 10 min followed by 40 cycles, each consisting of 40 s at 95 °C, 30 s at 45 °C, and 30 s at 72 °C, and a final extension step of 7 min at 72 °C. Products were analyzed by electrophoresis on 2% (w/v) agarose (Thermo Scientific, Mol Bio Grade, USA) gel, containing ethidium bromide (0.5 mg/mL in TAE buffer), 0.04 M Tris acetate, 0.001 M EDTA and photographed under UV illumination using Gel documentation system (Alpha Imager HP System Versatile Gel Imaging, Santa Clara, CA, USA). Negative controls were maintained using equal volume of deionized water instead of the DNA templates.

2.5. Statistical analyses

Statistical analyses at 95% confidence interval (CI) were calculated by online tool VassarStats [22]. The sensitivity and the specificity of the primer pairs were calculated according to the following statistical formulas:

$$\text{Sensitivity} = [a/(a+c)] \times 100$$

$$\text{Specificity} = [d/(b+d)] \times 100$$

where a, b, c and d represent numbers of true positive, false positive, false negative, and true negative samples respectively.

To determine the homogeneity among different diagnostic procedures, percent overall kappa agreement was used. Using Online Kappa Calculator the overall kappa agreement was calculated as 1) agreement among all diagnostic tests for tissue specimen, 2) agreement among all diagnostic tests for blood specimen, 3) agreement among PCR tests for tissue specimen, and 4) agreement among PCR tests for blood specimen [23–25].

3. Result

3.1. Immunochromatography

All of the clinically suspected VL patients (n = 26) were tested positive for an anti-rK39 antibody in patient's plasma against VL by the ICT test. However, none of the controls (including healthy and diseased individuals) was tested positive for rK39 (Table 2).

3.2. Microscopy and culture

Microscopic examination to detect *Leishmania* spp showed positive for 89% (17 out of 19) of the bone marrow and 86% (6 out of 7) of the splenic aspirates of the clinically suspected VL patients when subjected to Leishman staining (Figure 2A). However, *Leishmania* spp was not detected in the blood buffy coat specimens of either of the clinically suspected VL patients or the control individuals.

Seventy-four percent (14 out of 19) bone marrow aspirations and 86% (6 out of 7) splenic aspirations showed growth of promastigotes in NNN medium (Figure 2B). However, *Leishmania* spp was not detected in the blood buffy coat specimens of either of the clinically suspected VL patients or the control individuals by the growth in the NNN medium (Table 2).

3.3. PCR

DNAs collected both from the bone marrow and splenic aspirates of all of the 26 clinically suspected patients, produced the expected amplicon of 102 bp in a PCR probed with MK1F/R primers (Figure 3A). This finding was no different when DNAs, collected from buffy coat preparations of blood specimens from corresponding patients were used as template (Figure 3B).

The diagnostic sensitivity of VL using peripheral blood as specimens was measured 98.08% when compared to that of bone marrow and splenic aspirations. Further, none of the 66 control samples produced the amplicon, indicating 100% specificity of the analyses (Table 2). While the calculated

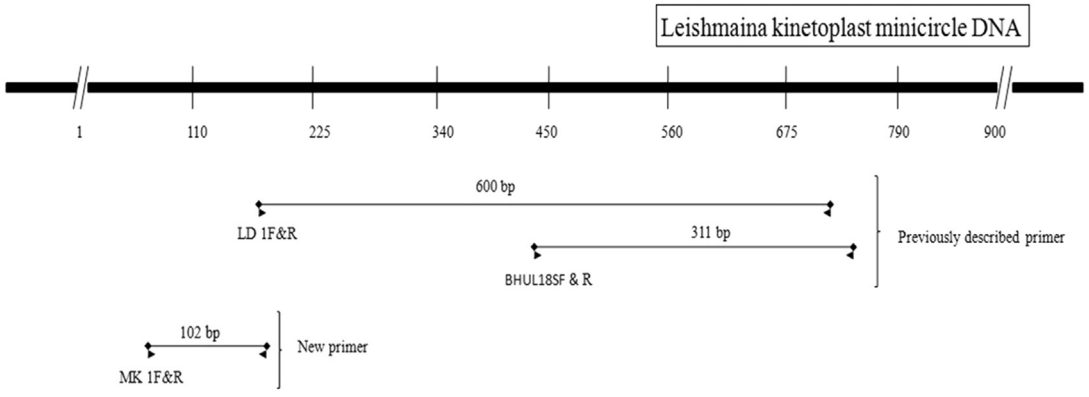

Figure 1. Location of PCR primers (LD1 F&R, BHUL18S F&R, MK1 F&R) used in this study on the *L. donovani* complex kinetoplast minicircle DNA.

Table 1

Main characteristics of the three PCR methods used in the study.

Method	PCR DNA target	Product size	Reference(s)	Primer sequence 5′ to 3′	PCR conditions		
					Amount of *Taq* polymerase	Annealing temperature	Extension time
MK1F, MK1R	Minicircles of kinetoplastic DNA of *Leishmania*	102	This study	CCC AAA CTT TTC TGG TCC TC GAG CCG ATT TTT GGC ATT T	1.50	45	30
LD1F, LD1R	Minicircles of kinetoplastic DNA of *Leishmania*	600	Maurya *et al*., 2005 [15]	AAA TCG GCT CCG AGG CGG GAA AC GGT ACA CTC TAT CAG TAG CAC	1.25	45	120
BHUL18SF, BHUL 18SR	Nuclear rRNA gene of *Leishmania*	311	Srivastava *et al*., 2011 [16]	CGT AAC GCC TTT TCA ACT CAC GCC GAA TAG AAA AGA TAC GTA AG	1.50	62	60

Product size (bp); amount of *Taq* polymerase (IU); annealing temperature (°C); Extension time (s).

Table 2

Comparative efficiency of rk39 ICT, microscopic examination, parasite culture in NNN medium, and PCR based assays to diagnose VL using blood buffy coat, spleen, and bone marrow specimens of clinically suspected patients.

Sample (*n*)	Sample source (*n*)	rk39 ICT positives (95% *CI*)	Microscopic test positives (95% *CI*)	Growth in NNN medium positives (95% *CI*)	PCR test positives (95% *CI*)		
					MK1F/R (newly designed)	LD1F/R	BHUL18SF/R
Blood buffy coat (26)	Clinically suspected VL (26)	52/52 (91.43–100%)	0/26 (0.00–16.02%)	0/26 (0.00–16.02%)	26/26 (83.98–100.00%)	21/26 (60.02–92.70%)	22/26 (64.28–94.96%)
Spleen (7)			6/7 (42.00–99.25%)	6/7 (42.00–99.25%)	6/7 (42.00–99.25%)	ND	ND
Bone marrow (19)			17/19 (65.46–98.15%)	14/19 (48.58–89.88%)	18/19 (71.90–99.73%	ND	ND
Healthy non endemic control (25)	Control (blood buffy coat) (66)	0/66 (0–6.85%)	0/66 (0.00–6.85%)	0/66 (0.00–06.85%)	0/66 (0.00–06.85%)	0/10 (0.00–34.45%)	0/10 (0.00–34.45%)
Healthy endemic control (16)							
Similar disease control (25)							

ND stands for not done.

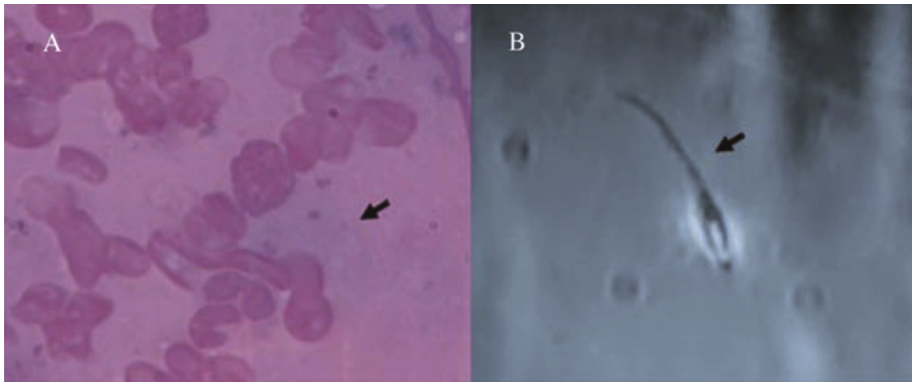

Figure 2. *Leishmania* spp. in splenic aspiration smear at 100× (oil immersion) lens under microscope (pointed by an arrow) (A) and promastigotes under microscope (pointed by an arrow) obtained from culture in NNN medium (B).

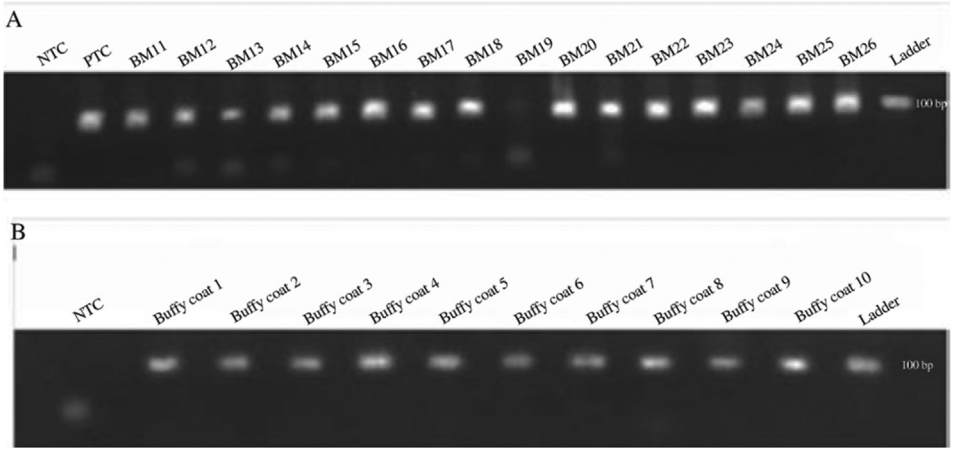

Figure 3. A 2% agarose gel showing amplification of a 102 bp band from DNAs, collected from 16 bone marrow samples labeled BM11 through BM26 (A), and 10 buffy coat preparations, labeled Buffy coat 1 through 10 (B), as representative samples from suspected patients when subjected to PCR with primers MK1F/R. Negative template control (NTC) having no DNA template loaded at far left lane and positive template control (PTC) where DNAs obtained from cultured promastigotes were used as indicated.
DNA marker is loaded in far right lane.

specificities for both LD1F/R and BHUL18SF/R primer pairs were 100%, the sensitivity results varied: 80.76% and 84.6% for LD1F/R and BHUL18SF/R respectively; indicating the edge of the designed oligos of this study over the other primer pairs.

The authenticity of the amplicons by the primers designed for this study was checked by DNA sequencing using a Thermo Sequenase premixed ABI Big Dye Terminator 3.1 cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions with ABI3130 genetic analyzer followed by BLAST search, and the identity of the amplicons was found matched with database sequences of *L. donovani* complex.

The overall kappa agreement (%) in detecting *Leishmania* using tissue and blood specimens among all diagnostic tests was estimated 83.07%, and 45.89% respectively. When PCR method was considered, the agreement (%) for the same was calculated as 97.43% and 84.61% respectively.

4. Discussion

Clinical diagnosis of VL is generally based on the presence of the parasite in bone marrow or splenic tissue specimens by microscopic observation; the detection of *L. donovani* complex DNA in tissue samples by PCR; or serodiagnosis by detection of antibodies against *L. donovani* complex. Sensitivity and specificity of the detection methods varies depending on the type and tissue origin of the samples that are analyzed as well as on the performance of the technique used. Here we report a novel primer MK1F/R based PCR detection of VL, while comparing its sensitivity and specificity with other routinely used techniques such as microscopic examination [4], rk39 ICT dipstick test [4], *in vitro* culture in NNN medium [4], and PCR based techniques [18,19].

In this study, rk39 ICT dipstick test was found 100% positive in suspected VL cases and 100% negative for all control individuals included in the study. Khan *et al.* reported that the sensitivity and specificity of the rK39 strip test using urine samples were 95% and 93.3%, respectively [26]. Similarly, the rK39 ICT dipstick test showed 93%–100% sensitivity and 96%–100% specificity using serum samples of VL patients [27]. The absolute level of sensitivity and specificity rates of ICT test in this study could be due to selection of cases, which have most florid clinical features of VL. The rK39 ICT dipstick test however, has limited uses as the target anti leishmanial antibodies: (1) may not be detectable in early stage of illness, (2) tend to persist in the circulation several years even after cure of VL and hence could be misleading for diagnosis, and (3) could be found among residents of endemic areas who had no history of leishmaniasis [28].

This study observed *Leishmania* amastigotes in 89% bone marrow and 86% spleen samples in microscopy; close to the sensitivity pattern of 60%–85%, described by Sundar and Rai from bone marrow samples [3]. Nevertheless, *Leishmania* spp. could not be detected in blood buffy coat samples by microscopy in our study. In culture, 74% and 86% samples from bone marrow and spleen specimens respectively produced promastigotes in NNN media. It was interesting to note that, though *Leishmania* spp. was present in 92% of cases in microscopy, the culture positivity rate was 76%. Similarly, another study reported that the isolation rate in culture from bone marrow-aspirated specimens was 76.2% [29]. Like microscopy, *Leishmania* spp. remained unculturable from blood in NNN medium indicating the parasite load in circulating blood was too low to be detected during the period of investigation.

In order to use 'blood buffy coat' as a specimen to replace bone marrow or spleen for diagnosis of *L. donovani* complex, thereby reducing the risk and pain during the sample collection, a PCR-based diagnosis could be useful. Since *L. donovani* complex are commonly found in myeloid cells, this study therefore used buffy coat preparation of peripheral blood mononuclear cells for DNA isolation which can be used as template for amplification in a PCR setting [15]. The primer pair, MK1F/R is 98–100% identical to corresponding position of *L. donovani* complex [*L. donovani* and *L. infantum*, and *L. infantum chagasi* (old classification *Leishmania chagasi*)]. Despite some degree of similarity of sequences with some other species, the pair is unique only for *L. donovani* complex when probed by both the forward and reverse primers. Using BLAST, clustal omega and in silico hybridization assay with alignment and free energy calculation tool (IDT oligo analyzer & NCBI primer BLAST) [30–32], it revealed that the designed primer would be specific for *L. donovani* complex. Indeed, the PCR analyses of buffy coat blood specimens yielded 98% sensitivity when compared to bone marrow and spleen specimens while producing absolute (100%) specificity as no product amplified from any of the control specimens in an identical PCR setting.

It was found that overall kappa agreement among all diagnostic tests is lower in blood than that of tissue specimens. This could be due to inability of some test methods like microscopy, culture in NNN medium to give test positives from blood samples. When PCR-based detection method was analyzed, again the agreement level (kappa) was recorded higher in tissue specimens compared to blood samples. This probably happened due to relatively low DNA load in the latter and varying strength of different PCR primers to detect VL. The greater disagreement among different PCR test results using blood specimen denotes varied sensitivity of PCR primers to give the same positive result.

In conclusion, specimens like bone marrow or splenic aspiration could be substituted by the peripheral blood buffy coat to score the infection with circulating *L. donovani* complex in patients by PCR without compromising the diagnostic efficiency of VL in Bangladesh.

Conflict of interest statement

We declare that we have no conflict of interest.

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