Peripheral blood buffy coat smear: A promising tool for diagnosis of visceral leishmaniasis

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ABSTRACT

Confirmative diagnosis of Visceral Leishmaniasis (VL) is still being a challenge at the primary health care facilities in most of the rural endemic areas in the Indian sub-continent. Conventional methods for parasitological confirmation are risky and require skilled personnel hence, unreachable to the poor people in the endemic region. Buffy coat smear microscopy, as a minimally invasive simple alternative, for the parasitological diagnosis of VL was evaluated in this prospective study. One hundred twelve VL patients were enrolled in this study. Buffy coat was separated from peripheral blood of all enrolled subjects using the Histopaque-1119 solution. Leishman-stained buffy coat smear was examined for Leishmania Donovani (LD) body and buffy coat was also utilized for detection of parasite DNA by Leishmania-nested polymerase chain reaction (Ln-PCR) for all cases. Concomitant splenic smears could be examined for LD body in 66 cases and parasite load was graded in a scale of 1+ to 6+ for LD-positive smears. All
splenic smear-positive cases were also found positive by Ln-PCR. Of 112 enrolled VL cases, 103
were found positive (92%) for LD bodies in buffy coat smear microscopy which is promising as
a confirmative diagnosis tool. We have also found a significant association in the buffy coat
smear positivity with parasitic burden in the spleen smear. In this preliminary observation in
Bangladesh, buffy coat smear microscopy has been found to be very simple, minimally invasive
and risk-free method of parasitological diagnosis for VL with a good diagnostic accuracy for its
potentiality in field use.

**Keywords:** Visceral leishmaniasis, Buffy coat smear microscopy, Parasitological diagnosis, Ln-
PCR, Diagnostic sensitivity
INTRODUCTION:

Visceral leishmaniasis (VL) or Indian kala-azar is a vector borne parasitic disease caused by an obligate intracellular haemoflagellate of the genus *Leishmania* (10). Not all leishmanial infections lead to overt clinical disease, but in those infected persons who do develop the disease, multiplication of the parasite in the reticuloendothelial system causes prolonged fever, anaemia, hepatosplenomegaly and weight loss (3). VL is fatal if it is not adequately treated. The current prevalence is estimated to be 45,000 cases with more than 40.6 million populations are at risk of developing the disease in Bangladesh. Out of 64 districts, at least 34 districts including 105 upazillas have been reportedly affected by kala-azar (17).

Diagnostic tests for VL need to be highly sensitive and specific because of the fatal evolution of the disease without adequate treatment and the serious toxicity of antimonials (25), the most commonly used first-line therapy. Moreover, tests must be cheap and easy-to-perform since VL occurs in poor and remote rural communities with limited access to referral hospitals. The development of diagnostic tests for improved case management of VL has been rated as one of the most needed among the infectious diseases prevalent in the developing world (12). Currently diagnostic options for VL include parasitological, serological and molecular methods but each has its own merits and demerits (23, 24). Although the need for accurate VL diagnostics is obvious, innovation in this field has been slow. There is always a search for new diagnostic tool particularly suitable for field with high sensitivity and minimal invasiveness. Isolation of the parasite in culture or demonstration in relevant tissues like spleen or bone-marrow by light microscopic examination of the stained specimen remain “gold standard” and naturally, leads to the definitive diagnosis of leishmaniasis. However, for the parasitological diagnosis to be made
from these tissues, painful and sometimes fatal invasive procedures limit their scope in routine clinical practice and not feasible in field. Serodiagnosis is a valid and attractive choice in kala-azar like many other infectious diseases and detection of anti-leishmanial antibody using serum and urine against recombinant antigens like rK39 by rapid Immunochromatographic test has been carried out in different endemic regions including India, Bangladesh and Nepal (8, 20, 26). Although this rapid test has been found highly sensitive in Indian subcontinent but its specificity is yet to be established (26). Further, its use is limited to discriminate disease and asymptomatic infection, because antibody titers may vary with the infecting species, tissue tropism, and the immunocompetence of the host (1). The development of Polymerase Chain Reaction (PCR) has provided a powerful approach to the application of molecular biology techniques to the diagnosis of leishmaniasis for more than a decade (2). PCR assay with buffy coat preparations to detect *Leishmania* DNA has been found to be 10 times more sensitive than that with whole-blood preparation (9, 19). However, for PCR, sophisticated machines, trained personnel and cost are limiting factors.

Detection of *Leishmania donovani* (LD) body in the peripheral blood buffy coat smear is an alternative and minimally invasive procedure for the parasitological diagnosis of VL. In a few studies its sensitivity ranged from 50 to 99% (15). Buffy coat is the portion of blood that contains concentrates of white blood cells including monocytes and platelets and for obvious reason its smear can be examined for detection of LD body among suspected VL patients. There are clear advantages of buffy coat over conventional smears for parasitological diagnosis in terms of its minimal invasiveness, simplicity and cost-effectiveness. As an alternative but definitive diagnostic tool buffy coat smear has the potential to be carried out for point-of-care VL case management. We report this preliminary observation for usefulness of buffy coat smear...
microscopy as a simple and noble method for parasitological diagnosis of VL for the first time in Bangladeshi patients.

MATERIALS AND METHODS:

Patients:

Subjects are 112 cases of VL defined by the national kala-azar elimination guideline of Bangladesh (16). One of 112 was diagnosed as congenital VL and had been reported earlier (7). All of them were from VL endemic area of Bangladesh. On admission to the Rajshahi Medical College Hospital (RMCH) during June, 2009 to June, 2010, all patients had fever (perceived by patient or guardian) more than two weeks, splenomegaly and positive by rK39 immunochromatographic test on finger prick blood specimen and later confirmed by doing splenic smear microscopy and Ln-PCR.

Sample collection and laboratory methods:

Blood collection. Taking all aseptic precautions, 3.0 mL of blood was collected into a vacuette (K3 EDTA tube) from all cases.

Splenic aspiration. After relevant laboratory evaluations and following standard technique as described by Bryceson (4), splenic aspiration was possible to be carried out for 66 patients by experienced doctor. Two good quality smears were prepared at bed side for microscopic examination.
Buffy coat preparation. Buffy coat was separated following the principle of concentration gradient separation by using the Histopaque solution (Sigma-Aldrich Histopaque-1119, USA). Three (03) mL of collected blood was layered onto 3 mL of the Histopaque-1119 solution in a sterile 15 mL centrifuge tube. The tube was capped and then centrifuged in a tabletop centrifuge at 4000 x g for 10 min at ambient temperature. A diffuse and grey band of leukocytes (buffy coat) in between the Histopaque solution and plasma, above the erythrocyte pellet was aseptically removed with a pipette and transferred to a sterile 1.5 mL microcentrifuge tube to be utilized for smear preparation and sample for Ln-PCR.

DNA extraction. Buffy coat DNA was extracted for PCR using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The DNA was eluted in 0.2 mL of AE buffer (supplied with the Qiagen kit). The purity of the DNA was satisfactory since a ratio of OD at A_{260} / A_{280} was within 1.7-1.9 for all DNA samples. We used molecular grade water instead of blood as an extraction control to check for carry-over contamination in every run of DNA extraction and PCR amplification.

Laboratory method:

Ln-PCR. We used a previously reported *Leishmania* specific nested PCR (Ln-PCR) with primers targeting the parasite’s SSU-rRNA region (6). An advantage of this Ln-PCR is its high sensitivity and specificity due to the use of a second set of *Leishmania* specific primers (R223 5′-TCCCATCGCAACCTCGTT-3′ and R333 5′-AAAGCGGGCGGTGCTG-3′) designed to an internal sequence of the first PCR product. For the first PCR run, we used Kinetoplastida-specific primers (R221 5′-GGTTCTTTCTGATTACG-3′, and R332 5′-GCCGGTGAAAGGC
In the first PCR, 2 μL of extracted DNA were amplified in a final volume of 25 μL containing 12.5 μL of Biorad iQ Supermix (Catalog number 170-8862 that contains 100 mM KCl, 40 mM Tris-HCl pH 8.4, 1.6 mM deoxynucleoside triphosphates, 50 unit/mL iTaq DNA polymerase, 6.0 mM MgCl₂). 0.3 μmol/L of each Kinetoplastida-specific primers R221 and R332 and additional 3.0 mM MgCl₂ were also added. Amplification was performed on a BioRad’s MyCycler. The PCR programme was run for 40 cycles that consisted of denaturation at 94°C for 30s, annealing at 64°C for 30s, and extension at 72°C for 30s.

Prior to the second amplification or nested PCR, the amplified products from the first run were diluted at 1:50 with molecular grade water, and 1 μL was added to a 25 μL reaction volume, as described above, containing 0.15 μmol/L of the Leishmania specific primers R223 and R333. For the second round of amplification, 35 cycles were used consisting by denaturation at 94°C for 30s, 65°C for 30s, and 72°C for 30s. In both amplifications, initial Taq DNA polymerase activation was performed at 95°C for 3 min, and a final extension at 72°C for 5 min was included. Amplification products were separated by electrophoresis on 2% agarose gel with 50 bp DNA ‘ladder’ (Invitrogen, USA, Cat. No. 15628-019) as molecular size-marker, and stained with ethidium bromide (0.1 mg/mL). Stained gels were visualized and photographed under UV light emission with a UV transilluminator (BioRad, Milan, Italy, S.N. 75S/03589). Amplification products were visualized and positive samples yielded a PCR product of 350 kDa. In every run, molecular grade water and healthy human DNA were used as negative controls, and DNA from cultured promastigotes served as positive control.

**Buffy coat smear microscopy.** Two good quality smears prepared from buffy coat and stained with Leishman stain were examined under oil-immersion light microscope (Olympus CH-20,
LD bodies in the smear were confirmed independently by two experienced microscopists by seeing the standard parasite’s morphology using following guideline:

1. Amastigotes are usually seen extra cellular in the buffy coat smear under oil-immersion objective of a light microscope

2. The hallmark of identification of structures as amastigotes in the buffy coat smear relies upon typical conjugation of unequal size of nucleus and kinetoplast.

3. The typical morphology of amastigotes (oval or elliptical cell having 2-4 µm in size, bounded by cytoplasmic membrane containing nucleus and kinetoplast, which are bound together right angle to each other and nucleus is comparatively larger than the kinetoplast) as demonstrated in the splenic or bone marrow-smears may not be well preserved in all amastigotes seen in the buffy coat smear. But the distinct conjugation with variable size of nucleus and kinetoplast covered by the complete or partially complete cytoplasmic rim are characteristics for the presence of amastigotes and make them sufficiently different from that of the platelets, which are the only structures that may confuse with them. Platelets remain usually as cluster and they are smaller in size than amastigotes. Moreover, certainly there is no separate nucleus and structure like kinetoplast inside the platelets.

4. The number of amastigotes is scanty in the buffy coat smear in comparison to splenic smear, which is logical. So, careful and patient searching is always required for clear demonstration of amastigotes.

Splenic smear microscopy. Two experienced microscopists independently examined spleen smears taken from 66 patients out of 112 cases in whom contraindications for splenic aspiration were absent. Splenic smears were stained with Leishman stain, and read in a standard way under
10 × 100 magnification for the presence of *L. donovani* amastigote. Presence of LD bodies was graded on a scale from 1+ to 6+. If the number of amastigotes counted per field was >100, 10–100 or 1–10, it was graded as 6+, 5+ and 4+ respectively. Similarly, 1–10 amastigotes in 10, 100 or 1000 fields were graded as 3+, 2+ and 1+ respectively (5).

**Serological test.** rK39 immunochromatographic test had been done by Kala-azar Detect™ Rapid Test, InBios International, Seattle, WA, USA as per manufacture’s instruction (20).

**Case definition.** VL case was defined as per national kala-azar elimination guideline of Bangladesh as mentioned above.

**Data analysis.** Descriptive statistical analysis, Chi-square test, McNemar paired test, and ANOVA were used for data analysis using SPSS 11.5 and R software.

**Ethical consideration:**

Ethical issues relating to this research protocol were approved by the Institutional Review Board of Rajshahi Medical College, Bangladesh. Informed written consent was obtained from either each patient or from the legal guardian before splenic aspiration and venipuncture for the collection of blood samples.

**RESULTS:**

**Study population characteristics:** Out of 112 VL patients, 75 (67%) and 37 (33%) were respectively male and female. Median age was 276 months (Quartiles 120 months and 384 months). All patients had splenomegaly ranging from just palpable to 12 cm from the costal margin.
along mid-clavicular line. All had positive rK39 tests, and underwent buffy coat smear microscopy for LD body and buffy coat Ln-PCR analysis for *Leishmania* DNA. Sixty six out of 112 VL patients consented for spleen aspiration diagnosis. Patients were treated with sodium antimony gluconate (SAG) at dose of 10-20 mg/kg body weight with a maximum dose not exceeding 10 mL (800 mg), given I/V daily without any interruption. All patients were discharged from hospital after 30 days of treatment with clinical improvements.

*Laboratory results:* Ninety two percent (103/112), 95.5 % (107/112) and 100% (66/66) of VL patients were positive respectively for buffy coat microscopy, buffy coat PCR and spleen smear microscopy. Buffy coat microscopy results did not show any relation with sex and age of the patients. Mean age±se of patients with and without buffy coat microscopy positive test was respectively 243±72 and 276±16, p= 0.58. Ninety percent (68/75) of male patients and 94.6% (35/37) of female patients were positive by buffy coat microscopy (p=0.47). However, buffy coat PCR positivity was more common among female patients 93% (70/75) vs. 100% (37/37) p=0.16.

Buffy coat microscopy found *Leishmania* amastigote in 93.5 % (100/107) of those positive by buffy coat PCR and 92.4% (61/66) of those positive for *Leishmania* amastigote by spleen smear microscopy. Compared to spleen smear microscopy, the positivity rate of buffy coat PCR and buffy coat microscopy was comparable (66/66 for PCR vs. 61/66, p=0.06, McNemar paired test).

Of 66 spleen smear positive for amastigotes, most of them (54/66) had grade ≥2+ parasite burden. Rates of positivity of buffy coat smear correlated significantly with the spleen smear parasite burden. Buffy coat smears were found 100% positive among cases having higher (grade 2+ to 5+) parasite burden (p= 0.003, Fisher exact test) (Table 1).
DISCUSSION:

The most important finding of the study is that buffy coat smear microscopy excellently correlated with clinical diagnosis of VL as per national kala-azar elimination guideline, buffy coat PCR for *Leishmania donovani* DNA and confirmatory diagnostic tool of VL (spleen aspirate smear). Another important finding is that the recommendation for diagnosis of VL and its treatment based on clinical criteria in the national kala-azar elimination program is correct since clinical diagnosis in 66 VL patients was justified by spleen aspirate smear which is the gold standard for diagnosis of VL. So far this is the first report of comparison of national kala-azar elimination guideline criteria in Bangladesh for VL diagnosis with spleen aspirate microscopy.

The development of an accurate, practical, and affordable diagnostic test is essential for any attempt to control VL in the endemic areas. The conventional methods of parasite demonstration is not only associated with painful invasive procedure but may be fatal. Moreover, prior laboratory evaluation of the patient is a prerequisite for conventional invasive procedures, which are not feasible to be performed at point-of-care in the VL endemic regions. Therefore, alternative method for the parasitological diagnosis of VL with minimum invasiveness and risk for the patients needs to be explored. Buffy coat positivity rate among clinically defined VL patients was 92%. The positivity rate was also high among confirmed VL patients (92.4%). Further, buffy coat smear was comparable with buffy coat PCR for detection of *Leishmania donovani* DNA. Thus buffy coat smear is a less invasive, affordable, virtually risk free promising diagnostic tool for VL which can be used at point-of-care diagnosis in the sub-district hospital of Bangladesh. It is necessary to mention that currently most of the VL pateints are clinically diagnosed and treated in the sub-district hospitals of Bangladesh where facilities for
confirmatory diagnostic tool is not available. Buffy coat smear can be used as a confirmatory diagnostic tool in these health facilities. However, its sensitivity and specificity has to be validated by further study following the standards for field evaluation of VL diagnostics for its recommendation as confirmatory test (3).

The idea for diagnosis of VL using peripheral blood buffy coat smear originated from the studies in the early 1990s (13,14,11). These studies showed that the *Leishmania* parasite could be demonstrated in the peripheral blood smear by microscopy among HIV-infected patients with visceral leishmaniasis. *Leishmania* amastigotes in peripheral blood of Indian kala-azar patients with a rate of 46% to 66% was also demonstrated later, depending on the time of blood sampling (21). *Leishmania*-stained blood smears revealed 1.3% of parasitaemia among 450 healthy individuals in another study conducted by Sharma et al in VL endemic areas of Bihar, India (22).

We also examined conventionally prepared buffy coat smears of 200 asymptomatic VL patients (defined by a person from a household or nearest to a household with kala-azar patient(s) in the past, positive for rK39 test and clinically completely healthy) and found none positive by buffy coat smear microscopy (our unpublished data from ongoing cohort study of asymptomatic VL patients in Trishal, Mymensingh). The study by Sharma et al and our unpublished data indicated that the buffy coat smear should be a good diagnostic method for active VL since parasitaemia among asymptomatic VL patients and healthy endemic control was very low (0%-1.3%).

So far, only one study conducted in Bangladesh tried to investigate the diagnostic sensitivity and specificity of buffy coat smear for diagnosis of VL (18). The study found a positivity rate of 31% by buffy coat smear among clinically suspected 67 VL patients. Unfortunately the results of present study cannot be compared with the results of the study of Roy et al, because they did not
report the buffy coat smear positive rate among their 44/67 confirmed VL cases and also among those who had been positive by rK39 rapid test (57/67). Nevertheless, the low positive rate of buffy coat smear found by Roy et al might be due to the conventional method for preparation of buffy coat smear which they used.

The positivity rate of buffy coat smear correlated well with the spleen parasite burden and most of the cases were with grade 2+ and above spleen parasite burden during admission. The buffy coat smear was positive in 98% of VL patients with grade 2+ and above spleen parasite burden. This finding is very encouraging to recommend buffy coat as a routine confirmatory test for diagnosis of VL.

In conclusion we found buffy coat smear as a promising confirmatory diagnostic tool for VL which can be used as point-of-care diagnosis in resource limited health facilities. However a well designed study following the recommended standards for evaluation of VL diagnostic tool is highly desired for its recommendation as a confirmatory diagnostic tool for VL.

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FOOTNOTE:
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Table 1 Association of buffy coat smear positivity with spleen parasite burden

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<td>1+</td>
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<td>2+</td>
<td>26 (39.39)</td>
<td>25 (96.15%)</td>
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<td>3+</td>
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<td>4+</td>
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References


Legend Figure 1: LD amastigote in buffy coat smear