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Polymerase Chain Reaction in the Diagnosis of Visceral Leishmaniasis Recurrence in the Setting of Negative Splenic Smears

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Abstract. This report presents two cases of visceral leishmaniasis (VL) recurrence where the microscopy of the splenic smear failed in diagnosis. However, a strong clinical suspicion compelled further evaluation by polymerase chain reaction (PCR), which validated the etiology. This short report highlights the usefulness of PCR in diagnosing cases of suspected smear-negative VL recurrence.

INTRODUCTION

The dramatic decrease in the number of annual active cases and deaths related to visceral leishmaniasis (VL) in Bangladesh provides promise that the disease may realistically be eliminated.[1,2] Yet to maintain and accelerate this achievement, every new case will need to be promptly diagnosed and initiated on an early treatment strategy. Furthermore, the diagnosis and management of cases of VL recurrence are very important as they serve as one of the most important sources of disease transmission.[3] To confirm the diagnosis of VL recurrence, the conventional splenic aspiration microscopy is recommended by the kala-azar elimination program in Bangladesh.[4] However, a low parasite concentration in the aspirate can yield a negative microscopy result.[5] Polymerase chain reaction (PCR) has been recently proposed as an alternative diagnostic modality that may improve sensitivity in cases of low parasite burden.[6] While the technical expertise and expense limit the widespread implementation of PCR, cases of suspected VL recurrence may represent a suitable niche. Herein, we demonstrate two cases of VL recurrence where microscopy of splenic smear failed to make the diagnosis, but PCR from the peripheral blood buffy coat detected *Leishmania donovani*. However, both cases were successfully treated with multidose liposomal amphotericin B with clinical remission and negative follow-up testing by PCR.

Case 1. A 9-year-old girl from Alongjani Village, Kanihari Union and Trishal Subdistrict from Mymensingh District (one of the most endemic village of Bangladesh for VL), had previously suffered with VL after diagnosis by national guidelines and was treated with single-dose liposomal amphotericin B at 10 mg/kg body weight within a World Health Organization Research and Tropical Disease Research–funded clinical trial.[7] After her original treatment, she had remained afebrile with no splenomegaly for 12 months. Yet during scheduled follow-up, she described 4 weeks of fever and anorexia. On examination, there was no lymphadenopathy or hepatomegaly, but the spleen was enlarged to 6 cm below the costal margin. She was referred to Surya Kanta Kala-azar Research Center as a suspected case of recurrence.

At the referral center, additional laboratory examination showed no abnormality in hematological, renal, and hepatic functions except a high erythrocyte sedimentation rate (Table 1). Testing for malaria parasite and human immunodeficiency virus (HIV) was also negative. A chest radiograph showed no abnormalities, but abdominal ultrasonography confirmed splenic enlargement. Splenic aspiration was performed with a 5-mL syringe from the middle axis of the spleen. The slide was prepared using Giemsa stain and then examined under microscope, but no *L. donovani* body was found. The patient was treated supportively but no improvement was observed. The splenic aspiration was repeated but was also negative. Thus, a PCR was performed from the peripheral buffy coat of the collected blood sample following standard procedure,[10] which was positive for *L. donovani* DNA. The patient was treated with liposomal amphotericin B at 5 mg/kg body weight for three alternative days. Six months after completion of treatment, the patient had maintained complete clinical recovery and a follow-up PCR was done after 6 months of treatment completion and found negative for *L. donovani* DNA along with no clinical symptoms and signs.

Case 2. The second case was also reported from Noapara Village, Sakhua Union and Trishal Subdistrict from Mymensingh District (one of the most endemic village of Bangladesh for VL), with the complaints of fever and cough. He was a 6-year-old boy and also treated with single-dose liposomal amphotericin B under the same clinical trial. His spleen measured 4 cm below the costal margin on examination and initial laboratory results were similar to Case 1. He was also referred to the research center, and after 1 week of failed supportive care, a splenic aspiration was unrevealing. All other laboratory investigations performed same as in Case 1 including HIV and were found insignificant. Thus, on the basis of the experience gained from Case 1, PCR was performed from the peripheral blood buffy coat and was positive for *L. donovani* DNA. He was treated as Case 1, and similarly at 6 months, he had maintained complete clinical resolution. A successful treatment outcome was confirmed by a follow-up PCR after 6 months of treatment completion along with no clinical symptoms and signs.

Laboratory process (PCR). *Blood collection.* Of venous blood, 5 mL was collected into an ethylenediaminetetraacetic acid tube following standard aseptic procedure.

Collection of buffy coat. The blood, 30 minutes after blood collection, was centrifuged at 4,000 rpm for 10 minutes. Then 500 μL of buffy coat was aspirated by using the tip of the micropipette from the middle leukocyte layer of the tube and transferred into a 1.5-mL microcentrifuge tube and preserved at −20°C for PCR amplification.

DNA extraction. Extraction of DNA from buffy coat was performed with the Qiagen DNA Blood Mini Kit (Qiagen,

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TABLE 1
Laboratory investigation report

	Case 1	Case 2
Total count of WBC (per mm^3)	14,000	10,000
Differential count (%)		
Neutrophil	71	68
Lymphocyte	19	18
Basophil	00	00
Monocyte	04	03
Eosinophil	02	03
Hemoglobin (g/dL)	11.4	11.2
Total count of RBC (million/μL)	4.2	4.1
Hematocrit (%)	33.7	33.9
Mean corpuscular volume (fL)	79.5	77.5
Mean corpuscular hemoglobin (pg)	26.9	29.9
Mean corpuscular hemoglobin concentration (g/dL)	33.8	35.4
Erythrocyte sedimentation rate (mm/hour)	60	75
Platelet count (per μL)	220,000	280,000
Fasting blood sugar (mg/dL)	102	96
Blood urea nitrogen (mg/dL)	16	18
Serum creatinine (mg/dL)	0.8	0.7
Serum bilirubin (mg/dL)	0.9	0.6
Alanine aminotransferase (U/L)	26	23
Aspartate transaminase (U/L)	24	28

RBC = red blood cell; WBC = white blood cell.
Normal value reference.[8,9]

Hilden, Germany). Then DNA was eluted in 0.2 mL of AE buffer (supplied with the Qiagen kit). The purity of the DNA was identified by the ratio of optical density at A260/A280, which was within 1.7–1.9 for all DNA samples. Molecular grade water (Qiagen, Hilden, Germany) was used instead of blood as an extraction control to check for carryover contamination in every run of DNA extraction and PCR amplification.

PCR procedure. Previously reported *Leishmania*-specific nested PCR (Ln-PCR) with primers targeting the parasite's small-subunit ribosomal RNA was used. For the first PCR run, Kinetoplastida-specific primers (R221 5′-GGTTCCTTT CCTGATTTACG-3′ and R332 5′-GGCCGGTAAAGGCCG AATAG-3′) were used. In the first PCR, 2 μL of extracted DNA were amplified in a final volume of 25 μL containing 12.5 μL of Bio-Rad iQ Supermix (Bio-Rad, Milan, Italy). In addition, 0.3 μmol/L of each Kinetoplastida-specific primers R221 and R332 and additional 3.0 mM $MgCl_2$ were added. Amplification was performed on the Bio-Rad MyCycler. The PCR program was run for 40 cycles that consisted of denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, and extension at 72°C for 30 seconds.

Before the second amplification, the amplified products from the first run were diluted at 1:50 with molecular grade water and 1 μL of this was added to a 25 μL reaction volume containing 0.15 μmol/L of the *Leishmania*-specific primers R223 and R333. For the second round, the thermal condition used was 35 cycles of denaturation, annealing, and extension at 94°C, 65°C, and 72°C, respectively, for 30 seconds. In both amplifications, Taq DNA polymerase activation was performed at 95°C for 3 minutes and a final extension at 72°C for 5 minutes. Amplification products were separated by electrophoresis on 2% agarose gel with DNA "ladder" (cat. no.

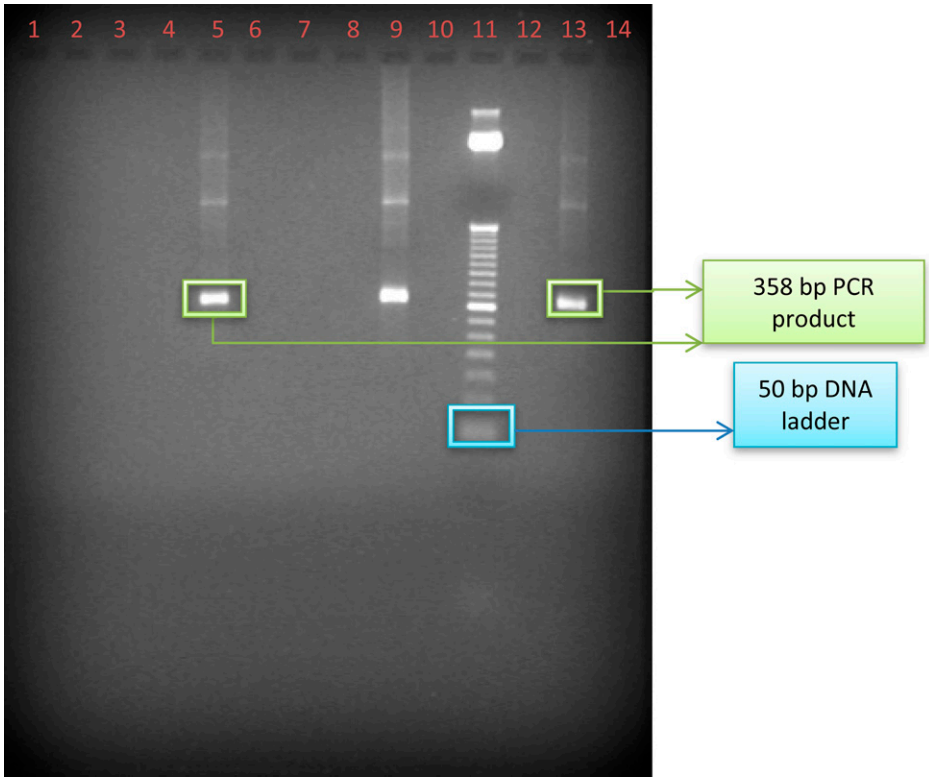

FIGURE 1. Gel electrophoresis pattern of *Leishmania*-specific nested polymerase chain reaction products amplified from extracted DNA samples. Lane 2, 3, 4, 7, 8, 10, and 12 = blank; lane 5 and 13 = 358-bp amplification products observed from case samples 1 and 2 before treatment; lane 6 and 14 = no amplification observed from case samples 1 and 2 after treatment; lane 9 = positive control; lane 1 = negative control (dH_2O); and lane 11 = 50-bp DNA ladder.

15628-019; Invitrogen, Waltham, MA) as molecular size marker, and stained with ethidium bromide (0.1 mg/mL). Stained gel was visualized and photographed under ultraviolet (UV) light emission with a UV transilluminator (S.N. 75S/03589; Bio-Rad, Milan, Italy). Amplification products were visualized and positive samples yielded a PCR product (Figure 1). In every run, molecular grade water was used as negative control and DNA from cultured promastigotes served as positive control.

DISCUSSION

To our knowledge, these two cases are the first reported VL recurrence cases diagnosed by Ln-PCR of the peripheral blood after a negative splenic microscopy. Importantly, a strong clinical suspicion guided this additional investigation and raises the possibility that other similar cases in endemic regions may remain undiagnosed.

Splenic aspiration and microscopy is a sensitive test that is of high yield in resource-limited settings.[5] However, on basis of these two cases, we therefore suggest that PCR should be used as a confirmatory diagnostic for cases of suspected recurrence and we provide the detailed laboratory procedure to complete these investigations. We advocate study of implementing this algorithm in Bangladesh including means of sample transportation and/or patient referral to centers equipped in PCR diagnosis and treatment of highly suspected splenic aspiration microscopy-negative recurrence. Furthermore, the government of Bangladesh is now promoting liposomal amphotericin B as the first-line treatment option for VL in various dosing schedules given its superiority in safety and early efficacy compared with other options (sodium stibogluconate, miltefosine, and paromomycin).[4,7] It is possible that late recurrence, such as observed in these cases, may be observed with increased frequency after this programmatic change, and requires a larger scale evaluation to determine the best approach to diagnosis and treatment of recurrence in this new era.

In Bangladesh, elimination of VL as a public health problem is a realistically achievable national priority. Thus, detection and treatment of every case is necessary to ultimately break the transmission cycle. Molecular diagnostic methods, such as the one we described, could likely accelerate this achievement.

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