

## Short Communication

# PCR-Based Detection of *Leishmania* DNA in Skin Samples of Post Kala-Azar Dermal Leishmaniasis Patients from an Endemic Area of Bangladesh

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**SUMMARY:** Post kala-azar dermal leishmaniasis (PKDL) is a sequel of visceral leishmaniasis (VL) and PKDL patients are an important reservoir for anthroponotic transmission of VL. Therefore, diagnosis and treatment of PKDL is important for the kala-azar elimination program in South Asia, including Bangladesh. While definitive diagnosis of PKDL is still based on microscopy, despite the low sensitivity of this method of diagnosis, PCR for identification of kinetoplast DNA (*kDNA*) from *Leishmania* parasites is expected to be a rapid and sensitive diagnostic method. We attempted PCR-based diagnosis from skin biopsy specimens and compared PCR to other available detection methods in order to determine the acceptability and feasibility of the PCR diagnostic method in an endemic area of VL in Bangladesh. Both skin biopsy specimens and blood samples were collected from 110 patients suspected to have PKDL from 6 subdistrict health complexes in Mymensingh, Bangladesh. Using microscopy, we identified 32 samples (29.1%) that were positive for *Leishmania*. Immunochromatography tests indicated that 85 samples (77.3%) were positive for *Leishmania*. In contrast, a total of 104 (94.5%) samples tested positive using nested PCR, while unaffected portions of skin from PKDL patients tested negative. Sequencing analysis of the PCR products indicated that the amplified portion had more than 98% nucleotide sequence identity to the *Leishmania donovani* reference strain, D10. These findings indicate that the PCR method using a skin biopsy is highly sensitive and useful for confirmatory diagnosis of PKDL.

Kala-azar or visceral leishmaniasis (VL) is a symptomatic infection of the liver, spleen, and bone marrow that is caused by *Leishmania donovani* complex, *L. donovani*, *L. infantum* (syn. *L. chagasi*). *Leishmania* currently infects about 12 million people in 88 countries, causing approximately 57,000 deaths annually, with 350 million individuals at risk. More than 90% of all VL cases are from India, Nepal, Bangladesh, southern Sudan, and northeast Brazil (1). In Bangladesh, the highest number of VL cases was recorded in the district of Mymensingh, where the average annual incidence rate between 1994 and 2004 was 5.8/10,000; currently, the incidence rate is as high as 300/10,000 in the most affected communities (2,3).

Post kala-azar dermal leishmaniasis (PKDL) is a complication or sequel of VL and occurs in nearly 10–20% of patients who have been cured of VL in India and approximately 50–56% of such patients in Sudan (4,5). In India, the disease occurs 1–20 years after recovery from VL. In contrast, in Sudan, PKDL most often develops during treatment or within months after completion of VL treatment and the symptoms may persist for decades

in some patients. Clinically, the condition is characterized by the appearance of macules, papules, or nodules in the skin (6). Affected persons, though clinically well, harbor the *Leishmania* parasite in their skin lesions, and thus become an important reservoir in anthroponotic transmission of leishmaniasis (7). Although PKDL has been commonly diagnosed by microscopy, the sensitivity of this technique is low due to the very low number of parasites in slit skin smears and skin biopsy specimens. The low sensitivity of the diagnostic technique prolongs the time to diagnosis. The immunochromatography test (ICT) with rK39, a recombinant antigen, has been shown to be highly sensitive and specific for detection of antibodies in patients with VL and PKDL that is caused by members of the *L. donovani* complex (8). A PCR-based diagnostic methods for leishmaniasis, in which conserved sequences in leishmanial kinetoplast mini-circle DNA is amplified, has been developed in recent years. This PCR-based method is anticipated to provide a powerful approach to the diagnosis of leishmaniasis (9). To detect *Leishmania* DNA using PCR in PKDL patients, bone marrow aspirates, lymph node aspirates, skin aspirates, skin scrapings, and skin biopsies have been used as test specimens in India and Sudan (10–13). These studies indicated that skin specimens were useful because the samples were easy to collect using noninvasive means and PCR assays of these samples resulted in a high positivity rate. However, the efficacy of PCR-based detection of *Leishmania* DNA in skin specimens

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from PKDL patients has not yet been fully evaluated because there were fewer PKDL patients tested, and different studies used different types of skin specimens (aspirates, scrapings, and biopsies). In Bangladesh, a high sensitivity and specificity of PCR-based diagnosis of VL using peripheral blood buffy coat was reported (14). However, the PCR method had a low detection rate for *Leishmania* when slit-skin specimens from PKDL patients were used (15). There are no reports of PCR-based diagnoses for PKDL using skin biopsies. In the present study, the PCR method for detection of *Leishmania* DNA was compared to other available detection methods and evaluated for acceptability and feasibility using skin biopsy specimens from PKDL patients in an endemic area for VL in Bangladesh.

Skin biopsy specimens and blood specimens were collected from 110 patients suspected of having PKDL. Patients had macular, papular, or nodular lesions (74, 33, and 3 patients, respectively). Patients were recruited from the medicine and pediatric outpatient departments of Mymensingh Medical College Hospital, Mymensingh, Bangladesh, and six kala-azar endemic sub-district health complexes of Mymensingh, from July 2009 to June 2010. All suspected PKDL patients were previously treated with anti-leishmanial drugs for VL. The time between treatment and development of the suspected PKDL lesion ranged between 1 month and 4 years (Table 1). Skin biopsy specimens were collected from the patients. To accomplish this, using surface anesthetic in the selected lesion, the skin was caught and held by sterile tooth forceps in an upright direction. A thin slit of skin (approximately 3 mm × 3 mm in size) was then cut out using a sharp BP blade. From some PKDL patients, skin tissues from an unaffected part of the trunk were also taken to provide a negative control. The study protocol was reviewed and approved by the Institutional Review Board at Mymensingh Medical College. Informed consent was obtained from all patients, following the guidelines of the ethical review committee of Mymensingh Medical College.

We performed three diagnostic tests for PKDL: (i)

microscopic identification of *L. donovani* bodies (LDB) by means of Leishman staining of skin smears prepared by rubbing glass slides on the raw surface of biopsy specimens, (ii) ICTs for detection of anti-leishmanial antibody in serum from patients using Onsite *Leishmania* IgG/IgM Combo Rapid Tests (CTK Biotech, Inc., San Diego, Calif., USA), and (iii) nested PCR for amplification of *L. donovani* kinetoplast DNA (kDNA). To extract DNA for PCR, skin biopsy specimens were collected in NET buffer (150 mM NaCl, 15 mM Tris-HCl [pH 8.30], and 1 mM EDTA) and cut into small pieces using a pair of sterile fine scissors. Specimens in NET buffer were lysed by adding proteinase-K (100 µg/ml), 1% sodium dodecyl sulfate, and 1% Triton-X 100 and incubating the samples in a 50°C water bath for 1 h. Finally, DNA was extracted by phenol-chloroform extraction and ethanol precipitation. Nested PCR to amplify a 385-bp product from *L. donovani* kinetoplast mini-circle DNA was performed using the 1st and 2nd PCR primers as described previously (11,13). Nucleotide sequences of selected PCR products from clinical samples of PKDL patients were determined using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, Calif., USA) on an automated sequencer (ABI PRISM 3100).

Among the 110 samples from suspected PKDL patients, microscopy indicated that 32 samples (29.1%) were positive, while ICT indicated that 85 samples (77.3%) were positive. In contrast, the nested PCR method, in which a product with expected size (385 bp) was amplified, indicated that 104 (94.5%) samples were positive. The results of PCR amplification from several representative specimens are shown in Fig. 1. All 32 microscopy-positive specimens were also PCR-positive. The samples of unaffected skin from PKDL patients (20 specimens) tested negative using PCR. Sequencing analysis of amplified products from the nested PCR of six randomly selected specimens indicated that the portion had more than 98% sequence identity to the reference strain, D10 (GenBank accession no. AB458389).

PCR exhibited a considerably higher positive rate

Table 1. Detection of *L. donovani* by microscopy, ICT, and PCR, in PKDL patients with different lesion types/durations to develop PKDL lesions after VL treatment

Patient group	No. of patients	No. of positive specimens (% in the patient group)		
		Microscopy	ICT	PCR
<b>Lesion type</b>				
macular	74	2 (2.7)	53 (71.6)	69 (93.2)
papular	33	27 (81.8)	29 (87.9)	32 (97.0)
nodular	3	3 (100)	3 (100)	3 (100)
<b>Time to develop PKDL lesions after VL treatment</b>				
0-6 months	11	0 (0)	3 (27.3)	9 (81.8)
>6-12 months	15	3 (20.0)	9 (60.0)	13 (86.7)
>1-2 years	45	13 (28.9)	37 (82.2)	44 (97.8)
>2-3 years	33	11 (33.3)	31 (93.9)	32 (97.0)
>3-4 years	6	5 (83.3)	5 (83.3)	6 (100)
<b>Total</b>	<b>110</b>	<b>32 (29.1)</b>	<b>85 (77.3)</b>	<b>104 (94.5)</b>

ICT, immunochromatography test; PKDL, post kala-azar dermal leishmaniasis; VL, visceral leishmaniasis.

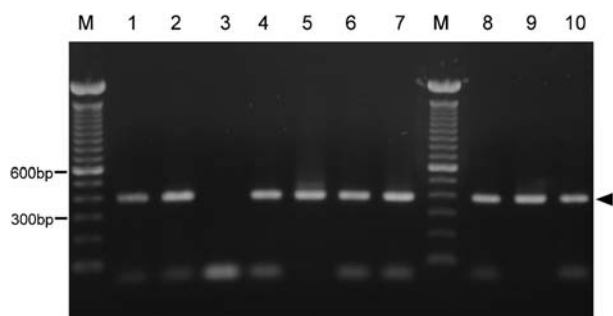


Fig. 1. *Leishmania donovani* kinetoplast DNA-specific PCR products (385 bp) indicated by an arrowhead from skin specimens of several suspected PKDL patients (lanes 1-10), except for lane 3 showing negative result. PCR products were visualized by staining with ethidium bromide after electrophoresis in 1% agarose gel. M, DNA size marker.

(93.2%) than microscopy (2.7%) and ICT (71.6%) among the macular lesion specimens, which were the most common. The detection rates in papular and nodular lesions were more than 80% using the three detection methods (Table 1). This finding suggests that macules do not contain as much *Leishmania* parasite as papules and nodules. In all patient groups, regardless of time after VL treatment, the highest detection rates were obtained using PCR, followed by ICT, and then microscopy. Positive rates generally increased for all the three detection methods as time between treatment and development of suspected PKDL increased, suggesting an increase in the number of parasites in lesions with time after VL treatment.

Previous reports indicated that microscopy had a detection rate between 4% and 58% for LDB in skin smears (5,15,16), and the detection rate was 29.1% in the present study. Such variable detection rates were considered to be caused because the diagnostic results of skin smear microscopy vary from person to person, and because it is prone to be influenced by the distribution of parasites throughout the skin tissue from which the skin biopsy was taken. Our results are in accordance with other authors who reported higher positive rates for PCR than for other detection methods (12,15). Although high detection rates of *Leishmania* DNA by PCR in suspected PKDL patients have been reported, lower rates were described when slit skin specimens were used for PCR; 42.9% in Bangladesh (12/28) (15), 82.7% in Sudan (19/23) (10). In contrast, in only two studies conducted in India, higher positivity rates, 93% (27/29) and 96% (24/25) were recorded by using skin biopsy specimens for PCR (12,13). However, the number of samples in these studies was too small to confirm the efficacy of PCR diagnosis. In the present study, a high positivity rate (94.5%) was demonstrated in a larger biopsy sample set ( $n = 110$ ). This finding confirms the advantage of using biopsy specimens from PKDL patients for PCR testing. It was especially noted that PCR was much more efficient for detection of *Leish-*

*mania* in macular lesions than microscopic diagnosis. We employed nested PCR, which might have increased the detection rate, as observed previously for PCR tests for leishmaniasis (13). In addition, the method of DNA extraction that we used, including cutting biopsy samples into small pieces, might be advantageous to efficient PCR.

In conclusion, the nested PCR method targeted at minicircle *kDNA* was highly effective and useful for detecting *L. donovani* genes in skin biopsy specimens from patients. The PCR method is recommended as a confirmatory diagnostic tool for PKDL.

**Conflict of interest** None to declare.

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