Leishmaniasis in India resulting from infection of the hemoflagellate protozoan parasite *Leishmania donovani* manifests in two different forms, visceral leishmaniasis (VL; also known as kala-azar) and its dermatologic sequel post–kala-azar dermal leishmaniasis (PKDL; discussed in ref. 1 and references therein). PKDL was first described by Brahmachari in 1922 in cured VL patients with eruptions and plaques in the skin, which was confirmed by demonstration of Leishman–Donovan bodies (LD) in slit skin smears, and it was termed dermal leishmanoid (discussed in ref. 1 and references therein). Later, the disease was renamed PKDL, because eruptions follow the visceral form, commonly called kala-azar. PKDL manifests in a variety of clinical forms ranging from hypopigmented macules to infiltrated plaques and nodules. Diagnosing PKDL has always been a challenge, because its geographical pocket is also endemic for leprosy, a disease that closely mimics PKDL. Because PKDL is the proposed reservoir, especially during the interepidemic periods of VL, diagnosing PKDL is of paramount importance to prevent further epidemics (discussed in ref. 1 and references therein). In more recent years, several diagnostic approaches have been developed ranging from serological tests and immunohistochemistry to polymerase chain reaction (PCR). Among the serological tests, the immunochromatographic strip test using recombinant kinesin 39 (rK39) is considered as a rapid, convenient, and useful test for diagnosis of Indian leishmaniasis. The K39 epitope is highly conserved in the visceralizing species of *Leishmania* and was used to develop a diagnostic test for VL and PKDL. In cases of VL, the rK39 nitrocellulose-based dipstick test showed high sensitivity and specificity and therefore, has gained substantial popularity for its ease of use, especially in the field setting. For detection of polymorphic PKDL (comprising both macular and papulo-nodular skin lesions), the sensitivity of the rK39 strip test is 95.6%, and for macular PKDL, the sensitivity is 86.3%. This report deals with three cases where relying on the rK39 strip test led to misdiagnosis and subsequent therapeutic delay. The study was approved by the Institutional Ethical Committee, and peripheral blood or skin biopsy was collected after obtaining informed consent from the parent/guardian of the minors.

**Abstract.** This report presents three cases where the rK39 strip test failed to diagnose two cases of post–kala-azar dermal leishmaniasis and one case of visceral leishmaniasis. However, a strong clinical suspicion prompted further evaluation by polymerase chain reaction (PCR), which established the etiology. The present case series highlights the usefulness of PCR in the diagnosis of leishmaniasis.

**CASE 1**

A 9-year-old boy from the Godda district of Jharkhand, India, presented with multiple hypopigmented macules occupying the face and upper torso as well as the upper and lower limbs (Figure 1). Five years ago, he had suffered from VL, for which he was treated with sodium stibogluconate (SSG). He complained of photosensitivity but had no sensory abnormality and no thickened or tender peripheral nerves. Cervical and axillary lymphadenopathy was present; however, no mucosal or genital lesions were present, and also, no systemic abnormality was found. Because the rK39 strip test was negative, he was not considered as a case of PKDL. Slit skin smear using modified Ziehl–Neelsen (ZN) stain and Giemsa was also negative for Acid Fast Bacilli (AFB) and LD body, respectively. However, on the grounds of a strong clinical suspicion of PKDL, indirect enzyme-linked immunosorbent assay (ELISA) was performed using crude *Leishmania* antigen as the coating antigen. The presence of antileishmanial antibody in serum was detected using horseradish peroxidase-conjugated Protein-A (Sigma-Aldrich Chemicals, St. Louis, MO), which reacted with substrate 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Roche Diagnostics, Mannheim, Germany), and optical density was measured at 405 nm (OD405) by a microplate reader (Model 680; Bio-Rad, Hercules, CA). The titer was considered as positive when the OD405 of the suspected case was at least twofold higher than the composite mean of 15 non-endemic controls (mean ± standard deviation [SD] was 0.08 ± 0.06). Because the OD405 was 0.26 and 3.3-fold higher, it was considered positive. We further confirmed the result by PCR (i.e., tested for parasite DNA in the skin biopsy sample [3-mm punch biopsy] using *Leishmania*-specific primers LITSR [5′-CTGGATCATTTTCCGATG-3′] and L5.8S [5′-TGATA CCACTTATCGACT-3′]) using Platinum Taq polymerase (Invitrogen, Carlsbad, CA), PCR buffer, and deoxyribonucleotide triphosphates (dNTPs; Fermentas, Glen Burnie, MD) in a Master cycler (Eppendorf, Hamburg, Germany). The PCR products were visualized by agarose gel electrophoresis (1.3%) and analyzed in G-BOX gel doc (Syngene, Cambridge, UK) using Gene Tools (version 4.01.04) software. DNA was isolated from both peripheral blood and skin biopsy by the QIAamp DNA mini kit (Qiagen, Hilden, Germany) and eluted in 200 μL elution buffer. For the PCR assay, a positive control...
DNA isolated from *L. donovani* promastigotes obtained from a patient with PKDL (SS10) and a negative control (water) were used. The PCR product length of the skin biopsy sample was 306 bp, which was comparable with the PCR product of the PKDL isolate (product length = 312 bp) (Figure 2). PCR was also performed in a peripheral blood sample obtained from a non-endemic human control, and it gave no band. The patient received SSG (20 mg/kg body weight per day intra-muscularly), because the patient was not from an area associated with resistance. Outcome of the treatment was assessed after 4 months and showed considerable improvement.

CASE 2

A 15-year-old girl from Murshidabad district of West Bengal, India, presented with multiple hypopigmented patches that were restricted to the face (Figure 3). The lesions were asymptomatic, and there was no sensory abnormality; importantly, they appeared 1 month after cure from VL. She had previously suffered from VL 1.5 years ago and had been treated with SSG. She gave no history of atopy, and peripheral nerves were not thickened. The slit skin smear did not reveal any AFB or LD body, and because rK39 was negative, the results posed a diagnostic dilemma; diagnosing PKDL solely on the basis of a positive history of VL was unjustified, especially because her lesions were restricted to the face. Indeterminate leprosy presenting with multiple lesions was also unlikely, and accordingly, the patient was offered ketoconazole shampoo and topical emollient, because seborrheic dermatitis was considered the most plausible cause. She was asked to return after 1 month but instead, returned after 2 months when the lesions had increased in size and number; again, there was no sensory abnormality or peripheral nerve thickening noted. Histopathology was performed, and it showed non-specific perivascular mononuclear infiltrate with no evidence of perineural/periappendageal localization that would be suggestive of leprosy.

With a strong clinical suspicion of PKDL, we performed indirect ELISA; the antileishmanial antibody titer was negative, OD<sub>405</sub> was 0.05, and the composite mean OD<sub>405</sub> of the control group was 0.08. PCR was done as previously described from a skin biopsy (3-mm punch biopsy) and was positive (having two bands of length of 318 and 415 bp) (Figure 2). Understandably, this was a case of false-negative rK39 strip.
test and ELISA, which delayed the diagnosis and led to unnecessary treatment for seborrheic dermatitis.

CASE 3

A 7-year-old boy from Canning, South 24 Parganas district of West Bengal, India, presented with fever without chill and rigor that was not associated with cough. He had previously suffered from VL and had received SSG, but for economic reasons, he was unable to complete the treatment. He remained afebrile for 4 months, and when he presented at the outpatient unit of the School of Tropical Medicine, he had been suffering for 7 months from a low-grade fever along with generalized weakness. There was no lymphadenopathy or hepatomegaly; spleen was enlarged 6 cm below the costal margin. The malaria parasite was not found, a chest radiograph showed no abnormalities; tests for human immunodeficiency virus (HIV) were negative, and there was no lymphadenopathy or hepatomegaly. Laboratory examination showed hemoglobin of 7.5 g/dL, hematocrit of 28.3%, mean corpuscular volume (MCV) of 74.9 fl, mean corpuscular hemoglobin (MCH) of 19.8 pg, and mean corpuscular hemoglobin concentration (MCHC) of 26.5 g/dL. The white blood cell count was 2,300 cells/mm³ (30% neutrophils, 62% lymphocytes, and 0.5% monocytes), platelet count was 210,000 cells/mm³, erythrocyte sedimentation rate (ESR) was 62 mm in the first hour, and prothrombin time was 11.0 seconds. Kidney function tests (urea = 19 mg/dL and creatinine = 0.7 mg/dL) and liver function tests (aspartate aminotransferase = 44 IU/L, alanine aminotransferase = 12 IU/L, alkaline phosphatase = 158 IU/L, total bilirubin = 1.0 mg/dL, and conjugated versus unconjugated = 0.4/0.6) were within normal limits. Total protein was 9.3 g/dL, and the albumin/globulin ratio was 2.7/6.6, indicating hypoalbuminemia and hypergammaglobulinemia. Blood sodium and potassium levels were normal as was blood sugar. Serological tests using dipstick rK39 showed no reactivity; however, the clinic and laboratory profile and the fact that the patient resided in an area where occurrence of VL has been reported prompted us to do ELISA, bone marrow aspiration for transformation of parasite, and PCR. The indirect ELISA OD₅₅₀ was 0.77, which was 9.6-fold higher than the composite mean of non-endemic healthy controls (0.08). Subsequently, bone marrow aspiration was done using a Salah's needle, wherein the posterior superior iliac spine was chosen as the site for aspiration. The aspirated material was collected, diluted 1:1 with Schneider's insect medium supplemented with 20% heat-inactivated fetal calf serum, penicillin G (50 IU/mL), and streptomycin (50 µg/mL), and kept in a tissue-culture flask (25 cm²) for transformation of parasites. Parasite transformation was evident 6 days later and was typed as L. donovani using a species-specific monoclonal antibody. Giemsa stain of bone marrow aspirate revealed 1–4 LD bodies per 10 oil immersion fields. Further confirmation was achieved by performing PCR using peripheral blood of this patient along with positive controls, which comprised of DNA isolated from promastigotes of three VL isolates (DD8, NS2, and YR08) confirmed to be L. donovani by ELISA using a species-specific monoclonal antibody along with negative control (DNA isolated from a peripheral blood sample of a healthy control from a non-endemic area of VL). The PCR product length of this patient was 306 bp, whereas the lengths of the L. donovani VL isolates DD8, NS2, and YR08 were 324, 422, and 419 bp, respectively (Figure 2).

The observed variation can, therefore, be attributed to a sequence variation known to occur between strains of L. donovani. The patient received a daily injection of amphotericin B (0.75 mg/kg body weight) for 4 weeks.

In case 1, it was very important to clinically differentiate it from lepromatous leprosy, because infiltration of peripheral nerves is symmetrical and asymptomatic, making the diagnosis difficult. Other tell-tale signs like photosensitivity and lymphadenopathy were important parameters to clinically differentiate PKDL from leprosy. In case 2, because both rK39 and indirect ELISA were negative, it suggests that the patient may have been immunocompromised (however, the patient tested negative for HIV) and therefore, only an antigen- or DNA-based test would be effective. In case 3, the clinical presentation prompted us to additionally evaluate by ELISA, PCR, and for the most definitive evidence, parasite transformation.

These three cases emphasize the need for relying on a high index of suspicion and clinical acumen in dealing with patients of VL and PKDL. The laboratory tests are always a supplement to clinical methods but relying too much on tests can be counterproductive. Slit skin smear, for detection of both AFB and LD bodies, lacks sensitivity, and here, the predictive value of a negative test is low. The rK39 is undoubtedly a useful test, especially in a field setting; however, if the rk39 is negative but clinical suspicion is high, one may consider performing a DNA-based test. In the Indian perspective, eradication of VL is presently a national priority. Because it is widely accepted that PKDL serves as the disease reservoir, it would, therefore, be pertinent to consider development of a referral system, where cases of PKDL can receive appropriate attention, to achieve the goal of eliminating VL.

Received October 18, 2010. Accepted for publication January 17, 2011.

Acknowledgments: The American Committee on Clinical Tropical Medicine and Travelers’ Health (ACCTMTH) assisted with publication expenses.

Financial support: This work received support from Department of Biotechnology and Council for Scientific and Industrial Research, Government of India.

Authors’ addresses: Nilay K. Das and Joyashree Nath Barbhuiya, Department of Dermatology, Calcutta Medical College, Kolkata, India, E-mails: drdasnilay@gmail.com and jnathbarbhuiya@yahoo.com. Sunny K. Singh and Bibhuti Saha, Department of Tropical Medicine, School of Tropical Medicine, Kolkata, India, E-mails: dr.sunnykssingh01@gmail.com and s.bibhuti@hotmail.com. Susmita Ghosh, Avijit Sarkar, Debanjan Mukhopadhyay, Susmita Roy, and Mitali Chatterjee, Department of Pharmacology, Institute of Postgraduate Medical Education and Research, Kolkata, India, E-mails: susmita_ghosh2010@yahoo.co.in, vijit2me@gmail.com, debmicro543@gmail.com, roy740@gmail.com, and ilatim@vsnl.net. Dwijendra Nath Ganguly, Department of Dermatology, School of Tropical Medicine, Kolkata, India, E-mail: malam022002@yahoo.com.

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