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Evaluation of PCR for Diagnosis of Indian Kala-Azar and Assessment of Cure

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This study was done to evaluate PCR with Ld1 primers for the diagnosis of Indian visceral leishmaniasis (VL) and to assess its role in prediction of the disease outcome. The PCR assay was performed with DNA isolated from the peripheral blood of parasitologically confirmed cases of VL before the initiation of treatment, just after the end of treatment, and at 3 and 6 months of follow-up. The pretreatment PCR result was positive for 100 of 101 patients (sensitivity, 99%; confidence interval [CI], 94 to 100%). None of the 150 negative controls tested were PCR positive (specificity, 100%; CI, 96.8 to 100%). Of 60 patients who were treated at our center, 51 (85%; CI, 73 to 93%) became negative immediately after treatment and continued to be negative at 3 and 6 months of follow-up. At the 3-month follow-up, two of the remaining nine patients were PCR positive, making 58 (96.7%; CI, 87 to 100%) patients PCR negative. At the 6-month follow-up, all patients became PCR negative. One patient who was PCR negative immediately after the end of treatment relapsed 11 months later. This limited prospective study with VL patients suggests that the PCR assay is a highly sensitive and specific (99% and 100%, respectively) tool for the diagnosis of VL. In the majority of patients, it can identify a successful disease outcome; however, its translation into the field setting remains a major challenge.

The diagnosis of visceral leishmaiasis (VL; kala-azar) rests upon the demonstration of parasites in splenic or bone marrow smears. Although the splenic smears are highly sensitive (>95%), the recovery of splenic tissue carries the risk of serious or fatal hemorrhage, while the sensitivity of bone marrow smears is unsatisfactory (13, 16). Culture of the aspirates might improve the sensitivity; but it is expensive and needs expertise and sophisticated equipment and, thus, is seldom used for routine clinical diagnosis (7, 16). The antibody-based diagnostics, like the direct agglutination test or rK39-based rapid immunochromatographic test, are highly sensitive; but they remain positive well beyond the time of cure, thus limiting their use for the diagnosis of relapses or reinfections (4, 17). Moreover, they also detect asymptomatic infections in areas of endemicity (15). In all forms of leishmaniasis, including VL, a sterile cure does not occur and it necessitates the definition of a cure by the use of clinical as well as parasitological parameters. Patients with VL are said to have achieved an "initial cure" at the end of treatment if there is resolution of fever, a reduction in spleen size, and the absence of parasites in splenic smears (5, 12). However, a few patients with an "initial cure" may relapse, and the majority of these do so within 6 months. Thus, for a patient to be defined as having a "final cure," the patient with an "initial cure" must remain free of signs or symptoms at the 6-month follow-up (5, 12, 14).

Recently, PCR assays with primers which amplify kinetoplast DNA (kDNA) have been evaluated for the diagnosis of VL and have been to shown to have excellent sensitivities and specificities (1, 3, 8), and they may prove to be useful for correlation of the findings at the time of diagnosis and the final outcome at the end of treatment (9). The aim of this prospective study was to evaluate the utility of PCR in the diagnosis of VL and to assess its value in identifying the disease outcome after treatment.

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MATERIALS AND METHODS

Patients. This study was conducted at the Kala-Azar Medical Research Center (Infectious Diseases Research Laboratory), Banaras Hindu University, Varanasi and Muzaffarpur, Bihar, India. The study was approved by the Ethical Committee of the Institute of Medical Sciences, Banaras Hindu University. Informed written consent was obtained from all patients. Pretreatment peripheral blood from 101 patients with parasitologically proven (by splenic smear and/or culture) kala-azar were included in the study of the PCR assay. Of these 101 patients, 60 patients opted for treatment at our center. PCR was repeated at the end of treatment and at 3 and 6 months of follow-up. Patients with a recurrence of the symptoms and signs of VL were subjected to splenic aspiration (smear and culture) and PCR of peripheral blood. Those with detectable parasites were considered to have relapses.

Controls. One hundred fifty controls were studied. These included 50 apparently healthy subjects each from regions of endemicity and nonendemicity and 50 patients with other diseases (malaria, 27 patients; tuberculosis, 15 patients; enteric fever, 5 patients; and leprosy, 2 patients). DNA extracted from *Leishmania donovani* DD8 (MHOM/IN/80/DD8) promastigotes from a purified culture were used as a positive control. The PCR mix with no DNA was used as a negative control.

Collection of material for PCR. Blood (1 to 2 ml) was collected and was either processed on the same day for the isolation of DNA or kept at −20°C for future use. Samples from the field site were brought to the laboratory on ice and processed in a fashion similar to that for the other samples.

DNA isolation. DNA was isolated by the phenol-chloroform method following the standard protocol. Briefly, fresh and frozen (after thawing) blood samples were treated with erythrocyte lysis buffer (pH 7.4; 155 mM NH_4Cl, 10 mM potassium bicarbonate, 0.1 mM EDTA). After centrifugation, the white blood cells pellets were suspended in extraction buffer (10 mM Tris HCl [pH 8.0], 0.1 mM EDTA, 20 μg/ml pancreatic RNase A, and 0.5% sodium dodecyl sulfate). Proteinase K (100 μg/ml) was used in both cases. DNA was extracted by phenol-chloroform extraction and ethanol precipitation. The DNA threads were washed with 70% ethanol and air dried. The DNA was dissolved in 50 μl TE buffer (10 mM Tris HCl [pH 7.5], 1 mM EDTA, autoclaved).

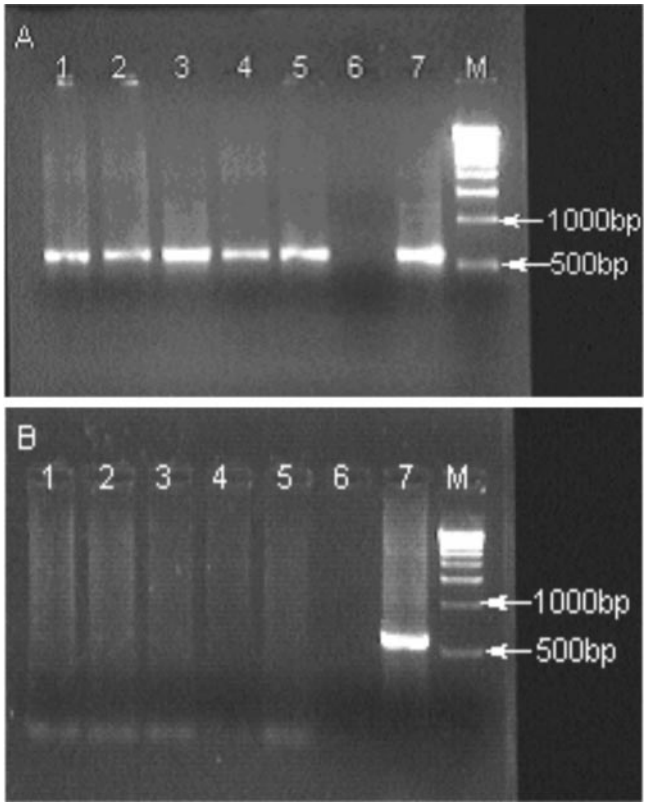

FIG. 1. PCR-amplified product of *Leishmania* minicircle kDNA isolated from a purified culture and peripheral blood of kala-azar patients. (A) Lanes 1 to 5, amplified DNA from the patients before the start of therapy; (B) lanes 1 to 5, negative PCR of the patients described for panel A after treatment; lanes 6 (A and B), negative control (PCR mix without template); lanes 7 (A and B), positive control (DNA from purified culture of *L. donovani*); lane M (A and B), marker (500-bp ladder).

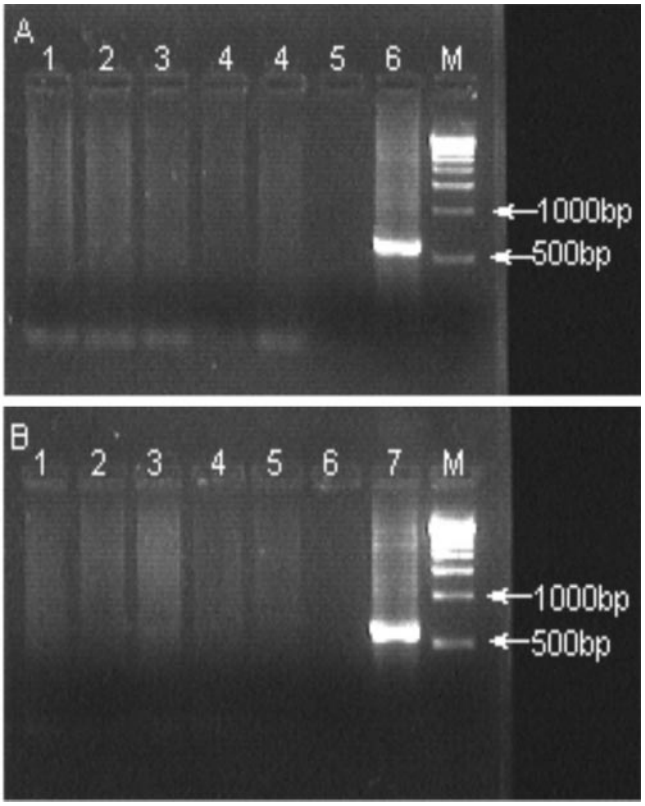

FIG. 2. PCR amplification of *Leishmania* minicircle kDNA isolated from a purified culture and peripheral blood of apparently healthy controls from areas of endemicity and nonendemicity. (A) Lanes 1 to 5, healthy controls from areas of nonendemicity; (B) lanes 1 to 5, controls from areas of endemicity; lanes 6 (A and B), negative control (PCR mix without template); lanes 7 (A and B), positive control (DNA from purified culture of *L. donovani*); lane M (A and B), marker (500-bp ladder).

Primers. The two *L. donovani* species-specific primers used were 5′-AAATC GGCTCCGAGGCGGGAAAC-3′ and 5′-GGTACACTCTATCAGTAGCAC-3′, together designated the Ld1 primers (11). These primers amplify a fragment of approximately 600 bp that is seen on gels (Fig. 1 and 2).

PCR amplification. DNA (5 μl) was added to 45 μl of a reaction mixture containing 10 mM Tris HCl (pH 8.3) and 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 50 ng of each primer, and 1.25 U of *Taq* DNA polymerase (Bangalore Genie, India). Amplification was performed with the heated lid option in a thermal cycler (MJ Research) programmed for 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 2 min, which were preceded by initial denaturation at 94°C for 2 min. Final extension was for 3 min at 72°C. The product was analyzed by electrophoresis in a 1% agarose gel containing ethidium bromide (0.5 μg/ml) in TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) and photographed under illumination on a gel documentation system (Alpha Imager, Alpha Innotech Corporation, San Leonardo, CA).

Statistical analysis. The sensitivity and specificity were calculated by using the following formulas: sensitivity = [number of samples with true-positive results/(number of samples with true-positive results + number of samples with false-negative results)] × 100; specificity = [number of samples with true-negative results/(number of samples with true-negative results + number of samples with false-positive results)] × 100. The positive and negative predictive values were calculated as the number of samples with true-positive results/(number of samples with true-positive results + number of samples with false-positive results) and as the number of samples with true-negative results/(number of samples with true-negative results + number of samples with false-negative results), respectively.

RESULTS

The results are listed in Table 1. All the negative controls were negative by PCR, whereas all positive controls were positive (Fig. 1 and 2), indicating the absence of contamination or inhibition of the PCR.

Pretreatment. Samples from 101 patients were subjected to PCR, and 100 of 101 (99%) were positive. For the only PCR-negative patient, the splenic smear was also negative, and the diagnosis of kala-azar was clinched by a positive culture result.

TABLE 1. Results of PCR with peripheral blood from 101 parasitologically proven visceral leishmaniasis cases before and after treatment

Patients with VL	No. of patients	No. (%) of samples with the following PCR result:	
		Positive	Negative
Pretreatment	101	100 (99)	1
Posttreatment			
1 day	60	9 (15)	51
3 mo	60	2 (3.3)	58
6 mo	60	0 (0)	60

TABLE 2. Specificity of PCR assay

Control group	No. of subjects	No. of subjects with the following PCR result:		Specificity (%)
		Positive	Negative	
Healthy controls (from regions of nonendemicity)	50	0	50	100
Diseased controls	50	0	50	100
Controls from regions of endemicity	50	0	50	100
Total	150		150	100

The sensitivity of PCR was 99%. All 150 controls, who were drawn from different populations, were negative by PCR; and this included 4 (8%) controls from areas of endemicity who tested positive by the rK39 rapid immunochromatographic test. These subjects with positive anti-rK39 antibodies were monitored for a period of 6 months and did not show any symptoms or signs suggestive of VL. Thus, the specificity of this PCR method was 100% (Table 2). The positive predictive value was 100%, whereas the negative predictive value was 99.3%.

After treatment. Sixty patients who opted for treatment at our center were available for follow-up. Samples from these patients were subjected to PCR a day after the completion of treatment. Of these, nine (15%) were still positive. However, all patients were asymptomatic and were monitored but received no further intervention.

Follow-up. After 3 months, samples from seven of the nine patients who were PCR positive at the end of treatment turned PCR negative and samples from only two patients remained positive. At the 6-month follow-up, these two patients also became negative, and thus, by 6 months after treatment all patients became PCR negative. One patient relapsed 11 months after the end of treatment. This patient had a recurrence of fever with enlargement of his spleen. His splenic aspirate was positive for *L. donovani* bodies, and PCR assay of peripheral blood was also positive. This patient was PCR negative at the end of treatment, and no signs or symptoms of relapse were noticed at the 6-month follow-up.

DISCUSSION

The highly sensitive rk39 antigen-based immunochromatographic strip test and direct agglutination test have been important steps forward in the diagnosis of kala-azar (16). However, the presence of antibodies in healthy controls from areas of endemicity is a major drawback to their use as diagnostic tools (15). The PCR assay has the capability of detecting low levels of parasitemia from the peripheral blood (6, 8). This capability can be exploited both for the diagnosis of the disease and for prediction of the disease outcome, since a negative PCR result at the end of treatment is likely to be associated with a favorable outcome (2, 10).

Although several studies that have developed PCR primers are available, none of the primers have been evaluated extensively for their utility in the actual diagnosis and management of kala-azar with peripheral blood. In our hands, the primers from Salotra et al. (11) performed well, and the sensitivity of the primers by PCR of peripheral blood was extremely satisfactory. No products were amplified from large numbers of samples drawn from healthy or diseased controls from regions of both endemicity and nonendemicity. Most importantly, immediately at the end of therapy, cure was indicated in 85% of the patients by a negative PCR result. Of the remaining nine patients, seven became negative at 3 months and two became negative at 6 months. Although one patient who was PCR negative posttreatment relapsed 11 months after the end of therapy, both the positive and the negative predictive values were very high: 100 and 99.3%, respectively.

The presence of DNA during follow-up was shown to be a predictor of the development of post-kala-azar dermal leishmaniasis (PKDL) in Sudan, where its incidence is very high following VL (9). In India, PKDL is an uncommon disease and develops 6 months to several years after the VL episode.

Another important feature of our study was the absence of amplification of a product from samples from any of the 50 controls from areas of endemicity, and it provides a significant advantage over antibody-based diagnostics. Although Salotra et al. (11) observed PCR amplification for 10% of their 20 controls, we did not observe any amplification for any of our 50 controls from areas of endemicity. Since it is not known whether the two controls of Salotra et al. (11) were monitored, it is possible that these two patients were in the early stages of developing clinical disease. The high specificity coupled with the very high sensitivity and noninvasive nature of the PCR method makes this method score over all available methods for the diagnosis of VL. Notwithstanding the high specificity obtained in this study, because of the high sensitivity of the PCR assay, there are chances that a product may be amplified from samples from healthy controls from regions of endemicity even in the absence of clinical disease (11). We plan to extend our experience with the PCR assay to a large sample of healthy controls from regions of endemicity.

This is also the first report of patients in a longitudinal follow-up study from India. Good concordance between the clinical outcome and the PCR results was observed. The only exceptional patient, who was PCR negative at the end of treatment and who fulfilled the criteria for a definitive cure at 6 months with no features suggestive of a relapse, had a recurrence of VL 5 months later. In the absence of definitive parameters, it is not possible to suggest with certainty whether this was a case of relapse or a case of reinfection. It does indicate the limitations of this method in predicting cure or relapse in all cases; nevertheless, in most patients the results of PCR correlated with the clinical state of the disease. Long-term follow-up studies with the subjects included in this study as well as previously cured VL patients will further delineate the role of PCR in the prognosis of the disease in terms of long-term cure, relapse, or the development of PKDL.

Thus, PCR is an important noninvasive tool which can be used for the diagnosis of VL with great accuracy. PCR can also be employed with significant precision to predict cure of the disease. The cost of one PCR works out to be 150 Indian national rupees (US$3.00) which, although it is slightly more expensive than the rK39 strip test (cost, US$1.50), the cost of the PCR is less than and/or comparable to those of other tests,

like splenic and bone marrow aspirate smears, enzyme-linked immunosorbent assay, and direct agglutination tests. Attempts should be made to establish nodal laboratories spread over the regions of endemicity to make this important diagnostic tool available in such regions.

REFERENCES

1. **Adhya, S., M. Chatterjee, M. Q. Hassan, S. Mukherjee, and S. Sen.** 1995. Detection of Leishmania in the blood of early kala-azar patients with the aid of the polymerase chain reaction. Trans. R. Soc. Trop. Med. Hyg. **89:**622–624.
2. **Cascio, A., S. Calattini, C. Colomba, C. Scalamogna, M. Galazzi, M. Pizzuto, R. Camilli, M. Gramiccia, L. Titone, M. Corbellino, and S. Antinori.** 2002. Polymerase chain reaction in the diagnosis and prognosis of Mediterranean visceral leishmaniasis in immunocompetent children. Pediatrics **109:**E27.
3. **Fisa, R., C. Riera, E. Ribera, M. Gallego, and M. Portus.** 2002. A nested polymerase chain reaction for diagnosis and follow-up of human visceral leishmaniasis patients using blood samples. Trans. R. Soc. Trop. Med. Hyg. **96**(Suppl. 1)**:**S191–S194.
4. **Hailu, A.** 1990. Pre- and post-treatment antibody levels in visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. **84:**673–675.
5. **Jha, T. K., S. Sundar, C. P. Thakur, P. Bachmann, J. Karbwang, C. Fischer, A. Voss, and J. Berman.** 1999. Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. N. Engl. J. Med. **341:**1795–1800.
6. **Lachaud, L., J. Dereure, E. Chabbert, J. Reynes, J. M. Mauboussin, E. Oziol, J. P. Dedet, and P. Bastien.** 2000. Optimized PCR using patient blood samples for diagnosis and follow-up of visceral leishmaniasis, with special reference to AIDS patients. J. Clin. Microbiol. **38:**236–240.
7. **Lightner, L. K., J. D. Chulay, and A. D. Bryceson.** 1983. Comparison of microscopy and culture in the detection of Leishmania donovani from splenic aspirates. Am. J. Trop. Med. Hyg. **32:**296–299.
8. **Nuzum, E., F. White III, C. Thakur, R. Dietze, J. Wages, M. Grogl, and J. Berman.** 1995. Diagnosis of symptomatic visceral leishmaniasis by use of the polymerase chain reaction on patient blood. J. Infect. Dis. **171:**751–754.
9. **Osman, O. F., L. Oskam, E. E. Zijlstra, A. M. el-Hassan, D. A. el-Naeim, and P. A. Kager.** 1998. Use of the polymerase chain reaction to assess the success of visceral leishmaniasis treatment. Trans. R. Soc. Trop. Med. Hyg. **92:**397–400.
10. **Pizzuto, M., M. Piazza, D. Senese, C. Scalamogna, S. Calattini, L. Corsico, T. Persico, B. Adriani, C. Magni, G. Guaraldi, G. Gaiera, A. Ludovisi, M. Gramiccia, M. Galli, M. Moroni, M. Corbellino, and S. Antinori.** 2001. Role of PCR in diagnosis and prognosis of visceral leishmaniasis in patients coinfected with human immunodeficiency virus type 1. J. Clin. Microbiol. **39:**357–361.
11. **Salotra, P., G. Sreenivas, G. P. Pogue, N. Lee, H. L. Nakhasi, V. Ramesh, and N. S. Negi.** 2001. Development of a species-specific PCR assay for detection of *Leishmania donovani* in clinical samples from patients with kala-azar and post-kala-azar dermal leishmaniasis. J. Clin. Microbiol. **39:**849–854.
12. **Sundar, S., T. K. Jha, C. P. Thakur, J. Engel, H. Sindermann, C. Fischer, K. Junge, A. Bryceson, and J. Berman.** 2002. Oral miltefosine for Indian visceral leishmaniasis. N. Engl. J. Med. **347:**1739–1746.
13. **Sundar, S., K. Kumar, V. P. Singh, and T. M. Mahopatra.** 1991. Diagnostic lag period in kala-azar: test for early diagnosis needed. J. Assoc. Physicians India **39:**651.
14. **Sundar, S., D. K. More, M. K. Singh, V. P. Singh, S. Sharma, A. Makharia, P. C. Kumar, and H. W. Murray.** 2000. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin. Infect. Dis. **31:**1104–1107.
15. **Sundar, S., K. Pai, M. Sahu, V. Kumar, and H. W. Murray.** 2002. Immunochromatographic strip-test detection of anti-K39 antibody in Indian visceral leishmaniasis. Ann. Trop. Med. Parasitol. **96:**19–23.
16. **Sundar, S., and M. Rai.** 2002. Laboratory diagnosis of visceral leishmaniasis. Clin. Diagn. Lab. Immunol. **9:**951–958.
17. **Sundar, S., S. G. Reed, V. P. Singh, P. C. Kumar, and H. W. Murray.** 1998. Rapid accurate field diagnosis of Indian visceral leishmaniasis. Lancet **351:** 563–565.