Potential of Direct Agglutination Test Based on Promastigote and Amastigote Antigens for Serodiagnosis of Post-Kala-azar Dermal Leishmaniasis

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Post-kala-azar dermal leishmaniasis (PKDL) is a dermal complication, a sequel to kala-azar. Diagnosis of PKDL presents a challenge due to the low parasite burden in the lesions. The direct agglutination test (DAT) based on promastigote and amastigote antigens of Leishmania donovani of indigenous isolates was developed to diagnose PKDL, and the results were compared with those of the rk39 strip test. The sensitivities of DAT for antileishmanial antibody detection, based on promastigote and amastigote antigens at a cutoff titer of 1:800 were 98.5% and 100%, respectively, with corresponding specificities of 96.5% and 100%. DAT could correctly detect 100% polymorphic cases and 95.4% macular PKDL cases. In comparison, the rk39 strip test was able to correctly diagnose 95.6% of polymorphic and 86.0% macular PKDL cases. DAT based on axenic amastigote antigen provided 100% sensitivity and specificity, making it particularly useful for macular PKDL cases, which are often missed by the rk39 strip test. Thus, DAT provides a simple, reliable, and inexpensive test for PKDL diagnosis with potential applicability in field conditions.

Post-kala-azar dermal leishmaniasis (PKDL), a dermal extension in treated kala-azar (KA) patients, is caused by the protozoan parasite Leishmania donovani. The disease develops in a variety of clinical forms from hypopigmented macules to infiltrated papules and nodules. In India, it manifests in 5 to 15% of KA cases after months or several years of remission from infection, while in Sudan it develops within weeks to a few months in 50 to 60% of cured KA cases (14, 25, 26). On the Indian subcontinent, untreated cases of KA and PKDL are considered the sole reservoir to house and disseminate the causative parasite in the absence of zoonotic transmission (4, 24).

Demonstration of parasite in the slit smear or by culture of the dermal tissue is considered the gold standard; however, the methods involved are invasive, poorly sensitive (58%), and difficult to perform in field conditions (20). In particular, the macular form of PKDL has often been misdiagnosed as leprosy or vitiligo, as the parasite load is low and not always proportional to the extent of dermal lesions (13). When the parasite is not demonstrated in skin biopsy specimens, the diagnosis of PKDL hinges on the endemicity of KA in the area and previous history of infection by the parasite. In 15 to 20% of PKDL patients, the absence of a history of KA suggests subclinical infection and poses further difficulty for diagnosis (14). With the use of techniques, such as enzyme-linked immunosorbent assay using crude and recombinant antigens (1, 18, 21), rk39 strip test (16), Western blotting (15), PCR (12, 17), and immunohistochemistry using monoclonal antibodies (8, 19), the sensitivity and specificity for the diagnosis of PKDL have been improved.

The direct agglutination test (DAT) with trypsinated, Coomassie brilliant blue-stained antigen has been shown to be a simple, highly specific, and sensitive test for diagnosis of KA in different geographic regions (2, 6, 10, 22). A study from Sudan has demonstrated the utility of DAT for diagnosis of PKDL (7); however, no such studies were performed in India. As the clinical manifestation of the disease is quite distinct in the two regions, this study aims to define the potential of DAT using antigens prepared from indigenous parasite isolates. In addition to the promastigote antigen, we included antigen from axenically grown amastigotes, the stage present in the mammalian host.

MATERIALS AND METHODS

Clinical samples. Blood samples were collected by venipuncture for sera from 128 individuals in various categories. Informed consent was obtained from all the individuals prior to sample collection.

PKDL patients. Sixty-eight clinically confirmed cases of PKDL, hailing from the state of Bihar where KA is endemic, and reporting to the Dermatology Department, Safdarjung Hospital, New Delhi, India, were included in the study. Of these 68, 62 reported a history of KA, while the remaining 6 had no history of KA. Dermal lesions manifested a few months to as long as 20 years after recovery from KA. Forty-six patients showed the polymorphic picture characterized by erythematous indurated areas, papulonodules, and hyperchromic macules scattered all over the body. Twenty-two patients had the macular presentation with characteristic symmetric and generalized distribution of hypopigmented macules to the extremities and trunk. Histopathological findings upon skin biopsies were similar to those reported earlier (11, 19). The dermis showed a diffuse infiltration by lymphocytes, histiocytes, and plasma cells. All patients responded well to therapy with sodium antimony gluconate.

Kala-azar patients. Ten confirmed cases of KA, reporting to the Department of Medicine of Safdarjung Hospital, with fever and splenomegaly and demonstrated to have Leishmania parasites in bone marrow aspirates, were included as positive controls in the study.

Leprosy and vitiligo patients. Twenty-six patients with skin diseases that are often confused with PKDL, i.e., lepromatous leprosy and vitiligo, were included in the study. Samples from 15 patients with lepromatous leprosy and 11 patients with vitiligo (confirmed by histopathology) who reported to the Department of Dermatology of Safdarjung Hospital were taken.
TABLE 1. Results of the direct agglutination test and rk39 strip test in various patient groups

| Patient category | No. of cases | No. of serum samples positive by DAT at the following dilution: | rk39 strip test result |
|------------------|--------------|---------------------------------------------------------------|-----------------------|
|                  |              | <1:100 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1,600 | 1:3,200 | 1:6,400 | >1:12,800 | Positive | Negative |
| PKDL             |              |        |        |        |        |        |        |        |        |          |          |          |
| Polymorphic      | 46           | 2      | 4      | 3      | 37     | 2      | 11     | 19     | 3      |          |          |          |
| Macular          | 22           | 1      | 7      | 1      | 1      | 11     | 19     | 3      | 10     |          |          |          |
| Kala-azar        | 10           | 10     | 10     |        |        |        |        |        |        |          |          |          |
| Leprosy          | 15           | 15     |        |        |        |        |        |        |        |          |          |          |
| Vitiligo         | 11           | 11     |        |        |        |        |        |        |        |          |          |          |
| Geographic controls* | 12     | 12     |        |        |        |        |        |        |        |          |          |          |
| Healthy controls | 12           | 12     |        |        |        |        |        |        |        |          |          |          |

* Healthy relatives of patients living in Bihar, India, where KA is endemic.

Healthy and geographic controls. Healthy controls (n = 12) were subjects living in Delhi, India. Geographic controls (n = 12) were the first-degree healthy relatives of patients living in Bihar, India, an area known for its endemcity for KA.

Parasite culture. Parasite cultures were set up from bone marrow samples from KA patients or dermal lesions of PKDL patients and propagated as promastigotes in M-199 (Sigma-Aldrich, Steinheim, Germany) supplemented with 25 mM HEPES (pH 7.5) and 10% fetal calf serum (Biological Industries, Israel) as described earlier (18). The indigenous isolates of parasites causing KA and PKDL were characterized as \textit{L. donovani} using monoclonal antibodies and a species-specific PCR (17, 23). Promastigotes of \textit{L. donovani} strain AG83 (MHOM/IN/AG83) originally isolated from a KA patient were also grown as described above. Axenic amastigotes from an indigenous isolate of \textit{L. donovani} were cultured by gradual adaptation of promastigotes to grow at pH 5.5 and 37°C in 6% CO2 atmosphere as described earlier (9, 23).

Antigen preparation. Promastigotes of \textit{L. donovani} isolated from KA and PKDL patients and AG83 strain (MHOM/IN/AG83) were mass cultured in M-199 with 10% fetal calf serum. The antigen was prepared by the method of Harith et al. (6, 7). Briefly, the promastigotes were harvested in logarithmic phase, washed repeatedly with cold Hanks balanced salt solution (HBSS) (In Vitrogen Corporation, Grand Island, NY), treated with 0.4% trypsin (Sigma) in HBSS at 10^6 parasites/ml for 1 h at 37°C. After the promastigotes were washed, they were fixed for 20 h at 4°C with 2% formaldehyde in HBSS. After fixation, the parasites were washed with citrate saline (0.15 M NaCl and 0.05 M sodium citrate, pH 7.4) to check the lysis of parasite during storage and stained with 0.02% Coomassie brilliant blue R250 (Sigma) in the same citrate-saline solution for 12 h at 4°C. After the promastigotes were washed, the stained, fixed promastigotes were resuspended in citrate saline containing 0.4% formaldehyde, the concentration was adjusted to 5 x 10^7 parasites/ml, and the antigen was stored at 4°C until further use. Logarithmic-phase axenic amastigotes were harvested and processed for antigen preparation essentially as the promastigote antigen was. The trypsinized, fixed, and stained amastigotes were resuspended in citrate saline containing 0.4% formaldehyde, and the concentration was adjusted to 5 x 10^7 parasites/ml.

DAT. The DAT was performed essentially as described earlier (6, 7). Serum samples were diluted in physiological saline (0.9% NaCl) containing 0.2% gelatin (Sigma) and 0.78% β-mercaptoethanol (Sigma). Twofold serial dilution of sera were made starting at a dilution of 1:50 and going up to 1:409,600. Fifty microliters of DAT antigen was added to each well containing 30 μl diluted serum in a V-shaped microtiter plate, and the results were read after 20 h of incubation at 25°C. For evaluation of the stability of antigen stored at 4°C, one positive control and one PKDL sample along with a negative control were tested every 15 days for up to 6 months by DAT.

rk39 strip test. The dipstick test using rk39 in the form of antigen-impregnated nitrocellulose paper strips (InBios, Inc., Seattle, WA) adapted for use under field conditions was used. The test was performed per the manufacturer’s instructions.

RESULTS

DAT. A total of 128 serum samples were collected and tested by DAT. initially, promastigote antigens derived from three different \textit{L. donovani} cultures (reference strain MHOM/IN/83/AG83, a field isolate from a KA patient, and an isolate from dermal lesions of a PKDL patient) were used. AG83 antigen gave titers of 1:204,800 to >1:409,600 for KA and 1:800 to >1:409,600 for PKDL. With PKDL-derived antigen, the titer for KA ranges from 1:12,800 to >1:204,800 and 1:800 to >1:204,800 for PKDL sera. The control groups were negative for all antigens, except for two geographic controls which gave a titer of 1:800 with both AG83 and PKDL-derived antigens. KA-derived antigen used for comprehensive study results in titers of >1:204,800 for KA sera and 1:800 to >1:204,800 for PKDL sera; results for the control group were similar to the results with AG83 and PKDL-derived antigens.

Subsequently, antigen derived from parasites isolated from bone marrow aspirates of a KA patient was used.

Test samples included samples from 68 PKDL patients and 50 controls along with 10 KA patients to serve as positive controls. The antileishmanial antibody titer of the PKDL cases with polymorphic presentation ranged from 1:800 to >1:409,600, while in patients with the macular form of the disease, the titer ranged from 1:800 to 1:204,800. In general, the titer was lower in macular cases; with a cutoff titer of 1:3,200, a majority of polymorphic PKDL cases, 40/46 cases (87%), were correctly diagnosed, while the corresponding figure in the macular PKDL was 13/22 (59%). The 1:800 dilution clearly differentiated the PKDL cases from the control groups, as the titer for controls ranged from <1:100 to 1:400, except for two samples from geographic controls that gave a titer of 1:800 (Table 1).

A set of PKDL samples including all the cases giving a titer of ≤1:6,400 (n = 20) with promastigote antigen (Table 1), along with all the controls, was examined with antigen derived from the amastigote stage of the parasite. The titer increased more than twofold for kala-azar and PKDL cases, while it remained <1:800 for all the controls.

Table 2 shows the sensitivity and specificity of DAT for the detection of PKDL cases with promastigote and amastigote antigens at various cutoff titers. With the cutoff titer of 1:800, the combination of 98.5% sensitivity and 96.0% specificity was achieved with promastigote antigen. DAT with the amastigote antigen gave 100% sensitivity and 100% specificity at 1:800, facilitating correct diagnosis in all patients, including those with macular PKDL, who in general showed low titers.

The titers for samples from PKDL and KA patients were found to be 1:51,200 and 1:204,800. When the samples were tested with stored DAT antigen, the titer was constant and did not fall over the time period of 6 months. The negative control
promastigote antigen were negative when tested with amastigote antigen.

The test was also specific in differential diagnosis of patients with other dermal diseases, such as leprosy and vitiligo, which are often difficult to separate on clinical grounds. Mostly hypopigmented macules, generalized nodules, and papules are common in patients with leprosy, and hypochromic patches are common in patients with vitiligo.

PKDL and KA are separate entities with distinct humoral and cellular immune responses (5, 15). A considerable relationship in the humoral immune response has been shown by positive serum sample reactivity of both KA and PKDL patients to rk39 antigen. The results of the present study also demonstrate a considerable serological relationship between the two, as similar results were obtained in DAT with antigen prepared using isolates from dermal lesions of PKDL patients, bone marrow aspirates of KA patients, or the reference strain Leishmania donovani AG83.

Therefore, we conclude that the use of indigenous parasite antigen-based DAT provides a useful test for detection of antileishmanial antibody in PKDL patients. The test would be applicable in field conditions, as it is easy to perform and requires no specific equipment and a small volume of serum is needed to perform the test. Moreover, the antigen was found to have a shelf life of ≥6 months. As PKDL is prevalent in areas with poor socioeconomic conditions, DAT using the indigenous parasite antigen would provide a more economical and practical assay for diagnosis. The use of amastigote antigen is recommended for the differential diagnosis of the disease from macular PKDL.

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REFERENCES

1. Badaro, R., D. Benson, M. C. Eulalia, M. Freire, S. Cumba, E. M. Netto, D. P. Sampaio, C. Madeira, J. M. Burns, R. L. David, and S. G. Reed. 1996. rk39: a cloned antigen of Leishmania chagasi that predicts active visceral leishmaniasis. J. Infect. Dis. 175:758–761.
2. Bern, C., S. N. Iha, A. R. Joshi, G. D. Thakir, and M. B. Bista, 2000. Use of recombinant K39 dipstick test and the direct agglutination test in a setting endemic for visceral leishmaniasis in Nepal. Am. J. Trop. Med. Hyg. 63:153–157.
3. Choudary, A., P. Y. Guru, A. Tandon, and K. C. Saxena. 1994. Enzyme linked immunosorbent assay in the diagnosis of kala-azar in Bhadohi (Varanasi), India. Trans. R. Soc. Trop. Med. Hyg. 84:363–366.
4. Djesjus, P. 2001. The increase in risk factors for leishmaniasis worldwide. Trans. R. Soc. Trop. Med. Hyg. 95:239–243.
5. Gasim, S., A. M. El Hassan, A. Kharrazi, E. A. Khalil, A. Ismail, and T. G. Theander. 2001. The development of post kala-azar dermal leishmaniasis (PKDL) is associated with acquisition of Leishmania reactivity by peripheral blood mononuclear cells (PBMC). Clin. Exp. Immunol. 119:523–529.
6. Harith, A. E., A. H. J. Kolak, J. Leeuwenberg, R. Muigai, E. Huigen, T. Jelsma, and P. A. Kager. 1988. Improvement of a direct agglutination test for field studies of visceral leishmaniasis. J. Clin. Microbiol. 26:1321–1325.
7. Harith, A. E., S. Choudary, A. Al-Masum, S. S. Santos, P. K. Das, S. Akhter, J. C. M. Vetter, and I. Haq. 1996. Reactivity of various leishmanial antigens in a direct agglutination test and their value in differentiating post-kala azar dermal leishmaniasis from leprosy and other skin conditions. J. Med. Microbiol. 44:141–146.
8. Ismail, A., A. Kharrazi, A. Permin, and A. M. El Hassan. 1997. Detection and characterization of Leishmania in tissues of patients with post kala-azar dermal leishmaniasis using specific monoclonal antibody. Trans. R. Soc. Trop. Med. Hyg. 91:263–265.
9. Joshi, M., D. M. Dwyer, and H. L. Nakhash. 1993. Cloning and characterization of differentially expressed genes from in vitro grown amastigotes of Leishmania donovani. Mol. Biochem. Parasitol. 58:345–354.

TABLE 2. Sensitivity and specificity of the direct agglutination test at various cutoff titers using promastigote and amastigote antigens

| Antigen and parameter | Value with the following cutoff titer: |
|-----------------------|---------------------------------------|
|                       | 1:400   | 1:800   | 1:1,600 | 1:3,200 |
| Promastigote antigen  | Sensitivity (%) | 98.5 | 98.5 | 94.1 | 77.9 |
|                       | Specificity (%) | 84.2 | 96.0 | 100 | 100 |
| Amastigote antigen    | Sensitivity (%) | 100 | 100 | 100 | 100 |
|                       | Specificity (%) | 100 | 100 | 100 | 100 |

always showed a compact blue dot at 1:50 dilution. These results indicate that there was no autolysis of the antigen and that it is stable for up to 6 months.

rk39 strip test. All 68 PKDL samples along with 60 control samples were subjected to the rk39 strip test. The strip test was positive for 63 of 68 patients, giving a sensitivity of detection of 92.6%. The sensitivity for detection of polymorphic PKDL patients was 95.6%, while for macular PKDL patients, it was 86.3%. The test gave negative results for all the controls and positive results for all cases of KA examined (Table 1).

DISCUSSION

Our study has shown that the DAT for detection of antileishmanial antibody provided a simple and reliable diagnostic test for PKDL. The utility of DAT in the diagnosis of PKDL was clearly demonstrated by the considerably high titer in both polymorphic and macular cases, with an overall sensitivity of 98.5% and specificity of 96%. The test exhibited 100% sensitivity for polymorphic PKDL and 95.4% sensitivity for macular PKDL compared to the rk39 strip test, which showed a sensitivity of 95.6% for polymorphic PKDL and a lower sensitivity (86.3%) for macular PKDL. Similar sensitivities of rk39 strips have been reported earlier, viz., 91% in general and 73% in macular PKDL cases (16).

With our set of serum samples, we found that the combination of sensitivity and specificity was optimal at a cutoff titer of 1:800 (Table 2). The titer for geographic controls was relatively higher than for controls from areas where KA was not endemic, indicating the possibility of previous exposure of the subjects to the parasite (3). Earlier studies from Sudan had shown that a cutoff titer of 1:3,200 was suitable for KA diagnosis and 1:1,600 was suitable for PKDL diagnosis (6, 7). Studies from India on DAT for KA used a cutoff titer of 1:800 (22), while no studies from India are available on DAT for the diagnosis of PKDL.

The use of the amastigote stage of the parasite in DAT led to improved antibody titer, further increasing the sensitivity and specificity of the test and proving the high potential of amastigote antigen in differential diagnosis. This is as expected, as this stage of the parasite is present inside the mammalian host and is responsible for inducing a specific immune response. Interestingly, a case of macular PKDL, which showed a faint positive band in the rk39 strip test, displayed a titer of 1:1,600 in DAT with amastigote antigen. Two of the geographic controls which were positive in DAT at 1:800 titer with...
10. Mbati, P. A., J. I. Githure, J. M. Kagai, G. Kirigi, F. Kibati, K. Wasunna, and D. K. Koech. 1999. Evaluation of a standardized direct agglutination test (DAT) for the diagnosis of visceral leishmaniasis in Kenya. Ann. Trop. Med. Parasitol. 93:703–710.
11. Mukherjee, A., V. Ramesh, and R. S. Misra. 1993. Post kala-azar dermal leishmaniasis: a light and electron microscopic study of 18 cases. J. Cutan. Pathol. 20:120–121.
12. Osman, O. F., L. Oskam, N. C. Kroon, G. J. Schoone, E. A. G. Khalil, A. M. El-Hassan, E. E. Zijlstra, and P. A. Kager. 1998. Use of PCR for diagnosis of post-kala-azar dermal leishmaniasis. J. Clin. Microbiol. 36:1621–1624.
13. Ramesh, V. 1994. On the difference between post kala-azar dermal leishmaniasis and leprosy. Trop. Doc. 24:120–121.
14. Ramesh, V., and A. Mukherjee. 1995. Post kala-azar dermal leishmaniasis. Int. J. Dermatol. 34:85–91.
15. Salotra, P., A. Raina, and V. Ramesh. 1999. Western blot analysis of humoral immune response to Leishmania donovani antigens in patients with post kala-azar dermal leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 93:98–101.
16. Salotra, P., G. Sreenivas, S. Sundar, and V. Ramesh. 2001. A simple and sensitive test for field diagnosis of post kala-azar dermal leishmaniasis. Br. J. Dermatol. 145:630–632.
17. Salotra, P., G. Sreenivas, G. P. Pogue, N. Lee, H. Nakhasi, V. Ramesh, and N. S. Negi. 2001. Development of a species-specific PCR assay for detection of Leishmania donovani in clinical samples of kala-azar and post-kala-azar dermal leishmaniasis. J. Clin. Microbiol. 39:849–854.
18. Salotra, P., G. Sreenivas, N. A. Ansari, B. V. Subha Raju, and V. Ramesh. 2002. Evaluation of ELISA for diagnosis of PKDL with crude or recombinant k39 antigen. Clin. Diagn. Lab. Immunol. 9:370–373.
19. Salotra, P., G. Sreenivas, K. R. Beena, A. Mukherjee, and V. Ramesh. 2003. Parasite detection in patients with post kala-azar dermal leishmaniasis in India: a comparison between molecular and immunological methods. J. Clin. Pathol. 56:840–843.
20. Singh, R. P. 1968. Observation on dermal leishmanoid in Bihar. Indian J. Dermatol. 13:59–63.
21. Singh, S., A. G. Sachs, K. P. Chang, and S. G. Reed. 1995. Diagnostic and prognostic value of k39 recombinant antigen in Indian leishmaniasis. J. Parasitol. 81:1000–1003.
22. Singla, N., G. S. Singh, S. Sundar, and V. K. Vinayak. 1993. Evaluation of the direct agglutination test as an immunodiagnostic tool for kala-azar in India. Trans. R. Soc. Trop. Med. Hyg. 87:276–278.
23. Sreenivas, G., R. Singh, A. Selvapandiyan, N. S. Negi, H. L. Nakhasi, and P. Salotra. 2004. Arbitrary-primed PCR for genomic fingerprinting and identification of differentially regulated genes in Indian isolates of L. donovani. Exp. Parasitol. 106:110–118.
24. Thakur, C. P., and K. Kumar. 1992. Post kala-azar dermal leishmaniasis: a neglected aspect of kala-azar control programmes. Ann. Trop. Med. Parasitol. 86:355–359.
25. Zijlstra, E. E., E. A. G. Khalil, P. A. Kager, and A. M. El Hassan. 2000. Post kala-azar dermal leishmaniasis in the Sudan: clinical presentation and differential diagnosis. Br. J. Dermatol. 143:136–143.
26. Zijlstra, E. E., A. M. Musa, E. A. G. Khalil, I. M. El-Hassan, and A. M. El-Hassan. 2003. Post-kala-azar dermal leishmaniasis. Lancet Infect. Dis. 3:87–98.