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Leish-KIT, a Stable Direct Agglutination Test Based on Freeze-Dried Antigen for Serodiagnosis of Visceral Leishmaniasis

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In order to increase the application potential of the direct agglutination test (DAT) for the detection of anti-*Leishmania* antibodies in human serum samples, we developed an antigen based on stained and freeze-dried *Leishmania donovani* promastigotes. We describe here the evaluation of the performance of the DAT based on this freeze-dried antigen. It was shown that the freeze-dried antigen remains fully active, even after storage at 56°C for 18 months. With a cutoff value of 1:1,600, the sensitivity of the DAT was shown to be 92% and the specificity of the test was 99.7%, which were comparable with the results found for the DAT based on liquid antigen. The major advantages of the freeze-dried antigen are that the production of a large batch of this antigen allows reproducible results in the DAT over a long period of time and that the freeze-dried antigen can be stored at ambient temperature, which, as was shown, makes the test a valuable diagnostic tool for use in the field.

Visceral leishmaniasis (VL), or kala-azar, is a disease caused by intracellular protozoan parasites of the *Leishmania donovani* complex. An estimated 200 million people are at risk of contracting the disease, with approximately 100,000 new cases annually (4). If the full-blown disease is not treated, mortality is almost 100%. Therefore, a rapid and accurate method for diagnosis is needed.

The routine diagnosis of VL may be based on one or more of the following methods: (i) the microscopical detection of the parasite in smears of lymph node, bone marrow, or splenic aspiration; (ii) the culturing of the parasite from patient material; or (iii) serological tests for the detection of anti-*Leishmania* antibodies. A relatively new and very sensitive technique for the detection of *Leishmania* parasites in blood, bone marrow, lymph node, or spleen material is PCR (19), but the application of this technique in the diagnosis of leishmaniasis is still in its infancy.

Compared with the first two categories mentioned above, serological tests have the advantage that blood sampling is relatively easy, with little inconvenience for the patient, and that many samples may be processed simultaneously. Over the years a number of tests for the detection of anti-*Leishmania* antibodies, such as the immunofluorescent antibody test (13), the enzyme-linked immunosorbent assay with either whole parasites or purified antigens (5, 13, 15), immunoblot analysis (14, 18), and the direct agglutination test (DAT), have been developed. This last test, making use of an aqueous suspension of stained *L. donovani* promastigotes (AQ antigen), was developed originally by Allain and Kagan (1) and further modified by Harith et al. (10, 11). The DAT is a fast and simple technique with a high sensitivity and specificity (11). This would make this technique very suitable for use in the field, as was demonstrated by Zijlstra et al. (20, 21), who used the DAT in field studies in the Sudan.

One of the major drawbacks of the DAT is the limited stability of the liquid antigen (11, 17). When no cooling facilities are available, as is often the case in the areas where VL is most frequently encountered, the availability of a stable form of the antigen used in the DAT would facilitate the use of this particular technique.

The aim of our research was to develop a DAT making use of an antigen that would be stable at ambient temperature for a prolonged period of time. We describe here the evaluation of a DAT based on freeze-dried antigen (FD antigen) for the detection of VL.

MATERIALS AND METHODS

Preparation of the antigen. AQ antigen was prepared essentially as described earlier by Harith et al. (11). Briefly, 15% fetal calf serum (Gibco BRL, Grand Island, N.Y.) and penicillin-streptomycin (Gibco BRL) were added to RPMI 1640 containing *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and L-glutamine (Gibco BRL). The resulting medium was inoculated 1:50 with *L. donovani* MHOM/SD/68/1S (10) promastigote culture grown from a cryopreserved stock and was incubated at 28°C. After harvesting, the promastigotes were washed with Locke solution (0.9% NaCl, 0.25% glucose, 0.04% KCl, 0.02% $CaCl_2$, 0.02% $NaHCO_3$) and treated with 0.4% trypsin in Locke solution at 37°C for 45 min, after which the promastigotes were fixed by treatment with 1% (wt/vol) formaldehyde in Locke solution for 20 h at 4°C. Following washing in cold saline (0.9% NaCl) with 1% (wt/vol) sodium citrate, the fixed promastigotes were stained for 90 min with the saline-citrate solution containing 0.02% Coomassie brilliant blue. Subsequently, the stained promastigotes were washed with the saline-citrate solution. Part of the antigen was put into freeze-drying solution, and 5-ml aliquots were freeze-dried and sealed under vacuum. This FD antigen forms the basis of a serodiagnostic kit for the detection of anti-*Leishmania* antibodies, designated *Leish*-KIT, which will soon be commercially available (Lyopharma, Bilthoven, The Netherlands). The remainder of the AQ antigen was stored at 4°C until further use.

Performance of the DAT. The DAT was performed essentially as described by Harith et al. (11). Briefly, serum samples were diluted in a dilution solution containing 0.9% (wt/vol) NaCl, 0.2% (wt/vol) gelatin (Difco Laboratories, Detroit, Mich.), and 0.78% (vol/vol) β-mercaptoethanol. To discriminate between agglutination and nonagglutination, the use of V-shaped microwell plates

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(Greiner, Frickenhausen, Germany) is mandatory (10). A twofold dilution series of the sera was made, starting at a dilution of 1:100 (step 1) and going up to a maximum dilution of 1:209.6 million (step 22). Prior to its use, aliquots of FD antigen were reconstituted in 5 ml of normal saline (0.9% [wt/vol] NaCl). Reconstituted antigen (50 μl) was added to each well of the microwell plate containing 50 μl of diluted serum. We routinely employed an 18-h incubation period at 18 to 20°C before the reading of the DAT.

The titer is defined as the highest dilution at which agglutination is still visible. In comparison to the blue dots present in the negative control wells, this agglutination shows as blue mats, enlarged blue dots with frayed edges, or enlarged blue dots.

Serum samples. A total of 501 serum specimens were collected from four different groups of subjects.

(i) There were 67 patients with parasitologically proven VL. All patients had clinical signs of VL, and parasites were found in smear material. In Amsterdam we tested 50 serum samples: 21 from patients from Perkerra region in Kenya and 29 from patients who presented at the kala-azar treatment center in Nimne in southern Sudan. Twenty serum samples from this last group plus four serum samples from other VL patients from Nimne were available for comparison of titers obtained with FD and AQ antigens in the field in southern Sudan.

(ii) There were 52 healthy controls living in West Africa.

(iii) There were 29 controls living in areas of Sudan in which VL is endemic; they were healthy individuals who had no history of VL and no clinical symptoms of the disease.

(iv) There were a total of 354 patients with a variety of diseases other than VL, namely, 71 with toxoplasmosis (Europe), 30 with malaria (Burkina Faso), 18 with sarcoidosis (Europe), 21 with schistosomiasis (Mali), 30 with African trypanosomiasis (Equatorial Guinea), 19 with Chagas' disease (Brazil), 20 with tuberculosis (Europe), 30 with toxocariasis (Europe), 23 with amoebiasis (Europe), 7 with giardiasis (Europe), 20 with Crohn's disease (Europe), 45 with onchocerciasis (Ivory Coast), 10 with leprosy (Philippines), and 10 with autoimmune diseases (vasculitis, purpura vasculitis, psoriasis, pemphigus, systemic lupus erythematosus, rheumatoid arthritis, and cutaneous discoid lupus erythematosus; Europe).

Stability of the antigen. AQ antigen was stored at room temperature (18 to 20°C) and at 37°C. After various periods of time the antigen was tested for its activity. In order to be able to predict the stability of the FD antigen provided in the *Leish*-KIT, we stored a number of aliquots at ambient temperature, 37°C, and 56°C as recommended by Jerne and Perry (16). After various periods of time one of these aliquots was reconstituted and tested for its activity.

Laboratory and field settings. Most experiments were performed in the laboratory in Amsterdam, The Netherlands. Experiments in the field were carried out by Médecins Sans Frontières staff in a kala-azar treatment center in the remotely located village of Nimne, Western Upper Nile, southern Sudan, where no electricity or running water was available.

Statistical analysis. The Wilcoxon signed rank sum test was used for comparing two groups of paired observations. In the case of more than two dependent groups, Friedman's two-way analysis of variance was used (2).

RESULTS

Stability of the AQ antigen at room temperature and at 37°C. After 3 weeks of storage at 37°C, the AQ antigen had deteriorated so far that reading of the test became impossible because of autoagglutination. Stored at room temperature, the quality of the AQ antigen remained unaltered for somewhat longer, but after 4 weeks the reading of the test became impossible.

Stability of the FD antigen and various solutions at various temperatures. After storage at room temperature, 37°C, or 56°C for 18 months, the FD antigen remained fully active in the DAT (results not shown).

All solutions were fully stable at 56°C, except for the serum dilution fluid. This problem was mastered by storing the β-mercaptoethanol as a 100% stock solution separate from the gelatin-saline solution. Prior to use, the β-mercaptoethanol was added to the solution.

The positive and negative control sera used in the *Leish*-KIT were freeze-dried and stable at 56°C.

Comparison of the performance of the FD and AQ antigens in the DAT. We tested 50 serum samples from patients with parasitologically proven VL with both FD and AQ antigens of the same batch. Titer comparisons are shown in Fig. 1. Statistical analysis revealed that titers were on the average 0.7 dilution step lower for the FD antigen than for the AQ antigen ($P < 0.001$).

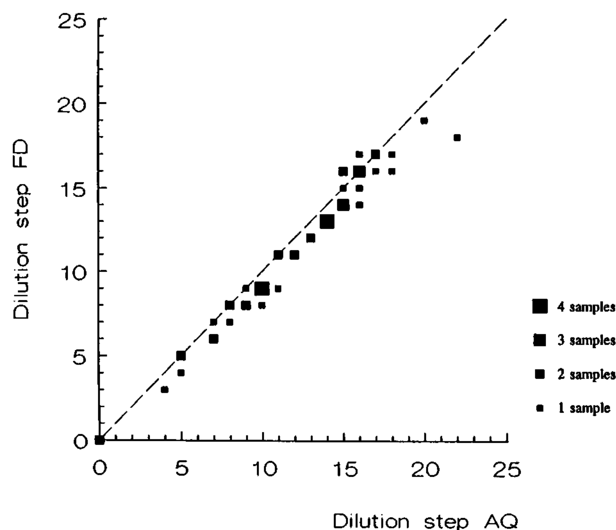

FIG. 1. Comparison of titers obtained with AQ and FD antigens in the DAT. Values on the *x* axis represent the titers obtained with AQ antigen, while the values on the *y* axis represent the titers obtained with FD antigen. All serum samples were from patients with parasitologically proven VL. For convenience, the titer is expressed as the last dilution step that still showed agglutination in the DAT. Twofold dilution steps were made ranging from 1:100 (step 1) to 1:209.6 million (step 22). Step 0 depicts titers of <1:100. The broken line represents the line obtained when the AQ and FD antigen titers are the same.

Sensitivity and specificity of the DAT using FD antigen. We determined the titers of a total of 485 serum samples from the different groups of subjects described in Materials and Methods in the DAT using FD antigen. Results are shown in Fig. 2. With the FD antigen, 4 of 50 serum samples from VL patients gave titers that were ≤1:800, 2 gave a titer that was 1:1,600, and 44 gave titers of ≥1:3,200. With a cutoff value of 1:1,600 in the DAT with FD antigen, 46 of 50 serum samples from proven VL patients would be classified as positive, representing a sensitivity of 92%. One serum sample from a patient with Crohn's disease gave a titer of 1:1,600, and 1 serum sample from a patient with Chagas' disease (American trypanosomiasis) gave a titer of 1:800; all other 433 serum samples gave titers of ≤1:400. With a cutoff value of 1:1,600, the specificity of the DAT using FD antigen was 99.7% (434 of 435 negative).

Performance of the DAT using FD and AQ antigens in a field setting. Twenty-four serum samples from VL patients were tested in the field with both AQ and FD antigens of the same batch. Results are shown in Fig. 3. Statistical analysis of the results revealed that there was no significant difference ($P = 0.21$) in the titers obtained with the AQ and FD antigens. Further analysis showed that the titers obtained with the FD antigen are the same in the field and in the laboratory (results not shown).

Reproducibility of the DAT. Three aliquots of FD antigen from the same batch, which had been stored at ambient temperature, were tested in the DAT on three consecutive days with 20 serum samples from VL patients. Statistical analysis showed no systematic day-to-day variation in the test.

DISCUSSION

Testing the stability of the AQ antigen, we found that it remained stable at 20°C for less than 4 weeks and at 37°C for less than 3 weeks: this is in agreement with previous findings

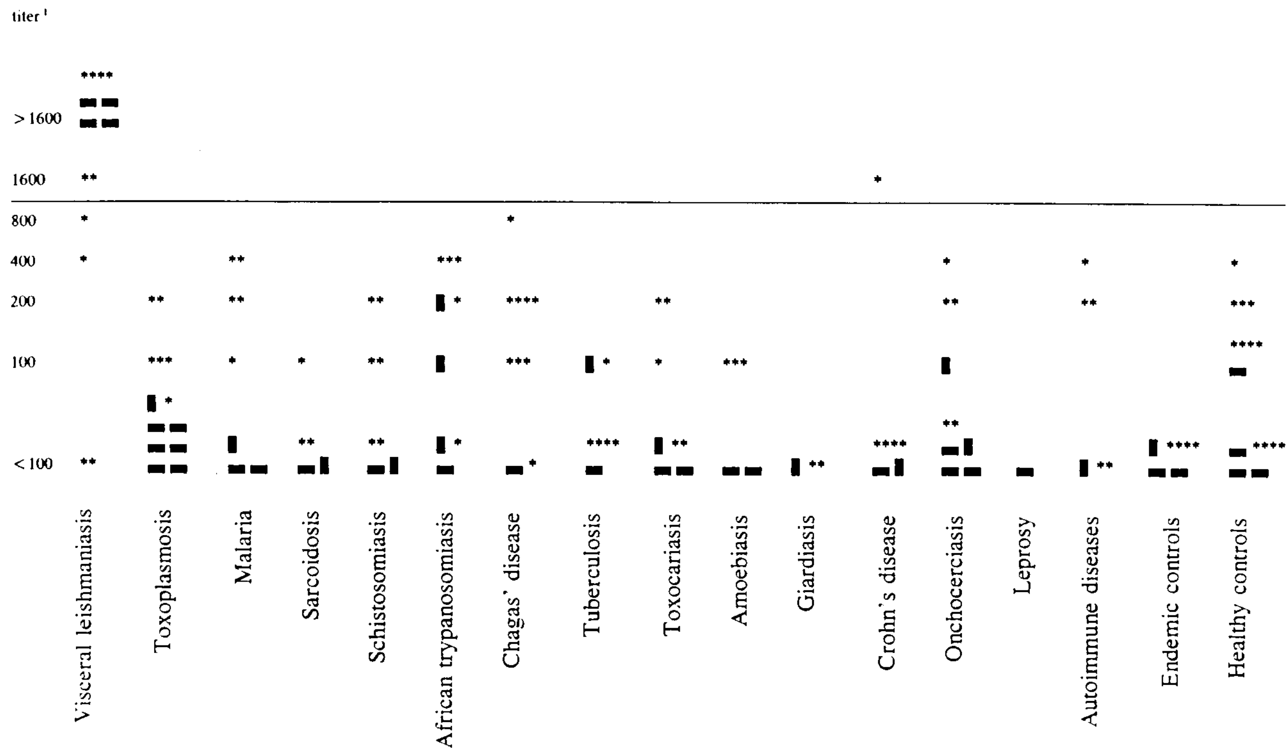

FIG. 2. Titers obtained in the DAT using FD antigen. Each column represents one group of subjects as indicated underneath. ▬, the titer of 10 serum samples; ▮, the titer of 5 serum samples; *, titer of 1 serum sample. The cutoff value is indicated by the horizontal line.

(11). In contrast, the FD antigen remained fully stable in the DAT even after storage for 18 months at 56°C. These results indicated that the use of FD antigen in the DAT is feasible.

Our next line of investigation concerned the comparison of the performance of the FD and AQ antigens in the DAT. We compared DAT titers of serum samples from parasitologically proven VL patients; the results, presented in Fig. 1, clearly indicate that in the laboratory situation the FD antigen generally gave a titer that was almost one dilution step lower than

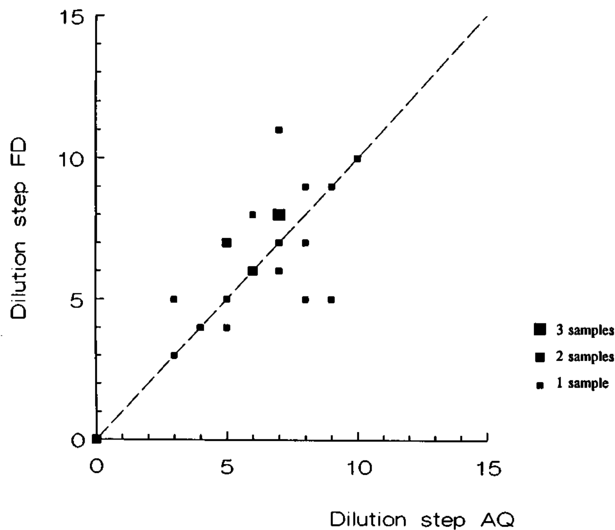

FIG. 3. Comparison of titers obtained with the DAT using FD or AQ antigen in a field setting. For further explanation, see the legend to Fig. 1.

the titer obtained with AQ antigen. However, for the interpretation of test results this is no problem when the cutoff value is changed accordingly. Previously, the cutoff value for the DAT based on AQ antigen was set at 1:3,200 (10). In order to test whether the cutoff value for the FD antigen could be set at 1:1,600, we tested 435 serum samples from different groups of subjects who did not suffer from VL. The results, presented in Fig. 2, clearly indicated that only two samples gave a titer of ≥1:800 and only one sample would be false positive with 1:1,600 used as the cutoff value. Moreover, titers with these serum samples were also generally almost one dilution step lower with the FD antigen than with the AQ antigen (results not shown).

With a cutoff value of 1:1,600 in the DAT with FD antigen, the sensitivity of the test is 92%. For the AQ antigen, with a cutoff value of 1:3,200, the sensitivity of the DAT with our set of serum samples would be 88%. The sensitivity of the DAT with AQ antigen was previously reported to be 100% (10, 11) and 94% (20). Thus, the sensitivity that we observed was lower than that described in literature. Four patients were false negative. We could not determine whether these patients would seroconvert, as collection of a second serum sample was not possible. Field experience in the epidemic situation in the Sudan taught us that in the marginally nourished population present in this area, titers of 1:200, 1:400, and 1:800 should be considered suspect (our unpublished observations).

With a cutoff value of 1:1,600, the specificity of the DAT using FD antigen was 99.7%. No cross-reactivity was observed with serum samples from patients suffering from malaria, schistosomiasis, Chagas' disease, and leprosy, diseases with clinical features that may be confused with either VL or post-kala-azar dermal leishmaniasis (6, 15, 18).

Trypanosomes are closely related to *Leishmania* organisms. It was reported previously that serum samples from Chagas' disease patients showed no cross-reactivity in the DAT (3), whereas approximately 30% of the serum samples from African trypanosomiasis patients cross-reacted in the DAT (9, 10). This problem was overcome by the addition of β-mercaptoethanol to the dilution solution (11). Using this dilution solution, we observed no cross-reactivity in the DAT using either FD or AQ antigen with serum samples from both African trypanosomiasis patients and Chagas' disease patients (Fig. 2).

These observations led us to investigate the performance of the DAT with FD antigen in a field setting lacking any technical sophistication. Under these circumstances, in contrast to the results obtained in the laboratory, no statistically significant difference was observed in titers between DATs using FD or AQ antigen (Fig. 3). This discrepancy may be explained by the deterioration of the AQ antigen due to the suboptimal cooling facilities present in the field.

In all our observations described above, we employed the generally accepted criterion that wells showing either a blue mat, an enlarged blue dot with a frayed edge, or an enlarged blue dot are all designated positive in the determination of the titer. The decision to consider a blue dot enlarged is subjective. Therefore, we also tried an alternative criterion in which the first two categories are still designated positive but wells containing enlarged blue dots are now designated negative. With this criterion, titers from all groups (VL patients, negative controls, and people with diseases other than VL) were read two steps lower than with the standard criterion. Thus, with the alternative criterion one can use a cutoff value which is two steps lower (i.e., 1:400) with the same sensitivity (92%) and specificity (99.7%) (our unpublished observations). The use of this alternative criterion does not change test interpretation but may facilitate test reading.

As indicated earlier, the DAT is not capable of distinguishing between past kala-azar, subclinical infection, and current disease (20). It is reported that increased levels of anti-*Leishmania* antibodies may be present for a long time after completion of treatment (8, 12). On the other hand, if VL is associated with human immunodeficiency virus infection or other sources of immunocompromise, a considerable number of patients lack detectable levels of antibodies (7). Therefore, as with all serodiagnostic tests, the significance of positive or negative results in the DAT should always be judged against clinical data and the results of other diagnostic methods.

We conclude that the DAT using heat-stable FD antigen is a highly specific and adequately sensitive addition to the diagnostic armament for VL but that the main gain lies in its reproducibility combined with a long shelf life even under harsh conditions.

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