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Improvement of a Direct Agglutination Test for Field Studies of Visceral Leishmaniasis

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To increase the potential for the wide-scale application of our direct agglutination test for visceral leishmaniasis, modifications in the components and procedures were introduced. Supplementation with 0.056 M citrate of the suspension medium stabilized the antigen for 9 weeks at 37°C. To circumvent the need for cooling systems in the field, 0.2% (wt/vol) gelatin was added to the serum diluent instead of fetal bovine serum, with reliable results. Specificity and sensitivity were improved by the incorporation of 0.1 M 2-mercaptoethanol in samples with borderline titers. The test could be performed on samples of whole blood; thus the difficulties of preparation and storage of serum, plasma, or filter paper blood are avoided. For mass screening programs, a single serum dilution of 1:6,400 could be employed, contributing to a further reduction in test expenses. Sera from different geographical areas showed equal reactivities in this direct agglutination test despite the nonhomologous *Leishmania donovani* antigens used.

A limited number of the available serological methods for provisional diagnosis of visceral leishmaniasis (VL) and for epidemiological studies of this disease are suitable for field application (7, 8, 11). Among the factors limiting the use of these methods are the instability of reagents and the need for sophisticated equipment and specialized skills.

A recently developed direct agglutination test (DAT) showed promise in fulfilling the requirements of a field test (4, 5), but further improvement and simplification were considered necessary for on-site field work. We report on a number of modifications in this DAT.

MATERIALS AND METHODS

Antigen production and test procedures. The detailed performance of the DAT has been previously reported (5). Briefly, promastigotes of *Leishmania donovani* were maintained in modified Dwyer medium (3), with beef extract substitution. Defibrinated rabbit blood, yeast extract (5.0 g/liter), and tryptose (0.1 g/liter; Difco Laboratories, Detroit, Mich.) were added to the basic medium. Mass production of the parasite was obtained by inoculating 1 liter of RPMI 1640 to which HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and fetal bovine serum (FBS) were added with 20 ml of rich promastigote maintenance culture. After harvest, the promastigotes were washed several times with cold Locke solution, treated at 37°C with 0.4% trypsin for 45 min, and fixed for 20 h at 4°C with 2% (wt/vol) formaldehyde in Locke solution. After being washed in saline, the parasites were stained with 0.1% Coomassie brilliant blue for 90 min. The parasite suspension was filtered through a nylon gauze, and the concentration was adjusted to 5×10^7 cells per ml in 0.4% (wt/vol) formaldehyde. Serial dilutions of test sera in normal saline with 1% FBS were made in V-shaped microdilution plates; 50 μl of antigen suspension was added. Results were read visually against a white background after 18 h of incubation at room temperature (5). This basic procedure was modified as described below.

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(i) Extension of antigen shelf life. The procedure for antigen production was the same up to the step of formaldehyde fixation before staining. After centrifugation, the parasites were suspended and washed two times with cold (4°C) saline (0.15 M NaCl) adjusted to pH 7.4 with sodium citrate (0.056 M). The parasites were then stained with Coomassie brilliant blue in the same citrate-saline solution. After being washed three times and suspended (at 6.5×10^7 to 7.5×10^7/ml) in citrate-saline plus formaldehyde (0.43% [wt/vol]), the antigen suspension was filtered. The antigen preparation was tested before storage and at several intervals after storage at 4, 21, 37, and 45°C with positive and negative reference sera.

(ii) Substitution of FBS by gelatin in serum diluent. Instead of the above-described serum diluent of normal saline with 1% FBS, a modified diluent was used consisting of an aqueous solution of sodium citrate (0.056 M) and sodium chloride (0.15 M) to which 0.2% gelatin (Difco) was added. The mixture was heated at 56°C for 10 min to dissolve the gelatin and then left to cool at room temperature. Sera diluted with this solution were used in the standard test procedure. When citrate had already been added to the antigen, it could be eliminated from the gelatin diluent without affecting the observed titers.

(iii) Addition of 2-ME. As reported for the test for Chagas' disease (6, 12, 15), 2-mercaptoethanol (2-ME) was added to sera showing borderline titers (1:1,600 to 1:6,400); 0.1 M 2-ME was added to the gelatin diluent, and the test was further performed as described above.

(iv) Use of whole blood and filter paper blood. For the mouse samples, 16 CBA mice were immunized with increasing doses of living *L. donovani* promastigotes (5.0×10^7 to 2×10^8) by four intraperitoneal inoculations at 2- to 3-day intervals. Twelve nonimmunized mice provided control samples. Four days after the last booster dose, blood samples were collected in heparin by cardiac puncture. Fifty μl of blood was transferred to Whatman no. 3 filter papers. These samples were air dried and stored, as were whole blood and plasma samples, at −20°C until needed.

The DAT was carried out on whole blood samples starting

at a dilution of 1:1,600 because, at lower dilutions, the concomitant sedimentation of erythrocytes made it difficult to distinguish positive from negative reactions.

Filter paper blood samples were extracted overnight at 4°C in 0.15 M saline; the eluates and the corresponding plasma samples were screened starting at a 1:50 or a 1:100 dilution.

For the field samples, both filter paper blood and serum samples were collected from 91 inhabitants during a field visit to the Marigat area, Baringo District, Kenya, in April and May 1986.

(v) Use of single serum dilution. With end-point titration, agglutination reactions at a serum dilution of 1:3,200 or higher are suggestive of VL (5). To assess the reliability of using a single sample dilution, 109 sera diluted at 1:3,200 or 1:6,400 in gelatin were tested for positive or negative readings.

The sera tested were obtained from patients with VL (n = 10), African trypanosomiasis (n = 10), and autoimmune disorders (n = 10) and from healthy Kenyans as controls (n = 79).

Human blood and serum samples. (i) VL. For all 36 Kenyan patients, the diagnosis was confirmed parasitologically (amastigotes were found in splenic aspirates). Sera were obtained before, during, or at the end of treatment (20 samples) and during follow-up at 1 to 14 months after cure (i.e., negative splenic aspirate and clinical and hematological improvement) (16 samples). Twenty-seven patients were male (4 to 40 years old), and nine patients were female (5 to 52 years old).

For 10 of 14 Brazilian patients, VL was parasitologically confirmed (bone marrows were positive for amastigotes); the sera from the other 4 were repeatedly strongly positive in immunofluorescence tests (P. P. Andrade, Recife, Brazil).

For the 12 Indian patients, VL was parasitologically confirmed (by bone marrow examination) (D. Sacks, Bethesda, Md.).

(ii) African trypanosomiasis. The disease in all 16 Zairian patients was parasitologically confirmed (E. Magnus and D. Le Ray, Antwerp, Belgium).

(iii) Chagas' disease. *Trypanosoma cruzi* infection was established by demonstration of the parasite in peripheral blood or by clinical tests and positive immunofluorescence results (M. E. Camargo, Sao Paulo, Brazil).

(iv) Malaria. All 22 patients (20 Dutch, 1 Tanzanian, and 1 Afghan) were diagnosed in the outpatient clinic of the University Hospital, Amsterdam. Nineteen patients had a *Plasmodium falciparum* infection, and three patients had a *P. vivax* infection.

(v) Toxoplasmosis. All 70 sera were from Dutch nationals. Twenty-one samples had elevated immunoglobulin titers in the enzyme-linked immunosorbent assay and positive tests with Sabin and Feldman dye (13), indicating active or recent toxoplasmosis; the others had positive tests with the dye and relatively high immunoglobulin G titers (≥3,200) in the enzyme-linked immunosorbent assay. Because no differences were found in the DAT, results are reported together.

(vi) Autoimmune disorders. Sera of 24 Dutch nationals, 10 with positive rheumatoid factor, 7 with antimitochondrial antibodies, and 7 with anti-smooth muscle antibodies, were provided by H. G. M. Geertzen, Amsterdam.

(vii) Controls. From Kenya, 546 serum samples were obtained in the Baringo District, where, since 1982, epidemiological studies in leishmaniasis have been carried out. During yearly house-to-house surveys, all persons were examined for splenomegaly. Specimens included those of family members and neighbors of patients with kala azar and patients with malaria or brucellosis. Several samples were collected from some individuals during different field visits, and all of these samples were included in the study.

Sera and blood collected on filter papers were obtained from 91 persons. In the field, the dried filter papers were kept at 4°C for 2 weeks; on their arrival in Amsterdam, the filter papers were frozen at −20°C. Filter papers were eluted to give a dilution of 1:50.

From Brazil, 251 sera from healthy Brazilian donors and patients with infections other than VL were tested. For India, 11 sera of attendants or family members of patients with VL were included.

RESULTS

Stabilization of antigen. The titers obtained with the four aliquots of antigen during the periods studied fluctuated within an acceptable range (Fig. 1). Longer storage resulted in the formation of clumps and the fading of antigen color. Even at temperatures as high as 37 to 45°C, the antigen suspension to which citrate had been added showed consistent reactivity for at least 3 weeks after preparation.

Serum diluent. No significant difference between readings was noticed with FBS or gelatin as protein source in the serum diluent (standard Pearson correlation coefficient, 0.9857; $P < 10^{-6}$) (Fig. 2).

2-ME addition to sera. Except for one sample from a treated patient with VL, all the other samples had significantly higher titers after 2-ME treatment (Table 1). In addition, nonspecific agglutination reactions were clearly reduced in sera of patients of all other conditions tested except African trypanosomiasis.

Whole blood and filter paper blood. Similar results were obtained with whole mouse blood, filter paper-collected blood, and the corresponding plasma samples. Titers of ≤1:1,600 and ≥1:12,800 were obtained for the nonimmunized control group (n = 12) and the *L. donovani*-immunized group (n = 16), respectively. Dilution of whole mouse blood at 1:1,600 presented no problems in the reading of DAT results. This improvement was also observed with human whole blood supplemented with sera from patients with kala azar (results not shown).

Results with filter paper-collected blood and with serum samples collected from people in the field were significantly correlated (standard Pearson correlation coefficient, 0.987; $P < 10^{-6}$). Among 91 samples collected by either procedure, 3 sera and 3 filter paper eluates revealed titers of ≥1:12,800. These samples were later disclosed to have been collected from patients with VL. In general, filter paper eluates had slightly lower titers than those of the corresponding serum samples, but patients with VL and noninfected persons (titers of ≤1:1,600) were clearly distinguished (Table 2).

Single dilution testing. Testing of 109 human serum samples at single dilutions of 1:3,200 or 1:6,400 by DAT (Table 3) revealed 15 and 9 seropositive samples at the former and latter dilutions, respectively. As expected, the sera of all 10 patients with kala azar were positive at a dilution of 1:3,200; 8 samples were also positive at a dilution of 1:6,400, but samples from 2 treated cases were negative at this dilution.

Sera from different areas where VL is endemic. Active and treated (up to 6 months) VL cases from Kenya, Brazil, and India had DAT titers indicative of the disease (≥1:12,800). Except for 18 samples, all other control sera (528 samples) from Kenya were clearly seronegative (titers of ≤1:1,600; Table 4).

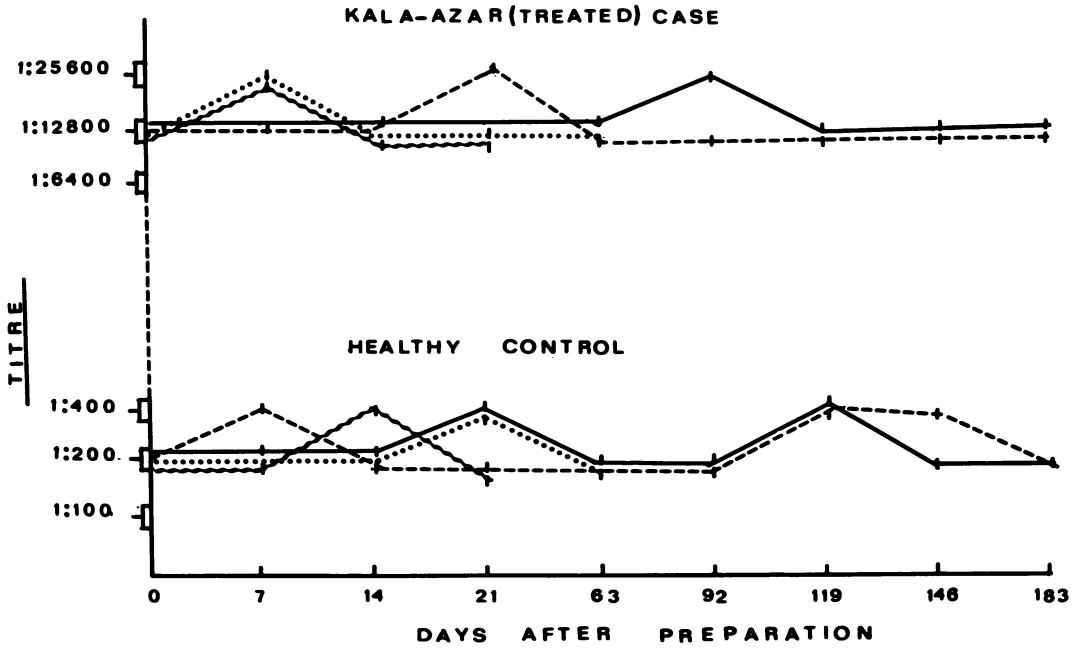

FIG. 1. Reactivity of *L. donovani* antigen after storage at 4°C (——), 21°C (- - - -), 37°C (· · · ·), and 45°C (∿∿∿).

DISCUSSION

The previously described DAT (1, 5) can be performed in central laboratories and moderately well-equipped clinical centers and was actually performed by three of us under field circumstances in Kenya. In the present study, we introduced further simplifications and improvements.

The addition of citrate to the antigen suspension ensured antigen stability even at temperatures as high as 45°C for up to 3 weeks. Lysis of promastigotes might occur earlier at temperatures of 37 to 45°C if sodium citrate is not included. Sodium citrate is used in isotonic buffer systems to stop lytic reactions in a hemolytic antibody titration technique (7a). Citrate is used for better long-term preservation of red blood cells for immunological tests and blood transfusion (2). The

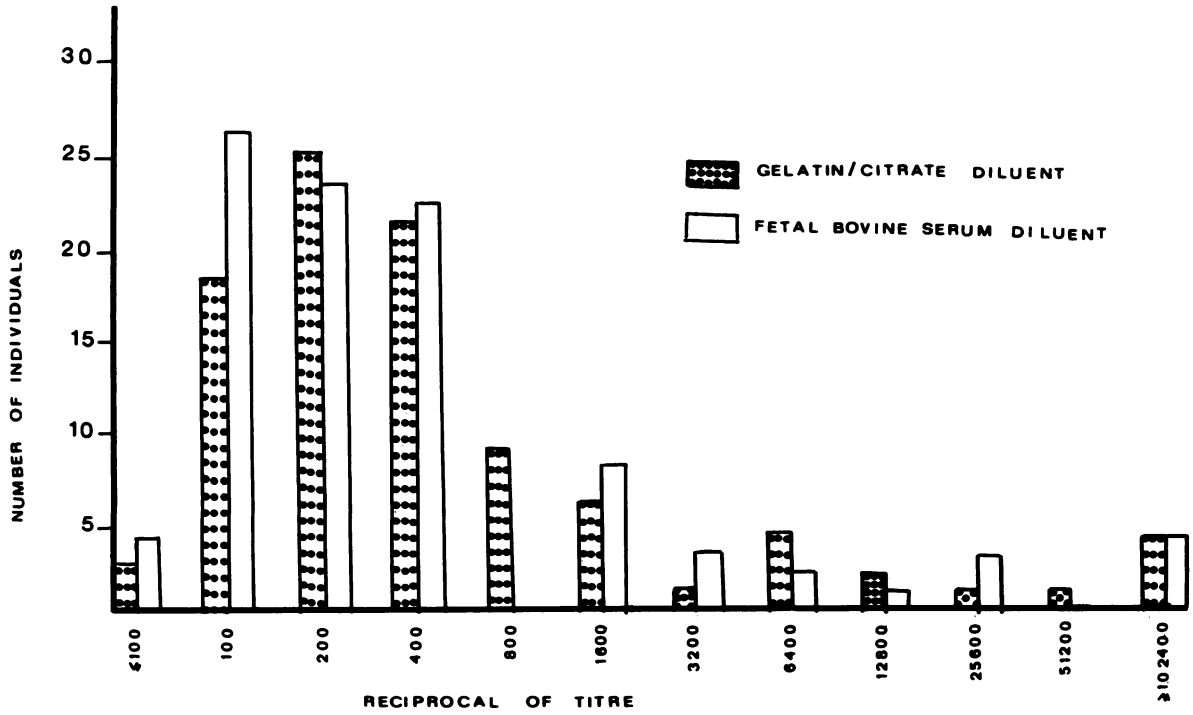

FIG. 2. Comparison of titer readings when FBS and gelatin are used as diluents. Standard Pearson correlation coefficient, 0.9857; $P < 10^{-6}$.

TABLE 1. Effect of 2-ME on titers of sera from patients with VL[a] and from individuals with other conditions

Diagnosis	No. of sera	2-ME[b]	No. of sera with indicated reciprocal of titer				
			≤800	1,600	3,200	6,400	≥12,800
VL	36	−	0	4	4	0	28
		+	0	0	1	0	35
African trypanosomiasis	16	−	7	6	2	1	0
		+	11	3	1	1	0
Chagas' disease	29	−	26	3	0	0	0
		+	29	0	0	0	0
Toxoplasmosis	70	−	59	9	2	0	0
		+	70	0	0	0	0
Malaria	22	−	19	2	1	0	0
		+	22	0	0	0	0
Autoimmune disorders	24	−	19	4	0	1	0
		+	24	0	0	0	0
Control	15	−	3	5	3	1	3
		+	15	0	0	0	0

[a] Sera from treated patients (4 to 14 months) were included.
[b] −, Without 2-ME; +, with 2-ME.

role of citrate in maintaining the intercellular repulsion forces, which are responsible for overcoming spontaneous clumping, may account for its contribution to the stabilization of the Formalin-fixed promastigote suspensions.

At tropical ambient temperatures, FBS undergoes deterioration and microbial contamination and is unsuitable as an ingredient in the diluent. The incorporation of a protein source in the DAT is crucial to the prevention of nonspecific agglutination. Gelatin is a denaturated protein free of immunoglobulins that may affect the DAT results; in powder form, gelatin can withstand temperatures of 30°C or higher. The replacement of FBS by gelatin did not alter the agglutination reaction and rendered the DAT more practical and cheaper for field application.

The addition of 2-ME resulted in the improvement of specificity (98.8%) and sensitivity (100%). Similar improvement in the specificities of previously developed direct agglutination assays for Chagas' disease has been reported (6, 12, 15). It is not yet clear how 2-ME removes nonspecific agglutination reactions while augmenting or at least maintaining specific ones. Experiments are in progress to investigate this effect of 2-ME. 2-ME can easily be incorporated in sera for the field application of the DAT.

Whole blood has not previously been used in the immunodiagnostic methods for VL. The results with mouse whole blood samples were similar to those with corresponding plasma samples. At the dilution chosen (1:1,600), erythrocyte sedimentation was so slight that it caused no confusion in the reading of the test. When sera from patients with kala azar were added to human whole blood, a dilution of 1:1,600 was also sufficient to avoid the problem of erythrocyte sedimentation (results not shown). On the basis of these results, we expect that the DAT could easily be carried out in places where facilities for the preparation and storage of serum or plasma samples are lacking. Whole blood has been used with success in a card agglutination test for African trypanosomiasis (C.A.T.T.) (10).

TABLE 2. Results for filter paper blood and serum samples from inhabitants of Marigat Area, Baringo District, Kenya

Sample[a]	No. of samples with indicated reciprocal titer			
	≤1,600	3,200	6,400	≥12,800
Filter paper blood	87	1	0	3[b]
Serum	82	2	4	3[b]

[a] $n = 91$.
[a] Samples were obtained from patients with proven kala azar (recent and treated cases).

TABLE 3. DAT readings with *L. donovani* antigen

Diagnosis	No. of sera	No. of sera with indicated DAT reading at the given serum dilution			
		1:3,200		1:6,400	
		Positive	Negative	Positive	Negative
VL	10	10	0	8	2[a]
African trypanosomiasis	10	0	10	0	10
Autoimmune disorders	10	1	9	0	10
Control[b]	79	4	75	1	78

[a] Sera were from treated patients.
[b] Control sera were obtained from healthy Kenyans.

Filter paper blood sampling is reportedly reliable for field research in VL (8), malaria (9), and onchocerciasis (14). In the present work, patients with VL were clearly detected by tests on both serum samples and filter paper eluates, whereas the control group gave negative results throughout. There was a significant correlation between the tests. If our laboratory results can be extrapolated to the field situation, even filter papers may not be necessary. A minute amount of blood collected from a finger prick could be tested directly in the DAT or allowed to run into a small vial containing citrate anticoagulant for later use.

If only positive and negative readings are required, the DAT can be performed on the basis of a single dilution. This method will enable the screening of large populations in a reasonable period. A dilution of 1:6,400 appears to be reliable for the detection of active kala azar cases in a population including patients with African trypanosomiasis. The cutoff point has to be established in every endemic area; it is likely to be around 1:6,400.

Irrespective of their geographic origin, active VL sera from Kenya, Brazil, or India showed titers indicative of the disease (≥1:3,200). We have worked with a *L. donovani* stock; it is not clear which subspecies this is, but it is clear that this antigen can be used to test sera from patients which were infected with different subspecies of *L. donovani*. The introduction of the above-described DAT into those areas where VL is endemic seems warranted. Further studies involving primary isolates of *L. donovani* from different areas where VL is endemic are in progress. Although reliable diagnostic information can be obtained within 4 to 5

TABLE 4. DAT titers of sera of patients with VL[a] and control sera collected from three areas where VL is endemic

Diagnosis	Area	No. of sera	No. of sera with indicated reciprocal of titer				
			≤800	1,600	3,200	6,400	≥12,800
VL	Kenya	36	0	4	4	0	28
	Brazil	14	0	0	0	0	14
	India	12	0	0	2	0	10
Control	Kenya	546	512	16	8	7	3
	Brazil	251	250	1	0	0	0
	India	11	11	0	0	0	0

[a] Sera from treated cases (4 to 14 months) were included.

h, we are continuing our efforts to modify this DAT for instant reading.

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