Use of a Newly Developed β-Mercaptoethanol Enzyme-Linked Immunosorbent Assay To Diagnose Visceral Leishmaniasis in Patients in Eastern Sudan

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Corroboration of serology results is essential for restricting the risk of inappropriate antileishmanial prescription. A direct agglutination test (DAT) and a recently developed β-mercaptoethanol-modified enzyme-linked immunosorbent assay (β-ME ELISA) based on the use of antigen prepared as described for the DAT were applied to 416 sera from two Sudanese populations with and without clinical evidence of visceral leishmaniasis (VL). Of 285 sera with the lowest antileishmanial DAT titers (≤1:100 to 1:1,600), 270 (94.7%) scored comparable minimum β-ME ELISA absorbance values (≤0.1 to 0.26). In 117 sera that demonstrated the highest DAT titers (1:12,800 to ≥1:25,600), 86 (73.5%) scored maximum (0.81 to ≥1.35) and 30 (25.6%) medium (0.27 to 0.80) β-ME ELISA absorbance values. VL diagnosis was established for 142 (44.1%) patients in the VL-symptomatic group (n = 322), based on positive microscopy for Leishmania donovani in lymph node aspirates or positive DAT (titer, ≥1:3,200). Of the 125 sera from the symptomatic patients for whom microscopy was positive for VL, 111 (88.8%) had comparable positive DAT and β-ME ELISA readings. In all 17 sera from the symptomatic DAT-positive patients for whom leishmaniasis was not established by microscopy but who responded favorably to antileishmanial therapy, absorbance values (≥0.27) indicative of VL were obtained by β-ME ELISA. Of 197 symptomatic patients for whom microscopy was negative for VL, 172 (87.3%) tested negative in β-ME ELISA and 180 (91.4%) in DAT. Based on the high reliability demonstrated here for VL detection, β-ME ELISA fulfills the requirement of confirming DAT results in patients manifesting suspected VL.

A prerequisite for sustainable control of visceral leishmaniasis (VL) in major areas of endemicity is the transfer of the necessary knowledge for reproducing established diagnostic tests. Although in several studies conducted in Sudan, properties of various imported tests were intensively evaluated, no measure regarding their long-term availability was addressed (2, 10, 11–14, 16).

At Ahfad University for Women (Omdurman, Sudan), direct agglutination tests (DAT) were successfully produced (7–9) through provision of modest laboratory facilities and training of medical personnel. National and international evaluations of the DAT produced in Sudan revealed excellent reliability for VL detection in both confirmed and unconfirmed cases in which patients responded favorably to antileishmanial therapy (9). However, despite the reports of high DAT reliability for VL detection, the decision to administer antileishmanial agents in unconfirmed cases should be adequately supported to avoid unnecessary health hazards.

By combining the use of a β-mercaptoethanol-modified antigen similar to that used in the DAT and an anti-human immunoglobulin G (IgG) conjugate for targeting specific IgG antibodies, a hybrid enzyme-linked immunosorbent assay (β-ME ELISA) was developed and successfully evaluated in a panel of reference VL and non-VL sera (1). The purpose of this study was to determine the reliability of the β-ME ELISA for detecting VL in patients suspected of having the disease presenting at a rural hospital in eastern Sudan.

MATERIALS AND METHODS

Study area and population. Three hundred twenty-two individuals suspected of having VL were received at Doka rural hospital during September 2004 to August 2006. Aside from fever (duration of ≥2 weeks), splenomegaly and lymphadenopathy were the most common manifestations in that group of patients. At our request, 56 other subjects residing in or around the same area and having no apparent manifestation or history of VL reported to the hospital and agreed to join the study as a healthy group from an area of endemicity. The necessary permission to conduct the study was granted by the Research Directorate, Federal Ministry of Health (Khartoum, Sudan).

VL diagnosis. Blood for serum was collected from all patients with suspected VL (n = 322) and the healthy subjects from the area of endemicity (n = 56). As routine diagnostic procedure, inguinal lymph specimens were collected from all patients with suspected VL. The aspirated lymph specimens were smeared onto glass slides and left to air dry. After methanol fixation, specimens were stained with Giemsa and examined under a microscope for amastigotes. Small portions of the sera collected from the patients with suspected VL and the healthy subjects were directly tested in the DAT (Doka rural hospital) by standard procedures described in detail previously (5, 6, 9). The remaining portions, designated for testing with the β-ME ELISA, were stored at −20°C until transportation to the central laboratory in Omdurman.

VL diagnosis was established either by demonstration of amastigotes in the Giemsa-stained lymph smears or by establishing titers of ≥1:3,200 in the DAT combined with positive disease presentation (15). Patients thus diagnosed with VL were then put on a 30-day treatment course with sodium stibogluconate (Pentostam; Albet-Davis). Patients determined not to have VL on the basis of
microscopy or DAT results were referred to the district hospital in Gedarif for further investigation. For comparison with the population from the area of endemicity, blood for serum was collected from 38 healthy female medical students (Khartoum state) who voluntarily agreed to serve as a control group. All serum samples were stored at −20°C until required.

**β-ME ELISA.** The antigen processing for β-ME ELISA was carried out as described for DAT with the exception of a Coomassie brilliant blue staining step (1). Following β-ME treatment and repeated washing in citrate saline solution, promastigotes were fixed as a stock suspension (1.5 × 10^7/ml) in a 1.2% (vol/vol) formaldehyde-citrate-saline solution. A working antigen promastigote concentration of 2.5 × 10^7/ml versus respective serum and goat anti-human IgG peroxidase conjugate (Jackson Immunoresearch Laboratories, Inc.) dilutions of 1:12800 and 1:100,000 was employed. Sera were assayed in duplicate along with appropriate negative and positive controls. The reaction was measured 15 min after addition of the substrate (5-aminosalicylic acid), at a 450-nm wavelength on a Titertek Multiscan.

**Data analysis.** For both β-ME ELISA and DAT, sensitivity was defined as the percentage of disease-positive patients with a positive test result (true positives/true positives + false negatives) × 100, and specificity was the percentage of the disease-negative individuals with a negative test result (true negative/false positive + true negative) × 100).

**RESULTS**

Table 1 presents the distribution of *Leishmania* antibody readings in a Sudanese study population comprising patients with suspected VL, healthy individuals from areas of endemicity, and controls from other areas.

### Table 1. Distribution of *Leishmania* antibody readings in a Sudanese study population comprising patients with suspected VL, healthy individuals from areas of endemicity, and controls from other areas

| DAT titera | No. (%) of samples with a β-ME ELISA absorbance ofb: | No. (%) of individuals |
|------------|------------------------------------------------------|-----------------------|
| ≤0.10–0.26 | 270                                                  | 15 (94.7)             |
| 0.27–0.80  | 11                                                    | 1 (5.3)               |
| ≥0.81–1.35 | 86                                                   | 4 (22.2)              |
| Total      | 273                                                  | 65 (56.5)             |

a A titer of ≥1:3,200 (DAT) or an absorbance of ≥0.27 (β-ME ELISA) is indicative of VL.

Supporting serological tests. The antigen for the urea-improved version of DAT was processed as described elsewhere (6). In order to confirm positive results obtained with β-ME ELISA in cases where microscopy was negative for VL, the urea-improved DAT and two commercially available VL diagnostic tests, namely, Leish-Kit (Lyopharma, Bilthoven, The Netherlands) and the rK39 strip test (DiaMed-AG, Cressier sur Morat, Switzerland), were applied. The urea-improved DAT was carried out as reported by el Harith et al. (6), and the Leish-Kit and rK39 strip test were used as instructed by the respective manufacturers.

Data analysis. For both β-ME ELISA and DAT, sensitivity was defined as the percentage of disease-positive patients with a positive test result (true positives/true positives + false negatives) × 100, and specificity was the percentage of the disease-negative individuals with a negative test result (true negative/false positive + true negative) × 100.

### Table 2. Comparison of β-ME ELISA results with the outcome of lymph node aspiration and DAT in 322 patients with suspected VL

| Lymph node aspiration result (no. of patients) | DAT result (no. of patients) | No. (%) with β-ME ELISA absorbance ofb: |
|-----------------------------------------------|-------------------------------|----------------------------------------|
| Positive (125)                                | Positive (114)                | ≤0.10–0.26: 3 (25.0) – 80%             |
| Negative (197)                                | Positive (17)                 | 0.27–0.80: 10 (58.8) – 7%              |
| Negative (180)                                | Negative (80)                 | ≥0.81–1.35: 8 (44.4) – 0%              |
| Total (322)                                   |                               | 53 (16.5) – 87 (27.0)                  |

b A titer of ≥1:3,200 (DAT) or an absorbance of ≥0.27 (β-ME ELISA) is indicative of VL.

Table 3 presents the differences in readings obtained with β-ME ELISA, DAT, and the rK39 strip test for eight patients with suspected VL and negative microscopy results for *L. donovani*.

### Table 3. Differences in readings obtained with β-ME ELISA, DAT, and the rK39 strip test for eight patients with suspected VL

| Patient code | β-ME ELISA absorbance | Standard DAT | Urea-improved DAT | Leish-Kit | rK39 strip test result |
|--------------|-----------------------|--------------|-------------------|----------|------------------------|
| 16           | 0.27                  | 1:800        | 1:800             | 1:1,600 (+) | +                      |
| 99           | 0.30                  | 1:100        | 1:100             | 1:100    | –                      |
| 157          | 0.37                  | 1:100        | 1:100             | 1:100    | –                      |
| 213          | 0.54                  | 1:100        | 1:100             | 1:100    | –                      |
| 225          | 0.37                  | 1:200        | 1:200             | 1:800    | –                      |
| 228          | 0.28                  | 1:1,600      | 1:1,600           | 1:1,600 (+) | +                      |
| 287          | 0.29                  | 1:100        | 1:100             | 1:100    | –                      |
| 301          | 0.34                  | 1:200        | 1:200             | 1:800    | –                      |

a All β-ME ELISA absorbance values are considered positive. Titters considered positive are indicated with a plus sign.
DISCUSSION

Given the potent toxicity involved, the decision to prescribe antileishmanial agents for unconfirmed VL cases in the Sudan is a difficult one. This problem is further complicated by the high prevalence of infections that bear clinical resemblance to VL, such as malaria, tuberculosis, brucellosis, and typhoid. Postponing or withholding treatment in such cases could increase mortality and VL transmission rates in these already affected areas.

Since the establishment of DAT production in our laboratory, hundreds of patients with unconfirmed VL have been successfully diagnosed and treated (7, 9). Most challenging in this category, however, were cases in which the patient had a high negative or low positive DAT titer (1:800 to 1:3,200). Reliable diagnosis of VL in this group is therefore dependent on the availability of a supporting test(s).

In the present study, concordant β-ME ELISA and DAT results were obtained for 89.5% of the VL-seropositive patients and in as many as 94.7% of the seronegative patients. Also highly promising in this study were the sensitivities (93.0% or 92.3%) demonstrated by the two tests for diagnosing VL; 132 cases were identified by β-ME ELISA and 131 by DAT. Of the 114 VL cases confirmed on the basis of microscopy in which DAT was also positive, 111 gave positive β-ME ELISA readings (97.4% concordance with DAT). Both tests showed superiority over microscopy in that they detected VL in 17 more patients. Had microscopy been used as the sole criterion for diagnosis in this study, all 17 patients would have been denied VL treatment.

The results obtained with β-ME ELISA in this field study confirmed results obtained previously at the laboratory level, where, in addition to being used for VL, it was compared to the DAT for testing sera from patients with tuberculosis, leukemia, and African trypanosomiasis (1). However, earlier results obtained with an IgG ELISA where the water-soluble fraction of Leishmania donovani promastigotes was used as the antigen (4) showed that it compared less favorably to the DAT. At a slightly higher cutoff absorbance value (0.28) than that used here, 10% to 20% of Kenyan patients who had lived in the district of Baringo (Kenya), where VL is endemic, and who had suffered from malaria or brucellosis gave positive ELISA readings. Consistent with DAT results, though, were those obtained with an improved ELISA utilizing recombinant Leish- mania antigen (rK39) in a Sudanese VL population from the eastern state (17). Efforts to further simplify VL diagnosis that included application of the same antigen to a strip device failed to match those of the DAT in two VL patient groups from an area in the Sudan where VL is endemic or epidemic (17). Further evaluation of the rK39 strip test in eastern and southern Sudan revealed significantly lower sensitivity (90.0%) and specificity (70.0%) than those reported for the DAT (respectively, 99% and 85%) (12, 14). In our opinion, the high specificity demonstrated for β-ME ELISA and DAT in this and a previous study (1) can be attributed only to the favorable effect brought about by β-ME on the promastigote membrane, whereby cross-reacting molecules were drastically reduced to elicit reaction against non-VL sera. However, as has been found with other versions of the ELISA, determination of the cutoff level in this β-ME ELISA can be influenced by the level of VL endemicity in the study area, the causative Leishmania subspecies involved, and the reagent lot employed.

The positive readings obtained with β-ME ELISA in eight patients with unconfirmed but strongly suspected VL suggest possible exposure to L. donovani. Early or subclinical VL infection cannot be excluded with certainty, since in this β-ME ELISA no antibodies other than those of the IgG class were targeted, and amastigotes may have escaped microscopic detection, possibly because they were scarce in the lymph aspirates. The positive readings obtained in samples from two of those patients with the rK39-strip test and/or Leish-Kit (Table 3) provided further evidence of this possibility. Notwithstanding the discrepancy observed here between DAT and the two commercial tests, recent analysis of data collected from several studies evidenced comparable diagnostic reliability for VL regardless of the parasitological outcome (3).

Plans for enhancing β-ME ELISA performance to provide early warning of VL include further modification for detecting antibody response in the IgM serum fraction of patients with suspected VL.

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