β -Mercaptoethanol-modified ELISA for diagnosis of visceral leishmaniasis

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Following antigen preparation procedures similar to those of the direct agglutination test (DAT), an IgG ELISA employing intact β -mercaptoethanol (β -ME)-treated *Leishmania donovani* promastigotes was developed. The performance of the β -ME ELISA thus developed was assessed in patients with confirmed visceral leishmaniasis (VL), revealing slightly lower sensitivity (39/40 = 97.5 %) than that of the DAT (40/40 = 100 %). When challenged with sera of individuals with non-VL conditions, including leukaemia and African trypanosomiasis, the specificity of the β -ME ELISA was 100 % (158/158), compared to 98.8 % (156/158) for DAT. In an endemic population ($n = 145$) manifesting a clinical suspicion of VL, results obtained with the β -ME ELISA were highly concordant with those of DAT, both in the seropositive (65/68 = 95.6 %) and seronegative (77/80 = 96.3 %) groups. Furthermore, the incorporated intact antigen demonstrated higher sensitivity in ELISA (16/18 = 88.9 %) than the water-soluble equivalent (13/18 = 72.2 %). The stability of the formaldehyde-fixed antigen (2 months at 4 °C) in β -ME ELISA, as well as the option for direct testing of whole-blood samples and visual reading of results (within 2 h, compared to 18 h for DAT), advocate the simultaneous application of the technique with DAT for confirmation of VL in laboratories with limited facilities.

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INTRODUCTION

Despite its high reliability for the diagnosis of visceral leishmaniasis (VL), marginal titres are occasionally recorded with the direct agglutination test (DAT) in non-VL patients (Harith *et al.*, 1988). Further improvements in test procedures, including the incorporation of β -mercaptoethanol (β -ME) in antigen processing and urea for denaturing non-specific agglutinating antibodies, have improved test sensitivity and specificity (Harith *et al.*, 1995). As an alternative to the conventional water-soluble antigen, an intact promastigote antigen has been evaluated in ELISA for the detection of anti-*Leishmania* IgG antibodies (Mohammed *et al.*, 1985). Possibly due to the crude nature of the antigen employed, difficulties were encountered, impeding the reproducibility of VL diagnosis. In this study, we have combined the use of promastigote surface antigen(s) that are favourably modified (cleaved or unfolded) by β -ME, as demonstrated earlier for DAT, with defined serum immunoglobulins described for ELISA, to further optimize the diagnosis of VL in laboratories with limited facilities.

METHODS

Strains and culture conditions. *Leishmania donovani* strain MHOM/68/1-S (an isolate from a VL case in Sudan), obtained from the Laboratory of Tropical Hygiene, Amsterdam, the Netherlands, was used throughout this study.

The strain was maintained by *in vitro* subculturing at 7–8 day intervals at 26 °C in liver infusion tryptose (LIT)/haemin medium (Sadigursky & Brodskyn, 1986). Promastigote mass production for antigen preparation was carried out by inoculating LIT/haemin-maintained parasites into RPMI 1640 medium supplemented with 5 mM HEPES, 100 IU penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 10 % heat-inactivated fetal calf serum. Incubation of the cultures was at 26 °C.

Patients and samples. Serum, plasma or whole-blood-spotted filter-paper specimens from subjects with the following conditions were included in the study:

Group A, proven VL ($n = 40$) from Gedaref, Eastern Sudan. VL was diagnosed by either bone-marrow or lymph-node aspiration (Kassab Treatment Centre, Gedaref State, Eastern Sudan).

Group B, high suspicion for VL ($n = 145$). Patients in this group manifested typical clinical signs and symptoms of VL, with negative diagnosis for malaria, typhoid and tuberculosis (Omdurman Hospital for Tropical Diseases, and Kassab Treatment Centre, Gedaref State, Eastern Sudan). In this patient group, the positive DAT results ($\geq 1:3200$) justified positive VL diagnosis.

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Abbreviations: DAT, direct agglutination test; β -ME, β -mercaptoethanol; VL, visceral leishmaniasis.

Group C, malaria ($n=28$), Omdurman Hospital for Tropical Diseases.

Group D, typhoid ($n=11$), Omdurman Hospital for Tropical Diseases.

Group E, undetermined liver or spleen disorders ($n=22$) with negative DAT ($\leq 1:100$), Omdurman Hospital for Tropical Diseases.

Group F, fever of unknown origin ($n=22$). Patients in this group had a negative DAT titre ($\leq 1:100$), Omdurman Hospital for Tropical Diseases.

Group G, African trypanosomiasis ($n=29$), National Medical Research Institute, Khartoum, Sudan.

Group H, pulmonary tuberculosis ($n=28$), Abuanja Academic Thorax Hospital, Omdurman, Sudan.

Group I, onchocerciasis ($n=10$), Institute of Endemic Diseases, University of Khartoum, Sudan.

Group J, leukaemia ($n=8$), Radiation and Isotopes Centre, Khartoum Central Hospital, Sudan.

Group K, apparently healthy non-endemic female students ($n=34$), Omdurman Hospital for Tropical Diseases.

From 73 patients in groups A and B, both serum- and blood-spotted filter-paper was collected. Only serum ($n=184$) or plasma ($n=8$) samples were collected from the patients in groups C–K. All samples were kept at -20°C .

Antigen processing and execution of serological tests. The antigen for DAT was prepared as described by Harith *et al.* (1995). The Coomassie Blue-stained promastigote suspension was washed and finally resuspended in 1.2% (v/v) formaldehyde/citrate/saline as $10\times$ concentrated (5×10^8 promastigotes ml^{-1}) stock antigen.

The antigen for β -ME ELISA was processed as described above for DAT, with the exception of the Coomassie Blue staining. The intact promastigote antigen so processed was fixed in formaldehyde/citrate/saline solution (1.2%, v/v) at 1.5×10^8 ml^{-1} . As control, a promastigote antigen suspension was processed in a similar manner, but without β -ME treatment. The water-soluble (strain 1-S) antigen for conventional ELISA was kindly provided by the Laboratory of Biomedical Research, the Royal Tropical Institute, Amsterdam, the Netherlands.

For executing ELISA and DAT, spotted-blood filter-paper samples were eluted in physiological saline (1:50 serum dilution) overnight at 4°C (Harith *et al.*, 1988). ELISA was performed according to the procedures reported by Voller *et al.* (1980), using intact or water-soluble promastigote antigen. After addition of substrate (5-aminosalicylic acid), the reaction was measured at a wavelength of 450 nm on a Titrek Multiscanner. In one experiment, the test was also read visually (1–1.5 h) by locating the highest sample dilution that still showed colour by comparison with the blank control well. The cutoff was defined as the mean absorbance value obtained in 34 sera from healthy subjects + 3SD.

In the conventional ELISA, the water-soluble antigen (protein content equivalent to that of a homogenate of 3×10^7 sonicated promastigotes ml^{-1}) was used in combination with optimal serum and anti-human IgG conjugate dilutions of 1:1600 or 1:100 000. A cutoff value of 0.50 (mean of test readings obtained in 25 healthy subjects + 3SD) was established.

Dilutions of the β -ME-treated or -untreated stock antigen suspensions were made in carbonate/bicarbonate coating buffer (pH 9.6), from 1×10^5 to 5×10^7 promastigotes ml^{-1} . A promastigote concentration

of 2.5×10^7 ml^{-1} for the β -ME-treated or -untreated intact antigen, and serum and conjugate dilutions of 1:12 800 or 1:100 000 were found to be optimal for executing the test. Absorbance values of ≥ 0.27 (mean of test readings obtained in 34 healthy subjects + 3SD) were considered to be ELISA positives with the β -ME-treated or -untreated antigen.

The DAT was executed according to the improved protocol, taking 1:3200 as the cutoff (Harith *et al.*, 1995). For confirmation or exclusion of VL, all samples showing marginal DAT titres (1:1600–1:3200) were retested after treatment with 0.03 M urea.

Data analysis. The sensitivity for each of three tests (β -ME ELISA, DAT and conventional ELISA) was defined as the proportion of true positives correctly identified [agreed positives/(agreed positives + false negatives) $\times 100\%$]. Specificity was defined as the proportion of true negatives correctly identified [agreed negatives/(false positives + agreed negatives) $\times 100\%$]. The paired sample *t* test was applied to compare results obtained in ELISA with the β -ME-treated or -untreated intact antigens, and Pearson's coefficient of correlation was employed to assess the performance of whole-blood sampling in comparison with serum.

RESULTS

Of 40 confirmed VL patients tested by β -ME ELISA, 39 (97.5%) had positive absorbance values in the range 0.28–2.44. No cross-reaction was observed in sera from healthy controls ($n=34$) or from patients with clinical conditions other than VL ($n=158$), revealing 100% specificity (Table 1). In contrast to results obtained with β -ME ELISA in non-VL sera ($n=158$), two patients with leukaemia and four others with African trypanosomiasis scored positive marginal titres (1:1600–1:3200) in DAT. By incorporating urea treatment into the DAT protocol, titres in all four trypanosomiasis patients were reduced to negative values ($< 1:3200$), but remained marginally positive (1:3200) in the two patients with leukaemia (results not shown).

In 65 (44.8%) of 145 suspected VL patients tested, both β -ME ELISA and DAT scored positive readings (Table 1). Three patients (2.1%) were positive in DAT but negative in β -ME ELISA. Despite the discrepancy, concordance between the two tests was 95.6% among seropositives and 96.3% among seronegatives.

By comparison with the untreated intact antigen, the β -ME-treated antigen showed slightly lower absorbance values in the non-VL patients. At a 1:100 serum dilution, eight non-VL sera scored absorbance values above the cutoff (0.27) with the untreated antigen, of which seven were negative with the β -ME-treated antigen. Eleven non-VL sera cross-reacted at a 1:800 dilution with the untreated antigen; negative ELISA readings were recorded in all 11 with the β -ME-treated antigen ($P<0.042$). Seven non-VL sera (from VL suspects, tuberculosis patients or healthy individuals) gave positive results with the untreated antigen at a 1:3200 dilution; only four of these tested positive with the β -ME-treated antigen. The difference between the two antigen preparations was even more pronounced ($P=0.017$) in the leukaemic sera. At a 1:12 800 serum dilution, however, no

Table 1. Performance of β -ME ELISA and DAT in patients with confirmed or clinical suspicion of VL and in individuals with other conditions

Group tested*	β -ME ELISA		DAT	
	Frequency of positive results†	Absorbance value (mean $\pm$ SD)	Frequency of positive results†	Reciprocal of titre
VL	97.5 % (39/40)	1.163 $\pm$ 0.651	100 % (40/40)	3200– $\geq$ 102 400
VLS	44.8 % (65/145)	0.666 $\pm$ 0.757	46.9 % (68/145)	100– $\geq$ 102 400
DC	0 % (0/158)	0.131 $\pm$ 0.042	1.3 % (2/158)	100–3200
HC	0 % (0/34)	0.181 $\pm$ 0.044	0 % (0/34)	$\leq$ 100–200

*Abbreviations: VL, visceral leishmaniasis; VLS, VL suspects; DC, diseased controls; HC, healthy controls.

†Percentage true positive (number positive/number tested).

cross-reaction was recorded in any of the 78 non-VL sera tested with the treated or untreated antigen. Also, irrespective of the antigen preparation applied, no significant difference ($P > 0.269$) in sensitivity was observed in 14 VL sera.

Based on the respective cutoffs, the absorbance values obtained in 18 VL sera with the β -ME-treated antigen were generally higher (0.19–1.86) than those with the water-soluble antigen (0.25–0.78).

Four VL samples (22.2 %) tested positive with the β -ME-treated antigen but negative with the water-soluble antigen. By comparison with the water-soluble antigen, the β -ME-treated antigen therefore showed a relatively higher sensitivity for VL detection (88.9 versus 72.2 %). At the same respective cutoffs, both antigen preparations gave negative absorbance values (β -ME treated, 0.06–0.24; water-soluble, 0.10–0.46) with all 65 non-VL sera tested, implying 100 % specificity.

In 24 VL whole-blood samples collected on filter paper, results of β -ME ELISA correlated favourably ($r = 0.711$) with those of the corresponding sera (results not shown). Absorbance values of 0.25–1.31 were recorded for filter-paper sampling compared to 0.28–2.44 with serum. Regardless of sampling procedure, comparable negative absorbance values of 0.08–0.23 were also recorded in all 49 non-VL sera tested.

By comparison with the negative control reactions observed in blank wells, 17 of 20 VL sera (85 %) developed (at $> 1:12\,800$ dilution) visible colour within 1–2 h of substrate addition (results not shown). At this dilution, however, no or hardly discernible coloration developed in the wells containing non-VL sera ($n = 25$); all these samples also gave negative DAT readings ($< 1:100$).

Throughout the 2-month storage period (at 4 °C), the reactivity of the formaldehyde-fixed antigen remained stable. Employing the same batch of β -ME-treated antigen suspension, comparable ELISA absorbance values of 0.27–2.35 were obtained at days 0, 30, 45 and 60 in 25 of 26 VL

samples evaluated (results not shown). Similarly consistent antigen performance, but in the negative range (< 0.27), was also observed in 32 non-VL sera tested on the same four occasions. Microscopic examination of specimens from the β -ME-treated antigen at day 60 of the storage period revealed normal promastigote morphology and a homogeneous antigen suspension.

DISCUSSION

At a cutoff of 0.27 based on optimal test conditions with respect to antigen concentration and serum and conjugate dilutions, the β -ME-modified ELISA showed a sensitivity of 97.5 %. Comparable sensitivity levels (97.2–100 %) have been reported earlier for ELISA with the conventional water-soluble or intact promastigote antigen (Shiddo, 1995; Hommel *et al.*, 1978; Mohammed *et al.*, 1985). However, in a similar Sudanese VL population tested with a recombinant *Leishmania* antigen in a strip test or conventional ELISA format, much lower sensitivity (67 %) has also been reported (Burns *et al.*, 1993; Zijlstra *et al.*, 2001). However, despite employing the same antigen to execute ELISA and DAT in this study, the sensitivity of the former technique was slightly lower (97.5 versus 100 %). Whether the negative ELISA reading obtained in one VL patient reflected our choice of the more commonly applied IgG ELISA will be answered in future trials with IgM for monitoring early infection.

In contrast to results obtained with DAT, no cross-reaction was observed in the 65 non-VL sera (including leukaemia and trypanosomiasis) tested against the β -ME-treated antigen, implying 100 % specificity. However, regardless of the *Leishmania* antigen employed in previous studies, cross-reactions in ELISA have been reported with African trypanosomiasis and malaria sera (Mohammed *et al.*, 1985; Voller *et al.*, 1980). Even by comparison with the current recommended techniques, namely the freeze-dried DAT kit, rK39 ELISA, rK39 strip test and urine latex agglutination (Burns *et al.*, 1993; Zijlstra *et al.*, 2001), β -ME ELISA showed higher reliability for differential VL diagnosis in the Sudan. In our opinion, this excellent specificity can

only be explained by the favourable cleaving or unfolding effect exerted by β -ME on complex promastigote surface antigens, whereby specific epitopes are optimally exposed for interaction with the corresponding IgG anti-*Leishmania* antibodies.

The high agreement ($r=0.711$; $P=0.000$) between results obtained by filter-paper (blood) and serum sampling, together with the advantage of visual reading of test results, imply good potential for successful application in laboratories with limited facilities. At a 1:12 800 serum dilution, cross-reaction with non-VL samples is significantly reduced, allowing for better discrimination of VL samples. Further optimization of the storage conditions, and of the temperature and duration of elution, is required for filter-paper whole-blood samples, to correct for the lower sensitivity (91.7%).

The formaldehyde-fixed antigen in β -ME ELISA proved stable during the 2-month storage period. This advantage was further enhanced by the absence of microscopic alterations in promastigote morphology and antigen homogeneity.

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