Validation of a β-ME ELISA for Detection of Anti *Leishmania donovani* Antibodies in Eastern Sudan

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ABSTRACT

Background: A β-mercaptoethnol (β-ME)-treated promastigote antigen of *L. donovani* was successfully employed in direct agglutination test (DAT) for the diagnosis of visceral leishmaniasis (VL). **Objective:** The β-ME-treated antigen was further incorporated into an enzyme-linked immunosorbent assay set-up (β-ME ELISA) and evaluated for VL diagnosis against outcome of reference freeze-dried DAT (FD-DAT) and rK39 strip test (RKT) commercial kits. **Methods:** Two-hundred and ninety-two sera from patients with high VL suspicion of whom 105 had confirmed *L. donovani* infection were tested. **Results:** Relatively higher sensitivities of 93.3% (95% CI: 88.4-98.2) and 92.4% (95% CI: 87.3-97.5) were determined for β-ME ELISA and FD-DAT as compared to 83.8% (95% CI: 76.7-90.8) for RKT. Of 73 VL sera that scored maximum absorbance values ($\geq$0.81) in β-ME ELISA, 66 (90.4%) tested at the highest agglutination titres ($\geq$1:51200) in FD-DAT as did 56 (76.7%) also at comparable reaction intensities (3 + colour intensity) in RKT. Compared with FD-DAT (94.7%, 95% CI: 91.5-97.9) or RKT (93.0%, 95% CI: 89.3-96.6), lower specificity was estimated for β-ME ELISA (90.4%, 95% CI: 86.1-94.6). Based both on positive and negative microscopy for *L. donovani* in organ aspirates of all VL suspects enrolled (292), significantly higher correlation ($p<0.01$, 0.919) was established between β-ME ELISA and FD-DAT than between β-ME ELISA and RKT ($p<0.01$, 0.824). Taking into calculation the combined estimates of sensitivity, specificity, positive and negative predictive values, higher agreement (94.8%) was determined between total performance of β-ME ELISA and FD-DAT than between that of β-ME ELISA and RKT (90.7%). **Conclusion:** Based on results and merits discussed, we recommend application of this β-ME ELISA both for diagnosis of VL at laboratory level and confirmation of results obtained with DAT or RKT in the field.

Keywords: β-ME, DAT, ELISA, rK39 Antigen, Visceral Leishmaniasis

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INTRODUCTION

Despite high efficiency reported for monitoring of visceral leishmaniasis (VL), results obtained with the direct agglutination (DAT) or rapid rK39 strip (RKT) tests in patients with less severe disease, could sometimes be marginal to justify or discourage prescription of chemotherapy (1-5). Even when simultaneously applied in patients with suspicion for VL in the field, outcome of these two procedures could disagree as to confirm or otherwise exclude infection (5,6,7). Such pitfalls in VL diagnosis were mostly reported in the field where conditions for maintaining test reagents or executing of the procedures were less favourable (5). Despite several practical merits reported, reactions in the DAT or RKT could thus be influenced by adverse conditions in the field to result in false negative or positive outcome resulting in either genuine cases with VL to be excluded from treatment or others free of VL to be unnecessarily subjected to toxic hazards of antimony preparations, respectively. Therefore, laboratory retesting of samples that present false reactions or marginal results in the field, with the same field procedure and/or with a laboratory-based alternative, can significantly minimize the chance for such a risk.

Of the several laboratory antibody-based procedures developed for diagnosis of VL, the enzyme-linked immunosorbent assay (ELISA) is considered as most appropriate (8,9,11,12). Performance of this assay was further optimized by the incorporation of highly purified proteins or recombinant *L.donovani* peptides to serve as antigens and of specific anti-human immunoglobulin conjugates to target antibodies directed to these antigens (5,12).

The use of a β-mercaptoethanol (β-ME) treated antigen of *L. donovani* promastigote in ELISA set-up as originally described for DAT (13), has successfully led to establishing of an assay version (β-ME ELISA) that evidenced excellent reliability for VL detection (14,15). Our objective here was to validate by comparison based on qualitative and semi-quantitative criteria, performance of this developed β-ME ELISA version against that of DAT and RKT as the current reference diagnostic procedures for VL.

MATERIALS AND METHODS

Specimens. Sera from 292 patients designated as VL suspects in a previous study carried out in the peripheral hospital of Doka (El Gedaref district, Eastern Sudan) were enrolled (16). Two-hundred and three samples were collected from males and 89 from females at ages from three to forty years. VL diagnosis was established by demonstration of *L. donovani* amastigotes in lymph node aspirates of 99 patients at Doka peripheral hospital and in bone-marrow (n=3) or spleen (n=3) aspirates of six patients at El Gedaref central hospital. Of 187 suspects that revealed negative aspirates, VL was established in ten based on positive clinical VL presentation (fever for 2 or more weeks, spleen and/or lymph node enlargement), positive titre reading (> 1:3200) with a locally produced DAT (17) and negative response to anti-malarial therapy. All 115 patients finally diagnosed as VL cases, responded favourably to a 30-day treatment regimen with sodium stibogluconate (17). Positive response to treatment was judged by the absence of *L. donovani* amastigotes in Giemsa-stained smears of aspirates taken from the inguinal lymph nodes after completion of the treatment, subsidence of fever, regression in spleen size and general improvement in physical condition of the patient.

Serum specimens collected from all 292 patients were kept frozen at -20˚C until transportation to the Laboratory of Biomedical Research in Ahfad (Omdurman) where they were constantly stored at the same temperature until required.

β-Mercaptoethanol ELISA. Processing of the antigen in β-ME ELISA and execution of the test was as described elaborately in earlier reports (14,15). An optimal working antigen suspension of 2.5 x 10^7 promastigotes /ml versus serum and goat anti-human IgG peroxidase conjugate (Jackson Immunoresearch Laboratories Inc.) dilutions of 1:12,800 or 1:100,000 were used. Serum samples were tested in duplicates and wells containing positive and negative control sera were included. Fifteen minutes after addition of the substrate (5-aminosalicylic acid), the reaction was measured at 450-nm wavelength on a Titertek Multiscan. Sera revealing absorbances ≥ 0.27 were considered as VL positives (14). Sera were then further classified according to intensities of the reactions developed and varied in absorbance from < 0.10-0.26 (0, negative) to 0.27-0.53 (1+, weak), 0.54-0.80 (2+, moderate) or 0.81- ≥1.35 (3+, strong).

Freeze-Dried Direct Agglutination Tests (FD DAT). The Leish-KIT, (Lyopharma, Bilthoven, The Netherlands) was procured via the East Mediterranean Regional Office of the World Health Organization in Cairo. Execution of the test was according to the manufacturer's instructions. Sera were tested in two-fold serial dilutions starting at 1:100 up to 1:102,400. Sera revealing titre readings ≥ 1:3200 were considered as VL positives (17). As for β-ME ELISA, sera were further classified according to intensities of the agglutination reactions developed and varied in titres from < 1:100-1:1600 (0, negative) to 1:3200-1:6400 (1+, weak), 1:12,800-1:25,600 (2+, moderate) or 1:51,200-≥ 1:102,400 (3+, strong).

rK39 Strip Test (RKT). The rapid VL IT-LEISH test (Diamed Ag, Cressier sur Morat, Switzerland) was procured through local agent in Khartoum. Execution of the procedure was as instructed by the manufacturer and the result was only considered valid when a control line appeared in the test strip. Test reactions were read visually (within 10-15 minutes) by three individuals relying on the presence or absence of a reaction line in the test band.

Positive reactions obtained with RKT were further graded according to the intensity of the colour developed in the test band that varied from dark purple (strong, 3+) to light purple (moderate, 2+), faint or barely visible colour (weak, 1+) (18). Samples revealing no reactive (test) bands were considered as VL negatives (0).

Data Analysis. The sensitivity and specificity of β-ME ELISA, FD-DAT and RKT were calculated as follows: sensitivity rate=TP/(TP+FN) × 100% and specificity rate=TN/(TN+FP) × 100% where TN represents true negative, TP true positive, FN false negative, and FP false positive. Positive predictive and negative predictive values were calculated for each of the three tests according to methods described by Fleiss (19) and Smith and Richard (20). Statistical analyses were conducted using SPSS software version 13.5 (SPSS Inc., Chicago, IL, USA), with a probability p value < 0.05 as statistically significant. The degree of correlation was determined by calculating Spearman's correlation coefficient values with 95% confidence intervals. Cohen's kappa values express the agreement beyond change, and a value of 0.21-0.60 represents a fair to moderate agreement, a value of 0.61-0.80 represents a substantial agreement, and a value ≥ 0.81 represents almost perfect agreement.

RESULTS

Of 105 VL cases that revealed positive microscopy for *L.donovani*, 85 (81.0%) tested positive in all three procedures. Seven (6.7%) of those confirmed cases tested negative in β-ME ELISA, as also did 8 (7.6%) in FD-DAT and 17 (16.2%) in RKT, implying highly matching sensitivities of 93.3% (95% CI: 88.4-98.2) and 92.4% (95% CI: 87.3-97.5) for β-ME ELISA and FD-DAT as compared to 83.8% (95% CI: 76.7-90.8) for RKT. Among ten (3.4%) diagnosed as VL cases on the basis of positive disease presentation and positive DAT results (titres ≥ 1:3200), 7 tested positive both in β-ME ELISA and RKT.

Of 187 suspects that revealed negative aspirates for L. donovani, 18 (9.6%) tested positive in β-ME ELISA as compared to 13 (7.0%) in RKT and 10 (5.3 %) in FD-DAT. Among the 18 suspects that had tested positive in β-ME ELISA, seven reacted also positive both in FD-DAT and RKT and four tested just marginally negative (titres=1:1600) in FD-DAT. All seven suspects tested positive in the three procedures plus three others diagnosed as VL cases on grounds of positive disease presentation and positive DAT outcome, responded favourably to sodium stibogluconate treatment. Based therefore on positive response to specific anti-leishmanial therapy rather than on microscopy, a specificity of 94.3 % (95% CI: 90.8-97.8) was determined for β-ME ELISA in this group of non-VL subjects (Table 1).

Table 1. Validity of β-ME ELISA for detection of visceral leishmaniasis (VL) as assessed against outcome of microscopy, freeze-dried direct agglutination test (FD-DAT) , rK39 strip test (RKT) or response to specific anti-leishmanial therapy.

Procedure taken as gold standard	β-ME ELISA				
	Sensitivity (95%CI)	Specificity (95% CI)	Agreement* (%)	Positive predictive value (%)	Negative Predictive predictive (%)
Positive microscopy	93.3(88.4-98.2)	90.4(86.1-94.6)	81.8	84.5	96.0
Freez-dried Direct Agglutination Test (FD-DAT)	92.4(87.3-97.5)	94.7(91.5-97.9)	87.7	90.7	95.7
rK39 strip test (RKT)	83.8(76.7-90.8)	93.0(89.3-96.6)	80.3	87.1	91.1
Positive response to Specific anti-Leishmanial therapy	92.2(87.2-97.2)	94.3(90.8-97.8)	87.6	91.4	94.9

*Agreement according to Cohen's kappa analysis.

Emerging from 0.5%-1.0% VL prevalence rate in these area, a positive predictive value slightly lower (84.5%) than that of RKT (87.1%) and a negative predictive value almost similar (96%) to that of FD-DAT (95.7%) was determined for β-ME ELISA.
Of 73 VL sera that tested at the strongest β-ME ELISA absorbance values (> 0.81), 66 (90.4%) scored the highest agglutination titres (≥ 1: 51200) in FD-DAT as also did 56 (76.7%) at comparable positive colour intensities (3+) in RKT (Table 2). Almost similar number of VL sera was revealed by β-ME ELISA or FD-DAT as least-reactive or non-reactive by comparison with at least twice as many by RKT (Table 2).

Table 2. Comparison among reaction intensities of β-ME ELISA, freeze-dried direct agglutination test (FD-DAT) and rK39 strip test (RKT) in 115 sera from patients with parasitologically confirmed or unconfirmed visceral leishmaniasis (VL).

		FD-DAT reaction intensity**				RKT reaction intensity***			
Absorbance range (reaction intensity)	Number of sera (%)	(-)	1+	2+	3+	(-)	1+	2+	3+
≤0.1-0.26 (-)	9* (7.8)	5	0	3	1	7	0	1	1
0.27-0.53 (1+)	8(7.0)	3	4	1	0	2	1	1	4
0.54-0.80 (2+)	25(21.7)	0	7	9	9	3	2	3	17
0.81-≥1.35(3+)	73(63.5)	0	2	5	66	7	5	5	56
Total number 115(100)		8* (6.9)	13 (113)	18 (15.7)	76 (66.1)	19* (16.5)	8 (7.0)	10 (8.7)	78 (67.8)

* Included sera from confirmed VL cases tested in β-ME ELISA (7), FD-DAT (8) and RKT (17).
** Agglutination intensities were according to titres ranging ≤ 1:100-1:1600 (negative -), 1:3200-1:6400 (weak 1+), 1:12800-1:25600 (moderate 2+) or 1:51200- ≥ 1:102,400 (high 3+).
***Colour intensities varied from dark purple (strong, 3+) to light purple (moderate, 2+), faint or barely visible (weak, 1+). Samples revealing no colour in the test band were considered negative (-).

Based both on positive and negative microscopy for *L. donovani* in organ aspirates of all VL suspects enrolled (292), a higher agreement ($p < 0.05$, 0.877) was established between β-ME ELISA and FD-DAT than between β-ME ELISA and RKT ($p < 0.05$,

0.803) (Table 1). By combining all estimates of sensitivity, specificity, positive and negative predictive values, a higher correlation was established between total results obtained with β-ME ELISA and FD-DAT (0.919) than between those with β-ME ELISA and RKT (0.824) (Table 3).

Table 3. Agreement and correlation between results obtained with β-ME ELISA, freeze-dried direct agglutination test (FD-DAT) or rK39 strip test (RKT) in 292 patients with suspicion for visceral leishmaniasis (VL) based on combined estimates of sensitivity, specificity, positive and negative predictive values.

Procedure	Statical test	β-ME ELISA	FD-DAT	RKT
β-ME ELISA	Agreement*	1.0	0.877	0.803
	Correlation**	1.0	0.919	0.824
FD-DAT	Agreement	0.877	1.0	0.851
	Correlation	0.919	1.0	0.838
RKT	Agreement	0.803	0.851	1.0
	Correlation	0.824	0.838	1.0
Outcome of Microscopy	Agreement	0.818	0.867	0.775
	Correlation	0.836	0.859	0.777

* Agreement calculated by Cohen's kappa test.
**Spearman correlation was significant at the 0.1 level (2-tailed).

DISSCUSION

Due to the unsettled dispute in designating a gold standard for VL diagnosis, validation of newly developed diagnostic procedures can no longer be justified by comparison with parasitological outcome only. Despite excellent results reported for the DAT or RKT in signalling the disease both at the early and advanced phases, neither procedure evidenced so far absolute reliability for VL diagnosis. For validating the performance of this β-ME ELISA version we have chosen therefore in addition to microscopy, comparison with results obtained with the DAT and RKT.

Unlike in reports published earlier, significant lower sensitivity (83.8%) was determined here for RKT in comparison with FD-DAT (92.4%) or β-ME ELISA (93.3%). Whether this unexpected negative outcome by all three procedures and in particular RKT, had to do with co-occurrence of immunosuppressive conditions is difficult to substantiate. We assumed nonetheless that these false negative results were most likely related to early VL infection in which, unlike for long-standing infection, antibodies belonging to the M- but not the G-class immunoglobulins were are most elevated (21). This and the objective behind the current test set-up to exclusively target IgG antibodies seem to have contributed to the failure of RKT and to a lesser extent to that of β-ME ELISA to

signal VL respectively in 17 or 8 of those confirmed cases (Table 2). Also, variability in the performance of RKT kits produced by different manufacturers and/or between batches produced at different time intervals by the same manufacturer can not entirely be excluded as reasons for this unexpected outcome. In our judgement, variability in RKT diagnostic performance is to a certain extent also expressed in the wide range of sensitivity (67% -96.0%) reported during the past decade by several research groups in this same endemic area (6,7,17,22).

Unlike in FD-DAT or β-ME ELISA where a homologous *L. donovani* strain was employed as an antigen, a heterologously cloned peptide of *L. chagasi* was used in RKT. In a successful attempt made earlier to optimize the sensitivity in DAT, it was demonstrated that by incorporating homologous or autochthonous *L. donovani* antigens, titres in VL sera were augmented to at least three folds (13). It seems possible therefore that due to the use of endemic *L. donovani* strains both in β-ME ELISA and FD-DAT that in addition to increase in sensitivity, significant enhancement in the intensities of the positive reactions was also achieved in these two procedures in comparison with the RKT (Table 2). No such correlation could be established between the positive intensities of RKT and the antibody response as semi-quantitatively assessed by titration of VL sera against either the homologous (*L. donovani*) or the heterologous (*L. chagasi*) antigen used in DAT and RKT procedures respectively, (18). Among future plans to further improving sensitivity and specificity of β-ME ELISA, are the introduction of a more effective proteolytic cleaving agent in antigen processing and the incorporation of a mono-specific anti-human conjugate for targeting IgM antibodies as a marker for early VL detection.

Even though slightly lower than levels estimated for FD-DAT or RKT, the specificity of β-ME ELISA in this study was still within the range (59.0%- 98.2%) earlier reported for these two aforementioned reference procedures in this endemic area (6,7,15,17,23). The apparent efficiency of β-ME ELISA for both sensitive and specific detection of VL is in our opinion expressed in its positive outcome in 18 of the 187 VL suspects that revealed negative microscopy for *L. donovani*. Nine of those 18 tested also positive in RKT and eight in FD-DAT; four other suspects (out of the same 18) though considered negative by FD-DAT, their titre readings were just marginal (1:1600). Unfortunately, no follow-up information was available on the remaining six suspects. Besides this potential for early VL detection, β-ME ELISA evidenced also desirable level of specificity against disorders that highly simulated VL in this study confirming our previous conclusion as to its reliability for discriminative VL detection even in areas that are also endemic for tuberculosis, malaria, typhoid or African trypanosomiasis (14).

Among the practical merits experienced with β-ME ELISA in this study is that antigen processing, unlike in other recently developed ELISA versions, does not require sophisticated equipments or special expertise in the field of biotechnology. Execution of the procedure is also simple and can successfully be achieved by a technician with average laboratory training. Storage of the antigen is also less demanding and as for its equivalent formaldehyde-fixed in DAT, does not require additional measures to maintain stability at 4°C (1). Although + 60 days was initially reported as being the shelf-life time, our latest observation is that antigen reactivity remains stable for at least one year at 4°C (15).

Taking all pertinent criteria into consideration, we think that this β-ME ELISA version is a reliable method both for routine diagnosis of VL at laboratory level and confirmation of results obtained with DAT or RKT in the field.

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