Qualitative and Semi-Quantitative Comparison of an Rk39 Strip Test and Direct Agglutination Test for Detection of Anti-*Leishmania Donovani* Antibodies in the Sudan

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ABSTRACT

Background: Until now, the comparison of the rK39 strip test (RKT) and direct agglutination test (DAT) for detection of visceral leishmaniasis (VL) is exclusively based on either positive or negative qualification of the reaction outcome. **Objective:** In this study, we compared the diagnostic performance of RKT and DAT for VL both qualitatively and semi-quantitatively. **Methods:** For comparison based on semi-quantitative grounds, the execution of RKT and DAT was according to the standard procedures. For comparison on semi-qualitative grounds with DAT, the RKT was applied to aliquots from positive samples that were two-fold serially diluted in saline to determine, as for the DAT, the end-point reaction in RKT. **Results:** While qualitatively both RKT and DAT demonstrated comparable reliability for VL detection (sensitivity = 96% and specificity = 98.7% or 99.3%), no significant correlation (r = 0.13) could be established between intensities of their positive reactions in 25 cases studied. A negative correlation was further determined in those 25 VL cases between the positive intensities of the RKT and antibody levels measured semi-quantitatively with the same procedure (r = -0.36) or the DAT (r = -0.30). Irrespective of the low, moderate or high antibody levels measured with RKT ($\leq$1:8 and 1:16-1:32 $\geq$1:256) or DAT ($\leq$ 1:25,600 and 1:51,200- 1:409,600 $\geq$ 1:3,276,800) in patients with confirmed or unconfirmed VL infection, exclusively strong positive intensities were obtained with RKT. **Conclusion:** For further optimizing diagnosis and simultaneously assessing magnitude of immune response to *L. donovani* infection in Sudanese patients, the combined application of RKT and DAT is recommended.

Keywords: Visceral Leishmaniasis, rK39, Agglutination Test

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INTRODUCTION

Of the several procedures developed for detection of visceral leishmaniasis (VL) in the past three decades, the direct agglutination test (DAT) and the rapid rK39 strip test (RKT) are considered as most appropriate (1-4). Almost absolute reliability for VL detection was reported with the latter procedure in the Indian sub-continent (3). Despite being relatively less rapid than RKT, the DAT was generally accepted as reliable and feasible diagnostic tool for VL in East Africa (2,5-7). In spite of the acknowledged high concordance, disagreement between results of the two procedures was also reported (6,8,9). Among others, variability in the performance of the test batches produced at different laboratories as in the case of the DAT or variability of manufacturers in the case of RKT has been reported as reason for the disagreement (3,6).
Our objective here was to investigate whether the antibody response to VL infection as qualitatively or semi-quantitatively assessed against the recombinant antigen (K39) involved in RKT, matches that against the β-ME improved equivalent in DAT. The specificities of RKT and DAT for detection of VL were challenged against conditions generally known as difficult to differentiate clinically or serologically from VL.

MATERIALS AND METHODS

Subjects and Specimens. Serum specimens were collected from the following subjects:
A. Confirmed VL cases (n = 25) diagnosed during a field study conducted in the period of September 2004 to October 2005 in Eastern Sudan by the demonstration of *L. donovani* amastigotes in lymph-node aspirates (10).
B. Reference pooled VL sera (n = 2) with moderate (1:51,200) or high (1:13,107,200) DAT titres prepared respectively from three and 15 serum samples collected from patients diagnosed by the demonstration of *L.donovani* amastigotes in lymph node aspirates (10,11).
C. Reference pooled VL sera (n = 2) with low (1:25,600) or high (1:3,276,800) DAT titres prepared from 15 serum samples each collected from patients diagnosed on the basis of positive VL clinical presentation, positive DAT result (titre $\geq$1:3200) and negative tests for typhoid, malaria and tuberculosis (10,11).
D. Untreated, smear positive tuberculosis patients received at Abuanja Academic Thorax hospital of Omdurman (n = 20).
E. Patients with positive blood-film for malaria; Laboratory of Biomedical Research, Ahfad University for Women, Omdurman (n = 20).
F. Patients with African trypanosomiasis having positive clinical symptoms and positive Card agglutination (CATT) or counter-immuno-electrophoresis test results obtained from the Tropical Medicine Research Institute, Khartoum (n = 12).
G. Cases with leukaemia; Radiation and Isotopes Centre, Khartoum Central Hospital (n = 20).
H. Reference pooled non-VL serum sample prepared from 30 individual sera of patients diagnosed with tuberculosis (n = 8), malaria (n = 8), African trypanosomiasis (n = 6) or leukaemia (n = 8) described in sections D, E, F and G.
I. Non-endemic and apparently healthy female students; Ahfad University for Women, Omdurman (n = 27)

J. Reference pooled non-VL endemic serum sample prepared from the individual sera of subjects described in section I.
K. Apparently healthy endemic subjects from Doka locality of Gedarif district, a well known endemic area for VL in the Eastern State of Sudan (n = 50).
L. Reference pooled endemic serum sample prepared from 30 out of fifty individual sera described in section K.

rK39 Strip Test (RKT). The rapid *VL IT-LEISH* test (Lot # 46240; Diamed AG, Cressier sur Morat, Switzerland) was procured through local agent in Khartoum. For mere determination of positive or negative (qualitative) result, the test was executed as instructed by the manufacturer. 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 observers relying on the presence or absence of the reaction line in the test band.
Positive reactions were further graded according to the intensity of colour developed and varied from dark purple (strong, 3+) to light purple (moderate, 2+), faint or barely visible (weak, 1+). Samples revealing no reactive (test) bands were considered as VL negatives (0).
All samples identified as RKT positives were then titrated through two-fold step serial dilution against the recombinant (rK39) antigen involved starting with 1: 2 up to 1: 2048. To investigate validity of using saline as diluent in RKT, serum samples from VL and non-VL subjects were diluted in normal saline or in non-VL pooled human serum before loading on the test strip for comparison. Further steps in RKT execution on the samples so diluted, were as instructed for neat sera using fresh test strip for every serial dilution. The value of the highest dilution preceded the one revealing negative RKT reaction was considered as end-point (titre) of the test sample. Reading of the test result and grading intensity of the positive reaction was performed independently by three different observers.

Direct Agglutination Test. Promastigotes of *Leishmania donovani* mass cultured in Liver Infusion Tryptose (LIT) medium were processed according to the improved DAT procedures previously described by el Harith et al. (12). The test was executed according to the standard procedures using β-ME as a reducing agent (13). Reference positive and negative sera were included in each occasion for test execution. Sera were tested in two-fold serial dilutions starting at 1:100 through full-out titration to determine end-point of agglutination reaction. Serum samples showing titres $\geq$ 1:3200 were considered as VL positives (13). Positive titres obtained, were further classified as high ($\geq$1:819,200), moderate (1:51,200-1:409,600) or low (1:3200-1: 25,600).

Statistical Analysis. The sensitivity for both RKT and DAT was defined as the percentage of disease-positive patients with a positive result: true positives/ (true positives + false negatives) x 100 and specificity was as the percentage of disease-negative individuals with a negative test result: true negatives/ (true negatives + false positives) x 100.
The relation between intensities of positive reactions and titre readings obtained with the RKT or DAT in the individual and pooled VL sera was evaluated via regression analysis in Excel 2003.

RESULTS

Of the 25 sera from VL cases enrolled, 24 tested positive both in RKT and DAT (Tables 1 & 3). The DAT missed one VL case and RKT missed another, implying identical sensitivity of 96% (with 95% confidence interval (CI) of 77.7% to 99.8%). Despite

discrepant outcome in three non-VL subjects, specificities of two procedures were desirably high, being 98.7% (with 95% CI of 94.7% to 99.8%) (147/149) for RKT and 99.3% (with 95% CI of 95.8% to 100%) (148/149) for DAT. The agreement rate (Kappa index) calculated from the patients data was 0.92 indicating an optimal agreement between the results of two tests. For any particular positive test result, the probability that it is "true positive" (Positive Predictive Value) was 0.92 (with 95% CI of 0.73 to 0.99) for RKT test and 0.96 (with 95% CI of 0.78 to 1.00) for DAT. For any particular negative test result, the probability that it is "true negative" (Negative Predictive Value) was 0.99 (with 95% CI of 0.96 to 1.00) for both tests. As in individuals sera, comparable diagnostic reliability was also demonstrated for the two procedures in the pooled sera from patients either with (48) or without (87) VL infection (Table 2).

There was a high correlation (r= 0.99) between the antibody titres measured with RKT in the pooled sera from the VL or the non-VL subjects that were diluted before testing in human serum or in normal saline, implying validity of using the latter as sample diluent in RKT execution (Table 2).

No significant correlation (r=0.13) could be established between the positive intensities of RKT and DAT in the 25 individual VL sera studied. A negative correlation in those VL sera was also found between RKT positive intensities and antibody levels semi-quantitatively measured with the same procedure (r= -0.36) or with the DAT (r= - 0.30) (Tables 1 & 3). Regardless of strong positive intensities (3+) determined with RKT in 19/25 (76%) of the individual VL sera, antibody response no higher than 1:32 titre level was recorded in 16/25 (64%) with the same procedure (Tables 1 and 3).

Of the individual VL sera that were tested by RKT, 5 (20%) had intensities < 2+ and were categorised as low, moderate, and high in DAT. One of the sera that tested negative by RKT, was categorized as high positive in DAT. On the other hand, nineteen (79.2%) were tested 3+ by RKT and positive by DAT of which, five sera were categorized at the lowest positive range (< 1: 25,600) (Table 1).

No match was observed between RKT positive intensities and antibody levels measured with the same procedure or DAT in the VL pooled sera from patients either with positive (18) or negative (30) microscopy for *L. donovani* (Table 2). Regardless of parasite presence, there was reasonable match between antibody titre levels measured with the RKT or DAT in those two VL groups (Table 2).

Table 1. Comparative performance of rK39 strip test (RKT) and direct agglutination test (DAT) in Sudanese patients with or without visceral leishmaniasis (VL)

		RKT reaction intensity:				RKT outcome
Study population	DAT titre range*	0	1+	2+	3+	
Subjects with VL (n= 25)	≤1: 1600		1			Positive (1)
	1:3200- 1: 25,600		1	1	5	Positive (7)
	1:51,200- 1: 409,600			1	4	Positive (5)
	≥ 1:819,200	1**		1	10	Positive (11)
						Negative (1)
Subjects with non-VL disorders (n= 72)	≤ 1:100- 1: 400	70	2***			Negative (70)
Subjects without VL (n= 70)	≤ 1:100-1: 1600 (69)	70				Negative (70)
	1: 3200 (1)					

*DAT cut-off value for VL = 1:3200. **Positive in DAT (titre=1:1 638 400) but negative (non-reactive) in RKT. ***Patients with malaria or leukaemia.

Table 2. Effect of sample diluent on rK39 strip test (RKT) outcome in pooled sera from Sudanese patients with or without visceral leishmaniasis (VL).

Study group	DAT titre	RKT	RKT titre of sample diluted in:	
(Number of pooled sera)	Level	Intensity	Human serum*	Saline
Confirmed VL (n= 15)	1:13,107,200			
	High	3+	1:128	1:256
Confirmed VL (n=3)	1:51,200			
	Moderate	3+	1:32	1:32
Unconfirmed VL (n= 15)	1:3,276,800			
	High	3+	1:64	1:64
Unconfirmed VL (n= 15)	1:25,600			
	Low	3+	1:4	1:8
Conditions other than VL**	1:100 -1:800			
(n= 87)	Negative	0	ND***	ND

*Pooled sample from sera of healthy, DAT negative Sudanese subjects living in non-endemic area. **Diseased (30), healthy endemic (30) and healthy non-endemic (27). ***ND: not done; none of the three (pooled) neat serum samples was reactive in RKT.

Table 3. Comparison of reaction intensities and antibody titre levels measured with rK39 strip test (RKT) or direct agglutination test (DAT) in sera from Sudanese patients with visceral leishmaniasis (VL).

Diagnostic test	Number of	RKT titre					
	VL cases	≤1:32	1:64	1:128	1:256	1:512	1:1024
RKT intensity							
0	1	1**					
1+	2	2					
2+	3	2				1	
3+	19	11		2	1	3	2
DAT titre range*							
≤1: 1600	1	1***					
1:3200- 1:25,600	7	3		1		2	1
1:51,200- 1:409,600	5	4				1	
≥1:819,200	12	8		1	1	1	1

*DAT cut-off for VL = 1:3200. **Positive in DAT (titre = 1:1,638,400) but negative (non-reactive) in RKT. ***Positive in RKT (intensity = 1+, titre = 1:4) but negative in DAT (titre = 1:1600).

DISCUSSION

Our results with RKT in this study are in line with those obtained with a similar test version (IT-Leish Diamed AG) in India, however, far less so with others reported in the Sudan and Nepal employing versions of Arista Biologicals, Inc or InBios International (3,6,14). Compared to the sensitivity and specificity of 67%-92% or 59%-97% for the RKT (Amrad ICT, and Arista Biologicals, Inc), those estimated for the DAT (84%-98% or 85%-98%) in Eastern Sudan, were considered to be higher (2,6). Irrespective of various RKT or DAT versions used, results of statistical analysis applied to sensitivity and specificity data obtained in the Sudan and other major endemic areas indicated comparable diagnostic efficiency for VL (4). Based on this and the additional merits of being

rapid and simple to execute, the RKT was recommended as a reference test for VL detection. Taking into account, however, the number of endemic and non-endemic subjects that were incorrectly identified as VL positives by RKT (1.3%, 3%, 8%, 13%, 29.0% and 30.0%) in this and previous studies (6,8,9,14,15) together with the risks involved in anti-leishmanial administration, corroboration of the results obtained by RKT seems appropriate.

The cross-reaction with malaria, observed in this study, requires special consideration as this parasitic infection is highly prevalent in almost all areas known to be endemic for VL in the Sudan. No cross-reaction as yet with malaria has been reported with this β-ME improved DAT version in Eastern Sudan (10,16). Simultaneous application of supporting diagnostic procedures such as DAT is expected therefore to be of great help in establishing more reliable and safer strategy for VL management in such areas of co-endemicity.

Despite the desired level of sensitivity (96%) demonstrated here for RKT, further qualification of its positive intensities in 25 confirmed VL cases versus antibody response semi-quantitatively assessed with the same procedure or the DAT, revealed significant discordance ($r = -0.36$ and $r = -0.30$, respectively). This inconsistency in expressing magnitude of immune response to *L. donovani* infection on account of RKT seems relevant in explaining why, as by comparison to India, less favourable performance was reported for the procedure in East Africa (6, 8, 9). Of relevance as well was the lower diagnostic efficiency reported versus an IgG ELISA based on the use of the same recombinant antigen in Eastern Sudan (6). Compared to 100% or 91% sensitivity for the mentioned ELISA version and DAT, a much lower level (67%) was determined for RKT. It seems most likely that the escape of 33% of genuine VL cases from detection by RKT, is not due to potency of the antigen involved but rather to the nature of the device used in antigen presentation. To ensure maximum sensitivity in VL detection among Ethiopian communities with high HIV sero-prevalence rates, DAT re-testing of VL suspects revealing negative RKT outcome was strongly recommended (9).

Despite the modest number of samples studied here, we believe that the current RKT version is as efficient as DAT in VL detection, it nevertheless lacks potential for expressing the magnitude of antibody response to *L. donovani* infection and therefore less capable of reflecting status or progress of the disease in the affected patients. Although the results obtained the DAT in Sudan and Bangladesh confirmed optimal sensitivity of the test at the chosen cut-off titre (1:3200), the immune response to parasite invasion was more pronounced (titres $\geq$ 1:52,100) in the patients who had harboured demonstrable number of amastigotes in organ aspirates (possibly an advance disease) (5,17). Unlike in these "advance" cases, titres at the marginal or low DAT positive range ($\leq$ 1:12800) were reported in patients who had high suspicion for VL but negative microscopy for *L. donovani*, in confirmed VL patients who had suffered from concurrent HIV or AIDS infection and in VL patients who had previously received anti-leishmanial therapy (2,5,9,11,18).

Whether the remarkable difference in antibody levels measured with RKT and DAT, respectively, against *L. chagasi* or *L. donovani* antigen (Tables 2 and 3) is related to the difference in taxonomy between the two sub-species, awaits further investigations with a similar recombinant antigen of *L. donovani* origin. It is worth mentioning that by comparison with heterologous antigens, the use of the homologous indigenous *L. donovani* in DAT has significantly improved the sensitivity of the test for the detection of VL in the

respective endemic regions (13). Therefore, it is reasonable to assume that due to the lesser availability of antibodies that are specifically reactive to *L. chagasi* antigen in the sera of East African endemic populations, relatively low sensitivity was reported for RKT in the Sudan, Ethiopia and Kenya where *L. donovani* is the principal agent for VL (6, 8, 9).
The criterion adopted for reading the reaction in RKT is dependent on individual visual judgment and could therefore lead to dubious qualification of outcome particularly in weak and marginally reacting samples such as the two (8%) included in this study (Table 1) or others described by Zijlstra et al. (6). The reported negative effect of high temperature ($\geq$ 40˚C) and low humidity (< 30%) on stability of the reagents and the preference for using serum over whole blood to execute the procedure (3,6) predicts negative impact on the application of RKT as a reference test in the poorly equipped rural hospitals of Sudan particularly during the hot/dry period (4-5 months/year). Unlike with RKT however, no problem was reported so far in using whole blood samples spotted on filter-paper for DAT execution in Sudanese or Ethiopian rural health settings (1,5,9). Stability of the antigen in DAT was successfully demonstrated with either freeze-dried or glycerol-preserved version in Eastern Sudan at ambient temperatures fluctuated between 28˚C and 47˚C (1,10,12).
Notwithstanding the practical merits experienced here with RKT, its recommendation as the reference test for VL diagnosis requires more assessment versus DAT particularly in clinical suspects that are designated as negatives by microscopy. Further evaluation in suspects revealing negative microscopy and marginal DAT readings (titres = 1:1600-1:6400) is essential for validating RKT performance in cases with early or sub-clinical VL infection. To meet with the urgent need for managing VL in such patients, we recommend the combined application of RKT and DAT in the Sudan.

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