Local Production of a Liquid Direct Agglutination Test as a Sustainable Measure for Control of Visceral Leishmaniasis in Sudan

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Abstract. A prerequisite for the control of visceral leishmaniasis (VL) is the accessibility to reference diagnostics. The high price of the freeze-dried direct agglutination test (FD-DAT) and the short shelf-life time of the rK39 strip test (rK39) have limited the application of these tests in Sudan. An original liquid DAT (LQ-DAT) with high reproducibility compared with the FD-DAT and rK39 has been routinely produced in our laboratory since 1999. In this study, a 3.4-year-old batch (of more than 90 test batches produced to date) was chosen to validate the diagnostic performance of this test against microscopy, FD-DAT, and rK39 in 96 VL and 42 non-VL serum samples. Relatively higher sensitivity (95/96, 99.0%) was recorded for the LQ-DAT than for the FD-DAT (92/96, 95.8%) and rK39 (76/96, 79.2%), probably because of the use of the endemic autochthonous *Leishmania donovani* isolate as the antigen. Experience with the LQ-DAT, its low cost of production, ease of providing this test, and diagnostic reliability compared with the FD-DAT suggest that widescale implementation of the LQ-DAT can contribute to sustainable VL control in Sudan.

INTRODUCTION

Proper control of visceral leishmaniasis (VL) in Sudan and other major endemic areas requires an uninterrupted supply of reliable diagnostic tests such as the freeze-dried direct agglutination test (FD-DAT) or the rapid rK39 strip test (rK39).[1,2] Despite the reported excellent performance for these two tests in both the laboratory and the field, they are not, more than 20 years after their development, routinely used in rural health settings. The main reasons for this include limited access to the FD-DAT and the short shelf-life time of rK39. VL control in Sudan can therefore only be achieved by making serious efforts to improve the properties of these two tests or through development of new tests that can cope with the unfavorable economic and climatic conditions. Building or strengthening the capacity for local production of such important tools would probably be the right approach to achieve this goal.

In 1999, following provision of the required equipment and reagents, the laboratory of Biomedical Research at Ahfad University for Women in Omdurman, Sudan, started producing the originally described liquid DAT (LQ-DAT) version.[3] The LQ-DAT has been used to diagnose VL in patients referred to us from various hospitals in Sudan. LQ-DAT supplies have also been offered to various Sudanese institutions that are involved in VL diagnosis and research. Glycerol preservation was introduced to further ensure safe long-term antigen storage under the harsh rural conditions prevailing in eastern Sudan.[4,5]

On the basis of the achievements thus far, we think that the LQ-DAT can significantly contribute to the control of VL in Sudan provided the finance is available to import the badly needed raw materials. Locally produced, ready-for-use LQ-DATs could then be dispatched to peripheral hospitals on a regular basis. Our ambition is to upscale production to a level sufficient for diagnosing 750–1,000 individuals per single test batch. This will enable us to provide services for implementing routine LQ-DAT application in rural health settings and for conducting periodic VL surveillance in all three major endemic areas in Sudan.

MATERIALS AND METHODS

LQ-DAT antigen. This study used a 3.4-year-old LQ-DAT batch from our antigen bank at the Biomedical Research Laboratory, Ahfad University for Women, Sudan. The batch had been continuously stored at 4°C. Processing of the antigen in this LQ-DAT batch, as for all others produced since 1999, was according to the improved protocol described by el Harith and others.[3,6] A *Leishmania donovani* strain isolated by bone marrow aspiration from a VL patient residing in the well-known VL-endemic area of Gedariff, eastern Sudan, was kindly provided by M. Mukhtar from the Institute of Endemic Diseases, University of Khartoum, Khartoum, Sudan. The strain was continuously maintained by subculture at 7- to 8-day intervals in liver infusion tryptose (LIT)–hemin medium.[7] Mass culture of the same strain that was raised in LIT-medium without the addition of hemin, but supplemented with fetal calf serum (10%), penicillin (100 IU), and streptomycin (100 μg/mL) was harvested 7 days later. After treatment with β-mercaptoethanol and fixation in 2% (w/v) formaldehyde–Locke's solution, the antigen was stained with Coomassie Brilliant Blue (Sigma Aldrich Co Ltd., Ayshire, United Kingdom) and finally resuspended in 1.2% (w/v) formaldehyde–citrate saline solution. After undergoing standard quality control procedures, the ready-for-use antigen was continuously stored, except for occasions of electric failure, at 4°C.

Execution of the LQ-DAT was as described for the improved version of the DAT.[6] Reference positive and negative sera were included at each test execution. Sera were tested in 2-fold serial dilutions ranging from 1:100 to 1:51,200. Serum samples showing titers of 1:3,200 or higher were considered to be VL positives.

Imported FD-DATs. ITMA-DAT kits (lot 11D1B1) were purchased from the Institute of Tropical Medicine, Antwerp (ITMA, Belgium). The freeze-dried antigen based on *L. donovani* strain MHOM/SD/68/1-S was reconstituted by adding 2.5 mL buffer to each antigen vial and the test was executed on

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96 V-shape microtiter plates (Greiner Bio-One, Frickenhausen, Germany) according to the manufacturer's instructions.[8] Sera were tested at 2-fold serial dilutions, ranging from 1: 100 to 1:51,200. The results were read after overnight incubation at room temperature. The DAT titer shown is the highest dilution at which agglutination was still visible. Samples with titers of 1:3,200 or higher were considered to be positive.

rK39 rapid test. Rapid rK39 strip tests (IT LEISH, ref. 710124) were purchased from Bio-Rad Laboratories (Marnes-la-Coquette, France) and stored at 4–8°C, as recommended. IT LEISH is a dipstick rapid test for the detection of anti-*Leishmania*–specific antibodies in human blood. The test device was precoated with *Leishmania chagasi* recombinant antigen. During testing, anti-VL–specific antibodies captured by a conjugate (protein A–colloidal gold) react with the coated rK39 antigen on the membrane. The reaction is indicated by the development of a specific color. The test was executed and interpreted according to the manufacturer's instructions using 10 μL serum samples. The test was considered positive when purple control and test bands appeared. A single band in the control area indicated a negative result.

Test sera. Aliquots of 138 serum samples from our serum bank were collected from the rural hospital of Doka, eastern Sudan.[5] The necessary permission and ethical clearance for the study were granted by the Ethical Review Committee of the Federal Ministry of Health in Khartoum, Sudan.[5] The nature and objectives of the study were explained through organizing orientation meetings with the local health authorities in Gedariff state. All patients and controls or their guardians had given consent for participation in the study. The samples belonged to the following subjects: 1) A total of 96 samples were from patients with VL and amastigote-positive lymph node aspirates. All of these patients resided in or near the Doka locality of Gedariff, a well-known endemic area for VL in eastern Sudan; 2) A total of 23 samples were from patients with the non-VL clinical conditions of malaria ($N = 7$), tuberculosis ($N = 10$), and leukemia ($N = 6$); 3) A total of 19 samples were from apparently healthy blood donors, nine from the VL-endemic locality of Doka (eastern Sudan) and 10 from the VL-free state of Khartoum.

Data analysis. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the LQ-DAT, FD-DAT, and rK39 were the estimated percentages of the true (parasitologically confirmed) positive or negative tests with exact binomial 95% confidence limits. To estimate the sensitivity, specificity, PPV, and NPV, the standard formulas were used as follows: 1) sensitivity = true positives/ (true positives + false negatives) × 100%; 2) specificity = true negatives/(true negatives + false positives) × 100%; 3) PPV = true positives/(true positives + false positives) × 100%; and 4) NPV = true negatives/true negatives + false negatives) × 100%.[9] For assessing the significance of differences in sensitivity and NPV between the LQ-DAT, FD-DAT, and rK39, the χ^2 test was applied. The statistical analysis software Statistica 7.1 (Stat Soft Inc., Tulsa, OK,) was used to calculate confidence intervals.

RESULTS

Our choice of LIT-medium to maintain and eventually mass culture the *Leishmania* antigen proved to be appropriate. With the exception of fetal calf serum, all other ingredients constituting LIT remained stable at widely fluctuating storage temperatures of 25–45°C for periods exceeding 12 months. Being autoclavable, LIT sterilization did not require the use of special microbial filters.

Substitution of the standard *L. donovani* strain MHOM/SD/68/1-S, routinely used in European laboratories, with the endemic autochthonous strain used in this study, delivered a reasonable parasite yield of approximately 2.5×10^7 promastigotes per mL, of which the majority (85%) consisted of the desired healthy spindle forms.[8,10] The promastigote population obtained through this parasite–medium combination showed no significant deformations or lysis during treatment with β-mercaptoethanol or repeated washing in Locke's solution. The avidity of the promastigotes to the Coomassie Brilliant Blue stain was satisfactory, as shown by the large proportion (> 90%) of parasites that picked up the stain. Microscopically, the formaldehyde–citrate saline preserved promastigote antigen showed the desired uniform dispersion with an insignificant presence of parasite clusters. The finished LQ-DAT antigen also demonstrated the required titer reading reproducibility in the quality control procedures that were consistently followed for validating all the batches produced.

Except for one serum sample that also tested negative in FD-DAT and rK39, the remaining 95 samples from patients with VL were correctly identified by LQ-DAT as VL positives (Table 1). Among the four samples that were incorrectly identified as VL negative by FD-DAT, two tested positive on both the LQ-DAT and rK39. Of 20 VL sera that were incorrectly identified as VL negative by the rK39, 18 scored positive readings on the FD-DAT and LQ-DAT. Higher sensitivities as compared with the rK39 were therefore estimated for LQ-DAT and FD-DAT (Table 2). Apart from a single VL case that was missed both by the LQ-DAT and FD-DAT, false-negative titers were recorded for three other samples with the FD-DAT (Figure 1). Overall, 80 of 96 VL samples (83.3%) tested strongly positive ($\geq$ 1:51,200) with the FD-DAT, compared with 86 samples (89.6%) with the LQ-DAT (Table 1). Despite comparable sensitivities ($P = 0.1740$), the LQ-DAT showed significant higher NPV than the FD-DAT ($P = 0.0485$); in comparison with rK39, however, the LQ-DAT showed both significant higher sensitivity and NPV (< 0.001).

Both the LQ-DAT and FD-DAT recorded unequivocally negative titers ($\leq$ 1:200) for 40 of the 42 non-VL sera included

TABLE 1
Performance of a locally produced liquid direct agglutination test (LQ-DAT), a commercial freeze-dried version (FD-DAT), and the rapid rK39 strip test in sera from 96 subjects with confirmed visceral leishmaniasis (VL)

DAT version and titer range*	DAT result	Number of sera	rK39 results	
			Positive	Negative
LQ-DAT				
$\leq$ 1:1,600	Negative	1	0	1
1:3,200–1:6,400	Positive	2	2	0
1:12,800–1:25,600	Positive	7	5	2
$\geq$ 1:51,200	Positive	86	69	17
FD-DAT				
$\leq$ 1:1,600	Negative	4	2	2
1:3,200–1:6,400	Positive	6	6	0
1:12,800–1:25,600	Positive	6	4	2
$\geq$ 1:51,200	Positive	80	64	16

*The cut-off titer for VL in the LQ-DAT and FD-DAT was 1:3,200.

TABLE 2
Diagnostic efficiency of a locally produced liquid direct agglutination test (LQ-DAT), a commercial freeze-dried version (FD-DAT), and the rapid rK39 strip test in sera from 96 subjects with confirmed visceral leishmaniasis (VL) and from 42 subjects with non-VL conditions

Diagnostic test	Sensitivity, n (%) (exact 95% CI)	Specificity, n (%) (exact 95% CI)	Positive predictive value (%)	Negative predictive value (%)
LQ-DAT	95 (99.0) (96.9–101.0)	42 (100.0) (−)	100.0	97.7
FD-DAT	92 (95.8) (91.8–100)	42 (100.0) (−)	100.0	91.3
rK39 strip test	76 (79.2) (70.9–87.4)	41 (97.6) (92.8–102.4)	98.7	67.2

CI = confidence interval.

(Figure 1). Both tests also recorded an identically high negative titer (1:800) in one of the malaria samples. Although both the LQ-DAT and FD-DAT reacted negatively for a second sample, a positive outcome was recorded for the rK39. All three tests, nonetheless, demonstrated excellent levels of specificity in the non-VL population (Table 2)

At the time of writing, the remaining aliquots of the same LQ-DAT batch used in this study maintained stability at 4°C.

DISCUSSION

Despite various shortcomings, locally produced generic brands of drugs, vaccines, and diagnostic tests have significantly contributed to the well-being of millions of people in developing countries. One example of this is a generic version of pentavalent stibogluconate that was developed and introduced to control VL in Asia and Africa. However, access to reliable (branded) diagnostic tests for VL, such as the FD-DAT and rK39, has remained problematic. Notwithstanding issues of standardization and thus the suggestion of centralizing production, we believe that building or strengthening the capacity to produce such important tools locally is the only way to achieve sustain-

able control of VL in affected areas. With its advantage of being technically less demanding than either the FD-DAT or the rK39, the LQ-DAT was adopted by our laboratory as the test of choice. Because of this merit of high reproducibility, processing of LQ-DAT was also successfully achieved with some modifications at central laboratory level in several other VL-endemic areas.[11–14]

Compared with some other Sudanese and African central laboratories, ours can be classified as moderately advanced, with basic facilities for culturing and processing LQ-DAT antigen. More than 90 valid antigen batches have been produced and used during the past 14 years. These have been used for the diagnosis of VL in individuals referred to our laboratory, and for VL research at both the laboratory and field levels.[5] All of the valid LQ-DAT antigen batches produced to date, including the 3.4-year-old batch used in this study, have demonstrated acceptable stability for periods of 12 months at 4°C. The introduction of an additional step of preservation in 50% glycerol has further improved stability of the LQ-DAT under the harsh field conditions encountered in eastern Sudan.[4,5]

Because of a lack of facilities for cryopreservation, and thus, an inability to continuously use the standard 1-S strain, our

FIGURE 1. Titer readings obtained with a locally produced liquid direct agglutination test (LQ-DAT) and a commercial freeze-dried version (FD-DAT) in 96 sera from individuals with confirmed visceral leishmaniasis (VL) and of 42 individuals with conditions other than VL. Titers are expressed as the number of 2-fold serial dilutions from 1:100 (dilution 1) up to 1:51,200 (dilution 10). DAT titers obtained with the LQ-DAT and FD-DAT antigens (Ags) overlapped in 84 VL and 39 non-VL sera.

efforts were directed toward recovering the parasite as part of the diagnostic procedures for patients presented at our laboratory or any of the collaborating laboratories. We observed relatively higher sensitivity for VL detection with the LQ-DAT as compared with the FD-DAT, probably because of the use of the endemic autochthonous *L. donovani* (eastern Sudan), and not the standard 1-S strain (Upper Nile, southern Sudan), for antigen processing. In agreement with this favorable observation are the previously reported results for antigens processed from indigenous *L. donovani* or *Leishmania infantum* isolates and tested against VL sera from corresponding endemic areas in Bangladesh, Sudan, or Morocco.[6] In contrast to the suggestion of using the standard 1-S *L. donovani* strain, the higher sensitivity observed here with a corresponding endemic strain is considered an advantage for the LQ-DAT over the FD-DAT. On the basis of the results obtained with this autochthonous *L. donovani* isolate, we suspect that the incorporation of the endemic isolates of *L. infantum*, *L. chagasi*, or perhaps even variants of *L. donovani* (not the standard [1-S]) as antigen in LQ-DAT, might improve the sensitivity for VL detection in the respective endemic areas.

It is relevant to mention that despite excellent diagnostic efficiency demonstrated for the LQ-DAT and FD-DAT in east Africa, lower sensitivities as compared with the rK39 (based on *L. chagasi*) have been reported for the same two tests in the Indian subcontinent.[1,15] Whether this lower sensitivity is associated with the absence of a genetic match between the causative subspecies of *L. donovani* in the two regions requires further research.[16] The higher degree of heterogeneity between the local endemic *L. donovani* subspecies in east Africa in comparison to India has been argued as a reason for the variation in rK39 performance in the two regions.[17] Further research is required to confirm whether the low sensitivity found with the rK39 in east Africa as compared with India has to do with the reported significantly lower antileishmanial IgG responses in the east African endemic population.[18]

In contrast with the results obtained here with the rK39, a recombinant polyprotein (rK28) antigen of *L. donovani* has been reported to be more sensitive for VL detection in Sudanese patients with negative or low anti-rK39 antibody readings.[19] To further enhance sensitivity in weakly reacting Sudanese VL patients, a recently developed recombinant protein (rKLO8) derived from the corresponding endemic *L. donovani* (eastern Sudan) strain has been used with success in an enzyme-linked immunosorbent assay.[9] These findings and our results with an endemic isolate, indicate that regardless of the candidate serodiagnostic test to be used, preliminary work has to be done, on the basis of homology, to determine whether a certain *L. donovani* strain does constitute the appropriate "variant" or "serotype" for the area under investigation.

Assuming that a significant proportion of individuals with a low reaction to the LQ-DAT, FD-DAT, or rK39 are in the early or subclinical phases of VL, incorporating an antigen from the right "variant" or "serotype" of the *L. donovani* subspecies might therefore enhance the detection sensitivity in these patients. Most probably because of the incorporation of the right variant of *L. donovani* in this study, a remarkable increase in the NPV of LQ-DAT was attained implying that the probability of excluding VL is significantly higher

with the LQ-DAT than with either the FD-DAT or rK39 (Table 2). However, regardless of the difference in the origin of *L. donovani* subspecies or the test format used here the probability of accurately predicting VL occurrence with the LQ-DAT, FD-DAT, or rK39 remained almost the same.

Because of the well-known high coprevalence of VL and malaria, VL-exclusive detection is considered to be an important characteristic when classifying a diagnostic test as being suitable for application in Sudan. Except for a false-positive reading for the rK39 in one of the malaria patients and a high negative titer (1:800) for both the LQ-DAT and FD-DAT in another patient, no other cross-reactions were observed against any of the four non-VL conditions included. However, given the high possibility of coinfection with VL in the assumed "VL-free" malaria patient group, it is difficult to determine the actual level of specificity for each of the three tests on the basis of data presented here. We can however conclude that not only specificity versus malaria, but also sensitivity in exclusively detecting early VL remain as the most challenging areas for these and future diagnostic tests.

The affordability of expenses related to VL diagnosis in financially less-privileged countries, such as Sudan, should be considered an important component of the disease-eradication strategy. With an average income of $70 a month, paying an approximately $30 to undergo FD-DAT testing per single patient is, by any measure, devastating for a family's budget. However, given a cost of $0.50 per patient for LQ-DAT, test availability throughout the year, and diagnostic reliability comparable with FD-DAT, individuals with suspected VL will seek a confirmed diagnosis and subsequent treatment. Should this be adequately synchronized with the ongoing free-of-charge treatment policy, much improved VL case management might be attained. Introducing the ready-for-use LQ-DAT at provincial or municipality levels is expected to contribute to a noticeable reduction in VL incidence in Sudan. Although local LQ-DAT production seems to be the right approach for the control of VL in Sudan, being economical and highly reliable for VL diagnosis, it is important to mention that this can only be achieved through the establishment of the required infrastructure, including accessibility to raw materials and training of laboratory personnel. It should also be noticed, that similar to all other current procedures, including the FD-DAT and rK39, the LQ-DAT is unable to differentiate between past and active VL infections.[1,10,15]

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