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REVIEW ARTICLE

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Are We Now Well Prepared for Another Major Visceral Leishmaniasis Epidemic in Sudan?

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To minimize the chance for future visceral leishmaniasis (VL) epidemics such as the 1988–1991 epidemic in Sudan, several VL detection tools have been introduced. There are many VL diagnostics with excellent sensitivities, specificities, and ease of use reported. However, additional test characteristics should be considered for use in the detection of future VL epidemics. The potential for local production or uninterrupted availability, low production and application costs, and stability at ≥45°C are of the utmost importance. Of the antibody-, antigen-, or DNA-based methods introduced, only a liquid direct agglutination test (LQ-DAT) remains in routine use. The LQ-DAT test may be the ideal diagnostic for detection of VL epidemics due to its low cost ($0.50/patient), stability under frequent and long-duration electric failures, and high level of reproducibility. The improved reliability for VL detection achieved locally through incorporating autochthonous *L. donovani* strains in antigen processing and precluding toxicants in test execution provides optimal sensitivity and safety for routine and mass application.

Keywords. antibody; antigen; diagnosis; epidemic; visceral leishmaniasis.

Aside from being 1 of the 6 most important countries endemic for visceral leishmaniasis (VL), Sudan has also previously been impacted by major epidemics (1936–1938 and 1956–1960) that took place in the former Upper-Nile and Blue-Nile states, respectively [1, 2]. The 2 important lessons learned from the third and the most devastating epidemic that took place in Southern Sudan (1989–1991) were the absence of reliable tools to accurately identify cases of VL and an inability to conduct mass screening to detect the infected individuals using such tools due to budget limitations [3, 4]. A conservative estimate of 100 000 deaths among 280 000 inhabitants exposed to VL was reported [5]. Such devastating effects could have significantly been attenuated had a functional surveillance program or alert system for monitoring VL transmission existed at the time. Fortunately, as of 2005, a Global Outbreak Alert and Response Network adjunct to the International Health Regulations (IHR) framework of the World Health Organization (WHO) was installed in order to provide timely warning of and response to disease outbreaks of such magnitude.

Thanks to foreign nongovernmental organizations' (NGOs') efforts, the etiology of the disease was finally identified as VL using the then newly developed direct agglutination test (DAT) [6, 7].The mass migration of the affected Nuer tribe on foot along the 900-km route to Khartoum seeking treatment led to the emergence of new VL foci in the states of Kordofan, Darfur, and White-Nile. Even though no disease mapping has recently been carried out, 16 years of uninterrupted monitoring through receiving suspects of VL referred from across Sudan, established by the Laboratory for Biomedical Research in Ahfad University for Women, strongly supports this observation (unpublished data).

In this report, we intend to address the effectiveness of several tools introduced for VL detection in Sudan to closely monitor VL transmission through both routine and mass screening in order to minimize the chance for upsurge, leading to an epidemic similar to that of the 1989–1991 epidemic.

VL FEATURES IN ENDEMIC AND EPIDEMIC SITUATIONS

The suspicion for VL in Sudan as currently based on clinical grounds proved extremely difficult if not misleading due to co-endemicity with other infections or disorders simulating VL. Regardless of age or sex, fever, anemia, splenomegaly, weight loss, and lymphadenopathy were reported both in endemic and epidemic situations of VL [8]. However, the presentation

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of these symptoms does not rule out occurrence of conditions other than VL, including malaria, typhoid, brucellosis, or tuberculosis. Even when taken in combination with case history and abnormalities in the hematologic or proteinogram outcomes, it remains difficult to confirm a VL diagnosis. The definitive diagnosis of VL may also become complicated should the suspect suffer from concurrent infections, as was the case in the 1989–1991 epidemic [4]. Possibly due to similar concurrent infections as those reported, combined with the absence of discriminative detection tools, the definitive identification of VL as the killing disease has been significantly delayed.

VL CONFIRMATION IN SUDAN

Spleen aspiration has been practiced in Sudan since 1911, demonstrating sensitivity of ≥90.0% [9]. Similar sensitivity levels were also reported in 3 other studies that followed [8, 10, 11]. Despite this relative high sensitivity, spleen aspiration is generally discouraged in Sudan due to the risk of internal bleeding. As it is less risky, albeit far less sensitive (≤70.0%), bone marrow aspiration is still performed in several Sudanese hospitals. The procedure is rather painful, and under current poor hygienic conditions in hospitals, it might also lead to septicemia. Inguinal lymph node aspiration is acknowledged as being the most tolerable and least invasive. It can easily be performed by a medical assistant at a rural hospital. Depending on the procedures followed for aspirate specimen processing, the VL phase, and possibly the geographical location, a sensitivity of 80%–100% has been achieved [11]. This method is currently performed on a routine basis at provincial and central hospitals. It is generally agreed that through enough training in proper specimen processing, use of the right fixative and stain, combined with developing the skill to correctly recognize *L. donovani* amastigote, sensitivity at the aforementioned range can consistently be achieved.

KNOW-HOW INTRODUCED FOR VL DETECTION IN SUDAN

During and after containing the 1989–1991 VL epidemic, teams from national and international expert organizations joined efforts to determine the factors that triggered the outbreak as the area affected had not previously been reported as VL endemic. Appreciable efforts were also made to explore new alternative methods and protocols to achieve better results in the diagnosis and treatment of VL. Of greatest significance were those taken to develop new antibody-, antigen-, or molecular-based techniques to provide timely warning of and contain a future VL upsurge or epidemic (Table 1).

MOLECULAR TOOLS

In spite of the suboptimal laboratory infrastructure and difficulty in accessing basic reagents, initiatives were nevertheless undertaken to transfer the know-how of applying molecular tools such as polymerase chain reaction (PCR), *Leishmania* OligoC-TesT, and NASBA-Oligochromatography (OC) to more sensitively detect VL [12, 13]. Unfortunately, up to now, nearly 8–14 years later, this objective has remained unachievable. To bypass the requirements for applying these procedures, a loop-mediated isothermal amplification (LAMP) method was developed and later evaluated in Sudan [14]. All reagents involved in the ready-for-use test kit are in powder form, ensuring stability at the ambient temperatures in Sudan. The procedure proved applicable on whole blood and buffy coat specimens, demonstrating sensitivity and specificity ranges of 97.6%–99.01% and 99.1%–100%, respectively. Due to ease in execution and stability at suboptimal conditions, the LAMP method also showed good diagnostic reliability at rural health settings. The needs, however, for equipment and special reagents to separate the buffy coat and extract the nucleic acid, respectively, are obstacles in routine application given the current financial situation in Sudan. More evaluation is also needed to independently confirm the level of the diagnostic efficiency reported and, more importantly, the feasibility and accessibility of the test kit.

Differently than the experience gained in Sudan, application of quantitative PCR (qPCR) in India has significantly helped in monitoring transmission of VL in 1 of the endemic areas and helped in determining the progress of the disease in the individuals who had acquired the infection with *L. donovani* [15].

ANTIGEN DETECTION TOOLS

Latex Agglutination Test (KATEX)

Having proven excellent sensitivity and specificity of 95.2% and 100%, respectively, in VL-endemic regions other than Sudan, the KATEX targeting *L. donovani* antigen(s) was introduced [16]. Apart from high diagnostic reliability, KATEX also demonstrated a number of merits that made the test optimal for routine application in Sudan. In contrast to antigen-based testing, the antibody-based KATEX is applicable on urine specimens, requires a short incubation period (~20 minutes), and the test result is easy to read. The test proved to be highly sensitive, as demonstrated in its efficiency for detecting VL in patients who also had HIV-positive test results. A prognostic merit due to its potential of signaling a matching negative outcome in successfully treated VL patients was additionally reported for KATEX. Because of difficulties in accessing the complete ready-for-use kit or the reactive antibody component, KATEX routine application could no longer be sustained in Sudan.

ANTIBODY DETECTION TOOLS

rK39 Antigen-Based Enzyme-Linked Immunosorbent Assay

Though it is much slower than the KATEX, an enzyme-linked immunosorbent assay (ELISA) version based on the well-known K39 *L. chagasi* recombinant antigen was evaluated in both endemic and epidemic populations, showing

Table 1. Performance of Antibody-, Antigen-, or DNA-Based Methods Introduced for the Detection of Visceral Leishmaniasis in Sudan During 1989–2018

Authors	VL Detection Method	Clinical Specimen	Sensitivity, %	Specificity, %
Mukhtar et al., 2018 [14]	LAMP	Blood	97.65	99.01
	Liquid direct agglutination	Serum	88.1	78.0
Osman et al., 2016 [24]	Liquid direct agglutination	Serum	95.0	100
	Freeze-dried direct agglutination	Serum	92.0	100
	rk39 rapid test	Serum	76.0	97.6
Mukhtar et al., 2015 [21]	rk28 rapid test	Serum	98.8	100
	Liquid direct agglutination	Serum	83.5	92.9
Abass et al., 2013 [20]	rkLO8 ELISA	Serum	98.1	96.1
	rk39 ELISA	Serum	96.2	94.8
	Liquid direct agglutination	Serum	94.3	100
	rk39 rapid test	Serum	81.1	98.7
Van Rij et al., 2013 [28]	rk39 rapid test	Urine	72.1	76.9
	Freeze-dried direct agglutination	Urine	62.8	69.2
Abass et al., 2011 [18]	β-ME ELISA[a]	Serum	92.4	94.7
	Freeze-dried direct agglutination	Serum	93.3	90.4
	rK39 rapid test	Serum	83.3	93.0
Saad et al., 2010 [13]	PCR-oligo c-test	Blood	96.2–96.9	90.0
	NASBA-oligo c-test	LN, BM aspirates	95.3–96.8	100
Mansour et al., 2009 [29]	Liquid direct agglutination	Serum	96.0	99.3
	rk39 rapid test	Serum	96.0	98.7
Andresen et al., 2009 [30]	PCR	Blood	92.5	nd
Boelaert et al., 2008 [31]	Freeze-dried direct agglutination	Serum	85.7	98.2
	rK39 rapid test	Serum	77.9	91.8
	KATEX	Urine	72.9	98.3
Mansour et al., 2007 [17]	Liquid direct agglutination	Serum	92.3	100.
	β-ME ELISA[a]	Serum	93	95.6
Ritmeijer et al., 2006 [32]	rk39 rapid test	Blood	89.6	99.2
	Liquid direct agglutination	Serum	98.0	nd
Abass et al., 2006 [17]	β-ME ELISA[a]	Serum	97.5	100
	Liquid direct agglutination	Serum	100	98.8
Abdallah et al., 2004 [33]	Freeze-dried direct agglutination	Blood	96.8	96.2
	Liquid direct agglutination	Serum	91.0	96.6
el Harith et al., 2003 [25]	Liquid direct agglutination	Serum	100	100
el Safi et al., 2003 [16]	Latex agglutination	Urine	95.2	100
Zijlstra et al., 2001 [14]	Liquid direct agglutination	Serum	94	72
	rk39 ELISA	Serum	93.0	97.0
Zijlstra et al., 2001[19]	rk39-strip test	Serum	67.0	nd
	Liquid direct agglutination	Serum	91.0	nd
Osman et al., 1997 [12]	PCR	Blood & LN, BM aspirates	100	100
Moody and el-Safi, 1996 [34]	Latex agglutination	Urine	88.0	96.0
de Beer et al., 1991 [4]	Liquid direct agglutination	Serum	100	100
Abdel-Hameed et al., 1989 [35]	Liquid direct agglutination	Serum	100	100

Abbreviations: BM, bone marrow aspirates; ELISA, enzyme-linked immunosorbent assay; LAMP, loop-mediated isothermal amplification method; LN, lymph node aspirates; nd, not done; PCR, polymerase chain reaction.

[a]ELISA version based on intact *L. donovani* promastigotes that were previously treated with β-ME and fixed in formaldehyde.

sensitivity exceeded that of DAT and conventional parasitological methods [14]. Based on processing procedures essentially similar to LQ-DAT by employing intact *L. donovani* promastigotes that were previously treated with β-mercaptoethanol and fixed in formaldehyde, an IgG ELISA version (β-ME ELISA) was developed and evaluated in Sudan. Excellent sensitivity of 97.5% and specificity of 100% were reported [17]. More advantageous than with the rK39-based test, the antigen in β-ME ELISA proved to be stable at 4°C for at least 1 year. The ease of reading the test outcome visually encouraged the routine application of β-ME ELISA to confirm LQ-DAT outcome [18]. Unfortunately, as experienced with other imported procedures, the application of β-ME ELISA was also interrupted by budget limitations to acquiring essential reagents such as branded antihuman immunoglobulin conjugates.

rK39 Rapid Strip Test

Despite excellent performance in the ELISA format, incorporation of the K39 recombinant antigen in a strip device did not deliver the desired sensitivity in Sudan [19]. Unlike in Sudan, however, the same rK39 strip did perform excellently in India, so much so that it has been included in the national program for VL control in the country. Among others, the heterogeneity among members of the *L. donovani* complex prevalent and the relatively lower values of IgG in Sudanese sera in comparison with Indian sera were reported as reasons for this lower sensitivity [20].

Although equally as fast for VL detection as KATEX, a significant drawback was encountered during evaluation of the rK39 rapid test in Sudan. Its shelf-life time at ambient temperatures proved to be rather short (±30 days). However, as with KATEX, accessing the branded test kit was difficult due to complexity of the logistics involved in purchasing.

rK28 Strip Rapid Test

As substitute to the relatively less sensitive rk39 rapid test, an equivalent (rK28) in which the *L. donovani* epitope density is increased by incorporating 2 *L. infantum* genes (K9 and K26) was developed. The multi-epitope strip test was introduced, revealing sensitivity of 97.6% and specificity of 94.5%, superior to the freeze-dried DAT (FD-DAT) and rK39 rapid tests [21]. Further evaluation is underway to confirm the initial merits reported for the rK28.

rKLO8-Based ELISA

Differently than with the multi-epitope antigen used in the rK28 strip test, a single-epitope derived from a Sudanese *L. donovani* isolate (rKLO8) was also evaluated in an IgG ELISA against VL and non-VL sera from the corresponding endemic area in Sudan. Both higher sensitivity (98.1%) and reaction intensity were reported for the single-epitope KLO8 antigen when further compared with the rK39 (96.2%) using the same sera [20]. It may therefore be of interest to assess the efficiency of a forthcoming rapid strip test wherein the KLO8 recombinant protein will be employed as the antigen instead of the K39.

Direct Agglutination Test

The DAT was first introduced in Sudan by the NGO organization Medecins Sans Frontiers to contain the VL epidemic of 1989–1991 [7]; thereafter, it was used in several projects sponsored by the Tropical Disease Research Program/WHO and European Union to evaluate its efficiency as an epidemiological tool in the major endemic area of Gedaref in Eastern Sudan [8, 19]. Having proven high diagnostic reliability and continuous availability either in liquid or freeze-dried form, it is fair to say that the introduction of the DAT has revolutionized VL diagnosis in Sudan. Being furthermore the 1 DAT version that has been extensively evaluated, the LQ-DAT has demonstrated sensitivity superior to microscopy, paving the way for timely administration of antileishmanials to genuine VL patients who otherwise would have died due to delayed intervention. Except for a study where a specificity of 72% was reported, taking the negative outcome of microscopy as a gold standard for VL absence, all others conducted independently during the past 28 years in Sudan have revealed specificities in the 78.0%–100% range (Table 3) [22]. Aside from being simple to execute, LQ-DAT's additional merits include ease of accessibility, stability of the antigen for periods of 12–72 months at 4°C, and low application cost of $0.50 per patient; these factors have justified its uninterrupted use as the routine VL diagnostic in Sudan until now.

To reduce interobserver and batch-to-batch variability and increase antigen stability at ambient temperatures in tropical countries, a commercialized freeze-dried version (FD-DAT) was developed and evaluated in Sudan [23]. Despite efficiency for VL detection similar to its LQ-DAT equivalent, application of the FD-DAT on a routine basis is seriously hampered by the extreme high cost of application ($32.0, vs a $70 average income per month). Also, because of the strict temperature (5°C–35°C) indicated for storage of the test kit vs the ambient of 18°C–46°C in Sudan, FD-DAT application is currently limited to only 1 VL treatment center run by Medecins Sans Frontiers in Eastern Sudan.

Because of its high diagnostic reliability and minimally invasive nature using finger prick or venous puncture for blood collection in comparison with organ aspiration, no resistance or implication either from the side of the VL suspects or the Sudanese health authority have been encountered in the application of the test.

As for all other serologic and molecular VL diagnostics that have been introduced, both LQ-DA and FD-DAT remain positive for some months or even years after "successful" treatment, raising the question of whether sterile VL cure is achievable through the administration of the generic antileishmanial sodium stibogluconate.

LOCAL PRODUCTION AND OPTIMIZATION OF LQ-DAT

As a measure to evade future VL disaster similar to 1988–1991, efforts were made to achieve production of LQ-DAT locally.

During the past 18 years, the Laboratory for Biomedical Research, Ahfad Univeristy for Women in Omdurman, has succeeded in the production of 102 valid LQ-DAT batches, employing for antigen production an autochthonous endemic *L. donovani* strain from the known VL-endemic area of Gedaref, Eastern Sudan, instead of the standard (MHOM/SD/68/1-S) from Southern Sudan [24]. Besides screening of cases referred from across the country and carrying out field studies, quantities of the ready-for-use LQ-DAT were offered to several health institutions that are also involved in the diagnosis and research of VL. All LQ-DAT batches

thus far produced have demonstrated stability for periods ranging from 1 to 6 years at 4°C. To ensure diagnostic reliability matching the European standard as well, a sample of the locally produced LQ-DAT was sent to the Hospital of Tropical Diseases in London (LHTD) for independent evaluation. The outcome of that evaluation revealed 100% sensitivity and specificity in 2 groups of VL suspects of 284 and 268 in whom the disease was diagnosed either by microscopy or on the basis of clinical findings and favorable response to specific antileishmanials (results not shown). To further confirm the efficiency of the locally produced LQ-DAT, a comparison was carried out against diagnostic procedures routinely applied for VL at LHTD (Table 2).

Further validation of the in-house-produced LQ-DAT was conducted by comparison with 2 WHO-recommended diagnostics, namely the FD-DAT and rapid rK39 strip tests [24]. Highly comparable with the results found earlier at LHTD, the outcome of that study revealed sensitivities of 99.0% for LQ-DAT as compared with 95.8% and 79.2% for the FD-DAT and rK39 rapid tests, respectively; all 3 tests showed comparably high specificity levels (Table 3). The stability of LQ-DAT in the adverse climate in Eastern Sudan was successfully achieved by further preserving the antigen in a suspension medium containing 50% glycerol [25].

The initial experiments using the endemic autochthonous *L. donovani* strain from Gedaref (Eastern Sudan) for antigen processing demonstrated an appreciable increase in both titer level and reaction intensity against the homologue VL sera when compared with the standard (MHOM/SD/68/1-S) from Southern Sudan [24]. These findings were further confirmed in a second study in which 2 antigens were processed from autochthonous *L. donovani* strains isolated either in Gedaref (Eastern Sudan) or Umrimta (Central Sudan) and tested against corresponding or alternate VL sera [26].

To further facilitate LQ-DAT application in routine and mass screening of VL but simultaneously ensure safety for the user and environment, formaldehyde and β-ME were successfully excluded without compromising the reliability of the test for VL detection (Figure 1) [27]. By further incorporating chlorine and urea, subsequently replacing the 2 aforementioned pollutants, LQ-DAT demonstrated efficiency comparable to the in currently used freeze-dried or liquid versions (Friedman ANOVA test: Fr = 0.3250; P = .8500) [27], thus providing the required safety for routine diagnosis and for mass screening programs.

Table 2. Performance of a Locally Produced Liquid Direct Agglutination Test for Diagnosis of Visceral Leishmaniasis as Assessed by Comparison With Other Diagnostics at the London Hospital for Tropical Diseases

	No. of VL Positives Detected/Total No. of Suspects					
	Latex	Rapid	Final Diagnosis			LQ-DAT
No. of Patients & Areas Visited	Agglutination Test	striprK39 Test	Micro*	Culture	PCR	(Cutoff Titer = 1:3200)
21 suspects: Dubai, Kosovo, Dominican Republic, Mali, Pakistan, India, Yemen, Spain, Cyprus, Portugal, and Belize	6/21	0/21	0/21	0/21	0/21	1:100–1:400 (21)
2 suspects: Tenerife and Mozambique	0/2	0/2	0/2	0/2	0/2	1:200–1:400 (2)
2 suspects: Egypt and Borneo	0/2	0/2	0/2	0/2	0/2	1:200–1:800 (2)
1 suspect[a]: Bolivia	0/1	0/1	0/1	0/1	0/1	1:3200 (1)
2 suspect: Greece and China	1/2	1/2	2/2	2/2	2/2	1:6400–1:12 800 (2)
7 suspects: India, Nepal, Sudan and Ethiopia	6/7	5/7	7/7	7/7	7/7	≥1:102 400 (7)

Abbreviations: LQ-DAT, liquid direct agglutination test; PCR, polymerase chain reaction; VL, visceral leishmaniasis.

[a]Patient with cutaneous leishmaniasis due to *L. viannia*.

Table 3. Diagnostic Efficiency of a Formaldehyde and β-Mercaptoethanol-Free Direct Agglutination Test Version in Comparison With the Liquid or Freeze-Dried Direct Agglutination Tests Currently in Use for Visceral Leishmaniasis Detection in Sudan

Direct Agglutination Test	DAT Titer Readings in Filter Paper–Collected Blood Samples[a]						
DAT version	≤1:400	1:800	1:16 00	1:32 00	1:64 00	1:12 800	≥1:25 600 (cutoff)
Current LQ-DAT (formaldehyde + β-ME, el Harith et al., 1988) [7]	15	8	2	6	1	6	22
Current FD-DAT (β-ME, Meredith et al. 1995) [22]	22	2	2	5	5	3	21
Improved LQ-DAT (formaldehyde and β-ME-free, Mahamoud et al. 2018) [26]	22	2	3	4	3	5	21

Abbreviations: ANOVA, analysis of variance; DAT, direct agglutination test; LQ-DAT, liquid direct agglutination test.

[a]Friedman ANOVA statistical test: Fr = 0.3250; P = .8500.

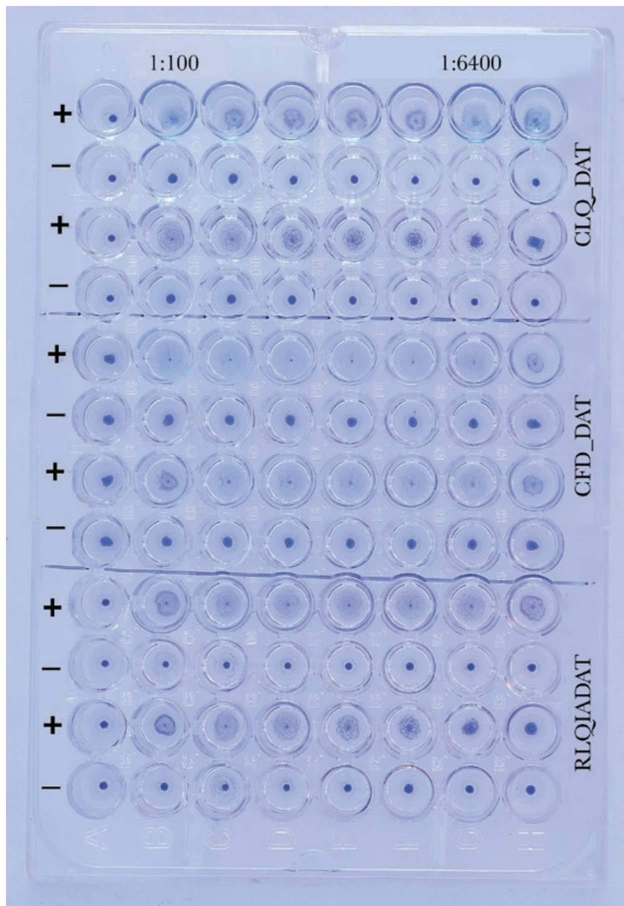

Figure 1. Three direct agglutination test (DAT) versions for the diagnosis of visceral leishmaniasis (VL). Two are currently in use, involving in execution the use of formaldehyde and β-mercaptoethanol (β-ME; C LQ-DAT) or β-ME only (C FD-DAT), and a third was recently developed (formaldehyde and β-ME-free; A LQ-DAT) and applied to 2 VL (+) and 2 non-VL (-) sera. All 4 sera were tested through 2-fold serial dilutions starting at 1:100, up to 1:6400. The test outcomes were similar for all 3 versions; they were read by locating a circumscribed blue spot (end point) in the titration row that resembles the 1 in the control well containing diluent only (extreme left column); the serum dilution that preceded the end point is considered the titer of the serum sample under investigation. Titers ≥ 1:3200 are indicative of VL. All 3 DAT versions showed comparable readings: positive titers of ≥1:6400 in the VL (+) and negative titers of ≤1:100 in the non-VL (-).

CONCLUSIONS

A prerequisite to proper VL control in the less privileged yet important VL-endemic areas such as Sudan is the uninterrupted accessibility to reliable detection tools. Of all the antibody-, antigen-, or parasite DNA–based diagnostics introduced in Sudan during the past 28 years, none has fulfilled all criteria required for routine or mass application. Aside from the drawback of a relatively longer incubation period (±2 hours), the locally produced LQ-DAT, because of its excellent diagnostic reliability, continuous availability, low cost of application, and longer shelf-life at suboptimal conditions, seems most appropriate for routine and mass application.

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