Diagnostic Accuracy of Two rK39 Antigen-Based Dipsticks and the Formol Gel Test for Rapid Diagnosis of Visceral Leishmaniasis in Northeastern Uganda

François Chappuis,[1,2]* Yolanda Mueller,[1] Alexandre Nguimfack,[1] John Bosco Rwakimari,[3] Sophie Couffignal,[1] Marleen Boelaert,[4] Philippe Cavailler,[5] Louis Loutan,[2] and Patrice Piola[5]

Médecins Sans Frontières, Swiss Section, Rue de Lausanne 78, 1202 Geneva, Switzerland[1]; Travel and Migration Medicine Unit, Geneva University Hospital, Rue Micheli-du-Crest 24, 1211 Geneva 14, Switzerland[2]; Ministry of Health of Uganda, Kampala, Uganda[3]; Institute of Tropical Medicine, Nationalestraat 155, Antwerp, 2000 Belgium[4]; and Epicentre, 8 Rue Saint-Sabin, 75011 Paris, France[5]

The development of an accurate, practical, and affordable diagnostic test is essential to improve the management of visceral leishmaniasis (VL) in remote health centers. We evaluated the Formol Gel test (FGT) and two rK39 antigen-based dipsticks, the DUAL-IT L/M, and the Kalazar Detect for VL diagnosis in Amudat Hospital in Uganda. The DUAL-IT L/M was also evaluated for the diagnosis of malaria. All patients clinically suspect of VL were prospectively included in the study between October 2003 and March 2004. The gold standard used to define a VL case was a positive spleen aspirate or a direct agglutination test titer of >1:12,800 with an appropriate clinical response to antileishmanial therapy. A total of 131 VL and 112 non-VL patients were included in the analysis. The DUAL IT L/M was found to be more sensitive than the Kalazar Detect: 97% (95% confidence interval [95%CI] = 92 to 99%) versus 82% (95%CI = 74 to 87%). The Kalazar Detect and the DUAL IT L/M were highly specific (99% [95%CI = 95 to 100%] and 97% [95%CI = 92 to 99%], respectively). The FGT lacked both sensitivity (66% [95%CI = 57 to 73%]) and specificity (90% [95%CI = 83 to 94%]). The sensitivity of the DUAL IT L/M for malaria was only 57% (95%CI = 37 to 76%). The two rK39 dipsticks can be used for diagnostic confirmation of VL in this region. The DUAL-IT L/M without its malaria diagnostic component (DiaMed-IT LEISH) will be adopted as first-line test for VL in Uganda.

Visceral leishmaniasis (VL) or kala-azar affects an estimated 500,000 persons yearly, predominantly in the poor rural areas of India, Bangladesh, Nepal, Sudan, and Brazil (10). Most patients present with prolonged fever, weight loss, and splenomegaly. Other tropical diseases such as malaria, disseminated tuberculosis, or enteric fever can share the same clinical presentation. Therefore, laboratory testing is necessary to confirm the diagnosis of VL. Diagnostic tests for VL need to be highly sensitive and specific because of the fatal evolution of the disease without adequate treatment and the serious toxicity of antimonials (18), the most commonly used first-line therapy. Moreover, tests must be cheap and easy-to-perform since VL occurs in poor and remote rural communities with limited access to referral hospitals. The development of diagnostic tests for improved case management of VL has been rated as one of the most needed among the infectious diseases prevalent in the developing world (13).

Direct microscopic examination of spleen aspirates is considered the gold standard for VL diagnosis, but both the aspiration procedure and the reading of slides require a high level of expertise that makes it unsuitable for generalized field use. Lymph node and bone marrow aspirates are alternative procedures but lack sensitivity (26). Serological tests have been developed to replace parasitological diagnosis in the field. The Direct Agglutination Test (DAT), developed in the 1980s (11, 12), has been validated in several areas of endemicity (1–3, 5). Unfortunately, the relative sophistication of the DAT procedure (e.g., need for micropipettes and microtitration plates) restricts its use to referral hospitals or well-supported health centers.

Simpler tests designed for field use exist. The Formol Gel Test (FGT) is a cheap, easy to perform but poorly sensitive test based on the detection of polyclonal immunoglobulins. The FGT remains the sole test available for VL diagnosis in many peripheral health centers in East Africa or in the Indian subcontinent (8). Recently, serological testing based on the detection of antibodies against a recombinant antigen derived from a 39-amino-acid repeat in *Leishmania chagasi* (rK39) was developed into a dipstick format. Validation studies have shown variable results depending on the location of the study site, the brand of the dipstick, and the study methodology (5–7, 9, 17, 19, 22, 27). Various brands of rK39 dipsticks have been evaluated in Sudan (16, 22, 27), but no validation studies have been published from other African countries. Since malaria is always present in the differential diagnosis of VL in Africa, a dipstick detecting both antibodies to rK39 antigen (for VL diagnosis) and specific plasmodial LDH (pLDH; for malaria diagnosis) was also recently developed (DUAL-IT L/M dipstick from DiaMed AG, Switzerland).

In Uganda, the area of endemicity of VL is restricted to Pokot County in the northeastern part of the country (24). This

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* Corresponding author. Mailing address: Travel and Migration Medicine Unit, Geneva University Hospital, Rue Micheli-du-Crest 24, 1211 Geneva 14, Switzerland. Phone: 41-22-3729620. Fax: 41-22-3729626. E-mail: francois.chappuis@hcuge.ch.
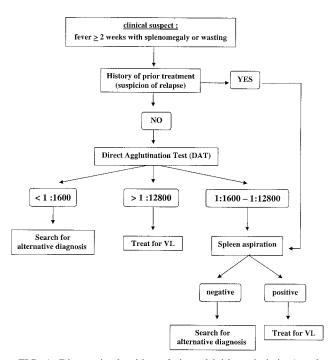
FIG. 1. Diagnostic algorithm of visceral leishmaniasis in Amudat Hospital, Pokot County.

endemic focus continues eastward in the West Pokot District of Kenya. Médecins Sans Frontières (MSF) has supported the clinical management of suspect VL patients in Amudat Hospital (AH) since 1997. The DAT and spleen aspiration were introduced in AH in 2000, and the DAT diagnostic thresholds (negative, borderline, positive) were validated in 2001 (unpublished data). However, cheaper and more practical diagnostic tests are needed to ensure a sustainable access to VL diagnosis in AH after the withdrawal of MSF support. Therefore, we conducted a diagnostic accuracy study in AH, comparing the performance of the FGT and two marketed rK39 dipsticks.

MATERIALS AND METHODS

Study area and population. The study was conducted in AH, a 120-bed hospital located in Pokot County, Nakapiripirit District, in northeastern Uganda. The aim was to enroll at least 100 true cases of VL and 100 true non-VL cases in the study to achieve adequate precision for the estimation of both the sensitivity and the specificity of the tests. The total sample size was fixed at 300 clinical suspects, taking into account an expected proportion of 40% true VL cases among clinical suspects.

Clinical suspect patients were defined as patients with a history of fever of ≥ 14 days with either clinical splenomegaly or wasting syndrome. All clinical suspect patients, adult and pediatric, who presented at the Outpatient Department of AH between October 2003 and March 2004 were eligible for the study. Consent was documented by signature of the written informed consent form by the patient or her or his tutor (for patients younger than 15 years old). All patients were guaranteed access to free care in AH. Patients were advised to come back to AH in case of recurrence of symptoms. The study protocol was approved by the Uganda National Ethics Committee Board in September 2003.

Laboratory investigations. The diagnostic work-up of all patients included in the study followed the diagnostic algorithm routinely in use at AH (Fig. 1). Personal and medical characteristics of the patient were recorded in a case reporting form. In the laboratory, 5 ml of blood was drawn by venous puncture. Two filter papers were impregnated, and the remaining blood was left to sedimate. The plasma was collected to perform the DAT, the FGT, and the Kalazar Detect dipstick (InBios International, Inc., Seattle, Wash.). An additional few drops of blood were obtained by finger pricking to perform the hemoglobin count, the DUAL-IT L/M dipstick (DiaMed AG, Switzerland) and the thick and thin smear for qualitative and quantitative assessment of *Plasmodium falciparum* and other *Plasmodium* species. The dipsticks were stored at room temperature that ranged between 24 to 32°C during the study duration.

Tests procedures. the spleen aspirate was performed by the physician in charge of the patient. The spleen material was spread on a glass slide, stained with Giemsa solution, and examined microscopically by an experienced laboratory technician. The DAT was performed by the same laboratory technician according to the method of Harith et al. (11). The FGT, the Kalazar Detect, and the DUAL IT L/M were performed on the day of plasma collection by another laboratory technician who was blinded to the results of both the DAT and the spleen aspiration. The results of the tests under evaluation were recorded on a separate log book and were not used by physicians for any decision regarding the management of the patient.

The FGT was performed by adding 20 μl of 40% formaldehyde to 200 μl of serum in a glass tube. The gelification reaction was observed after 30 min. The test was considered positive if a clot was seen at the bottom of the tube. The Kalazar Detect and the DUAL-IT L/M were performed according to manufacturers' instructions. The result of the tests was reported as positive or negative. For both tests, even a faint reactive line was considered positive.

Quality control. all thick and thin smears were kept in a separate box, transported to the Malaria Research Laboratory of Mbarara Hospital in Uganda and examined by laboratory technicians experts in malaria diagnosis. Blood-impregnated filter papers were sent for quality control of the DAT to the Royal Institute of Tropical Medicine in Amsterdam, Holland. All slides of spleen aspirates were cross-checked at the Laboratory of Parasitology of the Geneva University Hospital (GUH). Slides with discrepant results between AH and GUH were sent for identification by PCR analysis at the Swiss Tropical Institute in Basel, Switzerland (14).

Case definitions. A case of VL was defined as a patient with a positive spleen aspirate in two laboratories (by microscopic reading at AH and/or GUH or by PCR at the Swiss Tropical Institute) or as a patient with a DAT titer of >1:12,800 with an adequate clinical response to antimonial therapy (resolution of fever, improvement of general condition, and decreased spleen size at the end of treatment). A non-VL case was defined as a patient with a DAT titer of <1:1,600 or as a patient with a borderline DAT titer (1:1,600 to 1:12,800) with a negative spleen puncture confirmed in two laboratories with no diagnosis of VL made during the six following months. A malaria case was defined as a patient with a positive thick and thin smear examination at Mbarara Hospital laboratory, whereas a negative smear examination defined a nonmalaria case.

Data management and statistical analysis. Data from the case reporting form were entered in an Excel data sheet and later cross-checked by the principal investigator. The data were analyzed with SPSS 11.0 for Windows version (SPSS, Inc., Chicago, Ill.). Numerical variables were summarized by mean and standard deviation if normally distributed and, if they were not, by median and quartiles. Categorical variables were compared by using cross-tabulations and chi-square tests, whereas numerical variables (means) were compared to the Student t test, at a critical α-level of 0.05. All P values were two sided. Sensitivity, specificity, positive and negative predictive values, and their exact 95% binomial confidence intervals (95%CI) were calculated for the FGT and the two rK39 dipstick tests from the groups of VL and non-VL patients by using the Confidence Interval Analysis software for Windows.

RESULTS

Diagnostic accuracy of diagnostic tests for VL. A total of 276 clinical suspect patients were enrolled in the study between October 2003 and March 2004. Thirty-three patients were excluded from the analysis for the following reasons: failure to meet the definition of clinical suspect (21 patients), a difference of more than two dilution titers between AH and the Royal Institute of Tropical Medicine in Amsterdam, Holland, laboratories (10 patients), or death before completion of diagnostic investigations (one patient) or antileishmanial treatment (one patient). A total of 243 patients, 131 with VL (54%) and 112 with another diagnosis (46%), were included in the analysis. The demographic and clinical characteristics of the 131 VL and 112 non-VL patients are compared in Table 1. VL was

TABLE 1. Demographic and clinical characteristics of the 131 VL and 112 non-VL patients included in the diagnostic tests validation study in Amudat Hospital, Uganda

Characteristic	No. (%) or mean (SD)[a]		P
	VL patients ($n = 131$)	Non-VL patients ($n = 112$)	
Gender			0.001
Female	35 (27)	53 (47)	
Male	96 (73)	59 (53)	
Age	13.6 (11.1)	16.2 (12.5)	0.09
Country of residence			0.01
Uganda	36 (28)	54 (48)	
Kenya	95 (72)	58 (52)	
Previous treatment for VL	0	6 (6)	0.004
Symptoms (self-reported)			
Abdominal pain or swelling	125 (95)	102 (91)	0.17
Cough	89 (68)	59 (53)	0.015
Wt loss	40 (31)	22 (20)	0.052
Epistaxis	17 (13)	16 (14)	0.77
Headaches	65 (50)	47 (42)	0.23
Anorexia	21 (16)	22 (20)	0.46
Peripheral edema	8 (6)	3 (3)	0.2
Duration of fever (wk)	7.9 (9.4)	12 (17.1)	0.02
Spleen size from costal margin (cm)	13.8 (4.3)	13.2 (5)	0.33
Hemoglobin count (g/dl)	7.8 (1.7)	9.2 (1.7)	<0.001
Smear-proven malaria	1 (1)	20 (18)	<0.001

[a] Values indicate number of patients (%) except as noted.

confirmed by a positive spleen aspirate in 15 (11%) patients or by a DAT of ≥1:25,600 with a good response to antileishmanial treatment in 116 patients (89%). The final diagnosis of the 112 non-VL patients was hyper-reactive malarial splenomegaly in 68 patients (61%), smear-proven malaria in 17 patients (15%), brucellosis in 8 patients (7%), liver disease in 3 patients (3%), and another or unknown illness in 16 patients (14%). None of the initially diagnosed non-VL patients were diagnosed with VL within a 6-month period after discharge from AH.

The DUAL IT L/M was significantly more sensitive than the Kalazar Detect: 97% (95%CI = 92 to 99%) versus 82% (95%CI = 74 to 87%) (Table 2). The Kalazar Detect and the DUAL IT L/M were both highly specific: 99% (95%CI = 95 to 100%) and 97% (95%CI = 92 to 99%), respectively. The FGT lacked both sensitivity (66% [95%CI = 57 to 73%]) and specificity (90% [95%CI = 83 to 94%]). The performance of the tests was not significantly influenced by the mode of confirmation of VL diagnosis (data not shown).

Diagnostic accuracy of the DUAL IT L/M for malaria. Of the 276 patients enrolled in the study, 26 were excluded from analysis for the following reasons: failure to meet the definition of clinical suspect for VL (21 patients), thick and thin smear not available for examination at Mbarara laboratory (4 patients), or death before completion of diagnostic tests (1 patient). Of the remaining 250 patients, 21 (8.4%) had smear proven malaria, whereas 229 patients (91.6%) had a negative smear. Previous treatment of malaria within 2 weeks of admission was mentioned by 30 (12%) patients. A low parasitemia (≤200 parasites/mm^3) was found in nine (43%) of the positive cases. The sensitivity and specificity of the DUAL IT L/M for the diagnosis of malaria were, respectively, 57% (95%CI = 37 to 76%) and 86% (95%CI = 81 to 90%). We found a trend toward a higher sensitivity in patients with >200 parasites/mm^3 at 83% (95%CI = 55 to 95%) compared to patients with ≤200 parasites/mm^3 at 22% (95%CI = 6 to 55%). The performance of the test was not significantly affected by a history of previous antimalarial treatment before admission.

DISCUSSION

The DUAL IT L/M rK39 antigen-based dipstick from Dia-Med AG, Switzerland, was found to be highly sensitive (97%) and specific (97%) for the diagnosis of VL in northeastern Uganda. The Kalazar Detect dipstick was also highly specific (99%) but substantially less sensitive (82%). The FGT was less sensitive (66%) and specific (90%) than the rK39 dipsticks.

Our study is the first to evaluate the performance of these tests in Uganda and is one of the few diagnostic accuracy studies in VL using a prospective design in a clinical setting

TABLE 2. Sensitivity, specificity, positive and negative predictive values of the DiaMed-DUAL IT L/M, Kalazar Detect, and FGT for diagnosis of VL in Amudat, Uganda

Diagnostic test	% (95% CI)			
	Sensitivity	Specificity	Positive predictive value	Negative predictive value
DUAL-IT L/M	97 (92–99)	97 (92–99)	98 (93–99)	97 (91–99)
Kalazar Detect	82 (74–87)	99 (95–100)	99 (95–100)	82 (75–88)
Formol Gel Test	66 (57–73)	90 (83–94)	89 (81–94)	69 (61–76)

(23). The reference standard we used was a composite one: case ascertainment was based either on direct examination of Giemsa-stained splenic aspirates smears (close to 100% sensitivity and specificity) or on a combination of serology with observation of response to treatment. The diagnostic accuracy of DAT is known to be excellent and, since the therapeutic spectrum of antimonials is so narrow, a good response to it can safely be regarded as evidence of visceral leishmaniasis (25). All DAT results and spleen aspirates were reviewed in reference laboratories, and patients with discordant results were excluded from analysis. Therefore, we can safely claim that little or no misclassification occurred in the present study with regard to disease status.

The DiaMed-IT LEISH, the equivalent of the DUAL IT L/M but without the malaria diagnostic component, has been previously evaluated in India and Sudan. In India, the sensitivity of the DiaMed-IT LEISH was 99% and its overall specificity was 94% when tested on blood from patients with VL, patients with other diseases, and controls from areas of high and low endemicity (20). In Sudan, the DiaMed-IT LEISH was found to be 81% sensitive and 97% specific in a group of 341 clinical suspect individuals (16). Low sensitivity of rK39 antigen-based dipsticks was already reported in another study from Sudan, and the authors of that study suggested that Sudanese VL patients develop lower titers of antibodies against the K39 antigen (27). Other researchers suggest that the format of the immunochromatographic assay might be the cause (16). Therefore, we believe that the results of diagnostic accuracy studies for VL should only be extrapolated with caution between different epidemiological and ecological regions.

The Kalazar Detect dipstick from Inbios International, Inc., Seattle, Wash., has been previously evaluated (sometimes under another trade name) in the Indian subcontinent and Latin America, with reported sensitivities and specificities of 87 to 100% and 71 to 100%, respectively (5–7, 9, 19). The present study is the first published evaluation of the validity of the Kalazar Detect among African patients clinically suspect of VL. We found a disappointingly low sensitivity (81%) but an excellent specificity (99%). An ongoing multicenter diagnostic study conducted by the World Health Organization in several East African countries will add data on the validity of the Kalazar Detect. According to the manufacturer, a more sensitive generation of the Kalazar Detect dipstick is under development.

The poor sensitivity of the FGT confirmed the findings of previous studies (5, 8). The specificity of the FGT was good (90%) but lower than recently described in Nepal (5), most likely because hyper-reactive malarial syndrome, a classical cause of polyclonal hypergammaglobulinemia, was a frequent diagnosis (61%) in our non-VL patients. We considered the diagnostic performance of the FGT in this setting insufficient to recommend its use. Eleven percent of persons with a positive FGT would be wrongly started on antileishmanial therapy, an unacceptable proportion considering the heavy burden on the patient of 1 month of parenteral therapy and the relative toxicity and high cost of branded antimonials (Pentostam), the current first-line treatment in use in Uganda. Also, 34% of true kala-azar patients would be missed by the FGT, an unacceptably high proportion.

Practical issues in the utilization of the two rK39 dipsticks

also need to be considered. A major advantage of the DUAL IT L/M is that it can be performed with blood obtained by fingerprick. Moreover, all test components are included in individual packages. In contrast, the Kalazar Detect is performed with serum, requiring sampling of venous blood and extra materials such as a micropipette and test tubes. Another advantage of the DUAL IT L/M is that the test result remains stable and can be read up to several months later. This allows for external quality control when the test is performed at peripheral health units by workers with limited training. According to the manufacturers, the cost per unit of the Kalazar Detect and the DUAL IT L/M dipsticks for developing countries is $1.00 and $1.30 (U.S. dollars), respectively. This represents a very small fraction of the total expenses needed to manage patients with VL since the cost of test and treatment strategies depends mainly on the cost of hospitalization and treatment (4). The relatively high packaging volume of the DUAL IT L/M can indirectly increase transport cost if high numbers of dipsticks are needed.

We found no added value of the malaria antigen (pLDH) detection line present on the DUAL IT L/M in our population of VL suspect patients. Only few non-VL patients were diagnosed with smear-proven malaria (8.4%), and the sensitivity of the DiaMed DUAL IT L/M among these patients was only 57%. This poor sensitivity was most likely due to the high proportion (43%) of patients with low parasitemia (<200 parasites/mm^3). A low sensitivity of the Optimal test (DiaMed AG, Switzerland), using the same technology, has been previously reported in malaria patients with low parasitemia (15, 21). It must be emphasized that malaria has a clear seasonal pattern in the study area and that our study took place during a low-transmission season. The prevalence of smear-proven malaria and the intensity of parasitemia is likely to be higher during the high-transmission rainy season, thus influencing the performance of the malaria component of the DUAL IT L/M.

In summary, the DUAL IT L/M was found to be a highly accurate and practical diagnostic test for VL in Uganda. The malaria diagnostic component was not found to be useful. Therefore, we introduced the DiaMed IT LEISH as a first-line test for patients presenting with clinical suspicion of VL at AH in the first trimester 2005. If an ongoing 3- to 6-month post-study validation phase confirms the findings of the present study, the dipstick will replace the DAT for the diagnosis of VL in this setting in Uganda. Because of the long persistence of antibodies against rK39 antigen in the serum after treatment (27), spleen puncture will remain necessary to confirm the diagnosis of relapse in patients with a previous history of VL and a positive dipstick. The introduction of the dipstick in more peripheral health centers, leading to an earlier diagnosis and perhaps a better treatment outcome, should then be implemented. The major remaining challenge is for the Ugandan Ministry of Health to create and ensure a sustainable supply of this diagnostic tool to AH.

this study but had no role in the design, the implementation, or the analysis of this study or in the decision to publish it.

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