Comparison of Point-of-Care Tests for the Rapid Diagnosis of Visceral Leishmaniasis in East African Patients

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Abstract. The development of rK39-based rapid diagnostic tests (RDTs) has greatly aided the diagnosis of visceral leishmaniasis, especially in the Indian subcontinent and Brazil, by offering high sensitivity and specificity. However, these tests have been less sensitive and less specific in sub-Saharan Africa. To improve upon the performance of rK39 in Africa, we engineered the fusion molecule rK28, which retained some of the rK39 repeats and combined them with repeat sequences from two additional *Leishmania* genes. This polyprotein was used in the development of several prototype RDTs by different commercial manufacturers with the goal of assessing relative performance in inexpensive formats. Here, we report field studies showing that the rK28 antigen could be readily adapted to a variety of RDT formats to achieve high sensitivity, generally > 90%, and adequate specificity to aid in the diagnosis of human visceral leishmaniasis in East Africa, Asia, and South America.

INTRODUCTION

Leishmaniasis are a spectrum of infections caused by protozoan parasites of the genus *Leishmania*. *Leishmania* parasites are transmitted to mammals by the bite of phlebotomine sand flies and sometimes by blood transfusion, sharing of needles, or congenital transmission.^{1,2} In terms of global burden of the disease, leishmaniasis are the third most important vector-borne disease with an estimated 1.5–2 million cases worldwide and up to 350 million people at risk of infection in more than 98 countries.³ Leishmaniasis presents in three main clinical forms: cutaneous leishmaniasis has lesions confined primarily to the skin and is caused by parasites of the *Leishmania tropica* and *Leishmania major* complexes; mucosal leishmaniasis is caused by the *Leishmania braziliensis* complex and *Leishmania aethiopica* parasites; and visceral leishmaniasis (VL) is caused by the *Leishmania donovani* complex parasites called *L. donovani* and *Leishmania infantum*.

Visceral leishmaniasis, also referred to as kala-azar in the Indian subcontinent, is the most serious form of leishmaniasis with between 0.2 and 0.4 million cases annually. Ninety percent of the reported cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan.³ Mortality associated with VL is high in the absence of treatment, and accurate VL diagnosis is still problematic, because it is based on clinical presentations that are easily confused with other endemic disease symptoms. Clinical diagnosis relies on non-specific symptoms including long-standing fever, cachexia, anemia, and hepatosplenomegaly, which are only reliable in advanced cases and in epidemic situations. Parasitological confirmation by microscopy using Giemsa-stained aspirates from spleen, bone marrow, or lymph node^{3,4} remains the gold standard diagnostic procedure. However, the required tissue sampling for this technique is invasive, cumbersome, and time-consuming and must be performed by trained personnel at a health center. Furthermore, splenic aspiration carries the risk of

serious or fatal hemorrhage from an already enlarged spleen, and smears from lymph node or bone marrow aspirates are less sensitive than splenic aspirates.⁴

Serological tests for the detection of anti-leishmanial antibodies include indirect immunofluorescence, enzyme-linked immunosorbent assay (ELISA), a direct agglutination test (DAT), and an immunochromatographic lateral flow test (ICT) based on the recombinant antigen rK39.^{5–8} This antigen, a member of the kinesin family of proteins in *Leishmania* species, was first identified by Burns and others⁹ and shown to be abundantly expressed in tissue amastigotes of *Leishmania chagasi*. Using the ELISA format, the recombinant protein has been shown to be > 98% specific and > 98% sensitive for diagnosing VL in the Indian subcontinent.^{10,11} The use of the rK39 ICT has been shown in various parts of the world and has become the gold standard in India and Nepal.

The development of the rK39-based ICT RDT provided high sensitivity and specificity and greatly aided the diagnosis of VL in the Indian subcontinent and Brazil. For reasons that are not totally clear, these rapid tests have been less sensitive and less specific in sub-Saharan Africa (sensitivity range of 75–85%, specificity of 70–92%)¹² as compared with an rK39 ELISA. The increase in the number of VL/human immunodeficiency virus (HIV) co-infected patients has further imposed a limitation on the usefulness of serological diagnostic tests as these patients often present with lower antibody titers.¹³ We have addressed these limitations of the rK39 diagnostic with a new synthetic polyprotein, rK28, a fusion molecule comprising regions of *L. donovani* haspb1 (an *L. infantum* k26 homologue), *L. donovani* kinesin (an *L. infantum* k39 homologue), and *L. donovani* haspb2 (an *L. infantum* k9 homologue).¹⁴ This polyprotein was developed in prototype RDT platforms and evaluated under laboratory conditions. The Infectious Disease Research Institute (IDRI) has worked with a variety of manufacturers to develop and test the rK28 antigen in several simple, rapid, and inexpensive test formats known to exhibit optimal performance characteristics. Herein, we report the field evaluation and iterative refinement of rK28 RDTs from four different test manufacturers for the improved detection of VL in Ethiopia and Sudan.

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MATERIALS AND METHODS

Protein expression and purification of rK28. The recombinant protein rK28 was expressed and purified as previously described.¹⁴ Briefly, rK28 was produced in *Escherichia coli* strain HMS-174(DE3) with fed-batch fermentation using a 10 L bioreactor (New Brunswick Scientific, Edison, NJ) and induction with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 2 hours. The cells were harvested by centrifugation and lysed in 50 mM Tris, pH 8.0 using an 110S microfluidizer (Microfluidics, Newton, MA). The soluble expressed protein in the supernatant fraction was bound to Ni-NTA agarose (Qiagen, Valencia, CA) and, following extensive wash steps, eluted with 20 mM Tris-Cl pH 8.0 containing 400 mM imidazole. The rK28 in the eluted fraction was further purified by chromatography using Q Sepharose Fast Flow (GE Healthcare Biosciences, Piscataway, NJ) followed by OctylSepharose 4 Fast Flow (GE Healthcare Biosciences). The elution peak was dialyzed against 20 mM Tris-Cl pH 8.0 and sterile filtered through a 0.22 mm filter, and the final protein concentration determined using a bicinchoninic acid (BCA) assay (Pierce Chemical, Rockford, IL). The lipopolysaccharide content of each lot of purified protein was measured by a *Limulus* amoebocyte lysate test (BioWhittaker, Walkersville, MD) and shown to be below 10 endotoxin U/mg of protein.

Development of rK28 rapid test formats. For the purpose of developing RDTs, IDRI provided purified rK28 to four manufacturers: Chembio Diagnostic Systems (Medford, NY), CTK Biotech Inc. (San Diego, CA), EASE-Medtrend (Shanghai, China), and InBios International Inc. (Seattle, WA) as stated in Table 1. The DAT was performed using freeze-dried material purchased from KIT (Royal Tropical Institute, Amsterdam, The Netherlands).

Chembio uses what is termed the dual path platform (DPP) as their immunoassay format. The Chembio rK28-based prototype will be referred to here as DPP. The DPP differs from conventional lateral-flow systems in that the test serum sample with donor antibodies and the antibody-detecting conjugate are delivered to the test line area independent of each other using two laminated strips, connected to each other as a “T” shape inside a disposable plastic cassette. The first strip receives a serum sample and running buffer through the sample port. The sample migrates along the strip toward the second strip containing the test and control bands. Antibodies specific for the antigen in the test band, if present in the serum sample, will bind to the capture antigen immobilized on the second strip. Development of the assay is achieved by adding buffer to the second development port. This step releases the conjugate containing colloidal gold and facilitates its migration to the test area, and the conjugate will react with this complex, making the test band visually detectable. Irrespective of the

presence of antibodies in the test sample, the control band should develop to assure correct DPP assay performance.

The CTK Biotech's *Onsite Leishmania* Ab Combo Rapid Test (CTK) is an ICT for the qualitative detection of immunoglobulin G, (IgG), IgM, and IgA antibodies to *L. donovani* in human serum, plasma, or whole blood. The test cassette consists of a burgundy colored conjugate pad containing both recombinant K28 antigen conjugated with colloidal gold (rK28-conjugates) and rabbit IgG-gold conjugates. The cassette includes a nitrocellulose membrane strip with a test line pre-coated with unconjugated rK28 antigen and a control line pre-coated with goat anti-rabbit IgG antibodies. The test is performed by the addition of an adequate volume of serum, plasma, or fresh whole blood to the sample well, followed by the immediate addition of one drop (35–50 μ L) of sample diluent. If anti-*L. donovani* antibodies are present in the primary sample, they will bind to the rK28-conjugates, and the immune-complexes will migrate toward the test line on the membrane through capillary action. The immune complex is then captured on the membrane by the pre-coated antigen, forming a burgundy-colored test line indicative of a positive test result. The internal control line should develop a burgundy colored band of the immune complex of goat anti-rabbit IgG/rabbit IgG gold conjugate regardless of color development on the test line, indicative of a properly administered test.

The EASE-Medtrend single lateral flow test uses a proprietary dynamic flow principle. The EASE-Medtrend rK28-based prototype test will be referred to here as EMT. The test rK28 antigen is immobilized on a nitrocellulose membrane within the test zone. A solution with a conjugate is applied to the device through the reagent port, priming the device to facilitate the migration of serum applied in the sample port. The specific antibodies present in the serum are captured by the immobilized antigens and subsequently visualized in the form of a magenta-colored test line by the conjugate. In the control zone, a conjugate-binding reagent is immobilized on the membrane. A magenta line in the control zone appears in every valid test.

The InBios Kalazar Detect *Plus* (InBios⁺) rapid test uses the rK28 antigen pre-coated on the membrane test line. There is a separate control line to assure assay flow and performance. Testing is performed by the addition of serum or whole blood to the sample pad onto which is added a drop of a proprietary blend of a stable solution of conjugate labeled with protein A. The serum and conjugate mixture migrates by capillary action toward the recombinant antigen on the membrane. After 5 minutes, a drop of a secondary “chase buffer” is added to the sample pad to ensure proper migration and for development of the assay. If antibodies to the antigen are present in the primary sample, a red line will develop at the test line. A red line in the control region should always develop if the assay has been

TABLE 1
Manufacturers of the rK39 and rK28 rapid diagnostic tests used in these studies

Manufacturer	Kit name	Product abbreviation used here
Tests using rK39		
DiaMed AG (BioRad, USA)	DiaMed-IT Leish	DiaMed
InBios International, Inc.	InBios Kalazar Detect	InBios
Tests using rK28		
Chembio Diagnostic Systems, Inc., USA	Chembio Dual Path Platform rK28	DPP
CTK Biotech Inc., USA	<i>Onsite Leishmania</i> Ab Combo Rapid Test	CTK
EASE-Medtrend Biotech Ltd., China	EASE-Medtrend Dynamic Flow (rK28)	EMT
InBios International, Inc., USA	InBios Kalazar Detect <i>Plus</i>	InBios ⁺

TABLE 2
Ethiopia Study 1: Comparison of DAT and rK39 and rK28 RDTs using various disease and control sera*

Serum source	DAT	rK39		rK28		
		DiaMed	InBios	DPP	EMT	InBios ⁺
		Number positive/number tested (% positive)				
VL patients	77/82 (93.3%)	80/82 (97.6%)	77/82 (93.9%)	80/82 (97.6%)	81/82 (98.8%)	81/82 (98.8%)
HIV/VL co-infected patients	10/13 (76.9%)	12/13 (92.3%)	11/13 (84.6%)	11/13 (84.6%)	13/13 (100%)	12/13 (92.3%)
Healthy, non-endemic controls	0/35 (0%)	0/35 (0%)	0/35 (0%)	0/35 (0%)	0/35 (0%)	2/35 (5.7%)
Healthy, endemic controls	0/41 (0%)	0/41 (4.9%)	2/41 (4.9%)	3/41 (7.3%)	8/41 (19.5%)	8/41 (19.5%)
Other diseases	3/35 (9.4%)	0/35 (0%)	0/35 (0%)	3/35 (8.6%)	14/35 (40.0%)	9/35 (25.5%)

* RDT = rapid diagnostic test; DAT = direct agglutination test; DPP = dual path platform; EMT = EASE-Medtrend test; VL = visceral leishmaniasis; HIV = human immunodeficiency virus.

performed correctly, verifying that proper flow has occurred and catastrophic failure of the conjugate has not occurred.

Study 1 design: comparison in Ethiopia of DAT with two rK39 RDTs and three rK28 RDTs. Serum samples were obtained from different subjects and analyzed for reactivity against three different formats of rK28 RDTs from Chembio, EASE-Medtrend, and InBios; two rK39 RDTs from InBios and Diamed; and the direct agglutination test (DAT) from KIT. The following five panels of sera were tested: 82 parasitologically positive (splenic or lymph node aspirate) sera from VL patients; 35 sera from healthy controls from regions not endemic for VL (HNEC); 41 healthy control sera from VL-endemic regions (HEC); sera from 35 patients with other infectious diseases (ODs) including malaria, intestinal schistosomiasis, and pulmonary tuberculosis; and 13 sera from VL patients co-infected with HIV (HIV-VL). The RDT and DAT testing were carried out according to each of the manufacturers' procedures and guidelines.

Study 2 design: comparison in Ethiopia and Sudan of four improved rK28 RDTs using serum and fresh whole blood. In this series, the performance of the DPP, CTK, EMT, and InBios RDTs were evaluated in preparation for field evaluation in a phase 3 clinical study. The objectives of Study 2 were to examine these tests' sensitivity and specificity using archived sera and equivalency using matched serum and whole blood samples. Sera evaluated in Sudan included samples from 100 VL patients; 75 patients with ODs including malaria, intestinal schistosomiasis, and pulmonary tuberculosis; 50 HNEC; and

25 HEC. Matched serum and whole blood samples were evaluated for 25 confirmed VL patients and 20 OD controls in Sudan. Serum samples evaluated in Ethiopia included 100 parasitology confirmed VL patients; 100 patients with ODs (malaria, intestinal schistosomiasis, and pulmonary tuberculosis); and 50 HNEC.

The studies in Ethiopia were approved by the Institutional Review Board of the Department of Microbial, Cellular, and Molecular Biology in the College of Natural Sciences of Addis Ababa University and were incorporated into the Masters' thesis work of Asrat Bezuneh. The study in Sudan was approved by the Institute of Endemic Diseases Ethics Committee and by the National Ethics Committee at the Federal Ministry of Health. All patients gave informed consent for blood sample collection.

RESULTS

Ethiopia Study 1. Using control and disease sera from Ethiopia, prototypes of three different rK28 RDTs (DPP, EMT, InBios+) were compared with two commercially available rK39 RDTs (InBios, DiaMed) and DAT (Table 2). The sera tested were obtained from 95 parasitologically confirmed VL patients (82 confirmed VL and an additional 13 HIV/VL co-infected individuals) and 111 VL-negative control sera including 35 HNEC, 41 HEC, and 35 OD controls (13 malaria, 12 intestinal schistosomiasis, and 10 pulmonary tuberculosis). As shown in Table 3, all three of the rK28-based RDTs

TABLE 3
Ethiopia Study 1: VL sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for DAT and RDTs*

Target antigen	Product	Sensitivity	Specificity	PPV	NPV
		Percentage (95% confidence interval)			
rK39	DAT	91.6 (84.1–96.3)	97.3 (92.3–99.4)	96.7 (90.6–99.3)	93.1 (86.9–96.9)
	DiaMed	96.8 (91.1–99.3)	98.2 (93.6–99.8)	97.9 (92.5–99.7)	97.3 (92.4–99.4)
	InBios	92.6 (85.4–96.9)	98.2 (93.6–99.8)	97.8 (92.2–99.7)	93.8 (87.9–97.5)
rK28	DPP	95.8 (89.6–98.8)	94.6 (88.6–97.9)	93.8 (87.0–97.7)	96.3 (90.9–98.9)
	EMT	98.9 (94.3–99.9)	80.2 (71.5–87.1)	81.0 (72.7–87.7)	98.9 (93.9–99.9)
	InBios ⁺	97.9 (92.6–99.7)	82.9 (74.6–89.4)	83.0 (74.8–89.5)	97.9 (92.5–99.7)

* VL = visceral leishmaniasis; DAT = direct agglutination test; DPP = dual path platform; EMT = EASE-Medtrend test.

showed improvements in sensitivity (95.8–98.9%) when compared with DAT (91.6%) or the widely used InBios Kalazar Detect RDT using rK39 (92.6%). The DPP had the highest specificity (94.6%) and had good sensitivity (95.8%) of the three rK28 prototype RDTs. Even so, the Kalazar Detect RDT yielded a sensitivity of 92.6% and specificity of 98.1%, a marked improvement over previously published data on rK39 ICTs in East Africa.¹² In addition, the DiaMed-Leish diagnostic test that is also based on rK39, showed a 96.8% sensitivity and 98.2% specificity with our panel of sera from healthy endemic and non-VL endemic individuals and other endemic diseases (Table 3). The DAT assay was performed on these same serum samples and exhibited a sensitivity of 91.6% and specificity of 97.3% (Table 3). In an evaluation of a limited number of HIV/VL co-infected sera, all of the RDTs performed well (84.6–100% sensitivity). Nevertheless, there was clearly room for test improvement.

The results from Study 1 in Ethiopia were provided to the test manufacturers (Chembio, EASE-Medtrend, and InBios) to allow for further refinement of the rK28 test prototypes in anticipation of inclusion in the Study 2 design to be performed in both Sudan and Ethiopia. The IDRI also provided rK28 antigen to CTK Biotech Inc. for the development of an additional test kit to be evaluated in this second field study. The performance characteristics of the improved rK28 RDTs used in Study 2, as provided by the test manufacturers, are outlined in Table 4. The objectives of Study 2 were to 1) determine the sensitivity and specificity of the four tests compared with parasitology and DAT using archived sera, and 2) determine the performance of the tests using matched sera and whole blood samples in preparation for field evaluation in a phase 3 clinical study. Representative results of the 4 rK28 RDTs with visceral leishmaniasis (VL) patient serum are displayed in Figure 1.

Ethiopia Study 2. Serum samples evaluated in Ethiopia as part of Study 2 with improved rK28 RDTs included 100 VL patients; 94 patients with OD including malaria (47), schistosomiasis (25), and tuberculosis (22); and 50 Ethiopian HNEC. The compiled results for the Ethiopian serum analyses are given in Table 5. All four of the improved rK28-based RDTs detected 96–99% of VL cases ($N = 100$) versus 96% detected by DAT. The RDTs produced a few false positive signals, ranging from zero to three in the 50 samples assayed, whereas DAT had no false positives in these samples. Each of the tests detected one or more positive samples among the clinical cases of malaria, tuberculosis, and schistosomiasis, suggesting some of these individuals may also have recently been bitten by *Leishmania donovani*-infected sand flies or may have a subclinical co-infection. For this reason the RDT test specificity (Table 6) was calculated in two ways, one by calculating the

FIGURE 1. rK28 rapid diagnostic test (RDT) comparison on visceral leishmaniasis (VL) patient serum. RDTs were run according to instructions in each manufacturer's product insert. Each of the prototype tests shows clear and distinct development of both control (C) and test (T) lines.

observed negative responses/total number of samples combining the HEC, HNEC, and OD groups ($N = 144$) and one determined using negative responses/only the HNEC samples ($N = 100$). The presented negative and positive predictive values are calculated in a similar manner (Table 6).

Sudan Study 2. In Sudan 250 serum samples were evaluated including 100 VL cases; 75 patients with OD including malaria (25), intestinal schistosomiasis (25), and pulmonary tuberculosis (25); 50 Sudanese HNEC; and 25 Sudanese HEC. Matched serum and whole blood samples were evaluated for 25 confirmed VL patients and 20 OD controls. The results for the serum analysis are listed in Table 7. The RDT test Sensitivity (Table 8) was calculated based on the observed RDT positive responses/total confirmed VL cases ($N = 100$). The Specificity (Table 8) was determined by calculating the observed negative responses/total number of samples combining the HEC, HNEC, and OD groups ($N = 150$). The positive predictive value for these diagnostic devices as configured and tested in the field ranged from 97% to 100% in Ethiopia and 88.1% to 98.9% in Sudan using only the HNEC as negative serum samples.

As the intended field application of the test will likely use finger prick fresh whole blood, the four RDTs were evaluated for consistency of test performance between patient serum samples and whole blood. A total of 25 confirmed VL patients and 20 other disease patients were compared in Sudan. We observed 100% agreement in test performance (sensitivity, specificity) between whole blood and serum samples for VL patients (25 of 25 for both blood and serum) and blood and

TABLE 4
Performance characteristics of improved rK28 RDTs used in Study 2*

Product	Storage temperature (°C)	Sample type	Sample volume (μL)	Number of steps	Reading time (min)
DPP	2–30	Serum, plasma & blood	5	2	20
CTK	2–30	Serum, plasma & blood	serum, plasma 30–45; blood 40–50	1	10
EMT	Tests 2–30; conjugate 2–8	Serum, plasma & blood	5	2	10–15
InBios ⁺	2–30	Serum, plasma & blood	5	2	20

*RDTs = rapid diagnostic tests; DPP = dual path platform; EMT = EASE-Medtrend test.

TABLE 5

Study 2 (Ethiopia): sero-positivity of the four rk28 RDTs used in the study tested with healthy non-endemic controls and different patient serum panel

Serum panels	DAT	DPP	CTK	EMT	InBios*
Number positive/number tested (% positive)					
VL	95/99 (95.9)	99/100 (99.0)	98/100 (98.0)	98/100 (98.0)	96/100 (96.0)
Healthy, non- endemic controls	0/49 (0.0)	3/50 (6.0)	0/50 (0.0)	1/50 (2.0)	1/50 (2.0)
Malaria	0/42 (0.0)	13/47 (27.6)	4/47 (8.5)	20/47 (42.5)	23/47 (48.9)
Pulmonary TB	3/19 (15.7)	3/22 (13.6)	3/22 (13.6)	2/22 (9.1)	2/22 (9.1)
Intestinal schistosomiasis	0/23 (0.0)	5/25 (20.0)	1/25 (4.0)	3/25 (12.0)	3/25 (12.0)

* RDTs = rapid diagnostic tests; DAT = direct agglutination test; DPP = dual path platform; EMT = EASE-Medtrend test; VL = visceral leishmaniasis; TB = tuberculosis.

serum for other disease controls for the RDTs from all four test manufacturers, with no differences in test line interpretation noted between patient blood and serum samples.

DISCUSSION

Diagnosis of VL in Africa currently requires a clinical setting to perform an invasive and painful aspiration from bone marrow, lymph node, or spleen for microscopic or cell culture confirmation of parasites. Although highly specific, sensitivity determined by microscopic detection is influenced by the quality of the smear, the reagents used, and the experience of the individual reading the slides. Furthermore, microscopy is often not available outside of referral hospitals in endemic countries, contributing to the difficulty of accurate diagnosis of VL in primary healthcare settings.

The development of serological tests, such as the DAT and the rK39 ICTs, to quickly and accurately detect anti-leishmanial antibodies have aided the field testing for VL. Yet, each test still has its limitations, especially in East Africa. The DAT requires a laboratory equipped with a temperature-controlled incubator, a skilled technician, and an overnight incubation, keeping it from being a true point-of-care test. Although the DAT is able to detect low levels of antibodies by using the multiple antigens present in the preparation, in some

TABLE 7

Study 2 (Sudan): sero-positivity of the four rK28 RDTs to serum panels from different Sudanese patient groups and controls*

Serum panels	DAT	DPP	CTK	EMT	InBios*
Number positive/number tested (% positive)					
VL	95/100 (95.0)	95/100 (95.0)	94/100 (94.0)	97/100 (97.0)	96/100 (96.0)
Healthy, endemic controls	0/25 (0.0)	2/25 (8.0)	0/25 (0.0)	3/25 (12.0)	3/22 (12.0)
Healthy, non- endemic controls	0/50 (0.0)	0/50 (0.0)	0/50 (0.0)	2/50 (4.1)	1/50 (2.0)
Malaria	0/25 (0.0)	1/25 (4.0)	0/25 (0.0)	4/25 (16.0)	4/25 (16.0)
Pulmonary TB	0/25 (0.0)	0/25 (0.0)	0/25 (0.0)	1/25 (4.0)	1/25 (4.0)
Intestinal schistosomiasis	0/25 (0.0)	3/25 (12.0)	1/25 (4.0)	3/25 (12.0)	2/25 (8.0)

* RDTs = rapid diagnostic tests; DAT = direct agglutination test; DPP = dual path platform; EMT = EASE-Medtrend test; VL = visceral leishmaniasis.

instances the high sensitivity may come at the expense of specificity. Titers above 1:3200 are routinely considered positive and those below 1:400 negative, creating an indeterminate result when titers fall between these values. Lateral flow immunochromatographic assays, like other serologic test formats, still rely on an indirect measure of infection: detection of antibodies as a result of exposure to immunogenic molecules. Patients may have antibody present for many months or years after treatment of disease, and the tests may also detect antibodies in the sera of asymptomatic individuals and individuals who have recovered or self-cured after an exposure.^{15,16} Alternative direct detection approaches to serological tests are being developed, including laboratory-based nucleic acid amplification tests and the detection of antigens in bodily fluids.^{17,18} The current commercially available antigen detection test is a latex agglutination test (KATEX) for the detection of leishmanial antigen in the urine of VL patients.¹⁷ Although this test has modest sensitivity (60–80%), the general approach of testing for antigens instead of antibodies may provide a successful means of diagnosing VL in immune-compromised individuals who may have low antibody titers.^{19,20}

The identification of rK39 as a marker of active VL⁹ followed by its use in a rapid lateral flow test format exhibiting 96–98% sensitivity led to its incorporation into the

TABLE 6

Study 2 (Ethiopia): VL sensitivity, specificity, PPV and NPV for DAT and four rK28 RDTs using human sera

Test	Sensitivity	Specificity*	Specificity†	PPV*	PPV†	NPV*	NPV†
Percentage (95% confidence interval)							
DAT	95.9 (89.9–98.8)	97.7 (93.5–99.5)	100 (92.7–100)	96.9 (91.3–99.3)	100 (96.1–100)	97.0 (92.5–99.1)	92.4 (81.7–97.9)
DPP	99.0 (94.5–99.9)	83.3 (76.2–89.0)	94.0 (83.4–98.7)	80.4 (72.3–87.0)	97.0 (92.7–100)	99.1 (95.4–99.9)	97.9 (88.9–99.9)
CTK	98.0 (92.9–99.7)	94.4 (89.3–97.5)	100 (92.8–100)	92.4 (85.6–96.6)	100 (96.3–100)	98.5 (94.8–99.8)	96.1 (86.7–99.5)
EMT	98.0 (92.9–99.7)	81.9 (74.6–87.8)	98.0 (89.3–99.9)	79.0 (70.8–85.8)	98.9 (94.5–99.9)	93.6 (87.8–97.2)	96.0 (81.7–97.9)
InBios ⁺	96.0 (90.0–98.9)	80.5 (73.1–86.6)	98.0 (89.3–99.9)	77.4 (69.0–84.4)	98.9 (94.3–99.9)	96.6 (91.6–99.0)	92.4 (81.7–97.9)

* Values calculated using HNEC and OD serum samples.

† Values calculated using only HNEC serum samples.

VL = visceral leishmaniasis; PPV = positive predictive value; NPV = negative predictive value; DAT = direct agglutination test; RDTs = rapid diagnostic tests; DPP = dual path platform; EMT = EASE-Medtrend test.

TABLE 8

Study 2 (Sudan): sensitivity, specificity, PPV, and NPV for DAT and four improved rK28 RDTs*

Test	Sensitivity	Specificity	PPV	NPV
	Percentage (95% confidence interval)			
DAT	95.0 (88.7–98.3)	100 (97.5–100)	95.0 (96.1–100)	96.7 (92.6–98.9)
DPP	95.0 (88.7–98.3)	96.0 (91.5–98.5)	94.0 (87.5–97.7)	96.6 (92.3–98.9)
CTK	94.0 (87.4–97.7)	99.3 (96.3–99.9)	98.9 (94.2–99.9)	96.1 (91.7–98.5)
EMT	97.0 (91.4–99.3)	91.3 (85.6–95.3)	88.1 (80.6–93.5)	97.8 (93.8–99.5)
InBios ⁺	96.0 (90.0–98.9)	92.7 (87.2–96.2)	89.7 (82.3–94.7)	97.2 (92.9–99.2)

*Note: visceral leishmaniasis (VL) negative control panel consists of HNEC and OD. PPV = positive predictive value; NPV = negative predictive value; DAT = direct agglutination test; RDTs = rapid diagnostic tests; DPP = dual path platform; EMT = EASE-Medtrend test.

VL diagnostic algorithms that have been especially useful in the Indian subcontinent as a means of confirming diagnosis of VL patients.¹⁰ However, the generally lower sensitivity observed for rK39 RDTs in the VL-endemic regions of East Africa hampered the widespread use of these point-of-care serodiagnostic tests in that region. The reduced rK39 sensitivity in East Africa, as compared with the Indian subcontinent, may reflect molecular diversity of the rK39 homologous sequences among East African *L. donovani* strains or wider genetic differences in the human and parasite populations. In fact, molecular divergence in the rK39 kinesin repeat sequences was observed in homologues of East African *L. donovani* isolates as compared with sequences observed in Southeast Asian strains.²¹ The fact that the disease is mainly pediatric in the Indian continent and Brazil with mild morbidity compared with the East African disease that affects all age groups with very severe morbidity may also reflect genetic differences, as does the regional variation in response to treatment. Certain drug regimens that were successful in treating Indian kala-azar were not effective in treating VL in East Africa.^{22,23} Technological improvements in RDT assay formats may also improve the sensitivity of these tests, as shown with both the DiaMed-*Leishmania* test and the four prototypes evaluated in Study 2 in Ethiopia and Sudan compared with earlier versions tested in Study 1 of this report.

To increase the sensitivity of the rK39 RDT in detecting VL cases in East Africa, we evaluated additional serologically significant antigens and designed a fusion polypeptide composed of tandem repeat regions of the *haspb1*, *LdK39*, and *haspb2* genes.¹⁴ Designated as rK28, we previously showed an improved sensitivity and specificity of this fusion protein in ELISA format with sera from Sudanese VL patients as compared with rK39 alone.¹⁴ Incorporation of the rK28 antigen into a prototype RDT format gave superior results (95.9% sensitivity, 100% specificity) as compared with a commercial rK39 RDT (86.3% sensitivity, 98.4% specificity) in pilot studies conducted in Sudan.¹⁴

For the current studies, we have extended our evaluation of rK28 RDT kits produced by four different test manufacturers to multiple sites in Ethiopia and Sudan. The results reported herein show a modest improvement in sensitivity of the rK28 RDT assays over previous generations of rK39 rapid tests and merit further studies to facilitate adoption of these assays into

the VL diagnostic algorithm in East Africa. Although overall sensitivity was similar among the rK28 tests conducted in Sudan and Ethiopia, there were discrepancies in the specificity observed between site locations, particularly within the “other diseases” control sera. As noted previously, serological tests may give positive results within asymptomatically infected individuals; the rates of asymptomatic *Leishmania* parasite infection within endemic populations in Ethiopia have been estimated to be as high as 10–20%.²⁴ Of particular note, high rates of VL/malaria co-infection have also been reported within VL-endemic areas in East Africa.²⁵ The discrepancies observed between Ethiopia and Sudan for rK28 specificity in the “other diseases” control category cannot be fully explained; however, as noted previously, it seems highly likely that many patients with malaria, intestinal schistosomiasis, and tuberculosis either currently or previously had asymptomatic *L. donovani* infection, generating the immune reactivity seen among the Ethiopian OD patients.

Evaluating the performance of the rK28-based RDTs in VL patients with HIV co-infection is a high priority because all tests currently available are often less sensitive in these patients. In this report, 13 such patients were included. Although these numbers are too small to derive any definitive conclusions, we did observe that, depending on the RDT prototype, the reactivity to VL/HIV co-infected individuals was not necessarily that different from the VL patients when the first prototype rK28 RDTs were evaluated (98–99% versus 85–100%; see Table 2). We have initiated a follow-up study with the aim of comparing performance of the rK28-based RDTs in patients with and without HIV co-infection. In addition, based on the results presented herein, a larger phase 3 clinical study was initiated using fresh whole blood samples and serum in Sudan; this study is currently underway.

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