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Comparative Evaluation of Blood and Serum Samples in Rapid Immunochromatographic Tests for Visceral Leishmaniasis

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Rapid diagnostic tests (RDTs) based on the detection of specific antibodies in serum are commonly used for the diagnosis of visceral leishmaniasis (VL). Several commercial kits are available, and some of them allow the use of whole-blood samples instead of serum. An RDT is much more user-friendly for blood samples than for serum samples. In this study, we examined the sensitivities and specificities of six different commercially available immunochromatographic tests for their accuracy in detecting *Leishmania* infection in whole blood and serum of parasitologically confirmed VL cases. This study was performed in areas of India and Nepal where VL is endemic. A total of 177 confirmed VL cases, 208 healthy controls from areas of endemicity (EHCs), 26 malaria patients (MP), and 37 tuberculosis (TB) patients were enrolled. The reproducibilities of the blood and serum results and between-reader and between-laboratory results were tested. In India, the sensitivities of all the RDTs ranged between 94.7 and 100.0%, with no significant differences between whole blood and serum. The specificities ranged between 92.4 and 100.0%, except for the specificity of the Onsite *Leishmania* Ab RevB kit, which was lower (33.6 to 42.0%). No differences in specificities were observed for blood and serum. In Nepal, the sensitivities of all the test kits, for whole-blood as well as serum samples, ranged between 96.3 and 100.0%, and the specificities ranged between 90.1 and 96.1%, again with the exception of that of the Onsite *Leishmania* Ab RevB test, which was markedly lower (48.7 to 49.3%). The diagnostic accuracies of all the tests, except for one brand, were excellent for the whole-blood and serum samples. We conclude that whole blood is an adequate alternative for serum in RDTs for VL, with sensitivities and specificities comparable to those obtained in serum samples, provided that the test kit is of overall good quality.

Visceral leishmaniasis (VL), also known as kala azar, is caused by a protozoan parasite, *Leishmania donovani*, and is transmitted through the bite of an infected phlebotomine sand fly. Every year there are an estimated 0.2 to 0.4 million new cases and 90% of these are reported from India, Nepal, Bangladesh, Sudan, Brazil, and Ethiopia, where the disease disproportionally affects some of the poorest families. VL is a very severe systemic infection of the internal organs, such as spleen, liver, bone marrow, and lymph nodes. The clinical features are fever with rigors, fatigue, loss of weight and appetite, and hepatosplenomegaly (1). If untreated, the disease is almost always fatal. Accurate diagnosis is necessary to guide treatment, as the drug regimens are rather toxic and costly. Options to control VL are limited, especially in the Indian subcontinent, where there is no animal reservoir. As there is no vaccine, the only control options are vector control and early case finding and treatment.

However, in the clinic as well as in the control programs, diagnosis of VL remains a challenge. Parasitological diagnosis requires microscopic demonstration of *Leishmania* amastigotes in tissue biopsy specimens. The most sensitive tissue biopsy specimen for detection of amastigotes is a splenic aspirate, but this procedure carries a risk of fatal hemorrhage. Biopsy specimens from other tissues, like bone marrow or lymph glands, are associated with less risk, but their sensitivities are substantially lower. Alternative diagnostic procedures are serodiagnosis (2–5) and nucleic acid amplification techniques, such as PCR (6, 7). Although PCR seems reasonably sensitive and specific for the detection of leishmanial infections (6), this technology is difficult to apply and has not been standardized for field conditions. Moreover, in areas of endemicity, PCR may prove too sensitive in the clinical situation as it detects many asymptomatic infections (8). Several serological tests, such as immunofluorescence and enzyme-linked immunosorbent assay (ELISA), have been used for many years in the laboratory, but it was not until the development of the direct agglutination test (DAT) in the 1980s that serological diagnosis became feasible in field settings (9, 10). Among its disadvantages, DAT shares those of all antibody detection tests: it is not specific for acute disease as patients will remain positive for many months after their treatment and some asymptomatically infected persons will have positive results for the DAT. When used in combination with a clinical case definition, DAT proved a useful diagnostic tool with high sensitivity and specificity. Nevertheless, DAT is not ideal for use in a low-tech environment, as the procedure requires multiple pipetting and overnight incubation. Moreover, DAT has to be performed by a skilled laboratory technician with proper training; if not, reproducibility problems do arise.

The development of an immunochromatographic test (ICT) was the logical next step to make serology for VL more user-friendly. Several surface antigens, such as ribosomal antigens, histones, and nuclear and kinesin proteins, are known to elicit spe-

Received 10 May 2013 Returned for modification 14 June 2013
Accepted 9 September 2013
Published ahead of print 18 September 2013
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doi:10.1128/JCM.01232-13

TABLE 1 RDT characteristics

Manufacturer	Product	Catalog no.	Format	Storage temp (°C)	Expiration date (mo/yr)	Control line	Test line	Sample vol (µl) (type)	Buffer vol (no. of drops)	Reading times (min)	
										Minimum	Maximum
Span Diagnostics, Ltd. (Surat, India)	Signal KA	4000008267	Cassette	2–8	02/2013	Yes	rKE16	20 (serum/blood in 80 µl of saline), 50 (diluted sample)	2 (step 1), 2 (step 2), 3 (step 3)	2	10
	Crystal KA	4000008095	Dipstick	2–30	01/2013	Yes	rKE16	20 (blood), 20 (serum)	5	15	30
CTK Biotech, Inc. (San Diego, CA)	Onsite *Leishmania* Ab (RevA) rapid test	RD0360	Cassette	4–30	08/2013	Yes	rK39	40 (blood), 30 (serum)	1	1	15
	Onsite *Leishmania* Ab (RevB) rapid test	RD0361	Cassette	4–30	08/2013	Yes	rK39	40 (blood), 30 (serum)	1	1	15
InBios International, Inc. (Seattle, WA)	Kala-azar Detect	NB1060	Dipstick	4–30	01/2014	Yes	rK39	20 (blood), 20 (serum)	3	10	10
Bio-Rad Laboratories (Marnes-la-Coquette, France)	DiaMed-IT LEISH	2A0025L	Cassette	4–30	04/2013	Yes	rK39	10 (blood), 10 (serum)	1 (step 1, conjugate well), 4 (step 2, wash well)	5	Not specified

cific humoral immune responses in VL. A kinesin-related recombinant protein (rK) of 39 amino acid repeats proved a very promising diagnostic marker in ELISA and was used in an ICT format. The first large-scale evaluation of this rK39 ICT was done in India (11), and 100% sensitivity and 98% specificity were reported. Several other validation studies in different parts of the world, as well as a meta-analysis, confirmed the diagnostic performance of the rK39 ICT combined with a clinical case definition as good to excellent for VL, with a sensitivity in East Africa slightly lower than that in the Indian subcontinent (12). WHO–Tropical Disease Research (WHO/TDR) conducted a multicenter evaluation in 5 countries that confirmed the high diagnostic accuracy of this rK39 rapid diagnostic test (RDT) in India and Nepal (13). Subsequently, the VL elimination initiative in India, Nepal, and Bangladesh recommended treatment of patients with febrile splenomegaly and a positive RDT. An RDT based on a similar kinesin antigen, rKE16, was developed in India with good results (14). Currently, there are several commercial kits available. Two manufacturers, InBios International, Inc. (Kala-azar Detect), and Span Diagnostics, Ltd. (Signal-KA and Crystal-KA), endorse the use of their products only for serum samples. Other manufacturers, like CTK Biotech (Onsite *Leishmania* Ab RevA and RevB rapid tests) and Bio-Rad (DiaMed-IT LEISH), recommend use on either blood or serum samples. In peripheral health facilities, whole-blood samples are much simpler and easier to handle than serum samples. There are limited reports on the performances of RDTs in whole blood, and only one head-to-head comparison of whole blood versus serum was available up to 2013 (15, 16). In the present study, we evaluated the performances of six different types of RDTs, four based on rK39 and two on rKE16, on whole-blood samples and compared their results with results in serum samples.

MATERIALS AND METHODS

Study site. This study was conducted at two sites, (i) the Infectious Disease Research Laboratory of the Department of Medicine, Institute of Medical Sciences, Banaras Hindu University (BHU), Varanasi, India, and its field site, the Kala-Azar Medical Research Centre (KAMRC) in Muzaffarpur, Bihar, India, and (ii) the B.P. Koirala Institute of Health Sciences, Dharan, Nepal. This study was approved by the respective ethics committees of the two institutions. Written informed consent was obtained from all participating subjects.

Test selection. Based on the set of operational criteria developed by the VL Laboratory Network to define an RDT for VL (17), we used the following criteria to include an RDT in this evaluation study: (i) rapidness (test results should be available in less than 15 min), (ii) simplicity (the test can be performed in one or two steps and requires minimal training and equipment), and (iii) ease of interpretation (the test uses a card, cassette, or strip format with visual readout). Test inclusion criteria also included quality assurance certificates and the availability of a supply of sufficient quantities of RDTs of the same lot from the same manufacturer.

Sample size and selection of study subjects. The sample size was calculated to estimate the sensitivity and specificity of the rapid tests with a precision of at least 5% (as expressed by the half width of the 95% confidence interval, using Wilson score methods). Assuming a true sensitivity of 95%, a sample size of 177 VL cases (150 from India and 27 from Nepal) was required to ensure a power of 80% and a confidence level of 95%, with a confidence interval lower margin of at least 90%. For specificity estimation, the same formula applied, and we included 208 (85 from India and 123 from Nepal) samples from healthy controls from areas of endemicity (EHCs) with no history of VL and 63 samples from patients with potentially cross-reactive diseases, including 26 cases of malaria (14 from India and 12 from Nepal) and 37 cases of tuberculosis (TB) (20 from India and

TABLE 2 Characteristics of the subjects

Characteristic	Results for subjects from:			
	India		Nepal	
	VL patients ($n = 150$)	Controls ($n = 119$)	VL patients ($n = 27$)	Controls ($n = 152$)
Age (mean ± SD) (yr)	24.9 ± 12.1	35.2 ± 12.6	24.4 ± 19.28	35.34 ± 16.44
Sex (no. males/no. females)	84/66	47/72	9/18	75/77
Fever history (mean ± SD) (days)	51.5 ± 53.7		66.68 ± 58.26	
Spleen size (mean ± SD) (cm)	3.80 ± 1.92		6.07 ± 4.68	
HIV status	Negative	Negative	Negative	Negative

17 from Nepal). Consecutive patients of the desired target group presenting to the study clinic were invited to participate. Subjects who met the inclusion criteria for confirmed VL patients were subjects more than 2 years of age, living in areas of VL endemicity, with signs and symptoms suggestive of VL and confirmed by the presence of parasites in splenic smears. Pregnant patients were excluded from the study. Healthy controls from areas of endemicity were subjects living in areas of VL endemicity with no history of kala azar who were more than 18 years of age and also had negative serology in the DAT (titer, <1:1,600). Patients with TB had to be smear positive with acid-fast bacilli, and malaria patients needed to be parasitologically confirmed. Only patients older than 2 years of age were included.

Sample collection, transportation, and storage. From each patient with parasitologically confirmed VL, EHC, and patient with malaria or TB, 4 ml of blood was collected and immediately divided into two parts, one collected in an EDTA tube for whole blood and one in an untreated tube for serum. In the latter, blood was allowed to clot for 20 to 30 min at room temperature followed by centrifugation at 2,500 rpm for 5 min. The serum was removed from the clot and collected in cryogenic vials in duplicate and labeled accordingly. An aliquot of whole blood and serum was used immediately for performing the rapid tests per the manufacturer's instructions. In India only, the remaining blood and serum samples were stored at −20°C and transported frozen to the reference laboratory at BHU Varanasi for repeat RDT testing.

RDT shipments. The details of each manufacturer, product, and catalogue number and a comprehensive overview are listed in Table 1. All manufacturers shipped tests in the required amount to the participating sites. Upon arrival, tests were immediately unpacked and stored according to the manufacturers' instructions. Dates of arrival and conditions of each shipment were noted. Daily temperatures in the storage area of the RDTs were monitored with an electronic temperature recorder.

RDT procedures. The RDTs were brought to room temperature prior to use and labeled with the random sample code. Each sample was tested once against each product according to the manufacturers' instructions. RDT envelopes were opened immediately before use. The specified volume of sample was dispensed onto the RDT by micropipette. The buffer was applied using the dropper provided. Results were read and recorded on a standardized form by a first technician at the minimum reading time and within the maximum time recommended by the manufacturer, and then the test was read and recorded by a second technician, blinded to the first reading. Results of test and control lines were recorded as positive or negative by each technician. If the control line was recorded as absent by either technician, the same sample was repeated against a new RDT. If the control line was still absent, the test result was recorded as invalid.

In India, but not in Nepal, the study included a between-laboratory reproducibility assessment of the RDTs for results obtained in the field clinic (KAMRC) on fresh samples and in the reference laboratory (BHU) on stored samples of whole blood and serum.

Data analysis. Data were entered into an EPI Info database using a double data entry procedure. Data files were compared to identify typing errors. For data analysis we used STATA software. All source documents and electronic records of study data were maintained in secure storage until the study conclusion, data analyses, and report publication. Diagnostic accuracy was calculated using the RDT result from the first reading at the minimum reading time. Specificity estimates shown are the pooled results of EHCs and control patients with other diseases. We calculated 95% confidence intervals for sensitivities and specificities using exact binomial methods for proportions (18, 19). Cohen's kappa value was computed for assessing agreement between methods, including agreement between laboratories (KAMRC versus BHU) in India and between readers (two laboratory technicians for each RDT reading) in both countries.

RESULTS

Clinical data for the patients included in this study are given in Table 2.

Site and test selections. Six products from four manufacturers were evaluated in this study. Among them, four products detected anti-rK39 antibodies and two products detected anti-rKE16 antibodies.

Diagnostic performance. In India as well as Nepal, the sensitivities of all the RDT kits were excellent with fresh samples of whole blood (range, 96% to 100.0%) and serum (range, 96.3% to 100.0%) (Table 3). Most RDTs showed excellent specificity with whole blood (range, 90.8% to 100.0%). The exception was the Onsite *Leishmania* Ab RevB from CTK Biotech, which had specificities of only 33.6% in India and 49.3% in Nepal. The specificities in serum samples were similar to those in whole blood and ranged from 90.1% to 100%, with the exception of those of the Onsite *Leishmania* Ab RevB test, which showed specificities of 42.0% in India and 48.7% in Nepal (Table 3). Kappa indices for agreement of test results between fresh samples of whole blood and serum were excellent for all RDTs in both countries (kappa, >0.91), except with the Onsite *Leishmania* Ab RevB test, which showed only moderate agreement (kappas, 0.44 and 0.67 in India and Nepal, respectively) between blood and serum (Table 4).

Kappa indices for agreement between the readings of the different laboratory technicians with whole blood and serum ranged from 0.83 to 0.99 and 0.76 to 0.99, respectively.

Following storage and transportation of the Indian samples to the reference laboratory in BHU Varanasi, the sensitivities and specificities of nearly all the test kits were comparable. The sensitivities of RDTs for whole-blood samples ranged from 94.7% to 100.0%, and the specificities (with the exclusion of that of the Onsite *Leishmania* Ab RevB test) ranged from 95.8% to 99.2% in healthy controls from areas of endemicity and 95 to 100.0% in control patients with other diseases. The poor specificity of the Onsite *Leishmania* Ab RevB test was corroborated (52.9%). For serum samples, the sensitivities of all the tests ranged from 96.7% to 99.3% (Table 5), and the specificities ranged from 92.4% to 98.3% in healthy controls from areas of endemicity and 95.7 to

TABLE 3 Sensitivities and specificities of VL-RDTs in fresh samples of blood and serum from India and Nepal

Product	Sensitivity (n [%]) (95% CI[a]) for sample types from:				Specificity (n [%]) (95% CI) for sample types from:			
	India		Nepal		India		Nepal	
	Blood (n = 150)	Serum (n = 150)	Blood (n = 27)	Serum (n = 27)	Blood (n = 119)	Serum (n = 119)	Blood (n = 152)	Serum (n = 152)
Signal-KA	145 (96.7) (92.4, 98.9)	149 (99.3) (96.3, 100.0)	26 (96.3) (81.0, 99.9)	26 (96.3) (81.0, 99.9)	115 (96.6) (91.6, 99.1)	119 (100.0) (97.0, 100.0)	146 (96.1) (91.6, 98.5)	146 (96.1) (91.6, 98.5)
Crystal-KA	147 (98.0) (94.3, 99.6)	149 (99.3) (96.3, 100.0)	26 (96.3) (81.0, 99.9)	26 (96.3) (81.0, 99.9)	119 (100.0) (97.0, 100.0)	117 (98.3) (94.1, 99.8)	146 (96.1) (91.6, 98.5)	146 (96.1) (91.6, 98.5)
Onsite *Leishmania* Ab (RevA) rapid test	144 (96.0) (91.5, 98.5)	145 (96.7) (92.4, 98.9)	27 (100.0) (87.2, 100)	26 (96.3) (81.0, 99.9)	116 (97.5) (92.8, 99.5)	112 (94.1) (88.3, 97.6)	138 (90.8) (85.1, 94.9)	137 (90.1) (84.3, 94.4)
Onsite *Leishmania* Ab (RevB) rapid test	150 (100.0) (97.6, 100.0)	148 (98.7) (95.3, 99.8)	27 (100.0) (87.2, 100.0)	27 (100.0) (87.2, 100.0)	40 (33.6) (25.2, 42.9)	50 (42.0) (33.0, 51.4)	75 (49.3) (41.2, 57.6)	74 (48.7) (40.5, 56.9)
Kala-azar Detect	149 (99.3) (96.3, 100.0)	149 (99.3) (96.3, 100.0)	26 (96.3) (81.0, 99.9)	26 (96.3) (81.0, 99.9)	116 (97.5) (92.8, 99.5)	118 (99.2) (95.4, 100.0)	143 (94.1) (89.1, 97.3)	143 (94.1) (89.1, 97.3)
DiaMed-IT LEISH	148 (98.7) (95.3, 99.8)	148 (98.7) (95.3, 99.8)	27 (100.0) (87.2, 100)	26 (96.3) (81.0, 99.9)	118 (99.2) (95.4, 100.0)	118 (99.2) (95.4, 100.0)	142 (93.4) (88.2, 96.8)	141 (92.8) (87.4, 96.3)

[a] CI, confidence interval.

TABLE 4 Agreement between RDT results for blood and serum in fresh and stored samples

Product	Agreement index (Cohen's kappa) (% [95% CI]) for blood and serum in samples from:		
	India		Nepal
	Fresh	Stored	Fresh
Crystal-KA	94.5 (90.4, 98.5)	95.2 (91.5, 99.0)	100
Signal-KA	96.8 (93.7, 99.9)	96.8 (93.7, 99.9)	100
Onsite *Leishmania* Ab (RevA)	91.3 (86.3, 96,3)	92.1 (87.3, 96.8)	93.6 (87.5, 99.8)
Onsite *Leishmania* Ab (RevB)	44.5 (30.4, 58.5)	45.9 (33.3, 58.5)	66.7 (55.5, 77.7)
Kala-azar Detect	98.4 (96.2, 100)	96.8 (93.7, 99.9)	100
DiaMed-IT Leish	98.4 (96.2, 100)	95.2 (91.5, 99.0)	96.6 (91.9, 100)

100.0% in control patients with other diseases (these results exclude those of the Onsite *Leishmania* Ab RevB test, which showed 50.4% specificity) (Table 5). Agreement between tests carried out on stored samples of whole blood and serum was excellent (kappa, $>$0.92), except for the Onsite *Leishmania* Ab RevB test, which showed moderate agreement (kappa, 0.45) between blood and serum (Table 4). There was equally excellent agreement between tests performed on fresh material in the field clinic (KAMRC) and those performed on frozen material in the reference laboratory with blood (kappa, $>$0.93) as well as with serum (kappa, $>$0.89) except for the Onsite *Leishmania* Ab RevB test, which showed poor agreement (kappa, 0.30 to 0.42) between fresh and stored samples for both blood and serum.

DISCUSSION

This study showed excellent agreement between RDTs for VL done with blood and serum except for one brand that had inadequate specificities with serum as well as blood samples. Very encouraging was the fact that between-reader agreement in the same laboratory was excellent, as was the between-laboratory agreement observed in India, even in a comparison of fresh and stored samples. The sensitivity and specificity of RDTs in general are dependent on the quality of preparation and reading of the test. Generally, a simpler format with fewer steps or fewer required materials is likely to be conducted more reliably. According to Rennie et al. (20), RDTs with a cassette format are generally more reliably performed than those with a dipstick format. However, there were no apparent differences between the performances of the RDTs with dipstick and cassette formats in this study (putting aside the brand with low specificity).

This study is the first evaluation in which all the commercially available RDT kits for VL have been tested in a head-to-head comparison of fresh samples of whole blood and serum from a series of parasitologically confirmed VL cases and controls. Our findings contrast with those of Matlashewski et al. (15), who found up to 4% positive RDT results with serum that were negative with blood samples. However, in their study, the parasitological status of patients was not known and the authors attributed the discrepancy to low antibody titers in persons with an asymptomatic self-resolving infection. Our results clearly indicate that samples of whole blood can be used instead of serum for 5 of the 6 brands of RDT for VL tested, notwithstanding the fact that use of blood samples is not included in the package insert of three of these five

TABLE 5 Sensitivities and specificities of VL-RDTs of stored samples tested in the reference laboratory BHU, Varanasi, India

Product	Sensitivity (*n* [%]) (95% CI) for sample type:		Specificity (*n* [%]) (95% CI) for sample type:	
	Blood (*n* = 150)	Serum (*n* = 150)	Blood (*n* = 119)	Serum (*n* = 119)
Signal-KA	145 (96.7) (92.4, 98.9)	147 (98.0) (94.3, 99.6)	114 (95.8) (90.5, 98.6)	117 (98.3) (94.1, 99.8)
Crystal-KA	148 (98.7) (95.3, 99.8)	149 (99.3) (96.3, 100.0)	118 (99.2) (95.4, 100.0)	113 (95.0) (89.4, 98.1)
Onsite *Leishmania* Ab (RevA) rapid test	142 (94.7) (89.8, 97.7)	145 (96.7) (92.4, 98.9)	117 (98.3) (94.1, 99.8)	110 (92.4) (86.1, 96.5)
Onsite *Leishmania* Ab (RevB) rapid test	149 (99.3) (96.3, 100.0)	149 (99.3) (96.3, 100.0)	63 (52.9) (43.6, 62.2)	60 (50.4) (41.1, 59.7)
Kala-azar Detect	150 (100) (97.6, 100.0)	149 (99.3) (96.3, 100.0)	114 (95.8) (90.5, 98.6)	115 (96.6) (91.6, 99.1)
DiaMed-IT LEISH	149 (99.3) (96.3, 100.0)	148 (98.7) (95.3, 99.8)	117 (98.3) (94.1, 99.8)	112 (94.1) (88.3, 97.6)

brands. One of the 6 tests, the Onsite *Leishmania* Ab RevB kit, which was recommended for use on blood and serum in the manufacturer's instructions, performed poorly overall in India as well as Nepal, with low specificity, and clearly is not suitable for use for diagnosis of VL on the Indian subcontinent.

In conclusion, our results corroborate previous findings about the good performance of rK39- and rKE16-based RDTs in the Indian subcontinent. All the RDTs except one brand showed excellent sensitivities and specificities. Agreement between (i) serum and whole blood, (ii) two readers per test, and (iii) two Indian laboratories was excellent except for the brand with low specificity. In peripheral laboratories, whole-blood samples can be used with sensitivities and specificities similar to those of RDTs conducted on serum samples.

ACKNOWLEDGMENTS

We thank all the staff of the KalaAzar Medical Research Centre (KAMRC), a unit of the Sitaram Memorial Trust, for their assistance in collection of the samples used in this evaluation. We acknowledge, in Nepal, Saru Devkota, Iccha Ghale, Ganesh Sah, and other staff and nurses from the tropical ward at the B.P. Koirala Institute of Health Sciences, Dharan, Nepal.

This work was supported by the NIAID (NIH grant P50AI074321). Dinesh Kumar and Puja Tiwary thank the Council of Scientific and Industrial Research (CSIR), New Delhi, and Shyam Lal Mudavath thanks the University Grants Commission, New Delhi, India, for providing fellowships. The study was also supported by a grant from the Belgian Development Cooperation under the 3rd Framework Agreement DGDC-ITM, Strategic Project "3.04 VL control."

We declare no conflicts of interest.

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