

PERFORMANCE OF RECOMBINANT K39 ANTIGEN IN THE DIAGNOSIS OF BRAZILIAN VISCERAL LEISHMANIASIS

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Abstract. This study evaluated the performance of recombinant K39 (rK39) antigen in an immunochromatographic format (strip test) and a crude antigen enzyme-linked immunosorbent assay in the diagnosis of Brazilian visceral leishmaniasis (VL) in 128 consecutive patients with parasitologically proven infections (by microscopy and/or culture). For each patient, a medical history was obtained and a complete physical examination was performed. Controls included 10 healthy volunteers and 50 patients with other diseases: malaria (10), leprosy (9), Chagas' disease (10), tuberculosis (10), and cutaneous leishmaniasis (11). The sensitivities of the rK39 antigen strip test and the ELISA were 90% and 89%, respectively, while the specificities were 100% and 98%, respectively. Our study confirms the accuracy of the rK39 antigen strip test in the diagnosis of VL in a high prevalence population.

INTRODUCTION

Leishmaniasis is a group of infections of the skin, mucous membranes, and viscera caused by intracellular protozoa and transmitted by sandflies. Visceral leishmaniasis (VL, kala-azar) has a worldwide distribution and affects up to a half million children and adults in both the third and first world settings.¹ This disease, which presents with fever, gradual weight loss, splenomegaly, hypergammaglobulinemia, and pancytopenia, is uniformly fatal if left undiagnosed and untreated.² Thus, a simple method of diagnosis is essential. Equally important, however, is the accuracy of diagnosis since anti-*Leishmania* therapy often results in significant morbidity and even mortality.³

A definitive diagnosis of VL currently requires demonstration of parasites by smear or culture from tissue, usually bone marrow or spleen. Both require invasive procedures necessitating expertise to prevent pain and hemorrhage. Furthermore, the sensitivity is suboptimal, ranging from 70% for bone marrow aspirates to 90–95% for spleen aspirates.⁴

Much progress has been made in the development of less invasive tests to assist in the diagnosis of VL.^{5–8} A complement fixation test, an indirect fluorescent antibody test, an enzyme-linked immunosorbent assay (ELISA), and a polymerase chain reaction test that amplifies *Leishmania* DNA are a few examples of these efforts. Unfortunately, all of these tests have one of two drawbacks: significant non-specific cross-reactivity and a lack of portability as a result of substantial requirements for laboratory equipment.

A recent development has been the identification of the K39 *Leishmania* antigen, a member of the kinesin family of proteins. Detection of IgG antibodies to this antigen has been found to be extremely sensitive and specific in the diagnosis of VL. The cloning of the K39 antigen has resulted in the synthesis of recombinant K39 (rK39) antigen, which was then placed on cellulose strips to be used in the field for the diagnosis of VL.⁹ In this study, we compare the results of an rK39 antigen test (strip test) with an in house ELISA (using whole parasite as antigen) in 128 consecutive cases of VL from the states of Minas Gerais and Espírito Santo in Brazil.

MATERIALS AND METHODS

Patients. As part of ongoing evaluation studies of the epidemiology and treatment, serum were obtained from 128 con-

secutive patients with amastigote-positive bone marrow aspirates and stored at -70°C . Patients were enrolled at Universities Hospitals in Montes Claros, Minas Gerais and Vitória, Espírito Santo. For each patient, a medical history was obtained and a complete physical examination was performed. In addition, control serum from 60 patients with malaria (10), leprosy (9), Chagas' disease (10), tuberculosis (10), cutaneous leishmaniasis (11), and healthy controls (10) was similarly stored. All diagnoses were confirmed by microscopy and/or culture. Approvals for testing was obtained through the Institution Review Boards at both the Universidade Federal do Espírito Santo and the Universidade Estadual de Montes Claros. Informed consent was obtained from all patients and parents or legal guardians of minors.

Kala-azar Detect test. The Kala-azar Detect[®] test is an immunochromatographic test manufactured by InBios International (Seattle, WA). It was designed for the qualitative detection of antibodies against *Leishmania (L.) chagasi* rK39 antigen in serum during active infection.

Twenty microliters of serum were mixed with two drops of buffer provided with the test and placed on a cellulose strip. Following the manufacturer's instructions, a test result was positive when two bands, a control band and a positive test band, appeared within 10 minutes. The test result was negative if only the control band appeared. The test is qualitative and the manufacturer indicates that a faint band should be considered a positive result. An investigator blinded to the patients' underlying disease evaluated all tests.

ELISA. *Leishmania chagasi* (MHON/BR/74/PP75) parasites were cultivated in Schneider's medium supplemented with 25% fetal calf serum at 26°C . The promastigote pellet was collected by centrifugation, washed three times with phosphate-buffered saline (PBS), and lysed by freeze thawing. The parasite suspension was homogenized using a tissue grinder in an ice bath. The homogenized material was centrifuged at $10,000 \times g$ for 20 min at 4°C . The supernatant was collected and used as crude soluble antigen. The protein content of the sample was estimated by the Bradford method.¹⁰

Briefly, Immulon #4 plates (Dynatech, Chantilly, VA) were coated with $2 \mu\text{g}$ of *L. chagasi* crude antigen per well and incubated overnight at 4°C . The plates were then aspirated, blocked with 2% nonfat dried milk in PBS containing 0.05% Tween 20 (PBS-T) for one hour at 37°C , and washed four times with PBS-T. Sera diluted 1/200 in PBS-T containing 1%

nonfat dried milk were added to wells and incubated for 45 minutes at 37°C. The wells were washed four times and bound antibodies were detected with horseradish peroxidase-conjugated anti-IgG (Kirkegaard and Perry, Gaithersburg, MD) diluted 1/5,000 after incubation for 45 minutes at 37°C. Plates were then washed four times and incubated with *o*-phenylenediamine and H₂O₂ in citrate buffer for 15 minutes. The optical density was then read at 492 nm. Positive and negative controls sera were run in each plate to standardize the readings and plate variations. The cut-off point between negative and positive readings was calculated as the mean of the negative controls plus three standard deviations.

RESULTS

Serum from 128 patients with microscopy-confirmed VL underwent testing with both the Kala-azar Detect strip test and the ELISA. One hundred twenty-three of the 128 patients had positive cultures for *L. (L.) chagasi* as determined by isoenzyme analysis and testing with monoclonal antibodies. In addition, serum from 60 controls (10 healthy individuals and 50 patients with endemic diseases other than VL) underwent similar testing. Finally, serum gamma-globulin levels were determined in all patients with VL. Demographic data is shown in Table 1. Note that Brazilian VL is a disease of young children presenting early in the course of their disease, usually around the first month of illness.

The sensitivities of both the rK39 antigen strip test and the ELISA were high: 90% versus 89%, respectively, while the specificities of both tests was outstanding: 100% versus 98%, respectively (Table 2).

Of the 13 patients with VL and negative rK39 antigen strip test results, the duration of disease ranged from 14 to 300 days; (mean = 86.5, median = 30). The gamma-globulin levels ranged from 1.0 to 4.8 mg/dl (mean = 2.3, median = 2.2); and 31% (4 of 13) also had negative ELISA test results.

Follow-up testing at 12 months of all 128 patients with VL revealed a cure rate of 100%. The rK39 antigen strip testing was also performed on a random sample of previously positive patients at two months and six months. Nineteen (63%) of 30 patients still had positive test results at two months and 14 (46%) of 30 were still positive at six months. No further testing was performed at the 12-month follow-up.

DISCUSSION

Our results confirm the usefulness of the rK39 antigen strip test in diagnosis of VL. With a specificity of 100% in patients with VL, this simple diagnostic test may replace more invasive diagnostic methods in the initial evaluation of patients with suspected VL. Unfortunately, patients who have negative re-

TABLE 1
Demographic data of 128 patients with visceral leishmaniasis

Male/female ratio	1.4/1
Age (years)*	7.4/3 (2 months–48 years)
Duration of disease (days)*	64/30 (6–365)
Gamma-globulin (0.5–1.6 mg/dl)*	2.3/1.9 (0.8–5.9)

* Values are the mean/median (range).

TABLE 2

Performance of the recombinant K39 (rK39) antigen test and an ELISA in the diagnosis of visceral leishmaniasis (VL) caused by *Leishmania (L.) chagasi**

	VL patients (n = 128)	Healthy controls (n = 10)	Other infections† (n = 50)	Sensitivity/ specificity (%)
rK39 antigen test				
Positive	115	0	0	90/100
Negative	13	10	50	
ELISA				
Positive	114	0	1	89/98
Negative	14	10	49	

* ELISA = enzyme-linked immunosorbent assay.

† Malaria (10), leprosy (9), Chagas' disease (10), tuberculosis (10), and cutaneous leishmaniasis (11).

sults on an rK39 antigen strip test still have a significant incidence of VL (10%).

A comparison of our results with those of five previous studies showed significant variation. Sensitivities of the rK39 antigen strip test range from 67% to 100%. The highest sensitivities (100%) occurred in patients from India and Nepal.^{11–13} Patients in Venezuela had significantly lower percentages of true positive test results (88%), and the rK39 antigen strip test was least sensitive in patients from Sudan and other areas.^{12–14} The results of this comparison using sera or blood as a test specimen are shown in Table 3.

There are several hypotheses that could explain this regional variation of the results of the rK39 antigen strip test. First, there may be differences in the test accuracy between subspecies of the *L. donovani* complex. Similarly, within these subspecies, there may be regional differences as a result of variations in the rK39 antigen. Another possible explanation involves genetic differences in individual patients or in racial subgroups. Similar to differences in host response in patients exposed to *L. (L.) chagasi*, in which only 95% of the patients develop progressive disease, there may be unidentified genetic factors mediating the degree of host immune response.¹⁷

Other factors affecting the level of antibody response also may explain the regional differences observed. For example, Zijlstra and others showed that patients with positive rK39 antigen strip test results had significantly higher levels of IgG to rK39 antigen as determined by ELISA than patients with negative strip test results ($P < 0.0001$).⁸ This could be due to epidemiologic factors such as length or severity of disease. Unfortunately, although we know the length of illness in the present study (median = 30 days), similar data from the other five studies is unavailable. Interestingly, however, there was no difference in the present study in the duration of disease in the 13 patients with negative rK39 antigen strip test results and in the 115 with positive test results. In addition, there was no quantitation of disease severity or degree of malnutrition in any of the six studies compared.

In contrast to variations in sensitivities from region to region, the rK39 antigen strip test has uniformly high specificity. Of 379 patients with VL in these six studies, only six patients with positive rK39 antigen strip test results did not have microscopic/culture confirmation of the disease, and all six may have had active or incubating disease.

In evaluating the use of the rK39 antigen strip tests in patients who have completed treatment of VL, we found that a significant number (46%) remained positive for up to six

TABLE 3
Performance of the recombinant K39 (rK39) antigen in the diagnosis of visceral leishmaniasis (VL)*

Region (Reference)	No. of subjects enrolled	VL	rK39 positive	Sensitivity	Specificity
Nepal (11)	127	14	14	100%	100%
Sudan (13)	116	55	37	67%	98%
Venezuela (12)	117	41	36	88%	100%
Other (14)	96	14	10	71%	100%
India† (9)	348	127	127	100%	98%
Present study	188	128	115	90%	100%

* Results are summaries of published data using serum or blood as the test specimen.

† Whole blood instead of serum was used in this study. Four patients with presumed but unproven VL were false positives.

months. These results are similar to those of Zijlstra and others, in which a follow-up at 12 months failed to show a significant decrease in positivity rates.⁸ In contrast, rK39 antigen ELISA titers tend to mimic the clinical course that patients follow during and after treatment for VL.

In addition to evaluating the accuracy of the rK39 antigen test in the study population, we also evaluated the accuracy of the ELISA in the same 188 individuals. The sensitivity and specificity of the ELISA were similar to those of the rK39 antigen strip test (89% and 98%, respectively). Since 13 patients with negative rK39 antigen strip test results had positive ELISA results, combining the results of both tests increases the overall sensitivity to 96%. However, only a small change in the specificity (98%) was observed.

One area that has not been adequately evaluated in our study or in other studies is the accuracy of the rK39 antigen strip test in patients with VL who are also infected with human immunodeficiency virus (HIV). Infection with *Leishmania* is now occurring more frequently in patients infected with HIV. This presents the clinician with two difficulties in making a diagnosis. First, VL is easily confused with other complications of acquired immunodeficiency syndrome (AIDS), such as disseminated *Mycobacterium avium* complex and lymphoproliferative disorders. Second, the course of VL is more rapid in patients infected with HIV, thus complicating the diagnosis.¹⁶ Although none of the six studies compared had information concerning the HIV status of the study population, a study that tested the rK39 antigen ELISA in patients infected with HIV, both with and without AIDS, reported that the sensitivity of the ELISA decreased (76–87% in both groups), but did not report any effect on the specificity.¹⁷ Obviously, prospective analysis of the rK39 antigen strip test in HIV patients living in endemic areas for VL is badly needed.

A drawback to the present study is the relatively small number of controls. In a clinical setting, 90% or more of the patients tested will have diseases other than VL. Because diagnostic characteristics of a test (such as positive and negative predictive values) vary from population to population, it will be important to evaluate the rK39 antigen strip test in low prevalence populations, such as patients from non-endemic areas and travelers.

In conclusion, the present study confirms the accuracy of the rK39 antigen strip test in the diagnosis of patients with VL, without a concurrent infection with HIV, in a high prevalence population. More studies are needed to confirm its positive and negative predictability in more relevant populations. Only after these studies are completed will we be able to use the test with confidence in both endemic areas and in travelers.

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The diagnosis of visceral leishmaniasis (VL), a serious and often fatal parasitic disease caused by members of *Leishmania donovani* complex, remains problematic. Current methods rely on clinical criteria, parasite identification in aspirate material and serology. The latter methods use crude antigen preparations lacking in specificity. In fact in the case histories illustrated above, all the above-mentioned methods failed to clinch the diagnosis. The patient in Case 2 had a more rapid diagnosis because of our experience with the first patient.

3. Carvalho SF, Lemos EM, Corey R, Dietze R. Performance of recombinant K39 antigen in the diagnosis of Brazilian visceral leishmaniasis. *Am J Trop Med Hyg.* 2003;68:321-4. The ideal method for diagnosis of visceral leishmaniasis in all studies was a positive result on microscopical examination of lymph node, bone marrow, or spleen aspirate. Diagnosis was also confirmed by detection of parasites in culture (n = 4 studies) or by polymerase chain reaction (n = 1) or a good clinical response to antileishmanial drugs in patients with clinically suspected disease who had positive serology results (n = 4). Results of the quality assessment of studies of diagnostic accuracy evaluation are on bmj.com.

The direct agglutination test and the rK39 dipstick showed similar diagnostic performance for visceral leishmaniasis, ranging from good to excellent. The performance of dipstick tests for diagnosis in Sudan needs to be improved.

Footnotes. The serological diagnosis of visceral leishmaniasis relies on the recombinant K39 antigen expressed in *Escherichia coli* (Faria and de Andrade, 2012). The K39 protein is predominant in the amastigote stage of *L. infantum*, and it belongs to the kinesin superfamily. These proteins comprise a class of eukaryotic motor proteins involved in microtubule-based movements. The tandem repeats of the kinesins are responsible for protein-protein interaction and its oligomerization inside the cell (Verhey et al., 2011). The laboratory diagnosis of visceral leishmaniasis is based on microscopic examination, culture, serological tests, and molecular methods. In this study, w.

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A firm diagnosis of kala-azar/ visceral leishmaniasis (VL) requires demonstration of the parasite in splenic or bone marrow aspirate. But getting biopsy sample is an invasive test so different antigen/antibody detection methods are in use for the diagnosis of visceral leishmaniasis.

Antibody detection tests. Using K39 antigen-impregnated nitrocellulose strips developed for field conditions, fingerstick-obtained blood and serum samples demonstrated a sensitivity of 100% and a specificity of 97%. The strip testing proved simple to perform and yielded results within five minutes.

Antigen-detection tests A heat stable, low molecular weight carbohydrate antigen is detected by latex agglutination test in VL patients. It showed good specificity but only low to moderate (48-87%) sensitivity.