THE SENSITIVITY AND SPECIFICITY OF LEISHMANIA CHAGASI RECOMBINANT K39 ANTIGEN IN THE DIAGNOSIS OF AMERICAN VISCERAL LEISHMANIASIS AND IN DIFFERENTIATING ACTIVE FROM SUBCLINICAL INFECTION


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Abstract. The sensitivity and specificity of a Leishmania chagasi recombinant K39 (rK39)-based enzyme-linked immunosorbent assay (ELISA) for visceral leishmaniasis (VL) was assessed in Natal, Brazil. Anti-rK39 antibodies were detected in 93.3% of patients with parasitologically confirmed VL (n = 120) and in 33 others with clinically diagnosed disease. Anti-rK39 antibodies decreased significantly following treatment. The presence of antibodies was inversely correlated with development of a positive leishmanin skin test result. Anti-rK39 antibodies were detected in only 2.9% of asymptomatic subjects with a positive skin test result (n = 168). They were not detected in healthy controls (n = 30) or in persons with Chagas’ disease (n = 13) or active tuberculosis (n = 31). Antibodies were found in only one of 13 patients with cutaneous leishmaniasis. In contrast, an ELISA using total L. chagasi promastigote antigen was sensitive, but not specific. The results indicate that the rK39-based ELISA is a sensitive and specific diagnostic test for symptomatic VL and can differentiate progressive from self-resolving infection.

INTRODUCTION

Visceral leishmaniasis (VL) is an important protozoan disease in Latin America, Africa, India, and southern Europe. Although a number of Leishmania species can visceralize, three closely related ones account for the vast majority of cases of VL: L. donovani, L. infantum, and L. chagasi.1 During the past 20 years, major epidemics have occurred in the Sudan and western India.1 The likelihood of developing symptomatic infection is higher in persons immunocompromised by neoplastic diseases, chemotherapy, or infection with human immunodeficiency virus (HIV), as shown by the large number of cases of VL reported initially from southern Europe in persons with acquired immunodeficiency syndrome (AIDS).2–4 In Brazil, the pattern of VL has expanded from sporadic rural cases to include major periurban outbreaks in large cities, where thousands of people are now at risk of infection and disease.5,6

Studies conducted in northeastern Brazil indicate that the majority of those infected with L. chagasi are capable of mounting effective cell-mediated immune responses and have asymptomatic or subclinical, self-resolving courses.7,8 In a cohort of children who seroconverted in the state of Ceará, only 12% developed clinically apparent VL with the longest incubation period being 14 months.8–10

The standard approach to the diagnosis of VL has been the identification of amastigotes in tissue or promastigotes in cultures of splenic or bone marrow aspirates.1 Procedures used to obtain these specimens can be painful, and in the case of splenic aspiration, life-threatening hemorrhage has occurred in rare instances. Specialized laboratory equipment and staff are required to interpret results. Less invasive, quicker, and more reliable diagnostic measures are needed.

Although not protective, high titers of anti-leishmanial antibodies are typical in patients with VL. An indirect immunofluorescent antibody (IFA) test and a direct agglutination test (DAT) using Leishmania promastigotes have been developed for diagnosis.11,12 In Brazil the IFA test has been used by the Ministry of Health for testing dogs, which are presumed to be the most important reservoir for L. chagasi.1 In recent years, enzyme-linked immunosorbent assays (ELISAs) have replaced the IFA test and the DAT in humans.13 However, there have been concerns about the specificity and sensitivity of these assays, which have been done with crude leishmanial antigens.14–17

A major recent advance has been the identification and production of recombinant L. chagasi antigen K39 (rK39) that when used in ELISAs appears to be sensitive and specific for the diagnosis of active VL.18–22 K39 is a member of the kinesin family with repeats of 39 amino acids. This protein is conserved among the Leishmania species most commonly associated with VL. It is expressed predominantly by amastigotes.19

In this study, we compared the sensitivity and specificity of rK39 to total L. chagasi promastigote antigen in the diagnosis of VL in a large periurban outbreak of L. chagasi in Natal, Rio Grande do Norte, Brazil. We found an inverse correlation between anti-rK39 antibodies and the development of delayed-type hypersensitivity (DTH) responses among patients treated for VL. The findings confirm and extend earlier observations that rK39 used in an ELISA is a sensitive and specific test for the diagnosis of active VL.17–19 These data indicate two ways in which the rK39 ELISA has unique value. First, it is a sensitive diagnostic test with markedly improved specificity for active VL compared with the total promastigote antigen ELISA. Second, it has potential utility for following the response to chemotherapy.

MATERIALS AND METHODS

Study area and population. The state of Rio Grande do Norte is located in northeastern Brazil. An outbreak of VL due to L. chagasi is currently occurring in the suburbs surrounding Natal, which is located on the Atlantic coast. The metropolitan area includes seven municipalities. Most of the cases have occurred in rapidly growing suburbs to the north

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and west. Lutzomyia longipalpis, the major sand fly vector for *L. chagasi* in Brazil, is endemic in this area. Unless otherwise indicated, subjects were residents of the area.

**Informed consent.** Written consent was obtained from all adults and from parents or guardians of children less than 18 years of age. Consent forms and procedures were approved by human subjects committees at the Universidade Federal do Rio Grande do Norte, the University of Virginia, and the University of Iowa.

**Study population.** Documented VL. Serum was obtained from 120 patients with VL hospitalized at the Hospital de Doenças Infecciosas “Giselda Trigueiro” and Hospital Infantil “Varela Santiago” in Natal. Patients with presumed VL in Natal are typically referred to one of these hospitals. The diagnosis was confirmed by the identification of amastigotes in bone marrow aspirates using Giemsa-stained slides. Prior studies incriminated *L. chagasi* as the cause of VL in the area and recent isolates from patients typed by a polymerase chain reaction-based assay have been identified as *L. chagasi* (Nascimento ET, unpublished data).

Probable VL. Serum was obtained from 33 subjects who had an illness clinically consistent with VL, but who had negative bone marrow aspirates. The presumptive diagnosis was based on clinical findings (fever, hepatosplenomegaly, weight loss, etc.), exclusion of other possible diagnoses, and response to pentavalent antimony therapy (Glucantime).

**Individuals recovered from VL.** Ninety-five individuals from the community with a history of VL who had been treated within the past seven years also provided serum samples. All were clinically healthy without evidence of active disease at the time during the study.

Asymptomatic *L. chagasi* infection (DTH+). Among family members and neighbors of patients with VL, there were 168 individuals who were free of symptoms and had no history of clinically apparent VL, but had a positive leishmanin (Montenegro) skin test result.

**Cutaneous leishmaniasis (CL).** Sera were obtained from 13 individuals with clinically diagnosed CL in an area endemic for *L. braziliensis* in the southwestern corner of the state of Rio Grande do Norte, 480 km from Natal. In addition to CL, VL has been sporadically reported from the area.

Chagas’ disease. Sera from 14 patients with Chagas’ disease were kindly provided by the State Blood Bank (Hemonorte).

**Tuberculosis patients.** Sera from 31 patients with bacteriologically confirmed tuberculosis were obtained from the Hospital de Doenças Infecciosas “Giselda Trigueiro”.

**Healthy subjects.** Finally, sera were obtained from 30 healthy individuals living in Natal, but outside the suburban neighborhoods where cases of VL have been reported.

**Enzyme-linked immunosorbent assay.** Microaassay plates (Costar, Cambridge, MA) were coated overnight at 4°C with *L. chagasi* (MWHO/BR/00/L669) promastigote lysate (200 ng/well) or rK39 antigen (Infectious Diseases Research Institute, Seattle, WA) (50 ng/well) or rK39 antigen (Infectious Diseases Research Institute, Seattle, WA) (50 ng/well) in coating buffer (15 mM Na2CO3, 34 mM NaHCO3, pH 9.6). Blocking was done with phosphate-buffered saline (PBS)-1% Tween-20 for 1 hr at room temperature, followed by four washes with PBS-0.1% Tween-20. One hundred microliters of sera per well were diluted to 10^-2 to 10^-5 and incubated for 1 hr at room temperature. The well was then washed with PBS-0.1% Tween-20 three times and incubated with 100 μl of protein A conjugated with horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN) at a 10^-4 dilution for 1 hr and then rinsed three times with PBS-0.1% Tween-20. After incubation for 1 hr at 37°C with 100 μl of 1 mM 2,2'-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid in 70 mM of citrate-phosphate buffer, pH4.2 (Sigma, St Louis, MO), the reaction was stopped by the addition of 100 μl of 5% sodium dodecyl sulfate. The absorbance was determined at 405 nm using a Titertek Multiskan plate reader (ICN, Aurora, OH). The cutoff was determined as the mean + three standard deviations of the absorbance of control sera (n = 30). Each serum sample was assayed in triplicate and each plate had negative and positive controls.

Delayed-type hypersensitivity test. The DTH responses to leishmanial antigen were assessed using 25 μg of *L. chagasi* protein (Infectious Diseases Research Institute, Seattle, WA) administered intradermally. Skin tests were read after 48 hr. A positive test result was defined as induration with a maximum width ≥ 5 mm.

**Anti-rK39 levels in patients with VL.** Anti rK39 levels were determined in a subset of patients with acute VL followed prospectively after treatment and in subjects previously treated for VL. Sera were collected at different time points after successful treatment.

**Statistical analysis.** A log linear model of analysis was used to determine the significance of the inverse association between the presence of anti-rK39 antibodies and the development of a positive DTH response to leishmanial antigen. This was done using Statistica software, version 6.0 (Tulsa, OK).

**RESULTS**

The rK39-based ELISA result was positive in 93.3% of the 120 patients with VL confirmed by identification of amastigotes in bone marrow aspirates. In addition, 33 individuals suspected of having VL by clinical criteria, exclusion of other diagnoses, and response to pentavalent antimony therapy, but with negative bone marrow aspirates, were also anti-rK39 positive (Figure 1). When persons with documented and probable VL were combined, the sensitivity was 94.7%.

The cut-off value using rK39 was 0.416. The mean ± SD absorbance for 30 control sera obtained from healthy individuals residing in areas without known *L. chagasi* transmission was 0.101 ± 0.09 at a 10^-2 dilution. Proven VL patients had a mean ± SD absorbance of 2.5 ± 1.04 and those with probable VL had a mean ± SD value of 1.95 ± 1.05 at that dilution. Some patients were positive at dilutions up to 10^-5.

The cut-off value using an extract of total leishmania promastigotes as the ELISA antigen was 0.26. Healthy individuals had a mean ± SD of 0.086 ± 0.061 and persons with confirmed or probable VL had a mean ± SD of 0.93 ± 0.57. Among patients with proven or probable VL, 98% or 95.6% were positive using the total extract of *L. chagasi* promastigotes, respectively (Table 1). However, the specificity was poor with the crude promastigote antigen.

In a group of patients with VL (n = 95) assessed at different times following clinically successful treatment, the presence of anti-rK39 decreased with time (P = 0.001) (Figure 2) and was inversely associated with the development of a positive DTH response (P = 0.002) (Table 2). Six months after treatment 98% (47 of 48) of the patients still had detectable anti-K39 antibodies and three of those individuals had become DTH positive. Approximately 70% (11 of 16) of the
patients had positive anti-rK39 and positive DTH responses one year after treatment. Anti-rK39 antibodies levels decreased to undetectable levels ($P < 0.001$) two years after successful treatment in 90.5% of the patients studied. Only two of 22 subjects studied more than two years after initial therapy had detectable anti-rK39 antibodies. Both were DTH positive, and neither had evidence of VL (Table 2). In general, the level of antileishmanial antibodies, as detected by the total antigen ELISA, decreased concomitantly with the level of anti-rK39 antibodies.

The rK39 ELISA also distinguished patients with VL from those with CL, Chagas’ disease, tuberculosis, and healthy controls (Figure 1). All subjects with Chagas’ disease or tuberculosis had responses to rK39 below the cut-off value. Among 13 patients with CL, only one had a positive reaction with rK39 antigen. In contrast, 66.7% of the patients with Chagas’ disease, 41.7% of those with CL, and 55.2% of those with tuberculosis were seropositive when tested by the total $L. chagasi$ promastigote antigen ELISA, although the mean ± SD optical density observed for CL and tuberculosis patients was $0.424 ± 0.143$. Overall, the specificity with the total antigen was 45.3% for these groups. This compares with a 98.3% specificity with rK39 ELISA (Table 1). Additionally, among the group of 168 individuals with asymptomatic $L. chagasi$ infection determined by positive leishmanin skin test result, 2.9% had positive responses to rK39. The responses in those individuals were low (Figure 1), with a mean absorbance of 0.75. None of the individuals developed clinical signs of VL over a seven-year period of observation.

**DISCUSSION**

K39 was first identified through immunoscreening of an $L. chagasi$ genomic library in Lambda ZAP. Sequence analysis showed it to be related to the kinesin superfamily of motor proteins. It is comprised of repetitive 39 amino acids residues. $K39$ is highly conserved and expressed in amastigotes of both $L. chagasi$ and $L. donovani$.

**Table 1**

Sensitivity and specificity of rK39 and total $Leishmania chagasi$ antigen for detecting active visceral leishmaniasis (VL) or asymptomatic infection*

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Active VL (%)</th>
<th>Active VL + probable VL (%)</th>
<th>Asymptomatic infection (DTH+) (%)</th>
<th>Healthy individuals (%)</th>
<th>Other diseases (%)</th>
</tr>
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<tbody>
<tr>
<td>Total antigen</td>
<td>106/108 (98)</td>
<td>131/137 (95.6)</td>
<td>14/168 (8.3)</td>
<td>0/30 (0)</td>
<td>29/53 (54.7)</td>
</tr>
<tr>
<td>rK39</td>
<td>112/120 (93.3)</td>
<td>145/153 (94.7)</td>
<td>5/168 (2.9)</td>
<td>0/30 (0)</td>
<td>1/58 (1.7)</td>
</tr>
</tbody>
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* DTH = delayed-type hypersensitivity.
† Percent of total positive cases (sensitivity).
‡ Percent of cases of Chagas’ disease, tuberculosis, or cutaneous leishmaniasis that were positive.
Recombinant K39 has been used to diagnose acute VL in several geographic regions. The current study is the largest to date to determine the kinetics of anti-rK39 antibody responses in patients treated for VL and to assess its presence in individuals with subclinical \textit{L. chagasi} infection. A review of all patients treated for VL (n = 323) over a four-year period in Natal indicated that only 83% were confirmed parasitologically by bone marrow aspiration (Nascimento ET, unpublished data). The sensitivity of the rK39 ELISA in detecting parasitologically confirmed cases of VL was 93.3%. In addition, all 33 individuals suspected of having VL, but without parasitologic confirmation by bone marrow aspirate, were positive in the rK39 assay. All of those patients were successfully treated with pentavalent antimony, confirming the clinical diagnosis. When persons with documented and probable VL were combined, the sensitivity was 94.7%.

Cross-reactivity between leishmanial, trypanosomal, and mycobacterial antigens has been a substantial problem in serologic tests using crude promastigote antigens, but not with rK39. In our study, no cross-reactivity with rK39 antigen was observed in sera from healthy controls living outside the endemic area, patients with Chagas’ disease, or those with tuberculosis (Figure 1). Of note, one patient with CL was positive. The absorbance was low in that case, and the subject lived in a region far from Natal, where both \textit{L. braziliensis} and \textit{L. chagasi} are endemic. This may represent a cross-reacting response to \textit{L. braziliensis} or it could be due to subclinical infection with \textit{L. chagasi}. In our large group of patients with active VL, the sensitivity and specificity of rK39 ELISA were 94.7% and 98.2%, respectively, compared with a sensitivity of 98% and a specificity of 45.3% for the total \textit{L. chagasi} promastigote antigen ELISA in patients with Chagas’ disease, CL, or tuberculosis. However, concurrent infection with \textit{L. chagasi} cannot be ruled out in those groups.

Thus, the true value of the rK39 ELISA is that it provides a sensitive and highly specific assay for the diagnosis of active VL. It is far more sensitive, safer, and less painful than traditional parasitologic examination of bone marrow aspirates. It is hypothesized that the sensitivity of rK39 antigen is related to the high density of B cell epitopes on the protein. Of note, rK39 based-assays have also been reported to be sensitive in patients with active VL elsewhere, including those with HIV in southern Europe, where 1.5–9.5% of AIDS patients are co-infected with \textit{L. infantum}, although false-negative results may occur in that setting.

Only 2.9% of the asymptomatic patients with positive DTH responses to leishmanial antigens were positive for anti-rK39 antibodies. This group had lower levels of anti-rK39 antibodies; only one had an absorbance in the range of those with VL (Figure 1). Further studies are needed to determine the time course of development of DTH responses after infection in asymptomatic individuals, how long that response is maintained, and whether the response is maintained by a small number of parasites harbored by infected individuals or because of re-exposure.

The reciprocal relationship between antibodies to rK39 and the DTH response is intriguing (Table 2). This is reminiscent of findings in murine \textit{L. major} infection, in which a TH-2 response promoting production of certain antibodies is associated with disease exacerbation, whereas a TH-1 response, which correlates with the DTH reaction, is associated with cure. In the future, it would be of interest to test immunoglobulin subtypes to determine whether similar TH-1 and TH-2 reciprocity is seen in human subjects.

In conclusion, the findings extend earlier reports that rK39 used in an ELISA is a sensitive and specific test for the diagnosis of active VL and discriminates between persons with asymptomatic \textit{L. chagasi} infections and those with progressive disease. Our data further indicate that the rK39 ELISA is of similar sensitivity, but dramatically improved specificity in comparison with total leishmanial antigen. There is a progressive decrease in rK39 antibody levels in the months following clinically successful chemotherapy. It is therefore possible that the test could be useful not only for diagnostic purposes, but also to follow the progression of disease.

Acknowledgments: We thank all patients and subjects residing in the endemic area of perimetropolitan Natal for their participation in this study. We also thank Dr. Telma Souza (Secretaria de Saude do Estado) for the bone marrow data, Manoel Fernandes (Fundaçao Nacional de Saude) for location of remote VL patients, Professor José Wilton Queiroz for statistical analysis, and the collaboration of medical and pharmacy students Vanessa G. Pinheiro, Janaína A. Alves, Olivia M. N. Souza, and Luana C. Ferreira.

Financial support: This work was supported by a grant awarded by TMRC (National Institutes of Health [NIH]), by grant R03 TW01369 from the NIH Fogarty Foundation, and by Conselho Nacional de Pesquisa (Projeto Nordeste Brazil). Regina F. S. Braz received a fellowship from CAPES and PPG (UFRN) awarded medical and pharmacy students with fellowships.

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