Specificity of the rapid rK39 antigen-based immunochromatographic test Kalazar Detect® in patients with cutaneous leishmaniasis in Brazil

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The aim of this study was to evaluate the specificity of a rapid immunochromatographic test that was developed to detect antibodies against the rK39 antigen for the diagnosis of visceral leishmaniasis (VL). This evaluation was performed using sera from patients with a confirmed diagnosis of active cutaneous leishmaniasis. The sera from 272 patients with a confirmed diagnosis of localised cutaneous leishmaniasis (CL) who resided in an area endemic for Leishmania braziliensis *in Brazil were obtained before the initiation of antileishmanial treatment. Kalazar Detect® (InBios, Seattle, WA) recombinant K39 antigen-based immunochromatographic strips were used according to the manufacturer's instructions. The test results were evaluated independently by two examiners in sequential order. The positive controls for the test included five serum samples from five patients with parasitologically confirmed diagnosis of VL caused by* Leishmania infantum *in Brazil. Overall, 100% of the samples obtained from patients with CL were negative, confirming the absence of a serological cross-reaction for individuals with cutaneous disease when these patients were evaluated using the rapid test. The lack of a cross-reaction in patients who were infected by parasites of the same genus highlights the specificity of the rK39 antigen for the diagnosis of VL in areas with the sympatric circulation of* L. braziliensis *and* L. infantum.

Key words: cutaneous leishmaniasis - *Leishmania braziliensis* - *Leishmania infantum* - rK39 antigen - specificity - sympatry

According to recent estimates, approximately 0.2-0.4 million visceral leishmaniasis (VL) cases and 0.7-1.2 cutaneous leishmaniasis (CL) cases occur each year worldwide. More than 90% of global VL cases occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Stauch et al. 2011, Alvar et al. 2012). In Brazil, VL is caused by the protozoan *Leishmania infantum* (syn. *Leishmania chagasi*) (Schönian et al. 2010). The geographical distribution of VL in Brazil has increased in recent years and affected metropolitan areas, with an increased trend in lethality from 3.4% in 1994 to 5.7% in 2009 (MS 2011). Moreover, an increasing number of regions are endemic for CL and VL (Carranza-Tamayo et al. 2010). Because the use of rapid, accurate and reproducible tests for the prompt diagnosis of VL is required, it is important to investigate the specificity of tests for VL in regions with the sympatric circulation of dermotropic *Leishmania* species. *Leishmania braziliensis* is of particular relevance because of its CL disease burden (Alvar et al. 2012) and its geographical distribution in Latin America, which is the widest among all New World *Leishmania* species (Grimaldi & Tesh 1993).

Conventional VL diagnosis is based on the direct visualisation of amastigotes in bone marrow smears, lymph node aspirates and liver biopsy specimens. In addition, the culture and isolation of the parasite can be used for the diagnosis of VL using these tissues and, more recently, the amplification of several DNA sequences by polymerase chain reaction (PCR) has been used. In addition to being invasive, these methods require well-equipped laboratories, which are not available in most endemic areas. Another limitation in the diagnosis of VL is the low specificity of the serological tests that use crude antigens (de Assis et al. 2008). However, several purified, synthetic or recombinant antigens have been identified. Among them, the K39 recombinant protein has been extensively evaluated and has exhibited high specificity and sensitivity when used in immunoenzymatic assays (ELISAs). Using the K39 antigen in immunochromatographic platforms has many advantages. These tests are fast and easy to perform and the results are available in less than 20 min on average (Boelaert et al. 2007). Studies on rapid tests for VL have found sensitivity and specificity values that range from 67-100% and 59-100%, respectively (Schallig et al. 2002, Carvalho et al. 2003). However, there are variations among geographic regions and commercially available tests (WHO 2011). Studies have reported false positives when using the rK39 antigen due to cross-reactivity with other protozoans (Schallig et al. 2002, Sundar et al. 2002). A meta-analysis that involved 13 research centres that used the rK39 rapid test for the diagnosis of VL yielded average sensitivity and specificity values of 93.9% and 90.6%, respectively (Chappuis et al. 2006). Studies in the Middle East that used the rapid test based on the rK39 antigen indicated that the positivity rate was as high as 20% using serum from patients with CL (Hartzell et al. 2008). The aim of this study was

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to evaluate the specificity of the rapid test for VL in patients with a confirmed diagnosis of localised CL (LCL) in an area that is endemic for *L. braziliensis*.

SUBJECTS, MATERIALS AND METHODS

Participants - Serum samples from 272 patients with a confirmed diagnosis of LCL were evaluated. The LCL diagnosis was confirmed by parasite culture or by kDNA detection with PCR using material that was obtained from ulcerated lesions, as previously reported (Ampuero et al. 2009). The patients were from the south mesoregion of the state of Bahia, Brazil, an area that is endemic for *L. brazi-liensis* (Rosa et al. 1988, Romero et al. 2001). The patients were of both sexes (182 men and 90 women) and they had active disease with single or multiple skin lesions. The mean age was 23.4 years (7-50 years) and skin lesions developed over an average of 42.8 days (8-120 days). The number of lesions ranged from one-nine. Overall, 75% of patients exhibited some form of intestinal helminthiasis. Blood was obtained from these patients by venipuncture. The serum was separated at room temperature and kept at -20ºC until the completion of the serological analysis. Sera were collected before the initiation of specific treatment for the cutaneous disease. All of the patients participated in a clinical trial to test the efficacy of low doses of pentavalent antimony (ClinicalTrials.gov - NCT00317980).

Measures and data analysis - Kalazar Detect® (InBios, Seattle, WA) recombinant rK39 antigen-based immunochromatographic strips were used according to the manufacturer's instructions. The test results were evaluated independently by two examiners in sequential order. A total of five serum samples from patients with parasitologically confirmed diagnosis of VL caused by *L. infantum* were used as positive controls. The statistical analysis was performed using Statistical Package for the Social Sciences software 15.0 (Chicago, IL).

Ethics - The study was conducted according to the Declaration of Helsinki and the recommendations of Resolutions 196/96 and 347/2005 of the National Health Council. The project was approved by the Ethical Committee on Human Subjects Research at the Faculty of Medicine of the University of Brasilia (process CEP-FM 012/2009). The authors have no competing interests to disclose.

RESULTS

All of the 272 serum samples from patients with LCL were negative according to the rapid test. The five samples from patients with VL were positive according to the rapid test. Overall, four samples from patients with LCL had inconclusive results because the internal control band on the strip could not be properly visualised. The four sera with inconclusive results were retested using new strips and the results were negative. Therefore, there was no serological cross-reaction with *L. braziliensis* in patients with LCL submitted to VL testing. The results determined by the two independent examiners had a crude concordance of 100% for the evaluation of the 272 samples from patients with LCL. The estimated point specificity of the rapid test was 100%.

DISCUSSION

Studies have demonstrated the high sensitivity and specificity of the rapid immunochromatographic test using the rK39 recombinant antigen for the diagnosis of VL (Brandonisio et al. 2002, Rouf et al. 2009). The lack of cross-reaction in 100% of the samples that were analysed in the present study highlights the high specificity for patients with LCL in areas that are endemic for *L. (V.) braziliensis*. To our knowledge, our study is the first to evaluate a large number of sera from patients with CL to estimate the specificity of this rK39-based immunochromatographic test. This result is in agreement with the results of previous studies that were conducted in Brazil for the validation of this test. A multicentre study that was conducted in four regions in northeastern Brazil and used the rK39 rapid test to evaluate patients with symptoms that were suggestive of VL revealed that this test had a sensitivity of 93% and a specificity of 97%. In addition, the serum samples from patients were tested using an immunoenzymatic assay with the rK39 antigen (ELISArK-39), which demonstrated a higher sensitivity (97%) than the rapid rK39-based test (de Assis et al. 2008). In another study that was conducted in two areas of southeastern Brazil, sera from 128 patients with parasitologically confirmed diagnosis of *L. infantum* infection were tested using the Kalazar Detect® rapid test and ELISA with a crude *Leishmania* antigen; these two tests were found to have sensitivities of 90% and 89% and specificities of 100% and 98%, respectively (Carvalho et al. 2003). That study included sera from 50 patients with infectious diseases to evaluate specificity; however, only 11 samples included in that analysis were from patients with LC. In Sudan, Uganda, India and Afghanistan, validation studies of the rK39 rapid test yielded sensitivities that ranged from 67-100%. Despite the variability in the sensitivity, these studies reported similar specificities (95-100%) (Zijlstra et al. 2001, Singh et al. 2002, Chappuis et al. 2005, Ritmeijer et al. 2006) and this finding is in agreement with the results of the current study.

The World Health Organization (WHO) recently published a comprehensive study that evaluated rapid tests for VL in three endemic regions: the Indian subcontinent, East Africa and Brazil. The recombinant antigens rK39 and rKE16 were used in that study. In these three regions, the rapid tests exhibited specificities that exceeded 95% for both antigens. Both antigens exhibited a high sensitivity (over 93%) in the Indian subcontinent. In Africa and Brazil, the sensitivities of the rK39 antigen were 77.4% and 88.4%, respectively. In both regions, the sensitivities of the rKE16 antigen were very low, ranging from 36.8-79.2% (WHO 2011). The poor sensitivity of the rKE16 test in Africa and Brazil may have been due to the use of a recombinant rkE16 antigen that was derived from a strain of *Leishmania donovani* (MHOM/IN/KE16/1998) from India. The variability in the sensitivity of the rapid tests for VL across different regions of the world could be the result of the diversity of parasite strains and serum antibody levels, which are closely related to individual or between-population subgroup genetic factors, age and the immunological and nutritional status of the patient (WHO 2011). In a study of United States soldiers

who were stationed in Afghanistan and Iraq and had contracted CL, the rK39 rapid test and ELISA (Kala-zar Detect ELISA, InBios) demonstrated a positivity of 10.2% and 28.8%, respectively. Notably, patients with a CL-positive rapid test had a greater number of large skin lesions (Hartzell et al. 2008). This result could be due to the high serum antibody levels that are associated with the greater duration and severity of the cutaneous form of the disease. Moreover, in contrast to other regions of the world, Afghanistan and Iraq exhibit greater phylogenetic proximity between *L. donovani, Leishmania tropica* and *Leishmania major* (Mahdy et al. 2010), which could explain the increased number of cross-reactions between VL and LCL observed in this region.

In Brazil, the positivity of the rK39 rapid test due to cross-reaction with other protozoan species is low. A specificity study conducted in the state of São Paulo that used the rK39 test to analyse serum samples from patients with Chagas disease indicated 100% specificity for patients with Chagas disease diagnosed by parasite culture and 96% specificity for patients who were diagnosed by serological tests (Amato-Neto et al. 2009).

The specificity of this rapid test should ideally be evaluated using serum samples from patients infected with other dermotropic *Leishmania* species, particularly those that exhibit sympatric circulation and have closer phylogenetic relationships with *L. infantum*. In the Americas, *Leishmania mexicana* and *Leishmania amazonensis* would have a high probability of causing cross-reactions and sera from patients infected with these species need to be tested.

In conclusion, the rK39 rapid test is a valuable diagnostic tool. The fast and simple format of this test facilitates its use in regions with large populations and a lack of conventional diagnostic methods. Its specificity underscores the usefulness of this test in areas that are endemic for both *L. braziliensis* and *L. infantum*. This result is relevant because the geographical distribution of human VL is increasing and the overlapping distribution of CL and VL remains a challenge.

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