Tropical Biomedicine 32(2): 247–256 (2015)

Evaluation of an rK39-based immunochromatographic test for the diagnosis of visceral leishmaniasis in human saliva

da Silva, M.R.B.[1,2], Brandão, N.A.A.[1], Dorta, M.L.[1], Fátima Ribeiro-Dias[1], Costa, D.L.[2], Costa, C.H.N.[2] and Oliveira, M.A.P.[1*]

[1]Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Rua 235 S/N – Setor Universitário, 74605-050 Goiânia, GO, Brasil

[2]Instituto de Doenças Tropicais Natan Portela, R. Artur de Vasconcelos 151-Sul, 64001-450 Teresina, PI, Brasil

[*]Corresponding author email: miltonoliveira.ufg@gmail.com

Received 3 June 2014; received in revised form 9 September 2014; accepted 10 September 2014

Abstract. Visceral leishmaniasis (VL) is a tropical neglected disease endemic in 98 countries and affects more than 58 000 individuals per year. Several serological tests are available for VL diagnosis, including an immunochromatographic (IC) test with the rK39 antigen and finger prick-collected blood, a rapid and low-invasive test. Here, we investigate the possibility to use saliva as a non-invasive source of biological material for the rK39 IC test. Blood samples from 84 patients with suspected VL were screened by the rK39 IC test, and 29 were confirmed as being infected by a positive rK39 IC test and the presence of amastigotes on smears slides or parasite DNA (detected using PCR-RFLP) from bone marrow aspirate. The rK39 IC test using saliva samples was positive for 17 of the 29 confirmed VL cases (58.6%). The amount of *Leishmania*-specific IgG or total IgG, as evaluated by an immunoenzymatic assay, was higher in the saliva of patients who had rK39 IC test positivity using saliva, whereas the amount of *Leishmania*-specific IgA or total IgA was similar to the healthy donors. These results suggest that saliva is not an appropriated material for diagnosing VL with this test.

INTRODUCTION

Visceral leishmaniasis (VL) is an endemic infectious disease present in 98 countries on five continents, affecting more than 58 000 individuals per year (WHO, 2012). In Brazil, VL is caused by *Leishmania (Leishmania) infantum* (Syn. *Leishmania (Leishmania) chagasi*) and affected approximately 1.97/100 000 inhabitants between 1998 and 2009 (Brasil, 2011b). The laboratory diagnosis of VL is variable and the lack of a gold standard makes diagnostic research difficult (de Assis *et al.*, 2012). Demonstration of the parasite in a smear or culture is still the reference parasitological standard test for the diagnosis of VL in Brazil (Brasil, 2011b, de Assis *et al.*, 2012). The most common source used to search for the parasite is bone marrow aspirate, with sensitivity varying from 40 to 95% and specificity close to 100% (da Silva *et al.*, 2005, WHO, 2010). However, it is important to note that to reach a good sensitivity in smear analyses requires time-consuming steps in examining each slide (da Silva *et al.*, 2005). In addition to the variability in their sensitivity, parasitological tests are invasive, making them difficult to perform under field conditions.

Several serological tests are available for the diagnosis of VL. An immunofluorescence test is recommended by the Ministry of Health in Brazil and has a sensitivity ranging from 50 to 95% (Brasil, 2011b, Brasil, 2011a). The rapid immunochromatographic (IC) test using the rK39 antigen was validated in several countries and is most likely the best assay for the diagnosis of VL in peripheral services and reference centers (de Assis *et al.*, 2012).

The rK39 IC test shows different sensitivities in Africa and India, with a sensitivity of 90-95% and specificity of 93-100% in Brazil (de Assis *et al.*, 2008, de Assis *et al.*, 2011). The test is less invasive than the others described here because it uses a finger prick to sample blood. However, there is a possibility to use non-invasive sources of biological material for the VL diagnosis. Indeed, the rK39 IC test was evaluated in Bangladesh using urine and showed a sensitivity and specificity of 95% and 93.3%, respectively, in patients that were positive for VL by the rK-39 IC test using serum samples (Khan *et al.*, 2010). Using sputum for the diagnosis of VL, the rK39 IC test showed a sensitivity of 99.2% in parasitologically confirmed patients (Singh *et al.*, 2009). More recently, the rK39 IC test was evaluated using saliva from Indian patients, detecting 82.5% of VL cases in rK-39 serum-positive patients (Vaish *et al.*, 2012).

Here, we evaluated the efficiency of the rK39 IC test for diagnosing VL using saliva in positive finger prick-tested Brazilian VL patients. Additionally, the amount of total or Leishmania-specific IgG and IgA was also analyzed in serum and saliva.

MATERIALS AND METHODS

Patients

All suspected VL patients (n = 84) were attended at the Instituto de Doenças Tropicais Natan Portella – IDTNP, Terezina, Piauí, Brazil, from July 2011 to May 2012. Control samples (n = 20) from a non-endemic area were obtained from adult healthy donors at the Blood Bank of Clinical Hospital of Goiás, Goiânia, Goiás, Brazil. The study and informed consent forms were approved by the Ethical Committee of the Universidade Estadual do Piauí and by the Committee for Research of the Universidade Federal do Piauí. After patients underwent a clinical examination, venous peripheral blood and saliva were collected for serological tests, and bone marrow aspirate was collected for parasitological and PCR-RFLP tests. Clinical suspicion for VL was defined as a fever, anemia, and hepatosplenomegaly. Patients were excluded if an insufficient amount of saliva was collected or no saliva was. An HIV test using the Genscreen ultra HIV ab-Ag test (Bio-Rad Laboratories, Hercules, CA, USA) and a test for Chagas Disease using the ELISA Chagas III (Grupo-Bios, Santiago, Chile) were performed. Both HIV and Chagas Disease testing was performed at the Central Laboratory of the Piauí (LACEN).

Samples

A finger-pricked blood sample was collected onto a slide and immediately transferred to cassette for the rk39 IC assay. A volume of 1.0 to 5.0 mL of saliva was collected in the afternoon at two or more hours after the last meal into a 50 mL polypropylene tube. The patients received a small piece of Parafilm® to chew for 2-5 minutes to increase the amount of saliva. Peripheral blood (4 mL) was collected in 5 mL Vaccutainer® tubes to obtain serum. Both the serum and saliva were kept at -80ºC, with at least 3 replicates, until use. The samples were thawed only once prior to use.

rK39 IC test

The rK39 IC test (Orangelife, Rio de Janeiro, RJ, Brazil) was performed at IDTNP according to the manufacturer's instructions. Briefly, 20 µl of finger-pricked blood or saliva was added to the cassette at room temperature, followed by two drops of the chase buffer. The results were determined after 5-15 minutes. The test was considered positive when both the control and the test line appeared black in color.

Parasitological exam

Bone marrow (1-2 mL) was aspirated from the sternum or ileum to prepare smears by slides apposition. The slides were stained with panoptic (Ranylab, Barbacena, Brazil) and evaluated under a light microscope (1000 X). At least three bone marrow smears were evaluated for each patient.

PCR-RFLP

DNA was extracted from bone marrow aspirates using QIAamp® DNA Mini Kit following the described protocol (Qiagen Inc., Hilden, Germany). PCR was performed

using the primers 150: 5' GGG(G/T)AGGGG CGTTCT(C/G)CGAA 3' and 152: 5' (C/G)(C/G)(C/G)(A/T)CTAT(A/T)TTACACCAACCCC 3' (Volpini *et al.*, 2004). The reactions were carried out in a final volume of 20 µl containing 2 µl of DNA preparation, buffer (10 mM Tris–HCl pH 8.6, 50 mM KCl) 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µmol of each primer and 0,8U of Taq DNA polymerase (Invitrogen, Camarillo, CA, USA). The PCR amplification condition were an initial denaturation at 94ºC for 5 min, 35 cycles of denaturation at 94ºC for 45 sec, annealing at 59ºC for 45 sec, and extension at 72ºC for 30 sec, and a final extension at 72ºC for 7 min. After amplification, the samples were electrophoresed through an 8% polyacrylamide gel and silver-stained to identify the PCR products.

PCR-RFLP mkDNA to discriminate *L. infantum* was carried out according to a described protocol (de Andrade *et al.*, 2006). Briefly, 5 µl of PCR product was digested with 1 U Hae III (Invitrogen) and incubated for 3 h at 37ºC in the manufacturer's buffer. The restriction fragments were separated using a 15% polyacrylamide gel to identify the PCR products. The fragments generated were compared with those from the DNA of a *Leishmania* reference strain, *L. (L.) infantum* (MHOM/BR/74/PP75).

Immunoenzymatic assay (ELISA)

IgA and IgG antibodies were assayed in saliva or serum by sandwich ELISA using antibodies obtained from Bethyl laboratories (Bethyl laboratories Inc., Montgomery, TX, USA). To detect *Leishmania*-specific IgG and IgA by ELISA, promastigote parasites of *L. (L.) infantum* (MHOM/BR/74/PP75) were cultured in 75 cm^2 culture flasks (TPP, Trasadingen, Switzerland), starting at 2×10^5 promastigotes per mL in Grace's Insect Medium (Sigma Chemical Co., USA) supplemented with inactivated 20% fetal bovine serum (FBS, Cripion, Andradina, SP, Brazil), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma). The parasites were harvested at 5 days after starting the cultures, washed three times in hosphate-buffered saline (PBS), suspended in 1% of paraformaldehyde solution to 1 x 10^8 *Leishmania*/mL and stored at 4ºC until use. The parasite suspension was washed with PBS and, diluted in carbonate/bicarbonate buffer and 1×10^6 promastigotes per 50 µL was incubated in 96-well ELISA plates (Costar) overnight at 37ºC to dry completely. The wells were washed with 0.05% PBS-Tween and then blocked with 3% of FBS in PBS for 1 h at room temperature (RT). The wells were washed again, and successively diluted (5-fold) human serum (from 1:100 to 1: 62 500) or saliva (from 1:10 to 1: 6 250) was added for 2 h at RT, then wells were washed with 0.05% PBS-Tween. Fifty microliters of HRP-conjugated anti-human IgG (1:5 000) or anti-human IgA (1:2 000) (both from Bethyl laboratories) diluted in 3% FBS in PBS was added for 20 min at RT, followed by washing of the plate with PBS-Tween. The substrate (50 µL of TMB, Invitrogen) was then added for 10 min at RT, the reaction was stopped with 20 µL of 1 N H_2SO_4, and the optical density was measured at 450 nm.

Total IgA and IgG antibodies were detected as described above, except that the ELISA plates were coated with anti-human IgG or IgA antibodies (both from Bethyl laboratories) in carbonate/bicarbonate buffer instead of promastigote parasites. The protocol for total immunoglobulin was used to prepare standard curves for specific or total IgG and IgA using a standard serum with a known amount each isotype of immunoglobulin.

Hemoglobin assay

The amount of hemoglobin in saliva was quantified using a colorimetric Hemoglobin kit (Doles, Goiânia, Brazil) following the manufacture's instructions.

Statistical analyses

The data are presented as the mean ± SD. The data were compared for significance using Student's *t* test or ANOVA followed by Tukey's multiple comparison test using the GraphPad Prism Software 5.0 (Inc. San Diego, CA, USA). $p < 0.05$ was considered significant.

RESULTS

During the period from July 2011 to May 2012, blood samples from 84 patients with suspected VL were screened by the rK39 IC test (Figure 1). Thirty-nine patients had a positive IC test using serum, and VL was confirmed in 25 patients by PCR-RFLP and in 4 patients by amastigote observation on smear slides prepared from bone marrow aspirate. The confirmatory tests were not performed in 10 patients because no bone marrow aspirate was collected, and these patient were excluded from further analysis. Of the 29 VL-confirmed cases, the rK39 IC test using saliva was positive for 17 samples (SalPos) (58.6%; 95% CI = 40.71-74.51%) and negative for 12 samples (SalNeg) (41.4%; 95% CI = 25.49-59.29%). Twenty healthy donors from a non-endemic area (CT) were included in this study, and all were negative by the rK39 IC test using blood and saliva, showing a specificity of 100%. The rK39 IC test was also negative for the saliva of all patients who had a negative rK39 IC test using serum.

The studied groups were composed mainly largely of males, with ages ranging from 18 to 68 years old (Table 1). All VL patients presented splenomegaly, and more than 58% presented hepatomegaly. Serological cross reaction with *Trypanosoma cruzi* was similar in the SalPos and SalNeg groups and was present in more than 52% of the VL patients. HIV infection was higher the in SalNeg group (66.67%, 95% CI = 38.38–86.45%) than in the SalPos group (23.53%; 95% CI = 9.05–47.77%).

The amount of total and *Leishmania*-specific IgG in the serum of the VL patients was significantly higher than in the healthy donors from the non-endemic area (Figure 2). The levels of specific IgG in the SalNeg group was lower than in the SalPos group but was not statistically significant. The amount of total or specific IgA was similar in all groups. The highest amount of total IgG was also observed in the saliva of the VL patients (Figure 3) but was statistically significant only in the SalPos group. The amount of *Leishmania*-specific IgG observed in the saliva of both the SalPos and SalNeg groups was higher than in the control group, but SalPos saliva presented more *Leishmania*-specific IgGs than the SalNeg saliva. The levels of specific and total IgA were similar in the saliva of all groups. The specificity of

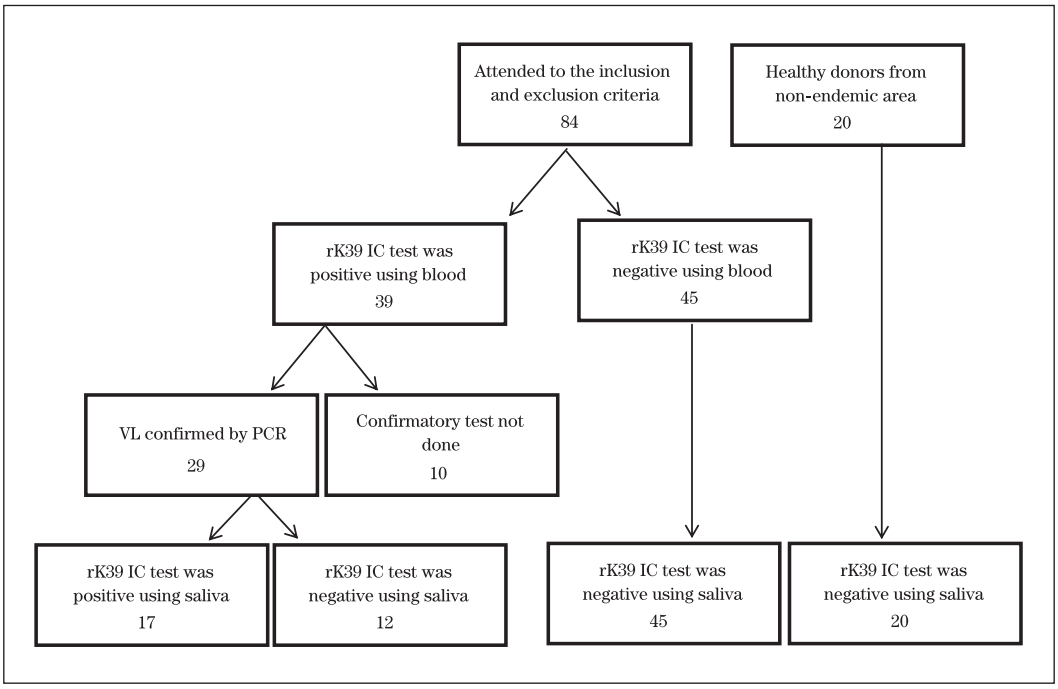

Figure 1. Flow diagram describing the flow of patients enrolled in this study.

Table 1. Characteristics of the patients and healthy donors from non-endemic area

	Sal Pos* (n=17)	Sal Neg* (n=12)	CT* (n=20)
Gender			
Female	2 (11.8%)	3 (25.00%)	6 (30.00%)
Male	15 (88.2%)	9 (75.00%)	14 (70.00%)
Age (years)			
16-20	3 (17.64%)	4 (33.33%)	4 (20.00%)
21-40	6 (35.29%)	4 (33.33%)	13 (65.00%)
41-70	8 (47.05%)	4 (33.33%)	3 (15.00%)
HIV infection	4 (23.52%)	8 (66.67%)	0

* Sal Pos (rK39 IC was positive using saliva); Sal Neg (rK39 IC was negative using saliva); CT (healthy donors from non-endemic area)

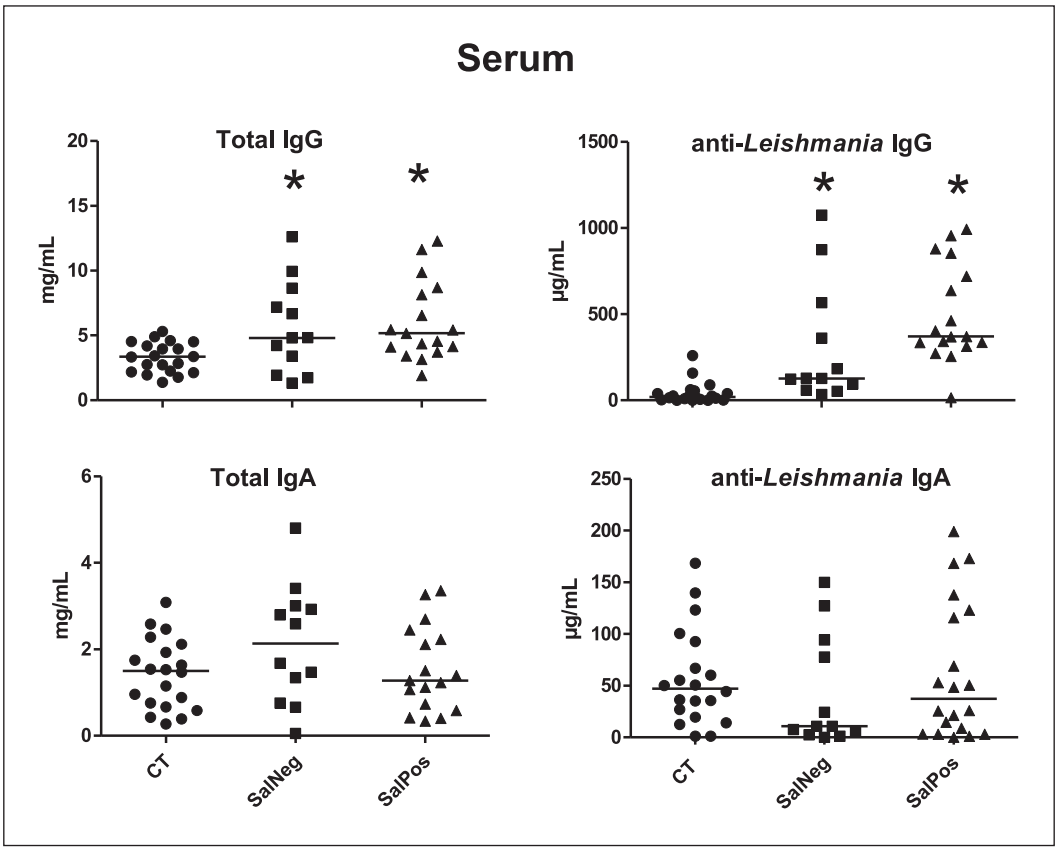

Figure 2. Total and VL-specific IgA and IgG in serum.
Total or *Leishmania*-specific IgG or IgA in the serum of healthy donors from a non-endemic area (CT, n = 20) or *Leishmania infantum*-infected patients was quantified by ELISA. Patients were divided into a positive (SalPos, n =17) or negative (SalNeg, n =12) group according to the result of the rK39 IC test using saliva. The symbols represent the amount of immunoglobulin from each subject, and the line represents the mean of the amount of immunoglobulin in each group. * indicates significant difference from the CT group ($p < 0.05$, ANOVA followed by Tukey's test)

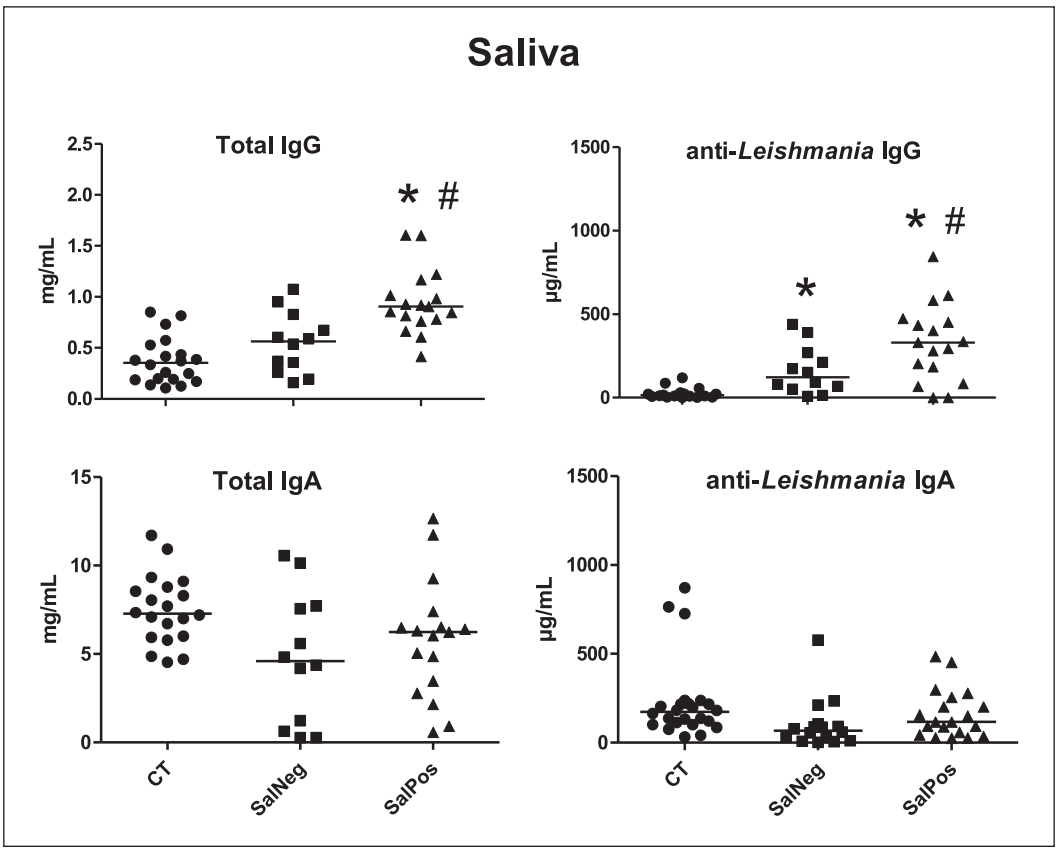

Figure 3. Total and VL-specific IgA and IgG in saliva.
Total or *Leishmania*-specific IgG or IgA in the saliva of healthy donors from a non-endemic area (CT) or *Leishmania infantum*- infected patients was quantified by ELISA. Patients were divided into a positive (SalPos, 17) or negative (SalNeg, 12) group according to the result of the rK39 IC test using saliva. The symbols represent the amount of immunoglobulin from each subject, and the line represents the mean of the amount of immunoglobulin in each group. * indicates significant difference from CT group and # indicates significant difference between the SalPos and SalNeg groups ($p < 0.05$, ANOVA, followed by Tukey's test)

the *Leishmania*-specific IgG ELISA with regard to serum and saliva was 95% (95% CI = 74.59-99.99%) and 90% (95% CI = 68.68-98.43%), respectively.

Some of the VL patients enrolled in this study had a precarious oral hygiene condition, and some saliva collected was contaminated with blood. To evaluate whether the differences in positivity by kK39 IC test using saliva were due blood contamination, we compared the amount of hemoglobin present in the saliva of the different groups. Hemoglobin was slightly higher in the saliva from the SalPos group than in the saliva from the SalNeg group (Figure 4), but this difference was not statistically significant ($p = 0.37$).

DISCUSSION

This study evaluated the ability of the rK39 IC test to diagnose VL using the saliva of VL-confirmed patients who had a positive rK39 IC test using finger-pricked blood samples, 58.6% positivity was detected using saliva. All 45 patients with suspected VL that had a negative rK39 IC test using finger-pricked blood samples were also negative using saliva, showing that the detection of VL using saliva in the rK39 IC test was not better than the detection using blood. A similar study performed in India showed that the rK39 IC test and rK39 ELISA using saliva were able to detect 82.5% and 83.3% of VL cases, respectively, while both tests detected 100%

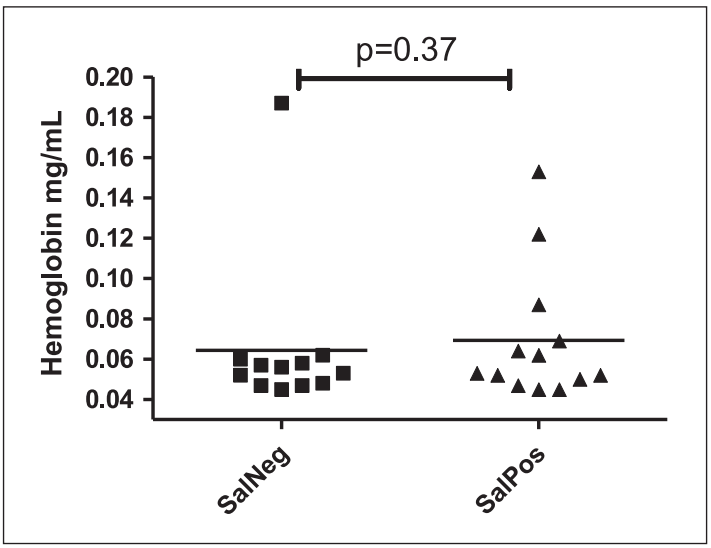

Figure 4. Amount of hemoglobin in saliva.
Hemoglobin in the saliva of VL-infected patients was quantified by a colorimetric assay. VL patients were divided into a positive (SalPos, n = 17) or negative (SalNeg, n = 12) group according to the result of the rK39 IC test using saliva. The symbols represent the amount of hemoglobin in the saliva and the line represents the mean of the amount of hemoglobin in each group.

of VL cases when using serum (Vaish *et al.*, 2012).

In an attempt to understand why the rK39 IC test using saliva did not confirm the presence of specific antibodies detected in blood, we investigated the levels of non-specific and *Leishmania*-specific antibodies in the serum and saliva of VL patients. An increase in *Leishmania*-specific and non-specific circulating antibodies in serum, which is a hallmark of VL infection (Ghose *et al.*, 1980), was observed for the patients in this study. In addition to the increase in serum IgG, we demonstrate here that *Leishmania*-specific and total IgG also increased in the saliva of several VL-infected patients. The amount of total IgG in the saliva of the SalNeg group was lower than in the SalPos group, suggesting that the low sensitivity of the rK39 IC test using the saliva was partially due to the lack of an increase in the IgG secretion in the saliva of some patients.

It is important to note that some patients from the SalNeg group presented a higher amount of *Leishmania*-specific IgG in their saliva, as detected by ELISA, than the average observed in the SalPos group. This finding suggests a low avidity of the antibodies from these patients to the antigen present in the rK39 IC test. It was recently proposed that a polymorphism in the K39 region of the parasite is responsible for variability in the results among subjects living in different areas and using different brands (Bhattacharyya *et al.*, 2013). Additionally, a lower amount of K39-specific antibody was also described in some VL-infected patients in Africa (Chappuis *et al.*, 2006). Although variability in the K39 region in Brazilian strains has not yet been studied (Bhattacharyya *et al.*, 2013), the K39 polymorphism and/or the low production of K39 specific IgG by some patients can be responsible for a decrease in the sensitivity of the test when using saliva.

The Orangelife IC test platform was designed to detect IgG against the rK39 antigen. Because saliva is enriched with IgA antibodies, we supposed that the presence of *Leishmania*-specific IgA would bind to the antigen and decrease the sensitivity of the test. Indeed, it was demonstrated that

Leishmania-specific IgA was increased in serum in the early phase of the infection in VL patients from Espírito Santo and Bahia/ Brazil (da Matta *et al.*, 2000). Conversely, changes in the IgA profile in VL patients from Africa and India were not observed (Ghose *et al.*, 1980, el Amin *et al.*, 1986, Elassad *et al.*, 1994, Anam *et al.*, 1999). In the present study, we were unable to observe a significant alteration of total or specific IgA in serum or saliva during VL infection, suggesting that the negative results in the SalNeg group were not due to IgA interference. IgM is another isotype that could bind to the rK39 antigen and decrease the sensitivity of the test, but it was not tested in our experiments.

The patients in both the SalPos and SalNeg groups were similar with regard to most parameters used to compare the groups, though the SalPos did present a smaller number of HIV^+ subjects than the SalNeg group. It is not clear whether HIV infection could interfere with the sensitivity of the rK39 IC test (Cota *et al.*, 2013), but our data suggest that HIV infection can decrease the sensitivity of rK39 IC test, even though the amount of *Leishmania*-specific IgG in serum and saliva was similar in patients infected or not with HIV (data not shown).

The salivary concentration of immunoglobulin is higher in the early morning, with a minimal variation of IgA and IgG concentrations from 10:00 am to 5:00 pm (Rantonen & Meurman, 2000). Because the VL patients attended at the IDTNP arrive all day long, we collected the saliva in the afternoon. Although it is possible that collecting saliva early in the morning would increase the sensitivity of the test, this does not reflect what is observed under routine hospital conditions. Additionally, we froze the saliva to be able to collect sufficient material for immediately to performing the ELISA assay. Indeed, successive freezing and thawing of saliva can denature immunoglobulin and decrease the sensitivity of serological tests; however, the saliva in our study was frozen in replicate and thawed only once to avoid denaturation.

It is known that the incidence of asymptomatic and subclinical leishmaniasis can outnumber clinical cases in endemic areas (Hasker *et al.*, 2014). A recent cohort study in India and Nepal showed that asymptomatic individuals who present a strong rK39 IC test reaction have an increased risk of progression to severe disease (Hasker *et al.*, 2014). During the screening of the patients in the present study, we also observed that the rK39 IC test performed with blood developed a strong or weak reaction, but we considered all of them to be positive because it was difficult to define parameters to rank the results of such a test. Furthermore, the difficulty in evaluating the density of the bands formed in the rK39 IC test is increased by the background observed when blood is used (Matlashewski *et al.*, 2013). Because our results suggest that there is more *Leishmania*-specific IgG in the sera from the SalPos group than SalNeg group ($p = 0.055$, Student's t test) and high serum titers in asymptomatic individuals appears to be a risk factor for developing more severe VL disease, it is possible that rK39 IC test positivity in asymptomatic individuals can be a risk factor for VL progression.

In conclusion, our study demonstrates that the saliva of VL-infected patients has limited use for the diagnosis of VL. We also suggest that IgA in saliva and serum has no value for VL diagnosis.

Acknowledgements. The author thanks Dr. Marco Collovati from OrangeLife, Rio de Janeiro, Brazil for the donation of rK39 IC test Kits. This work was supported by CNPq and CAPES. MRBS had a fellowship from CAPES and CHNC and FRD are CNPq fellows.

REFERENCES

Anam, K., Afrin, F., Banerjee, D., Pramanik, N., Guha, S.K., Goswami, R.P., Gupta, P.N., Saha, S.K. & Ali, N. (1999). Immunoglobulin subclass distribution and diagnostic value of Leishmania donovani antigen-specific immunoglobulin G3 in Indian kala-azar patients. *Clinical and Diagnostic Laboratory Immunology* **6**(2): 231-235.

Bhattacharyya, T., Boelaert, M. & Miles, M.A. (2013). Comparison of visceral leishmaniasis diagnostic antigens in African and Asian Leishmania donovani reveals extensive diversity and region-specific polymorphisms. *PLoS Negected Tropical Diseases* **7**(2): e2057.

Brasil. (2011a). Leishmaniose Visceral: Recomendações Clínicas para Redução da Letalidade In: SdVe Saúde., Ed. Ministério da Saúde do Brasil, pp. 77.

Brasil. (2011b). Manual de recomendacões para diagnostico, tratamento e acompanhamento de pacientes com a coinfeccao Leishmania-HIV In: SdVe Saúde, Ed. Ministério da Saúde do Brasil, pp. 106.

Chappuis, F., Rijal, S., Soto, A., Menten, J. & Boelaert, M. (2006). A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. *British Medical Journal* **333**(7571): 723.

Cota, G.F., de Sousa, M.R., de Freitas Nogueira, B.M., Gomes, L.I., Oliveira, E., Assis, T.S., de Mendonca, A.L., Pinto, B.F., Saliba, J.W. & Rabello, A. (2013). Comparison of parasitological, serological, and molecular tests for visceral leishmaniasis in HIV-infected patients: a cross-sectional delayed-type study. *The American Journal of Tropical Medicine and Hygiene* **89**(3): 570-577.

da Matta, V.L., Hoshino-Shimizu, S., Dietze, R. & Corbett, C.E. (2000). Detection of specific antibody isotypes and subtypes before and after treatment of American visceral leishmaniasis. *Journal of Clinical Laboratory Analysis* **14**(1): 5-12.

da Silva, M.R., Stewart, J.M. & Costa, C.H. (2005). Sensitivity of bone marrow aspirates in the diagnosis of visceral leishmaniasis. *The American Journal of Tropical Medicine and Hygiene* **72**(6): 811-814.

de Andrade, H.M., Reis, A.B., dos Santos, S.L., Volpini, A.C., Marques, M.J. & Romanha, A.J. (2006). Use of PCR-RFLP to identify Leishmania species in naturally-infected dogs. *Veterinary Parasitology* **140**(3-4): 231-238.

de Assis, T.S., Braga, A.S., Pedras, M.J., Oliveira, E., Barral, A., de Siqueira, I.C., Costa, C.H., Costa, D.L., Holanda, T.A., Soares, V.Y., Bia, M., Caldas Ade, J., Romero, G.A. & Rabello, A. (2011). Multicentric prospective evaluation of rk39 rapid test and direct agglutination test for the diagnosis of visceral leishmaniasis in Brazil. *Transactions of the Royal Society of Tropical Medicine Hygiene* **105**(2): 81-85.

de Assis, T.S.M., Braga, A.S., Pedras, M.J., Barral, A., Siqueira, I.C., Costa, C.H., Costa, D.L., Holanda, T.A., Soares, V.Y., Bia, M., Caldas, Ade, J., Romero, G.A. & Rabello, A. (2008). Validação do teste imunocromatográfico rápido IT-LEISH® para o diagnóstico da leishmaniose visceral humana. *Epidemiologia e Serviços de Saúde* **17**(2): 9.

de Assis, T.S.M., Rabello, A. & Werneck, G.L. (2012). Predictive models for the diagnostic of human visceral leishmaniasis in Brazil. *PLoS Neglected Tropical Diseases* **6**(2): e1542.

el Amin, E.M., Wright, E.P. & Vlug, A. (1986). Characterization of the humoral immune response in Sudanese leishmaniasis: specific antibody detected by class- and subclass-specific reagents. *Clinical and Experimental Immunology* **64**(1): 14-19.

Elassad, A.M., Younis, S.A., Siddig, M., Grayson, J., Petersen, E. & Ghalib, H.W. (1994). The significance of blood levels of IgM, IgA, IgG and IgG subclasses in Sudanese visceral leishmaniasis patients. *Clinical and Experimental Immunology* **95**(2): 294-299.

Ghose, A.C., Haldar, J.P., Pal, S.C., Mishra, B.P. & Mishra, K.K. (1980). Serological investigations on Indian kala-azar. *Clinical and Experimental Immunology* **40**(2): 318-326.

Hasker, E., Malaviya, P., Gidwani, K., Picado, A., Ostyn, B., Kansal, S., Singh, R.P., Singh, O.P., Chourasia, A., Kumar Singh, A., Shankar, R., Wilson, M.E., Khanal, B., Rijal, S., Boelaert, M. & Sundar, S. (2014). Strong association between serological status and probability of progression to clinical visceral leishmaniasis in prospective cohort studies in India and

Nepal. *PLoS Neglected Tropical Diseases* **8**(1): e2657.

Khan, M.G., Alam, M.S., Podder, M.P., Itoh, M., Jamil, K.M., Haque, R. & Wagatsuma, Y. (2010). Evaluation of rK-39 strip test using urine for diagnosis of visceral leishmaniasis in an endemic area in Bangladesh. *Parasite and Vectors* **3**: 114.

Matlashewski, G., Das, V.N., Pandey, K., Singh, D., Das, S., Ghosh, A.K., Pandey, R.N. & Das, P. (2013). Diagnosis of visceral leishmaniasis in Bihar India: comparison of the rK39 rapid diagnostic test on whole blood versus serum. *PLoS Neglected Tropical Diseases* **7**(5): e2233.

Rantonen, P.J. & Meurman, J.H. (2000). Correlations between total protein, lysozyme, immunoglobulins, amylase, and albumin in stimulated whole saliva during daytime. *Acta Odontologica Scandinavica* **58**(4): 160-165.

Singh, D., Pandey, K., Das, V.N., Das, S., Kumar, S., Topno, R.K. & Das, P. (2009). Novel noninvasive method for diagnosis of visceral leishmaniasis by rK39 testing of sputum samples. *Journal of Clinical Microbiology* **47**(8): 2684-2685.

Vaish, M., Singh, O.P., Chakravarty, J. & Sundar, S. (2012). rK39 antigen for the diagnosis of visceral leishmaniasis by using human saliva *The American Journal of Tropical Medicine and Hygiene* **86**(4): 598-600.

Volpini, A.C., Passos, V.M., Oliveira, G.C. & Romanha, A.J. (2004). PCR-RFLP to identify Leishmania (Viannia) braziliensis and L. (Leishmania) amazonensis causing American cutaneous leishmaniasis. *Acta Tropica* **90**(1): 31-37.

WHO (2010). Control of the Leishmaniasis. Geneva, pp. 199. from http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf.

WHO. (2012). Leishmaniasis: epidemiology and access to medicines.Retrieved February 30, 2014, from http://www.who.int/leishmaniasis/resources/leishmaniasis_epidemiology_access_to_medicine/en/