Parasitology Research (2019) 118:2317–2323
https://doi.org/10.1007/s00436-019-06352-4

PROTOZOOLOGY - SHORT COMMUNICATION

# *Leishmania infantum* exo-antigens: application toward serological diagnosis of visceral leishmaniasis

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Received: 24 September 2018 / Accepted: 9 May 2019 / Published online: 29 May 2019


## Abstract

The laboratory diagnosis of visceral leishmaniasis (VL) presents limitations related to its sensibility and/or specificity. In this context, the aim of this study was to evaluate an enzyme-linked immunoassay to detect IgG antibodies against *Leishmania infantum* exo-antigens for diagnosis of VL, called ELISA-Exo. This assay was applied in 309 masked serum samples from VL, tegumentary leishmaniasis, Chagas disease, schistosomiasis mansoni, malaria patients, and healthy individuals. The results were compared with those from ELISA using rK39 as antigen (ELISA-rK39). The ELISA assays presented sensitivity of 96.8% and 98.4% ($p = 0.68$), specificity of 92.4% for both, and diagnostic accuracy of 94.2% and 94.8% ($p = 0.48$) by the ELISA-Exo and ELISA-rK39, respectively. An excellent agreement beyond chance (Kappa index = 0.82) was obtained when the results from ELISA assays were cross-tabulated. The Western blotting showed that false-positive results presented by ELISA-Exo probably were produced by cross-reactivity of antigens shared with the species of the family *Trypanosomatidae.* In the future, an immunoproteomic approach can contribute for identification of main immunoreactive *L. infantum* exo-antigens.

**Keywords** Visceral leishmaniasis · Serological tests · ELISA-Exo · Immunoreactive exo-antigens

## Introduction

In Brazil, the visceral leishmaniasis (VL) is caused by the protozoan *Leishmania* (*Leishmania*) *infantum* (Syn. *L. chagasi*) and constitutes a severe problem of public health.

VL is usually diagnosed by the detection of parasite in bone marrow aspirate smears and/or in bone marrow, lymph node, or spleen aspirate cultures. However, the accuracy of the microscopic examination is influenced by the biological sample and ability of the physician and the laboratory technician. In Brazil, parasitological diagnosis is usually performed through the examination of bone marrow aspirate. Although it has a good safety, it is an invasive exam and presents lower sensitivity (53–86%) (Ministério da Saúde 2014).

Specific antibodies may be detected by serological tests using crude, purified, and recombinant antigens. Different recombinant antigens have been proposed in the last years, including the antigen K39 from *L. infantum* (Burns et al. 1993); the rKLO8 from a native strain *L. donovani* in Sudan, containing conserved domains with high similarity to the immunodominant kinesin proteins of *Leishmania* (Abass et al. 2013); two hydrophilic antigens (K9 and K26) from *L. infantum* (Bhatia et al. 1999); the rKE16 from an Indian *L. donovani* strain (Sivakumar et al. 2008); and the A2 antigen (Akhoundi et al. 2013). From all of these antigens, a significant advance was the identification and production of a recombinant K39 (rK39), which is a protein composed by 39 amino acid-repetitive immunodominant B cell epitope of kinesin-related protein from *L. infantum* (Burns et al. 1993). When used for ELISA test, the rK39 shown to be more sensitive and specific for the diagnosis of active VL than the soluble antigens (Pedras et al. 2008). A high diagnostic accuracy was also reported for the rK39 immunochromatographic strip test, better known as the rK39 Rapid Test (Chappuis et al.

Section Editor: Nawal Hijjawi

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2006; Machado de Assis et al. 2011; Peruhype-Magalhães et al. 2012). However, some studies had showed a lower sensitivity of rK39 Rapid Test when it was employed for diagnosis VL in the Sudanese population (Chappuis et al. 2006).

Additionally, secreted-excreted antigens (exo-antigens) of *Leishmania* spp. can be used in the ELISA for VL diagnosis. In previous works, it was evaluated by the ELISA and Western-blotting assays with *Leishmania* spp. exo-antigens in serum samples from VL patients (Martin et al. 1998; Ryan et al. 2002; Pindedo-Cancino et al. 2013), canine VL (Ryan et al. 2002; Rajasekariah et al. 2001), and tegumentary leishmaniasis patients (Ryan et al. 2002; Pindedo-Cancino et al. 2013).

Here, we report the applicability of *L. infantum* exo-antigens, and we compare its accuracy with the rK39, using ELISA assays for VL diagnosis.

## Methodology

### Serum samples

The equation recommended by Banoo et al. (2010) was applied to obtain a representative number of positive and negative serum samples:

$$n = z^2 \cdot p \cdot (1-p)/x^2$$

where $n$ = positive or negative numbers, $p$ = sensitivity (or specificity) index, and $x^2$ = confidence interval. Considering 95% of confidence interval, 0.97 of sensitivity and 0.98 of specificity index, reported previously (Rajasekariah et al. 2001; Ryan et al. 2002), were calculated a minimal 45 positive and 30 negative serum samples.

To determine the sensitivity, the specificity and the diagnostic accuracy of the ELISA assays, we used serum samples recovered from the serum collection adequately stored in freezer − 80 °C at the laboratory of our research group.

To determine the two ELISA assays sensitivity, a visceral leishmaniasis group (VL group) was composed by 124 serum samples collected from patients with clinical and parasitological VL diagnosis. Positive diagnosis of VL was defined when clinical and epidemiological features and amastigotes were seen at Giemsa-stained smears or promastigote forms were isolated from culture of bone marrow aspirates.

The specificity of the ELISA assays was evaluated in the control group formed by 105 samples of healthy individuals living in Belo Horizonte, Minas Gerais, (H group) plus 80 serum samples collected from individuals with other infectious diseases and placed into the following subgroups:

TL group: Composed by serum samples of 20 patients who were diagnosed with tegumentary leishmaniasis at the Reference Center for Leishmaniasis of the Instituto René Rachou/Fiocruz through clinical and parasitological examinations

CD group: Constituted by serum samples of 20 patients living in Uberaba and Belo Horizonte, Minas Gerais, who had been previously diagnosed with Chagas disease by immunofluorescence antibodies test (IFAT) and ELISA

SM group: Containing serum samples of 20 patients living in Pedra Preta community of Northeast of Minas Gerais state who had schistosomiasis mansoni, previously diagnosed by the parasitological technique of Kato-Katz

M group: Composed by serum samples of 20 patients living in Minas Gerais and Mato Grosso states, with a clinical and parasitological diagnosis of malaria

The serum samples were recovered, blended, aliquoted, and stored in freezer − 20 °C up to be tested in the ELISA assays.

### *L. infantum* exo-antigens preparation

*L. infantum* promastigotes (MHOM/BR/2002/LPC-RPV) were grown in 0.5-L flasks at 26 °C in NNN/LIT medium containing 20% of heat-inactivated fetal bovine serum (FBS) (Gibco/Invitrogen, New York, NY, USA). The promastigotes in logarithm phase were centrifuged for 15 min at 1.500×*g* and washed three times with protein-free medium (RPMI containing 0.076 mM Xylose, 25 mM HEPES, 30 mM sodium bicarbonate). In the next step, $2.0 \times 10^7$ parasites/ml in the protein-free medium were maintained in an incubator shaker at 26 °C and 100 rpm for 24 h. Then, the parasites were centrifuged for 30 min at 9000×*g* and 4 °C, and the supernatant was recovered and filtered using a cellulose acetate membrane (Pore size 0.22 μm, Nalgene, Rochester, NY, USA). The protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### rK39 antigen

The recombinant K39 antigen used in this work was kindly given from S.G. Reed, Infectious Disease Research Institute, Seattle, WA, USA.

### ELISA assay with *L. infantum* exo-antigens (ELISA-Exo)

After conducting the checkerboard titration tests, the best ELISA protocol was defined as follows: Each well of the MaxiSorp® polystyrene microplates (Nunc ThermoScientific, Vernon Hills, IL, USA) were coated with 50 ng of *L. infantum* exo-antigens diluted in sodium carbonate/bicarbonate buffer 0.1 M, pH 9.6, containing metilgliaxol 1% and, incubated in humid chamber at 37 °C for 30 min followed by further overnight incubation in refrigerator (2 to 8 °C). Then, the microplates were washed four

times with phosphate buffered saline (0.01 M PBS, pH 7.3) containing 0.05% Tween 20 (PBS-T) and 200 μl of 2% nonfat milk diluted in PBS-T (PBS-T-Milk 2%) was added to each well and incubated for 2 h in a humid chamber at 37 °C to saturate the free sites of the microplates. Soon after that, the microplates were again washed four times with PBS-T, and 50 μl of sera diluted 1:500 was deposited in duplicate into wells from the microplates, which was incubated for 1 h in a humid chamber at 37 °C. After new washings, 50 μl of conjugate (anti-IgG human–peroxidase, Sigma Chemical Co., St. Louis, MO, USA) diluted 1:2000 in PBS-T-milk 1% was added in each well and incubated for 1 h in a humid chamber at 37 °C. New washes were performed, and 50 μl/well of the substrate solution (Tetramethylbenzidine and Hydrogen Peroxide, Sigma Chemical Co.) was added. The plates were incubated during 5 min at room temperature in the dark, and the enzymatic reaction was stopped with the addition of 50 μl/well of sulfuric acid 1N. The absorbance readings were performed at 450 nm in a microplate reader (Model 550, Bio-Rad Laboratories, Tokyo, Japan). Considered as positive samples were those samples that presented absorbance readings over cut-off, defined by ROC curve analysis.

### Enzyme-linked immunosorbent assay with rK39 (ELISA-rK39)

Polystyrene microplates NUNC Maxisorp (NUNC, ThermoScientific) were coated with 50 ng/well of rK39 diluted in carbonate-bicarbonate buffer 0.1 M (pH 9.6) by 18 h in the refrigerator (2–8 °C). Following, the microplates were washed three times with PBS-T and 200 μl of PBS-T-milk 2% were deposited into each well of the microplates, which were incubated for two hours in a humid chamber at 37 °C. Then, the ELISA was performed employing the same protocol of ELISA-Exo, but with some modifications: the serum samples were diluted to 1:100 and the anti-IgG human conjugated to peroxidase was diluted 1:30.000.

### SDS-PAGE and Western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 44 μg of *L. infantum* exo-antigens diluted in the sample buffer and electrophoresed at 150 V in a 12% preparative polyacrylamide gel. We used molecular weight markers from 12 to 225 kDa (Amersham ECL High-Range Rainbow Molecular Weigh Marker, GE Healthcare, Boston, MA, USA). Next, the gel containing exo-antigens was electroblotted to the polyvinylidene difluoride (PVDF) membrane (Immunolon-P™, pore size 0.45 μm, Millipore, Birellica, MA, USA) at 300 mA for 2 h. After that, the PVDF membrane was incubated with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% of Tween 20 for 2 h. Then, the PVDF membrane was washed three times with TBS-0.05% Tween 20 (TBS-T) and cut in strip of 3 mm. The strips were incubated for 1 h at room temperature with a pool of 10 VL-positive samples, a pool of five tegumentary leishmaniasis (TL), a pool of seven Chagas disease (CD), those presented cross-reactivity in the ELISA-Exo, and a pool of 10 healthy individuals group (HG) samples diluted 1:1000 in TBS-T containing 1% nonfat milk (TBS-T-milk). After three washes, the strips were incubated during 1 h with anti-human IgG-horseradish peroxidase conjugate (Sigma Chemical Co., St Louis, MO, USA) diluted 1:10,000 in TBS-T-milk. Finally, the strips were washed three times and incubated for 5 min at room temperature with chromogenic solution (TBS containing 2 mg/ml of diaminebenzidine and 0.01% hydrogen peroxide). The immunoreactivity of *L. infantum* exo-antigens was then visually analyzed.

### Data analysis

A data bank was created into Microsoft Excel 2007 spreadsheets and analyzed using GraphPad Prism 6.0 software (San Diego, CA, USA) or Open-Epi Software (Dean et al. 2015). The cut-off values of the ELISA-Exo and ELISA-rK39 assays were established for optimal sensitivity, specificity, and accuracy (area under the curve) using receiver operating characteristic (ROC) curve analysis in GraphPad Prism. The absorbance readings produced by the ELISA assays were individually assessed using the Shapiro-Wilk normality test (W test) and compared using the Mann-Whitney *U* test, considering a level of significance of $p < 0.05$. Chi-square test ($\chi^2$) was employed for comparison of sensitivities, specificities, and diagnostic accuracy presented by the ELISA assays, considering a 5% significance level (Bhattacharyya and Johnson 1977). Agreement beyond chance was assessed using the Cohen coefficient (Cohen 1968) and interpreted according to the scale of Landis and Koch (1977): 0.81–1.00, excellent; 0.61–0.80, good; 0.41–0.60, moderate; 0.21–0.40, weak; and 0.0–0.20, negligible.

## Results

ROC curve analysis defined cut-off values of 0.290 and 0.288 and the areas under the curves were of 0.98 (95%CI 0.97 to 1.0) and 0.99 (95%CI 0.98–1.0), for ELISA-Exo and ELISA-rK39, respectively (Fig. 1a).

The median of the absorbance readings produced by the ELISA-Exo in the VL group was lower than in the ELISA-rK39, with statistical difference ($p < 0.0001$, Fig. 1b). Four (4/124) and two (2/124) serum samples from the VL group showed false-negative results by the ELISA-Exo and the ELISA-rK39, respectively. Fourteen serum samples (14/185) from the control group, of which one was of the H group, five were of the TL group, seven were of the CD group, and one

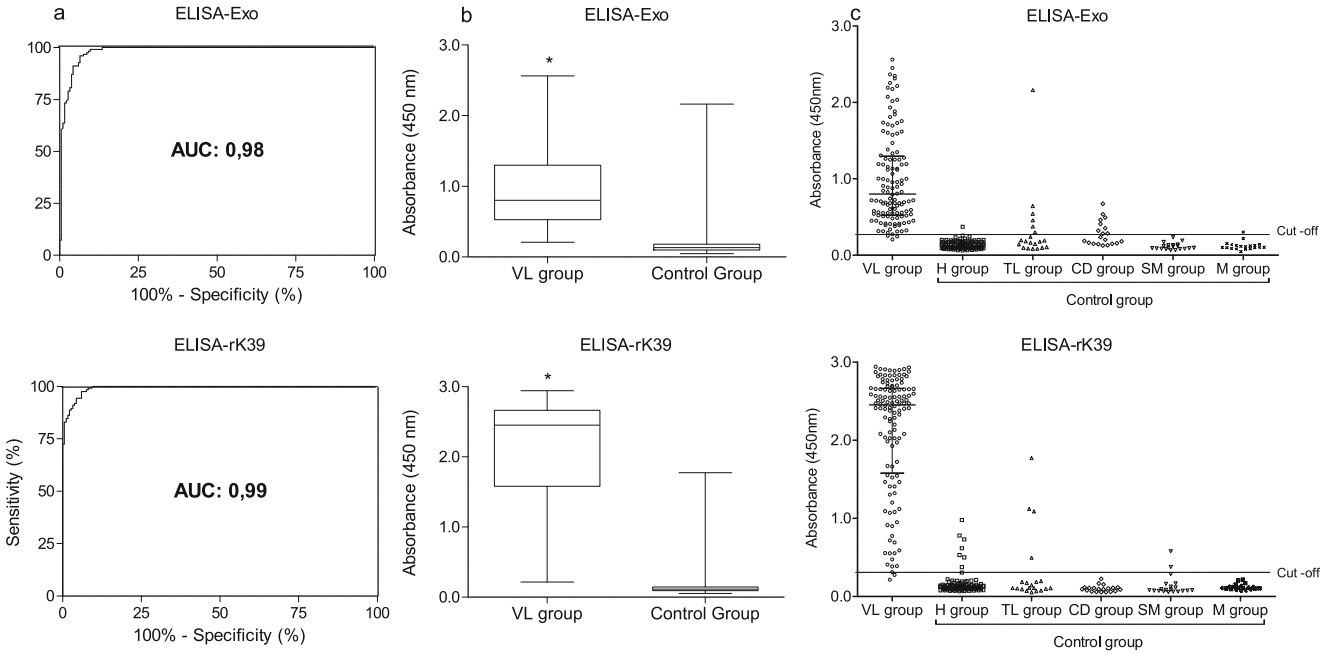


**Fig. 1** **a** ROC curve showing the areas under the curve (AUC) of the ELISA-Exo and ELISA-rK39. **b** Boxplots of the absorbance readings from the sera in the VL group and control group that presented statistically significant differences (*Mann-Whitney *U*: $p < 0.0001$). **c** Reactivity and positivity of the ELISA-Exo and ELISA-rK39 measured in serum samples of VL group ($n = 124$) and control group (H group, $n = 105$; TL group, $n = 20$; CD group, $n = 20$; SM group, $n = 20$; M group, $n = 20$). The median of absorbance readings showed by ELISA-Exo in the VL group was 0.801, 25% percentile = 0.529, 75 percentile = 1.292. In contrast, the median of absorbance readings showed by ELISA-rK39 in the VL group was of 2.451, 25% percentile = 1.607, 75% percentile = 2.661 (Mann-Whitney *U*: $p < 0.0001$)

was of the M group, showed false-positive results by ELISA-Exo. Fourteen serum samples (14/185) from the control group presented false-positive results in the ELISA-rK39, of which eight were from the H group, four were from the TL group, and two were from the SM group (Fig. 1c).

Table 1 shows serologic parameters demonstrated by the two serological tests. The sensitivity of the ELISA-Exo was of 96.8%, lower than the ELISA-rK39 (98.4%). However, there was no statistical difference between them ($p = 0.68$).

The specificity was determined for the total group of non-visceral leishmaniasis patients (control group). The specificity value observed was 92.4% for both ELISA assays. The moderate frequency of false-positive results (14/185) reduced the specificity of both ELISA assays. The diagnostic accuracy rates measured in the total number of samples were of 94.2% and 94.8% for the ELISA-Exo and the ELISA-rK39, respectively. Consequently, the positive likelihood ratios presented by the two ELISA assays were of 12.8 and 13. The observed difference in these results was not statistically significant ($p > 0.05$).

An excellent agreement beyond chance (Kappa index = 0.82) was obtained when the results from ELISA assays were cross-tabulated. In this analysis, 13 serum samples presented positive results using ELISA-Exo and were negative by the ELISA-rK39. On the other hand, 15 serum samples presented negative results by the ELISA-Exo, but were positive when the ELISA-rK39 was used (data not shown).

The Western blotting showed three immunoreactive bands of around 14, 30, and 73 kDa against sera pool of TL patients in the Western blotting. Additionally, four bands of around 14,

**Table 1** Serological parameters of the ELISA-Exo and ELISA-rK39 applied in serum samples from the VL group and control group (other infectious diseases and healthy individuals)

| Serological tests | Sensitivity ($n = 124$) | Specificity ($n = 185$) | Diagnostic accuracy ($n = 309$) | Positive likelihood ratio |
|---|---|---|---|---|
| ELISA-Exo | (120) 96.8%[a] (92–99.1) | (171) 92.4% (87.6–95.8) | (291) 94.2%[b] (91–96.3) | 12.8 (11.1–14.7) |
| ELISA-rK39 | (122) 98.4 (92–99.1) | (171) 92.4 (87.6–95.8) | (293) 94.8 (91.8–96.8) | 13 (11.1–14.7) |

[a] Difference between sensitivity: $p = 0.68$

[b] Difference between diagnostic accuracy: $p = 0.48$

() 95% confidence interval

20, 30, and 50 kDa were visualized when it was tested with sera pool of Chagas patients. The health sera pool did not present immunoreactivity in the Western blotting (Fig. 2).

## Discussion

In this study, we proposed an ELISA test using *L. infantum* exo-antigens for the diagnosis of VL (ELISA-Exo). The sensitivity presented by the ELISA-Exo was similar to that by the ELISA-rK39 with no statistically significant difference ($p = 0.68$). Other author reported sensitivity of 95.1% when *L. donovani* exo-antigens were used in ELISA to diagnosis of VL in samples from Brazil, Italy, North Africa, and Nepal (Ryan et al. 2002). An ELISA with *L. chagasi* exo-antigens showed 100% of positivity when 53 serum samples from VL patients were tested (Pindedo-Cancino et al. 2013). Furthermore, these authors reported that the antibodies recognized *L. infantum* exo-antigen bands of molecular weight ranging from 26.5 to 31.5 kDa. The results from the ELISA-rK39 test showed here were similar to the one reported by other researchers (Burns et al. 1993; Zijlstra et al. 1998; Braz et al. 2002), who showed sensitivities ranging from 93.4 to 97%, when this test was applied in VL-endemic countries.

In this study, the median of absorbance reading was lower with ELISA-Exo than with ELISA-rK39. There were samples with absorbance readings higher in ELISA-Exo that presented lower values in ELISA-rK39. In contrast, the frequency of samples with lower absorbance readings in the ELISA-Exo was more abundant than in the ELISA-rK39 with a statistically significant difference, $p < 0.0001$ (Fig. 1b, c). Probably this difference in the absorbance readings happens due to the characteristic of the antigens used in the ELISA assays. The *L. infantum* exo-antigen is constituted by a big number of proteins, enzymes, and glycoproteins (Braga et al. 2014), while the rK39 is a synthetic antigen produced from *L. chagasi* (Burns et al. 1993). However, this fact did not affect the ability of the ELISA-Exo to discriminate the positive and the negative samples.

Despite the specificity of 92.4% showed by the ELISA-Exo has been equal to the one presented with by ELISA-rK39, it was observed a heterogeneity in the false-positive results. ELISA-Exo presented many cross-reactivity with sera of the TL ($n = 5$) and the CD ($n = 7$) groups while ELISA-rK39 showed larger number of false-positive results (8/105) in the H group. These false-positive results compromised the specificities and the diagnostic accuracies of both ELISA assays. Due to the overlap of VL, tegumentary leishmaniasis, and Chagas disease in many Brazilian areas and the phylogenetic proximity of your infectious agents, the occurrence of false-positive results with sera from patients with these diseases are already well known. Other study reported 25 and 50.8% of cross reactions when an ELISA with *L. chagasi* exo-antigens was tested against sera from acute and chronic Chagas disease and cutaneous and mucosal forms of tegumentary leishmaniasis (Pindedo-Cancino et al. 2013). This happens due to the antigenic similarities among the species of the family Trypanosomatidae. Certainly, the immunoreactive bands of the *L. infantum* exo-antigens showed in the Western blotting against serum samples from patients with LT and

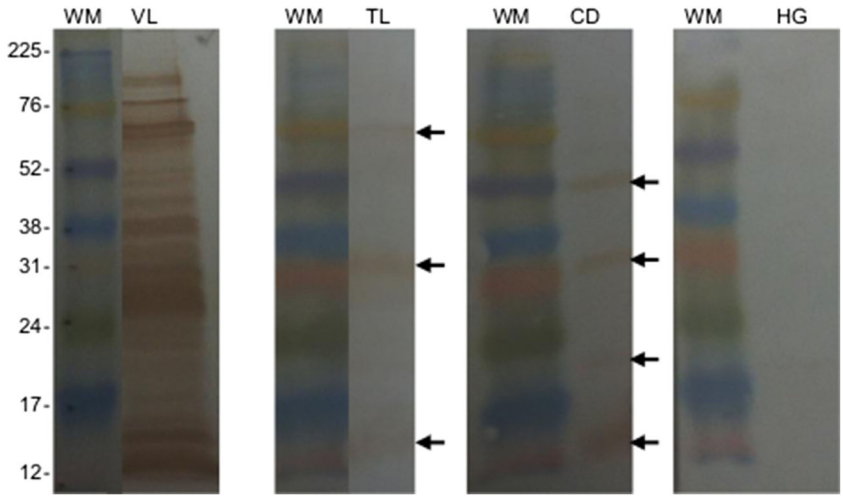


**Fig. 2** Western blotting with sera pool of VL, CD, and TL patients and healthy individuals (HG). Immunoreactive bands ranging from 10 to 100 kDa were revealed against VL sera pool. Three bands of around 14, 30, and 75 kDa and four bands of around 14, 18, 25, and 50 kDa showed immunoreactivity against sera pool from the TL patients and sera pool from the Chagas disease patients, respectively. None band was visualized in the strip assayed with health individual sera pool. WM, weight marker (Amersham ECL High-Range Rainbow Molecular Weigh marker, GE, Boston, MA, USA)

CD (Fig. 2) were responsible for the cross reactions presented in the ELISA-Exo. The frequent false-positive results observed in the H group, surely by unspecific reactions, when it was assayed by ELISA-rK39, have caused us concern and deserve further investigation.

## Conclusion

In this study, we confirmed the potential of the *L. infantum* exo-antigens for the VL diagnosis. It, when used in ELISA, presented an accuracy diagnostic similar to the ELISA with rK39. In the future, an immunoproteomic approach can contribute for the identification of main immunoreactive *L. infantum* exo-antigens.

**Acknowledgements** We thank the Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG, grant: APQ-00318-13), Belo Horizonte, Minas Gerais, Brazil, for funding this work. We also thank the Bidimensional Electrophoresis Platform, Rede de Plataformas Tecnológicas, Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais, Brazil, for the technical support in the proteomic experiments. E.O. is a research fellow supported by National Council for the Development of Research of Brazil (CNPq, grant: 301159/206-5), Brasília, Distrito Federal, Brazil.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All procedures performed in this study were in accordance with the ethical standards of the Research Ethics Committee of the René Rachou Institute, Belo Horizonte, Minas Gerais, Brazil (Protocol No. 14/2011) and with the 1964 Helsinki declaration. An informed consent was obtained from the patients or guardians and from the healthy participants at the time of blood collection.

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