

***Leptomonas seymouri* and *Crithidia fasciculata* exoantigens can discriminate human cases of visceral leishmaniasis from American tegumentary leishmaniasis ones**

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ABSTRACT

Exoantigens (exo) from *Leptomonas seymouri* and *Crithidia fasciculata* were used in an enzyme linked immunosorbent assay (ELISA), showing 100% reactivity with sera from visceral leishmaniasis (VL) cases, and no reactivity with American tegumentary leishmaniasis (ATL) ones. Our results have indicated that these exoantigens can be applied in the discrimination of VL and ATL cases.

KEYWORDS: *Leptomonas seymouri*. *Crithidia fasciculata*. *Leishmania* (*Leishmania*) *infantum chagasi*. Exoantigens. *Leishmaniasis*.

SHORT COMMUNICATION

Within the Trypanosomatidae family, plant and insect trypanosomatids have been used as laboratory models for biochemistry and molecular biology studies as they share homologous molecules with pathogenic species such as *Leishmania* spp. and *Trypanosoma cruzi*, the causative agents of leishmaniasis and Chagas disease, respectively¹⁻⁴. The high degree of conserved antigens between plant and insect *trypanosomatids* may explain their cross-immunoreactivity with sera from Chagas disease (CD)⁵⁻⁸, visceral leishmaniasis (VL)^{6,9-11} and American tegumentary leishmaniasis (ATL) patients^{6,12}. To date few studies have investigated this interesting feature of trypanosomatids, the majority being limited to phylogenetic analyses¹³.

Despite the numerous studies on exoantigens of insect and plant trypanosomatids^{1,3,4,14}, the immunogenicity of these protozoans and the fact that they can be recognized by anti-*Leishmania* spp. and anti-*T. cruzi* antibodies has been overlooked. In contrast, exoantigens of *T. cruzi*¹⁵, and *Leishmania* spp¹⁶ have been studied, have performed well in the diagnosis of CD^{17,18}, and have also been promising for the diagnosis of VL¹⁹⁻²¹ and ATL²⁰⁻²³.

In this study, exoantigens from the insect trypanosomatids *L. seymouri* and *C. fasciculata* were tested by ELISA to evaluate the reactivity of IgG antibodies from patients with CD, VL and ATL.

Exoantigens from *L. seymouri* (TCC011E) and *C. fasciculata* (TCC039) were obtained as previously described for excreted-secreted antigens (ESA) of *L. (L.) infantum chagasi*²⁰ (MHOM/BR/1972/LD). Briefly, exoantigens from *L. seymouri* and *C. fasciculata* were recovered from RPMI-1640 medium containing 1-5 x 10⁸ cultured parasites/mL after incubation for 24 h at 26 °C without agitation, and stored in small aliquots at -40 °C. They were then used without any further purification. None of the exoantigen batches contained tubulin molecules that may have been

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released from the lysed parasites, as attested by the absence of reactivity with a monoclonal anti- α tubulin antibody (data not shown). Trypomastigote excreted-secreted antigens (TESA) of *T. cruzi* (Y strain) were obtained as described elsewhere¹⁷. Alkaline extracts (AEs) from *L. seymouri*, *C. fasciculata* and *L. (L.) infantum chagasi* promastigotas and *T. cruzi* epimastigotes were prepared as previously described^{6,18,20}. Briefly, the parasite pellets were solubilized in 0.3 N NaOH for 18 h at 4 °C, neutralized with 0.3 N HCl to pH 7-8, and centrifuged at 12,000 g for 1 min. at 4 °C. The protein concentration of the supernatants was quantified (Macro-bicinchoninic acid protein assay reagent kit; Pierce Co, Rockford, USA) and aliquots were stored at -20 °C.

ELISAs were performed as previously described^{6,20} using diluted sera 1:200 and incubated with 4 μ g/mL of AEs from the parasites analyzed and with 2 mg/mL of exoantigens from *L. seymouri* and *C. fasciculata*, and 1 mg/mL of ESA from *L. (L.) infantum chagasi* and TESA. Serum samples were recorded as positive or negative based on the cut-off values calculated from the receiver operating characteristics (ROC) for sera collected from 20 healthy blood donors from endemic and non-endemic areas of CD and *Leishmania* spp.

One-way analysis of variance with Bonferroni multiple comparison adjustment was used to compare the mean titers from the evaluated antigens and parasites. The results were considered significant when $p < 0.05$ with a 95% confidence interval. Statistical analyses were performed with the GraphPad Prism 3.0 for Windows (GraphPad Software, USA).

Figure 1 shows the reactivity of serum IgG antibodies according to the antigenic preparation and results were expressed as the absorbance at 492 nm ($Abs_{492\text{ nm}}$). Data from ELISA-Exo revealed that molecules released from *L. seymouri* and *C. fasciculata* reacted with all of the sera ($n=30$) from patients with active VL, living in the State of Piauí, Brazil, whose clinical and diagnostic data had been previously reported²⁴, and the reactivity was similar to that obtained with ESA from *L. (L.) infantum chagasi* (Fig. 1A, Table 1), with no statistical difference between the two tests results ($p > 0.05$), while TESA from *T. cruzi* cross-reacted with only 13% of the sera from VL patients (Fig. 1A, Table 1). AEs from *L. seymouri*, *C. fasciculata* and *L. (L.) infantum chagasi* reacted with IgG antibodies from 100% of VL cases, with no statistical difference among them ($p > 0.05$), while AE from *T. cruzi* reacted with 93% of VL cases (Fig 1B, Table 1), confirming previously described data⁶.

It is known that antibodies found in VL cases show a high degree of cross-reactivity with antigens of different trypanosome species^{6,20}. Indeed, several authors found

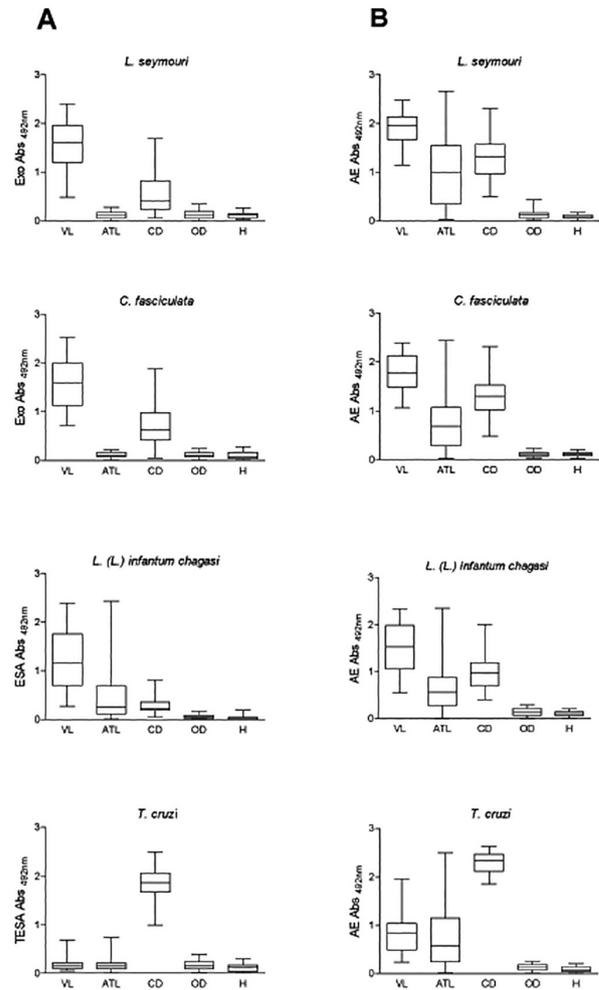


Figure 1 - Box-and-whisker plots of levels of specific IgG antibodies against Exo, ESA, TESA (A) and AE antigens (B) of *L. seymouri*, *C. fasciculata*, *L. (L.) infantum chagasi* and *T. cruzi* expressed as the absorbance at 492 nm ($Abs_{492\text{ nm}}$) in sera from patients with human visceral leishmaniasis (VL), American tegumentary leishmaniasis (ATL), Chagas disease (CD) and other diseases (OD), as well as in sera from healthy individuals (H). The horizontal line inside the box-whisker plot indicates the median.

that serological methods for the detection of VL or ATL cases failed to discriminate between VL and ATL, even with the use of species-specific antigens^{12,20,25}. Thirty-two sera from Brazilian ATL patients from the *Instituto de Infectologia Emilio Ribas* in São Paulo, presenting with clinical diagnosis, as well as a positive Montenegro skin test and identification of the parasites in either skin or mucosal biopsies showed no reactivity with exoantigens from *L. seymouri* or *C. fasciculata*, while 53% of them reacted with ESA from *L. (L.) infantum chagasi*, a significant statistical difference ($p < 0.05$), and 13% reacted with TESA from *T. cruzi* although titers were lower (Fig. 1A, Table 1). ATL sera cross-reacted with AE from *L. seymouri* (63%), *C. fasciculata* (56%), *L. (L.) infantum chagasi* (66%) and

Table 1 - Number of positive cases (*n*) and percentage of positivity (P%) of human sera from patients with visceral leishmaniasis (VL), American tegumentary leishmaniasis (ATL), Chagas disease (CD) and other diseases (OD), as well as from healthy individuals (H). Sera were tested by ELISA using exoantigens (Exos), ESA, TESA and alkaline extracts (AEs) of *L. seymouri*, *C. fasciculata*, *L. (L.) infantum chagasi*, and *T. cruzi*.

Group	<i>n</i>	<i>n</i> (%)							
		<i>L. seymouri</i>		<i>C. fasciculata</i>		<i>L. (L.) infantum chagasi</i>		<i>T. cruzi</i>	
		Exo	AE	Exo	AE	ESA	AE	TESA	AE
VL	30	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)	4 (13)	28 (93)
ATL	32	0	20 (63)	0	18 (56)	17 (53)	21 (66)	4 (13)	19 (77)
CD	27	15 (56)	26 (96)	17 (63)	26 (96)	11 (41)	27 (100)	27 (100)	27 (100)
OD	29	0	0	0	0	0	0	0	0
H	20	0	0	0	0	0	0	0	0
cut off		0.38	0.66	0.50	0.64	0.23	0.38	0.36	0.34

T. cruzi (77%), with no statistical difference among them ($p > 0.05$) (Fig 1B, Table 1).

In this study, exoantigens from *C. fasciculata* and *L. seymouri* did not exhibit antigenic molecules that react with ATL antibodies, however, it was not possible to determine the *Leishmania* species causing lesions in our casuistic so as to affirm that the results presented herewith could be extended for all species occurring in Brazil. Nonetheless, our results have suggested that these exoantigens may constitute a potential alternative for discriminating between VL and ATL.

Cross-reactivity was evaluated using sera from 27 chronic CD patients whose positivity was confirmed by serology. High reactivity levels were observed with AEs from *L. seymouri* and *C. fasciculata* (96%) and *L. (L.) infantum chagasi* (100%) with no statistical difference among them ($p > 0.05$), while for *T. cruzi* (100%) higher mean titers ($p < 0.05$) were detected (Fig 1B, Table 1).

A total of 56% and 63% of these sera were reactive to exoantigens from *L. seymouri* and *C. fasciculata* ($p > 0.05$); and 41% with ESA from *L. (L.) infantum chagasi* ($p < 0.05$); while reactivity was 100% with TESA (Fig. 1A, Table 1).

Despite the cross-reactivity with sera from CD patients, the mean ELISA titers using non-pathogenic trypanosomatid antigens were always lower ($p < 0.05$) when compared to the ones from ELISA using *T. cruzi* antigens.

As expected, none of the 29 sera from patients with other diseases (OD), as defined by clinical, epidemiological and serological diagnosis (4 with toxoplasmosis, 4 with malaria, 4 with schistosomiasis, 6 with tuberculosis, 5 with autoimmune diseases (presence of antinuclear antibodies), 3 with histoplasmosis and 3 with toxocariasis, or 20 healthy (H) blood donors, showed cross-reactivity with exoantigens, ESA, TESA or AE antigens (Fig. 1A, B; Table 1).

This finding represents an advance in the study of

diagnostic techniques for leishmaniasis and it opens up the possibility of using molecules released by *L. seymouri* and *C. fasciculata* in the ELISA format to develop a rapid, accurate and sensitive diagnostic procedure for differentiating between VL and ATL. Moreover, insect trypanosomatids are non-pathogenic to humans and could be used instead of pathogenic *Leishmania* species. The ability to differentiate infection caused by dermatropic *Leishmania* from viscerotropic *Leishmania* in areas where there is an overlap of these parasites, and also the fact that this antigen could be also applied to seroprevalence studies in non-endemic areas for *T. cruzi* are very exciting. However, future accuracy studies in larger populations must be conducted in order to state the real utility of the tested techniques.

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