Immunological characterization of antigens with serological cross-reactivity in trypanosomes

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Abstract

Trypanosomatidae is a group of protozoa belonging to Trypanosomatida order, where the genres of medical importance Leishmania and Trypanosoma are inserted. These genres include parasites Leishmania spp., Trypanosoma cruzi and Trypanosoma brucei, etiologic agents of leishmaniasis, Chagas disease and sleeping sickness, respectively classified as Neglected Tropical Diseases (NTDs) by the World Health Organization (WHO). The diagnostic methods used so far for the detection of these diseases are essentially serological tests, where cross-reactions often occur, since there are antigens shared among the three populations of trypanosomatids. In this sense, this work aimed to characterize and identify antigens of the three trypanosomatids (Leishmania spp., T. cruzi and T. brucei) responsible for the development and maintenance of humoral immunity during the stages of infection and involved in serological cross-reactivity and serological specific reactivity (anti-Leishmania spp., anti-T. cruzi and anti-T. brucei) of different populations. To this end, samples of experimentally infected mice sera and of naturally infected human and canine, with one of three types of trypanosomatids, were used. The results obtained after characterization and crossing of the polyclonal antisera produced, demonstrated the existence of serological cross-reactivity essentially between the four species of Leishmania studied and between T. cruzi Y and T. b. brucei. In what regards the characterization and identification of antigens, there have been identified some antigens with cross-reactivity, where the antigens with molecular weight of approximately 88, 77, 75, 25 and 20 kDa have special relevance. Later, by conducting an analysis in silico, it should be possible to identify the proteins highlighted in the context of this study and there could contribute for the development of new diagnosis methods.

Key-words: Leishmania spp., Trypanosoma cruzi, Trypanosoma brucei, Leishmaniasis, Chagas Disease, Sleeping sickness, cross-reactivity.

1. Introduction

In 2006, the World Health Organization (WHO) proposed the classification of diseases as global, neglected and most neglected. This classification represents an improvement of the term tropical diseases, since it contemplates the political, economic, and social development contexts[1]. Neglected tropical diseases (NTDs) are a medically diverse group of infections caused by a variety of pathogens such as viruses, bacteria, protozoa and helminthes, that develop mainly among the poorest populations. The 17 NTDs prioritized by WHO affect more than one billion people worldwide and are endemic in 149 countries[2]. A special feature of the NTDs in study is that their etiological agents all belong to the same taxonomic family: Trypanosomatidae[2], which are protozoa belonging to Kinetoplastida order. Of the nine genera which constitute this family, Trypanosoma and Leishmania are the only ones with medical importance, due to their unique capability of infecting humans[3]. The sharing of antigens between trypanosomatids has been known and described, since the 60s. In 1969 it was confirmed the presence of cross immunoreactivity between T. cruzi and Leishmania[4]. In fact, this feature for trypanosomatids of medical importance is well documented in the literature and has been confirmed by various immunodiagnostic methods[4–7]. Based in previous studies, an immunochimical characterization of antigens with serological cross-reactivity between different trypanosomatids will be made. Then, it will be possible to use these antigens for the development of serological diagnosis for leishmaniasis, Chagas disease and African trypanosomiasis. Also, these antigens could be used in the development of vaccine prototypes.
2. Materials and Methods

2.1 Animals and parasites

Female Balb/C mice between 5 and 8 weeks of age used in this work were obtained and maintained in approved animal facilities at the Instituto de Higiene e Medicina Tropical (IHMT), Lisbon, Portugal.

2.2 Parasites

L. amazonensis MHOM/BR/1973/M2269: The parasite was isolated in 1973 from a patient with anergic diffuse cutaneous leishmaniasis in the State of Pará, north of Brazil. This strain was identified at the Leishmaniasis laboratory of Evandro Chagas Institute (Belém, Pará state, Brazil) using monoclonal antibodies and isoenzyme electrophoretic profiles. Amastigotes have been maintained in BALB/c mice footpad, isolated and grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS)[9].

L. guyanensis MHOM/BR/2001/M19663: The parasite was isolated in 2001 from human cases of localized cutaneous leishmaniasis, in the lower Amazon mesoregion of Pará state, Brazil. Triturated tissue from punch biopsies was inoculated into the feet of hamsters and cultivated in Difco B45 culture medium. The Evandro Chagas Institute (Belém, Pará state, Brazil) proceeded to the identification using monoclonal antibodies and and isoenzyme electrophoretic profiles[9].

L. infantum MCAN/2012/IHMT0003SG: The parasite was obtained by aspiration using fine needle lymph node of a canine kennel in Lisbon, Portugal and maintained at IHMT by successive passages in Balb/C mice. Promastigotes were isolated in Schneider's Drosophila medium (Sigma-Aldrich, Germany) supplemented with 10% FBS[10].

L. shawi - MHOM/BR/96/M15789: The parasite was isolated from a patient with American tegumentary leishmaniasis in Buriticupu County, State of Maranhão, Brazil, and identified by monoclonal antibodies and multilocus enzyme electrophoresis at the Evandro Chagas Institute in Belém, State of Pará, Brazil. The parasites maintained in BALB/c mice footpads were isolated and grown in RPMI-1640 medium. On day 6 of culture, promastigote forms were centrifuged (1200g, 10 min) with phosphate buffer saline solution (PBS, pH 7.4) and used for antigen production and mouse infection[11].

Trypanosoma cruzi Y: Strain isolated from an acute case of Chagas disease by Silva and Nussenzweig in 1953. Trypomastigote forms were kept in Swiss mice and epimastigotes in LIT culture (Liver Infusion Tryptose) developed by Camargo (1964)[12] and modified by Martinez (2004)[13,14].

Trypanosoma brucei brucei: Strain isolated from wild animals in 1966 from Serengeti National Park, Tanzania.

2.3 Leishmania spp. culture

Virulent promastigotes forms of four Leishmania species were cryopreserved in liquid nitrogen in IHMT facilities. After thawing, the cryopreserved samples were centrifuged at 1800g for 10min, and the parasites (pellets) resuspended in 1 mL of Schneider medium. The parasites were transferred to T-flasks containing 1 mL of Schneider medium and maintained at 24°C. For obtaining virulent L. infantum respect to the five passages limit suggested by the literature[10].

2.4 Trypanosoma cruzi culture

T. cruzi epimastigotes culture started with transferring 1 mL of a pre-established culture for a T-flask containing 5mL LIT medium. The culture was maintained at 24°C and the volume was being duplicated up to a maximum volume supported by T-flask.

2.5 Total protein extract from Trypanosoma cruzi and Leishmania spp.

After reaching the maximum volume capacity of T-flasks and confirm that they were in the stationary phase of growth, the cultures of Leishmania spp. and T. cruzi were centrifuged at 3000g for 10 min, at 4°C. The pellets were washed three times with PBS-20 mM glucose, by centrifugation at 3000g for 10min, at 4°C. The resulting pellets were resuspended in lysis buffer containing protease inhibitor and preserved at -20°C until further use. Total protein content was quantified using Bradford reagent (Bio-Rad, USA) and bovine albumin used as standard.
2.6 Infection of Balb/C Mice with *T. b. brucei*

Balb/C mice were infected with *T. b. brucei* cryopreserved. The intraperitoneal infection was performed, following protocol already developed\(^\text{[15]}\), with a minimum infective dose where the usual clinical manifestations of disease were observed. One week after infection, blood samples were collected for evaluation of the presence of parasites in the blood by optical microscopy. When the infection reached the peak of parasitemia, whole blood was collected by cardiac puncture.

2.7 Total protein extract from *Trypanosoma brucei brucei*

Blood collected from mice infected with *T. b. brucei* was used to purify the bloodstream forms of the parasites using a DEAE Sepharose Fast Flow (Amersham Pharmacia Biotech, USA) anionic exchange chromatography column equilibrated with PBS-glucose 20 mM pH7.4\(^\text{[16]}\). The fractions obtained from eluting the column were observed by optical microscopy in order to verify the presence of the bloodstream forms of *T. b. brucei*. The parasite-containing fractions were centrifuged at 10000g for 10min, and washed three times with PBS-20 mM glucose in order to clean the extract from any residual blood or serum components. The resulting pellets were resuspended in sterile PBS and aliquots were kept at -20°C until further use. The total protein content was measured using the BCA Protein Assay (Sigma, USA).

2.8 Production of monospecific polyclonal antibodies by immunization of Balb/C mice

Groups of three Balb/C mice were immunized subcutaneously with a mixture of protein extract of the respective parasites and adjuvant in order to induce an effective immune response. In the first immunization, an emulsion composed of 100 µg of protein extract and CFA (Freund's Complete Adjuvant) (Sigma, USA) were inoculated. Subsequently, four and six weeks after the first inoculation, the second and third immunization was performed, respectively, with 100 µg of protein extract and IFA (Freund's Incomplete Adjuvant) (Sigma, USA). The animals were sacrificed for whole blood collection to obtain polyclonal sera. Blood samples were incubated at 37°C for 30 min and then at +4°C for 1h. To obtain the serum, blood samples were centrifuged at 10000g for 10min and the serum (supernatant) was removed and stored at -20°C until further use. When necessary, sera was thawed at room temperature and a pool of sera of different groups was used for the determination of anti-*Leishmania* spp., anti-*T. cruzi* Y e anti-*T. b. brucei* antibodies.

2.9 Determination of anti-trypanosomatids antibodies by Enzyme-linked immunosorbent assay (ELISA)

Total IgG antibodies were measured by ELISA using sera samples from mice in the immunized and control groups. Previously, 96-well microplates (Nunc\(^\text{TM}\), Roskilde – Denmark) were coated with 100 ng/well of total protein extract from different parasites in carbonate buffer (0.1M pH 8.5) overnight at 4°C. The microplates were washed three times with PBS-Tween 20 (Promega, EUA) 0.05% (v/v) and blocked for 1h at room temperature with 5% (w/v) powder milk suspension in PBS. After three washes, serial dilution of each pool of the different immunized mice sera were added in duplicate and incubated for 1h at room temperature. After incubation with serum samples, the plate was washed five times. For detection of mouse anti-trypanosome antibodies, the plate was incubated with 100 µL/well of horseradish peroxidase (HRP) conjugated anti-mouse IgG (Sigma, USA) diluted 1:4000 (v/v) for one hour at room temperature. Then, five washings were made. To reveal the presence of conjugated the plate was incubated with 100 µL/well of a substrate solution made up of 10 mL of citrate buffer pH 5.0 with 10 mg of OPD and 10 µL of hydrogen peroxide 30% (v/v); (both from Sigma—USA) for 30min at room temperature and protected from light. Finally, the reaction was stopped by the addition of 4N sulfuric acid and the absorbance was measured at 490 nm.

2.10 Immunocharacterization of antigens using anti-trypanosomatids antibodies

To perform the antigenic analysis by Western Blotting technique, total extract from parasites were used. The proteins were separated by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDSPAGE) with a 10% polyacrylamide gel. The determination of the proteins molecular mass was estimated by comparison with a commercial HyperPAGEW (Bioline, UK) molecular weight marker. After SDS-PAGE, gels were blotted into PVDF membranes (GE Healthcare, United Kingdom) by electric transference at 4°C (30 V) in blotting buffer, overnight. The membranes were then washed two
times with PBS 1% and blocked with blocker buffer (3% (w/v) powder milk suspension in PBS) and incubated for 1h at room temperature. After this, the membranes were washed two times with PBS 0.05% Tween-20 and one time with PBS 1% and incubated with different groups of sera samples all diluted 1/100 (v/v) for 1h at room temperature on shaking. After incubation with primary antibodies the membranes were washed again three times. The antigen-antibody reaction was developed with anti-mouse IgG diluted 1:10000 in blocking solution for 1h at room temperature and washed five times with PBS 0.05% Tween-20. Finally, the membranes were incubated with 10 mL of developing solution [DAB (3,3’ – Diaminobenzidine tablets, Sigma-Aldrich, USA) in PBS] at room temperature until colour development. The reaction was stopped with distilled water. After stopping the reaction membrane was dried and stored for later use in the identification of immunogenic proteins.

3. Results and Discussion

The main purpose of the present study is to evaluate the specific and cross immune reactivity between the different populations of trypanosomes: Leishmania spp., T. cruzi and T. b. brucei. Initially, it was required a production of total protein extracts from each of the parasites mentioned above. Then, these extracts were used to immunize mice, in order to produce monospecific polyclonal sera against each species. Serological reactivity of the respective sera was determined using ELISA tests. These allowed to determine the levels of antibodies with serological reactivity for each of the extracts and/or between extracts and then quantify the level of reactivity between the three trypanosomatids.

Afterwards, it sought to identify antigens present in each protein extracts and responsible for serological reactivity. By immunoblotting, it was intended to characterize immunochemically the monospecific polyclonal sera and the sera from infection caused by Leishmania spp., T. cruzi and T. b. brucei.

3.1 Immunocharacterization of polyclonal antibodies by ELISA

The results obtained in the ELISA assays, to characterize the produced monospecific IgG polyclonal are presented in Figure 1 and 2. These results were based on the immunoenzymatic reaction between the produced monospecific polyclonal antibodies of each species of parasites and respective protein extracts.

![Image](image.png)

**Figure 1** – Specific reactivity of anti-Leishmania polyclonal antibodies produced against the respective extracts. A - L. amazonensis; B - L. guyanensis; C - L. infantum; D - L. shawi; Negative controls represented in black.

In the presented figures, the results are expressed as the inverse of the ratio of dilution of polyclonal sera as a function of its absorbance (OD), at a wavelength of 490nm. In Figure 1 and Figure 2, it is possible to notice that the monospecific polyclonal sera showed high OD values (titers greater than 3200 for all polyclonal sera) when compared to sera obtained from non-immunized animals (control group). This confirmed the presence of anti-Leishmania, anti-T. cruzi and anti-T. b. brucei IgG antibodies, in the polyclonal sera produced by immunization process using the protein extracts from each parasite. In Figure 1, it can be observed that the anti-L. amazonensis, anti-L. guyanensis and anti-L. infantum polyclonal antibodies (represented in Figure 1A, B and C) present identical serological reactivity profiles. However, the serological reactivity profile of the anti-L. shawi polyclonal antibody is much higher, as shown in Figure 1D. The titer of anti-L. shawi antibodies, determined as the inverse of the dilution of the anti-L. shawi polyclonal serum, was only measurable from a dilution of 1/1600, since for lesser dilutions, the OD values were greater than 3.5. Its high reactivity, relatively to the other species of Leishmania, may be due to an enhanced expression of its protein antigens present in extracts or to the expression of more immunogenic antigens.
For the anti-\textit{T. cruzi} and anti-\textit{T. b. brucei} polyclonal shown in Figure 2, it appears that the observed serological reactivity profiles are higher than those observed for the anti-\textit{L. amazonensis}, anti-\textit{L. guyanensis} and anti-\textit{L. infantum} polyclonal antibodies. The high titer of anti-\textit{T. cruzi} and anti-\textit{T. b. brucei} polyclonal antibodies can be a consequence of the very constitution of the protein extracts and of the type of antigen they present.

3.2 Serological cross-reactivity between different polyclonal antibodies

Using the monospecific polyclonal antibodies produced against the parasite protein extracts, it was possible to study the specific and cross-reactivity and establish a comparison between the different species of \textit{Leishmania}, between \textit{T. cruzi} Y and \textit{T. b. brucei} and among all trypanosomatids. Figure 3 shows the results of specific and cross reactions among the four \textit{Leishmania} species. As expected, when compared with the reactivity of sera obtained from animals of control group, the specific reactivity of anti-\textit{Leishmania} polyclonal antibodies was, in all tests, higher, with OD values higher than 0.5 for up to dilutions of 1/400. Comparatively with cross-reactivity between the four species of \textit{Leishmania}, the specific reactivity of anti-\textit{L. amazonensis}, anti-\textit{L. guyanensis}, anti-\textit{L. infantum} and anti-\textit{L. shawi} polyclonal antibodies proved to be identical or higher. The high serologic reactivity observed in each assay (equivalent to highly reactive positive controls), along with a total of negative controls with residual values, ensure the validity of the results presented. It was also observed a reactivity profile common to all species, which titles are expressed in terms of dilution and where it can be found that higher titles correspond to lower dilutions and higher values of OD.

Analyzing the data present in Figure 3, it is noted that specific reactivity of anti-\textit{L. shawi} polyclonal antibody is quite high when compared to the one from the other species of \textit{Leishmania}, presenting values of OD higher than 3.5 for up to a dilution of 1/800. On the other hand, the cross-reactivity of anti-\textit{L. shawi} polyclonal antibody against extracts of \textit{L. amazonensis} and \textit{L. infantum} has lower OD values than the ones presented by cross-reactivity with the other species of \textit{Leishmania}. The slight difference observed could be due to the fact that \textit{L. shawi} belongs to the \textit{Viannia} subgenus, while \textit{L. amazonensis} and \textit{L. infantum} species belong to \textit{Leishmania} subgenus. In contrast, when \textit{L. shawi} is crossed with \textit{L. guyanensis}, it can be observed in Figure 3B and in Figure 3D a higher cross reactivity. This is possibly due to the affinity between the two species, since both belong to the \textit{Viannia} subgenus, therefore being responsible for the cutaneous and mucocutaneous leishmaniasis. In a similar way as for the group of the previous results, it is possible to observe in Figure 4, that the assays which aimed at the study of specific and cross-reactivity between \textit{T. cruzi} and \textit{T. b. brucei} were, based on the lower values of the negative controls and on the predominant activity of the specific reactions, properly executed. They also present the expected curved profile, with a decrease in absorbance as the dilution of the polyclonal antibody increases. Comparing the graphics A and B of Figure 4, it can be concluded that the specific serologic reactivity of the anti-\textit{T. cruzi} and anti-\textit{T. b. brucei} polyclonal antibodies
has similarly high values (with OD values higher than 1 for a dilution of up to 1/3200 of polyclonal sera), so it is only measurable for polyclonal antibody dilutions higher than 1/400. In what regards cross-reactivity between *T. cruzi* and *T. b. brucei*, it was verified that there is a higher recognition of anti-*T. b. brucei* polyclonal antibody by the protein extract from *T. cruzi* Y. When compared with the cross-reactivity observed between the four species of *Leishmania*, cross-reactivity between *T. cruzi* and *T. b. brucei* is lower. However, this might be due to the inherent differences between the two parasites. In fact, Stevens et al (1999) suggest that *T. cruzi* and *T. b. brucei* have different origins and fundamentally different patterns of evolution [17]. In addition to this, there has also been shown that there are differences in the ways of transmission and infection, thereby resulting in different life cycles and different clinical expressions [18,19]. Moreover, it should also be noted that the anti-*T. b. brucei* polyclonal antibody has been produced against extract of trypanmastigotes blood forms from the parasite. On the other hand, anti-*T. cruzi* polyclonal antibody has been produced against extract of epimastigote forms present in the digestive tube of the vector. Despite this, there still exists cross recognition by both polyclonal sera.

![Figure 4](image)

**Figure 4** – Specific and cross-reactivity of anti-*T. cruzi* (in orange) and anti-*T. b. brucei* (in red) polyclonal antibodies against each of extracts. A – *T. cruzi* Y extract; B – *T. b. brucei* extract; Negative controls represented in black.

In Figure 5, it is graphically represented the serological reactivity of anti-*T. cruzi* and anti-*T. b. brucei* polyclonal antibodies against the extracts of *Leishmania* spp. (Figure 5A-D) and the serological reactivity of anti-*Leishmania* spp. polyclonal antibodies against the extract of *T. cruzi* Y (Figure 5E) and the extract of *T. b. brucei* (Figure 5F). It is possible to observe a recognition of the protein extracts of different species of Leishmania by anti-*T. b. brucei* polyclonal antibody. However, since they are different parasites, the reactivity levels are lower than what is shown in Figure 3. For the anti-*T. cruzi* polyclonal antibody, the reactivity profile is close to the reactivity curve of the control groups, suggesting a lack of recognition of the parasitic extracts of *L. amazonensis*, *L. guyanensis*, *L. infantum* and *L. shawi*. From Figure 5E and Figure 5F, it is clear that none of the anti-*Leishmania* polyclonal antibodies recognizes the protein extracts of *T. cruzi* and *T. b. brucei*, since they present a reactivity identical to that of the animals of the control group. The results described Figure 5, in relation to anti-*T. cruzi* and anti-*Leishmania* polyclonal antibodies are not consistent with those described in the literature, where it is reported the occurrence of cross reactions between *Leishmania* and *Trypanosoma* [4], in particular, between *T. cruzi* and *Leishmania*.

![Figure 5](image)

**Figure 5** – Specific and cross-reactivity of anti-*L. amazonensis* (in pink), anti-*L. guyanensis* (in blue), anti-*L. infantum* (in green), anti-*L. shawi* (in purple), anti-*T. cruzi* (in orange) and anti-*T. b. brucei* (in red) polyclonal antibodies produced against respective extracts. A – *L. amazonensis* extract; B – *L. guyanensis* extract; C – *L. infantum* extract; D – *L. shawi* extract; E – *T. cruzi* Y extract; F – *T. b. brucei* extract; Negative controls represented in black.

### 3.3 Characterization of the antigens involved in specific serologic reactivity and serological cross-reactivity between different trypanosomatids

After confirming the existence of serological cross-reactivity between trypanosomatids, it sought to identify the antigens responsible for cross-
reactivity using the immunoblotting technique. In these tests it was decided to use sera from natural and experimental infections, instead of polyclonal sera, given the possibility to adapt the results to the clinical context. There were tested 3 sera from natural infection by *L. infantum* in dogs, 3 sera of natural infection by *T. cruzi* in individuals from Brazil and 2 sera from experimental infection by *T. b. brucei*, obtained 7 and 35 days after the infection of BALB/c mice. A polyclonal antibody specific from each extract used in the above ELISA assays, was used as positive control and a serum from healthy individuals was used as negative control, allowing the detection of nonspecific reactions.

Regarding the analysis of the results, it can be observed in the strips corresponding to the positive control in Figures 6 and 7, a protein profile characteristic of the reaction of each extract with respective polyclonal antisera (anti-*Leishmania* spp., anti-*T. cruzi* and anti-*T. b. brucei*). Additionally, the absence of bands in the strips corresponding to the negative controls in Figures 6 and 7, attests the validity of the assays, since it is not observed antigenic reactivity with the proteins of infection sera used, which ensures the absence of nonspecific reactions. In set of results related to the sera from experimental infection by *T. b. brucei*, it is not observed the existence of bands, as it can be found in the strips 7 and 8 of the membranes of each of parasites (Figures 6 and 7). The lack of detection by the sera of the proteins that constitute the different protein extracts may be due to the low titers of antibodies, an hypothesis that was subsequently confirmed by ELISA. The presented negative results relative to the cross reactivity with sera by *T. b. brucei* cannot, however, be supplemented or refuted by the literature, given the absence of studies in this area. In contrast with the infection sera from *T. b. brucei*, the sera related to infections by *L. infantum* and *T. cruzi* proved to be reactive at both the specific and cross levels, being possible to recognize in Figures 6 and 7, the proteins detected in the various assays of immunoblotting.

*Figure 6* – Immunoblotting profile of the membranes with *Leishmania* spp. extracts. (M) molecular weight marker; (C') positive control; Strip 1, 2 and 3 – Sera from infection by *L. infantum*; Strip 4, 5 and 6 – Sera from infection by *T. cruzi*; Strip 7 and 8 – Sera from infection by *T. b. brucei*; (C) negative control.
By using a protein molecular weight marker, it was possible to estimate the molecular weight of the antigens recognized by the infection sera. Regarding the extracts of L. amazonensis, there has been a recognition by the anti-L. amazonensis polyclonal of antigens with mol. wt. 77, 70, 67kDa (Figure 6 - L. amazonensis extract, strip C'). Sera from leishmaniasis infection recognized in the L. amazonensis extract the same antigens described before and antigens of mol. wt. 88, 25, 20kDa (Figure 6 - L. amazonensis extract, strips 1-3).

For the L. guyanensis extract, the anti-L. guyanensis polyclonal reacted with antigens of mol. wt. 185, 145, 142, 125, 77, 70, 67, 60kDa (Figure 6 - L. guyanensis extract, strip C'). Sera from infection by L. infantum, recognizes antigens of mol. wt. 145, 88, 77, 70, 35kDa (Figure 6 - L. guyanensis extract, strips 1-3).

Anti-L. infantum polyclonal recognizes in the L. infantum extract antigens of mol. wt. 77, 70, 67, 60, 35kDa (Figure 6 - L. infantum extract, strip C'), while sera from infection by L. infantum detects antigens of mol. wt. 88, 77, 50, 44, 42, 25, 20kDa (Figure 6 - L. infantum extract, strip 1-3).

In the extract of L. shawi, anti-L. shawi polyclonal recognizes antigens of mol. wt. 185, 166, 145, 113, 109kDa (Figure 6 - L. shawi extract, strip C'). On the other hand, the sera from leishmaniasis infection reacted with the antigens of mol. wt. 88, 77, 50, 44, 42, 25, 20kDa (Figure 6 - L. shawi extract, strip 1-3).

According to the description given above, proteins of mol. wt. 88, 77, 25, 20kDa stand out, since they are detected by sera infected by L. infantum in almost all Leishmania extracts, thus may be considered antigens with cross-reactivity between the four species of Leishmania.

In literature there can be found some studies that describe L. infantum antigens which constitute the basis of serological diagnosis. Aisa et al. (1998) analyzed by western blott, a set of 72 sera from dogs infected with L. infantum, which recognized antigens of mol. wt. 70, 65, 46, 30 28, 14, 12kDa [23]. Mary C. et al. (1992) found that antigens of mol. wt. 68, 46, 30-28, 16, 14kDa are recognized by sera from patients diagnosed positive for visceral leishmaniasis [24]. Analyzing exclusively the serological reactivity of the L. infantum extract, it was verified, in the set of infection sera from L. infantum and anti-L. infantum polyclonal, the detection of the antigens of mol. wt. 88, 77, 70, 67, 60, 50, 44, 42, 35, 25, 20kDa. Some of these antigens might correspond to the ones identified in the previous studies, namely the ones of mol. wt. 70, 67, 44, 35 e 25kDa, since there is always an error associated with the molecular weight estimation.

In the extract of T. cruzi Y, the anti-T. cruzi polyclonal recognizes antigens of mol. wt 87, 75, 71, 50, 47, 44, 28, 20kDa (Figure 7 - T. cruzi Y extract, strip C') and the sera from chagasic patients reacts with antigens of mol. wt. 80, 50, 47, 28, 20kDa (Figure 7 - T. cruzi Y extract, strips 4-6). A previous study of our research group identified a reactivity pattern in a set of 28 sera from chagasic patients living in Europe which included four reactivity zones: 15-20kDa, 25-30kDa, 40-50kDa
and 80-125kDa, matching the reactivity zones observed in this study.\textsuperscript{25}

In the extract of \textit{T. b. brucei}, the anti-\textit{T. b. brucei} polyclonal recognized the antigens with mol. wt. of 125, 118, 100, 92, 77, 71, 54, 47, 40kDa (Figure 7 - \textit{T. b. brucei} extract, strip C'), while the sera from chagasic patients reacted with the antigens of mol. wt. 118, 77, 54, 47, 36kDa (Figure 7 - \textit{T. b. brucei} extract, strips 4-6).

In order to analyze the serologic reactivity between \textit{Leishmania}, \textit{T. cruzi} and \textit{T. b. brucei}, it was taken into account the antigens recognized by infection sera by \textit{T. cruzi} present in the extracts of \textit{Leishmania} spp. and the antigens detected by the sera from infection by \textit{L. infantum} present in the extracts of \textit{T. cruzi} and of \textit{T. b. brucei}. The antigens present in the \textit{T. b. brucei} extract recognized by the infection sera by \textit{T. cruzi} had already been considered previously.

The sera from chagasic patients recognized in all extracts of \textit{Leishmania} spp. the antigens of mol. wt. 77, 35 and 25kDa, these being considered antigens with serological cross-reactivity. Besides the referred antigens, other proteins showed reactivity in some extracts. The sera from chagasic patients recognized, in the extract of \textit{L. amazonensis}, the antigen of mol. wt. 20kDa (Figure 6 - \textit{L. amazonensis} extract, strips 4-6), in the extract of \textit{L. guyanensis}, the antigens of mol. wt. 125, 70, 67, 30kDa (Figure 6 - \textit{L. guyanensis} extract, strips 4-6) and in the extract of \textit{L. infantum} the antigen of mol. wt. 30kDa. (Figure 6 - \textit{L. infantum} extract, strips 4-6). In the extract of \textit{T. cruzi} Y, the sera from \textit{L. infantum} infection reacted with antigens of mol. wt. 80, 75, 70, 35, 20kDa (Figure 7 - \textit{T. cruzi} Y extract, strips 1-3). At last, in the extract of \textit{T. b. brucei}, the sera from leishmaniasis infection, recognized the antigens of mol. wt. 88, 80, 77, 75, 60, 54kDa (Figure 7 - \textit{T. b. brucei} extract, strips 1-3).

Unlike what was observed in ELISA assays, in these assays it was possible to detect proteins with serological cross-reactivity between the three populations of trypanosomes, which is due to the fact that the immunoblotting shows greater sensitivity when compared with ELISA.

4. Conclusions

This study pretended to determine and identify the antigens common to the three populations of trypanosomes and therefore responsible for the serological cross-reactivity between \textit{Leishmania} spp., \textit{T. cruzi} and \textit{T. b. brucei}. By ELISA, it was possible to confirm the existence of cross-reactivity between the three trypanosomes and to show, as it was expected, a higher specific serologic reactivity. There was a greater cross-reactivity between the four species of \textit{Leishmania} and between \textit{T. cruzi} and \textit{T. b. brucei}.

Through immunoblotting profile analysis, antigens responsible for serological cross-reactivity were identified among \textit{Leishmania} species, which were the antigens of mol. wt. 88, 77, 25, 20kDa; it was also possible to identify the antigens responsible for serological cross-reactivity between \textit{T. cruzi} and \textit{T. b. brucei}, of mol. wt. 118, 77, 54, 47, 36kDa; also the antigens responsible for serological cross-reactivity between \textit{T. cruzi} and \textit{Leishmania} spp. were identified, of mol. wt. 80, 77, 75, 35, 25, 20kDa and, at last, the antigens responsible for serological cross-reactivity between \textit{Leishmania} spp. and \textit{T. b. brucei}, of mol. wt. 88, 80, 77, 75, 60, 54kDa.

In the future, to complement the work, it is suggested to carry out the sequencing of the main detected antigens involved in serological cross-reactivity between the three trypanosomes. It is expected that the results of an \textit{in silico} analysis contribute to the research and identification of new target antigens, which may be used as serological markers in the development of new laboratory tests.

It is important to note that this study is only a base model to extrapolate results based on a small sample of infection sera, being relevant to validate this model in a population with natural infection.

In order to improve the obtained results, it is suggested to use a larger number of samples of infection sera representative of each of the different pathologies and from the distinct endemic areas. This will contribute to confirm and validate the present study and to identify other proteins responsible for the antigenic cross-reactivity. In addition, it is useful to include the infection sera by \textit{T. brucei} with a sufficiently high reactivity, in order to effectively identify antigens which have cross reactivity with the \textit{Leishmania} spp and \textit{T. cruzi} extracts.

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6. References