

## LEISHMANIA BRAZILIENSIS: ISOLATION OF CARBOHYDRATE-CONTAINING ANTIGEN AND POSSIBILITY OF ITS USE IN THE IMMUNODIAGNOSIS OF AMERICAN CUTANEOUS LEISHMANIASIS

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### SUMMARY

*Leishmania braziliensis* is a causative agent of American Cutaneous Leishmaniasis (ACL). The 034-JCG strain, isolated from a patient from the northern region of Paraná State, Brazil, was cultivated in Blood Agar Base medium, lyophilized and submitted to phenol-water extraction. The extract was treated with RNase I. The carbohydrate containing-antigen (Ag-CHO) was immunogenic to rabbits and showed at least a fraction with some negative charge at pH 8.2. This antigen showed cross-reactivity with the phenol-water extract of the growth medium used for the culture of promastigotes and with the surface antigens of promastigotes. Its composition is: 24.3% of total sugars, from which 11.2% of galactose, 7.5% of mannose and 5.6% of ribose. Protein content was 5.4% and phosphate 18.5%. The antigenic activity was maintained after: repeated freezing-thawing; lyophilization; heating at 100°C for 30 minutes; treatment with RNase, trichloroacetic acid and sodium metaperiodate. The precipitin line obtained is Periodic Acid Schiff positive. The application of the Ag-CHO in counterimmunoelectrophoresis reaction for the immunodiagnosis of ACL showed 60% sensitivity, and no cross-reaction with the five sera of Chagas' disease patients tested. The use of this antigen in a more sensitive technique, with more samples of sera, may improve these results.

**KEYWORDS:** *Leishmania braziliensis*; Carbohydrate-containing antigen; Immunodiagnosis.

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### INTRODUCTION

Different species of the genus *Leishmania* are responsible for a large spectrum of cutaneous, mucocutaneous and visceral diseases that occur in the New World, becoming thus, one of the main problems in public health. Chemotherapy is normally difficult and not always effective. In Brazil, American Cutaneous Leishmaniasis (ACL) is endemic, and one of its agents, *Leishmania braziliensis*, can invade the nasal mucosa and the soft palate. When not properly treated it may lead to extensive destruction of cartilage <sup>20</sup>.

The diagnosis of ACL is based on clinical, epidemiological and laboratory evidences. The following laboratory methods can be practiced: parasitological diagnosis, cutaneous tests and serological reactions. Search for parasites in smears from cutaneous lesions and tissues presents positivity percentages inversely proportional to the duration of infection <sup>16</sup>. The Montenegro intradermal reaction presents positivity levels ranging from 82.4 to 100%, being negative or slightly positive in the early stage of

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infection<sup>16</sup>. Furthermore, the usual concept is that the intradermal reaction remains positive during the patient's life time, allowing a retrospective diagnosis of the disease in practically 100% of cases<sup>16</sup>.

The antibody production in response to the parasite has been demonstrated in all clinical forms of leishmaniasis<sup>29</sup>. Due to difficulties often met to establish parasitological diagnosis in leishmaniasis, the search for a serological test has been the objective of many researchers<sup>37</sup>. Among serological reactions for ACL diagnosis, complement fixation techniques<sup>16</sup> have been standardized and also those of passive hemagglutination<sup>42</sup>, counterimmunoelectrophoresis<sup>42</sup>, indirect immunofluorescence<sup>27,35</sup>, radio immunoassay<sup>29</sup>, and enzyme immunoassay<sup>20,28</sup>.

Several components of *Leishmania* have been studied. The surface components which show antigenicity<sup>25,30</sup> are glycoproteins<sup>8,13</sup> and glycolipids<sup>25,30</sup>. Excreted factors (EF) of the parasite in the culture medium<sup>23,34</sup>, rich in carbohydrate, presumably a glycoprotein<sup>43</sup>, are immunologically reactive. They may be bound by and precipitated with homologous antibodies, and show serological specificity<sup>14,15</sup>. A kinesin-related antigen of *Leishmania chagasi* specific in African and American visceral leishmaniasis has been characterized<sup>5</sup>. These studies have aimed at identifying and characterizing components potentially useful to differentiate species and strains of *Leishmania*, to show differences between phases of life cycle<sup>17</sup>, to propitiate ways for immunodiagnosis and immunoprophylaxy or to interfere in the interactions parasite-host<sup>31</sup>.

In this work a constituent fraction of promastigote forms of *Leishmania braziliensis* was obtained and partially characterized. The *Leishmania* was isolated in the northern region of Paraná State, Brazil and its immunogenicity and the possibility to be used in techniques for laboratory diagnosis of American Cutaneous Leishmaniasis were evaluated.

## MATERIALS AND METHODS

### *Culture and obtention of Leishmania*

The strain 034-JCG of *Leishmania* was isolated from a cutaneous lesion of a patient from the northern region of Paraná State, and characterized as *Leishmania (Viannia) braziliensis* through monoclonal antibodies

and enzymatic profile, by Dr. J. J. Shaw, Evandro Chagas Institute, Belém, Pará.

The parasite was cultivated at 25°C, for eight days, in Blood Agar Base Difco<sup>38</sup> medium, pH 7.2, containing 10% of defibrinated and lysed rabbit blood<sup>29</sup>. The parasites were washed three times at pH 7.2 in a phosphate buffered solution (PBS) at 1800g and 4°C for 20 minutes. The parasitic mass was kept in liquid nitrogen for late use.

### *Obtention of antigen containing carbohydrate (Ag-CHO)*

The parasitic mass (59.6g) was lyophilized (5.15g) and resuspended in distilled water (15 ml/g dry weight). Extraction was made in 5g of material with phenol 90% at 68°C, for 10 minutes. Afterwards, the material was centrifuged at 1900g and 10°C for 40 minutes<sup>40</sup>. The aqueous phase was precipitated with two volumes of ethanol containing 2% of potassium acetate, overnight at 4°C. The precipitate obtained by centrifuging at 3500g and 4°C for 10 minutes was resuspended in distilled water and dialysed at 4°C for 48 hours<sup>18</sup>. The insoluble material was decanted and the supernatant centrifuged at 2000g for 20 minutes, at 10°C and then lyophilized (0.23g).

The same procedure was used for carbohydrate extraction from the culture medium not used for the parasite culture (N) and from the culture medium used to cultivate the parasites (U), after removing them.

Presence of nucleic acids in Ag-CHO was quantitatively assessed using the diphenylamine and orcinol techniques<sup>10,11</sup>. For the removal of nucleic acids, Ag-CHO was dissolved in 100 ml of buffer TRIS-HCl 20mM, containing 5mM magnesium acetate, pH 7.4. The mixture was incubated with 50,000U of Ribonuclease T<sub>1</sub> Sigma at 37°C for 7 hours. It was then dialysed at 4°C against distilled water and lyophilized<sup>18</sup>. To remove RNase a new extraction was made with phenol, precipitated with ethanol and lyophilized. The lyophilized Ag-CHO (0.12g) was kept in a desiccator, under vacuum, at room temperature until its use.

### *Determining carbohydrates, proteins and phosphate*

Total sugars, except those aminated, were determined using the phenol-sulphuric method<sup>12</sup>. Neutral sugars were determined by gas-liquid chromatography (G. L. C.)<sup>1,32</sup>. The samples were injected in chloroform solution, and the retention times obtained were com-

pared to those of standards. Peak areas were calculated by triangulation.

Proteins were determined using the Lowry method <sup>26</sup> and total phosphate according to AMES (1966) <sup>2</sup>.

#### **Immunization of rabbits**

Male, albino rabbits were immunized with Ag-CHO or with whole forms of lyophilized *Leishmania braziliensis* (Ag-LI), both at 4mg/ml in PBS. The immunization scheme used for Ag-CHO (rabbit A) was intravenous doses of 0.5; 1.0 and 2.0 ml on the 1<sup>st</sup>, 3<sup>rd</sup>, and 5<sup>th</sup> days respectively. Additional subcutaneous doses of 0.5 ml with 0.5 ml of Freund's incomplete adjuvant (FIA) were given on days 15, 22, 29, 36, 57 and 64. For Ag-LI, two immunization schemes were used. Rabbit C was immunized with intravenous doses of 0.5; 1.0 and 2.0 ml on days 1, 3 and 5 respectively. Subcutaneous doses with FIA were also given on days 15, 22, 29, 36, 64 and 116<sup>th</sup> day. Rabbit D was immunized with 0.5 ml of Ag-LI with 0.5 ml of FIA, subcutaneously, on days 1, 8, 15, 22, 29, 36, 43, 71, 78 with an additional dose on the 123<sup>rd</sup> day. All rabbits were bled before the beginning and during the immunization scheme. Rabbits A, C and D were total bled by cardiac puncture on the 81<sup>st</sup>, 127<sup>th</sup> and 134<sup>th</sup> days respectively.

#### **Sera**

Rabbits' sera were obtained at several bleedings. Human sera, positive for American Cutaneous Leishmaniasis (ACL) were obtained from 10 individuals who had skin lesions, with microscopy of lesion material positive for *Leishmania*. The sera of individuals suffering from Chagas disease were obtained from 5 patients with positive hemoculture for *Trypanosoma cruzi*. Normal sera were obtained from 5 individuals without ACL history.

All sera were aliquoted and stored at -20°C until use. In some cases, the gammaglobulin fraction of serum was precipitated with ammonium sulphate, pH 8.2 at 40% <sup>39</sup>.

#### **Analysis of Ag-CHO Biochemical Properties**

Ag-CHO was submitted to several treatments <sup>18</sup> such as: heating in boiling water bath for 30 minutes; trichloroacetic acid at 10%, for one hour at 4°C, having the precipitate been removed by centrifugation and the supernatant neutralized with NaOH 0.5N; lyophilization; repeated freezing and thawing; treatment with

RNase or with sodium metaperiodate at 0.135M for two hours at room temperature. The products of each treatment were tested by immunoprecipitation methods, the reactions of which being stained with Periodic Acid of Schiff (PAS) <sup>4</sup>.

#### **Immunoprecipitation reactions**

The double diffusion reactions (DD) were made in agarose 1% in PBS. Immunoelectrophoresis reactions (IEP) were made in agarose 1% Veronal-HCl Buffer 0.05M, pH 8.2 with 0.5% of sodium azide and the electrophoresis was carried out with current of 6 mA. Counterimmunoelectrophoresis reaction (CIEP) was made in agarose 1% Veronal-HCl Buffer 0.05M, pH 8.2 and the electrophoretic run was carried out with a current of 6 mA. For the development of precipitation lines, incubations were performed in a damp chamber at 25°C for 24 hours. Reactions were stained alternatively, with Ponceau S.

#### **Indirect immunofluorescence test**

Antigen for the indirect immunofluorescence test (IIF) was prepared from a six-day culture of strain 034-JCG of *Leishmania braziliensis* in Blood Agar Base Difco medium. The promastigotes were washed three times in PBS at 1800g for 20 minutes, at 4°C. The parasitic mass obtained was suspended in 2% formalin in PBS, for two hours at room temperature. Afterwards, promastigote forms were washed three times in PBS at 1800g, for 10 minutes at room temperature. The suspension was standardized so as to contain 10<sup>7</sup> promastigotes/ml, aliquoted, lyophilized and stored at 4°C. For use, the parasites were resuspended in distilled water, distributed on square-lined slides and stored at -20°C for later use. The test was conducted according to GUIMARÃES et al. (1974) <sup>19</sup>. The sera were diluted in PBS pH 7.2 from 1/20 in ratio 2. The anti-rabbit total immunoglobulin and anti-human immunoglobulin G fluorescein isothiocyanate conjugates were standardized according to CAMARGO (1973) <sup>6</sup>.

## **RESULTS**

Table 1 shows Ag-CHO composition. The electrophoretic analysis at pH 8.2 shows that Ag-CHO is apparently composed by one antigenic fraction with few negative charges. After staining with Ponceau S, two precipitation lines were observed, but with different diffusion velocities.

The serum of rabbit A, immunized with Ag-CHO,

TABLE I

Composition of Ag-CHO of *Leishmania braziliensis*.

	mg%
Total Sugars <sup>1</sup>	24.3
Galactose <sup>2</sup>	11.2
Mannose <sup>2</sup>	7.5
Ribose <sup>2</sup>	5.6
Protein <sup>3</sup>	5.4
Total Phosphat <sup>4</sup>	18.5

<sup>1</sup> Phenol-sulfuric method.

<sup>2</sup> Gas-liquid chromatography.

<sup>3</sup> Lowry method.

<sup>4</sup> Ames method.

obtained at different bleed times, was tested by DD and CIEP against Ag-CHO at the concentration of 0.6 mg/ml. Antibodies were detected only from the 40<sup>th</sup> day of immunization (Figure 1.1), and the serum obtained on the 81<sup>st</sup> day of immunization only reacted at 1/1.

Figure 1.2 shows that the serum of rabbit A immunized with Ag-CHO reacts with Ag-CHO, like the sera of rabbits immunized with Ag-L1 (rabbits C and D). Through IIF test with promastigotes the titer of 1/1280 was obtained with serum of rabbit A and 1/640 with the sera of rabbits C and D.

Serum of rabbit A (Ab-CHO) reacts with extracts from the culture medium used for the cultivation of *Leishmania braziliensis* (U) (Figure 1.3), but it does not react with extracts from the culture medium unused for the cultivation of *Leishmania* (N) at the same concentrations. In Figure 1.4 the line of antigenic identity between U and Ag-CHO can be observed. The reaction specificity was revealed by normal serum of rabbit, which does not react with U, N or Ag-CHO (Figure 1.5).

The Ab-CHO, absorbed with formalin-treated promastigotes in the proportion of  $2 \times 10^8$  promastigotes/ml at 37°C, does not react in DD with Ag-CHO (Figure 1.6).

Ag-CHO kept its antigenic activity unchanged after treatments such as: lyophilization; repeated freezing-thawing; heating at 100°C for 30 minutes; trichloroacetic acid at 10%, and sodium metaperiodate 0.135M (Figure 1.7). The precipitation lines obtained were stained by PAS.

CIEP with Ag-CHO detected six patients from a group of ten ACL carriers with positive parasitological

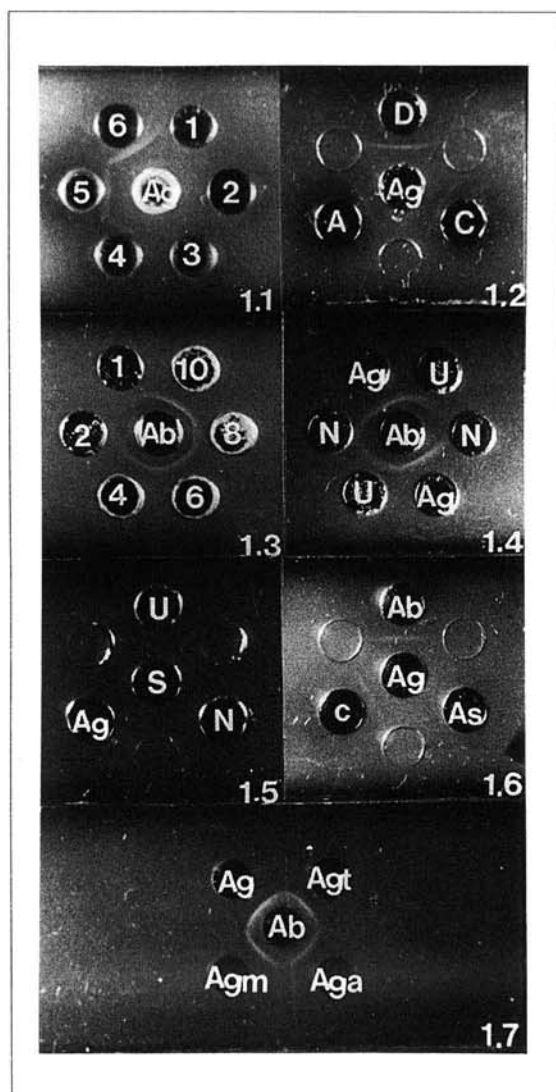


Fig. 1 - Double diffusion reaction.

1.1 Ag: Ag-CHO at 0.6 mg/ml. 1, 2, 3, 4, 5 and 6: sera of rabbit A on the 0, 12th, 19th, 40th, 68th and 81st days of the immunization, respectively.

1.2 Ag: Ag-CHO. A, C and D: Sera of rabbits A, C and D, respectively, on the total cardiac puncture.

1.3 Ab: Ab-CHO. 10, 8, 6, 4, 2 and 1: concentration of U at mg/ml.

1.4 Ab: Ab-CHO. Ag: Ag-CHO at 0.2 mg/ml. U: extract from the culture medium used to cultivate at 2 mg/ml. N: extract from the culture medium not used for the parasite culture at 2 mg/ml.

1.5 S: serum of normal rabbit. Ag: Ag-CHO at 0.2 mg/ml. U: extract from the culture medium used to cultivate at 2 mg/ml. N: extract from the culture medium not used for the parasite culture at 2 mg/ml.

1.6 Ag: Ag-CHO at 0.2 mg/ml. Ab: Ab-CHO. As: Ab-CHO absorbed with promastigotes of *Leishmania braziliensis*. c: absorption control.

1.7 Ab: Ab-CHO. Ag: Ag-CHO. Agt: Ag-CHO after heating at 100°C for 30 minutes. Aga: Ag-CHO treated with trichloroacetic acid at 10%. Agm: Ag-CHO treated with sodium metaperiodate at 0.135M.



diagnosis. The five sera of Chagas' disease carriers, with positive hemoculture for *Trypanosoma cruzi* and the five sera of normal patients did not present reaction by CIEP.

IIF was positive in all ten patients of ACL, when assuming as significant for the northern region of Paraná the titer 1/40<sup>36</sup>. It did not present any reaction for normal individuals, but it detected four crossed reactions in the group of five Chagas' disease carriers.

## DISCUSSION

The proportion of sugars obtained from *Leishmania braziliensis* by phenol-water extraction (24.3%) is close to that found in *Leishmania adleri* (24%)<sup>26</sup>. Using quantitative analysis in G. L. C. (Table 1) mannose and galactose were found (rate 1.5:1) as the Ag-CHO main constituents. The detected ribose may result from contamination by RNA, remanent of the extraction process. Several authors have detected ligand terminals to lectins on the surface of *Leishmania*<sup>3, 8, 13, 22, 37, 41</sup>. The protein found amounted to 5.4%. Similar quantities of proteins (7%) were found in *Leishmania adleri*<sup>26</sup>.

Three times of electrophoretic migration (60, 90 and 120 min) with Ag-CHO at 10 mg/ml concentrations were used for the IEP reaction. However, no significant differences were observed in the migration, probably due to the fact that Ag-CHO presents only few negative charges at pH 8.2. The Ag-CHO is, apparently, composed by one antigenic fraction. The parallel precipitation lines observed after staining with Ponceau S (data not shown) may reflect different classes of antibodies reacting with the same antigenic component, also observed with Ag-CHO of *Trypanosoma cruzi*<sup>18</sup>.

Among the times of electrophoretic migration (15, 30, 45 and 60 minutes), and the concentrations (0.4; 0.2; 0.1; 0.07; and 0.05 mg/ml) of Ag-CHO tested in the CIEP reaction, the best results were obtained with the concentration 0.1 mg/ml at 30 minutes.

The low reactivity of immune serum of rabbit against Ag-CHO may be due to the use of Freund's incomplete adjuvant (FIA). GOTTLIEB (1977)<sup>18</sup> used Freund's complete adjuvant to prepare hiper-immune serum against Ag-CHO of *Trypanosoma cruzi* and a titer at 1/8 by CIEP was obtained.

The Ab-CHO reacted up to the dilution of 1/1280 in IIF, whereas the rabbit's serum obtained prior to immunization did not react in dilution 1/20 (smallest dilution tested). The Ab-CHO absorbed with formalin-treated promastigotes stopped reacting by DD with Ag-CHO, showing that the antibodies were absorbed by antigens on the surface of promastigotes, during incubation (Fig. 1.6). The ability of formalin-treated promastigotes in absorbing antibodies of Ab-CHO, thus inhibiting the reaction with Ag-CHO, as well as the ability of Ab-CHO in binding itself to formalin-treated promastigotes in IIF, demonstrates crossed antigenic reactivity between Ag-CHO and antigens of the cellular surface externally oriented. The fact that Ab-L1 reacts with formalin-treated promastigotes (titer 1/640) and with Ag-CHO (Figure 1.2), suggests that Ag-CHO is a constituent of membrane.

The reaction of Ab-CHO with components present in the phenolic extract of the culture medium used for cultivation of *Leishmania* (U) (Figure 1.3), and the antigenic identity line observed (Figure 1.4) shows the presence of Ag-CHO in the medium used. These observations indicate that Ag-CHO is liberated in the culture medium, during the parasite's development. Presence of antigens in the culture medium, cross-reacting with soluble extracts of promastigotes of *Leishmania*, has been detected by several groups<sup>7, 33</sup> and referred to as exoantigens or excreted factors (EF).

GOTTLIEB (1977)<sup>18</sup> reports that Ag-CHO of *Trypanosoma cruzi* is immunogenic, it is found in serum of mice infected with *Trypanosoma cruzi*, and that the hiperimmune serum absorbed with intact epimastigotes loses the precipitating activity. Our results show that Ag-CHO of *Leishmania braziliensis* is immunogenic and is found in the culture medium; that Ab-CHO reacts with formalin-treated promastigotes; that Ab-CHO absorbed with formalin-treated promastigotes does not react with Ag-CHO. These facts lead us to suggest that, such as the Ag-CHO obtained from *Trypanosoma cruzi*, the Ag-CHO obtained from *Leishmania braziliensis* is component of the parasite's external surface, more complex than its excreted factors, since it is immunogenic and precipitinogenic, whose antigenic determinants are shared with their excreted factors.

Ag-CHO isolated from *Leishmania braziliensis* contains polysaccharidic antigenic determinants, as it was observed through its reactivity against Ab-CHO by

DD, even after treatments such as: repeated freezing and thawing; lyophilization, treatment with RNase, heating at 100°C for 30 minutes; treatment with trichloroacetic at 10% (Figure 1.7), and the staining precipitation lines by PAS. The permanence of precipitogenic reactivity after treatment with metaperiodate at 0.135M indicates that the sugars that compose the antigenic determinants do not contain residues of carbohydrates with vicinal hydroxyl groups. Moreover, the relative similarity of the extraction method used and the chemical constitution of Ag-CHO obtained in *Leishmania braziliensis* with that of the fraction extracted with phenol-water of *Leishmania adleri*<sup>26</sup>, in which galactose is bound 1→3, could, partly, justify the Ag-CHO resistance to treatment with periodate. It is also known that periodate attacks hydroxyl groups in aminoacids like serine, threonine, and tyrosine<sup>9</sup>. Thus, despite the presence of proteins in Ag-CHO, these aminoacids must not be part of the antigenic determinant.

The IIF test for the diagnosis of leishmaniasis presents positivity indexes that vary from 57.8 to 100%<sup>16,27</sup>, besides cross-reaction with Chagas' disease<sup>16,21</sup>. The reaction of CIEP with Ag-CHO from *Leishmania braziliensis*, in spite of low sensitivity (60%), did not reveal cross-reaction in any of the five sera of patients with Chagas' disease, whereas the reaction of IIF presented four cross-reactions among sera of Chagas' disease patients. The DOT-ELISA reaction using Ag-CHO was tested but, probably due to its biochemical characteristics, we were not successful in the fixation of the Ag-CHO at the nitrocellulose membranes used. These results, despite the small number of samples tested, lead us to believe in the possibility of using Ag-CHO, perhaps applied to more sensitive techniques, for the immunodiagnosis of ACL.

## RESUMO

### ***Leishmania braziliensis*: isolamento de antígeno contendo carboidrato e a possibilidade de sua aplicação no imunodiagnóstico da Leishmaniose Tegumentar Americana**

A *Leishmania braziliensis* é um dos agentes causadores da Leishmaniose Tegumentar Americana (LTA). A cepa 034-JCG, isolada de paciente da região norte do estado do Paraná, Brasil, foi cultivada em meio Blood Agar Base, liofilizada e submetida a extração com fenol-água e o extrato obtido foi tratado com RNase I. O antígeno contendo carboidrato (Ag-

CHO) mostrou-se imunogênico para coelhos e apresentou pelo menos uma fração com poucas cargas negativas em pH 8,2. Este antígeno apresenta reação cruzada com extrato fenólico do meio de cultura usado para o cultivo de promastigotas e com antígenos de superfície de promastigotas. É composto por 24,3% de açúcares totais, dos quais 11,2% de galactose, 7,5% de manose e 5,6% de ribose. As proteínas foram estimadas em 5,4% e fosfato em 18,5%. A atividade antigênica foi mantida após: congelamentos e descongelamentos repetidos; liofilização; aquecimento a 100°C durante 30 minutos; tratamento com RNase, ácido tricloroacético e periodato de sódio. As linhas obtidas nas reações de precipitação coram-se pelo Ácido Periódico de Schiff. A utilização do Ag-CHO na reação de contraímuno-elektroforese para o imunodiagnóstico de LTA mostrou 60% de sensibilidade, e nenhuma reação cruzada com os 5 soros de pacientes com Doença de Chagas testados. O uso deste antígeno com técnicas mais sensíveis, e com um maior número de amostras poderá melhorar estes resultados.

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