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Validation of *Trypanosoma cruzi*-GPI Anchored Membrane Proteins for Specific Sero-Diagnosis of Chagas Disease

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Abstract
Trypanosoma cruzi-GPI anchored membrane proteins (*T. cruzi*-GPI-AMP) were isolated, purified, characterized and antigenically validated to be proposed as candidates to serologically diagnose Chagas disease. The partition Triton-114 protein fractioning method allowed recovering nearly 7% *T. cruzi*-GPI-AMP from the total parasite proteins. The immunogenic capability of *T. cruzi*-GPI-AMP was demonstrated in experimental hosts. Validation of *T. cruzi*-GPI-AMP, as an antigenic product to make up an alternative test for serologic diagnosis of Chagas disease, was performed in 174 sera samples: 112 from unquestionable chagasic patients and 62 from non-chagasic to estimate its sensitivity and specificity, respectively. Statistical analysis revealed 100% sensitivity, specificity, positive and negative predictive values, with $P<0.005$. In addition, estimation of the Kappa index revealed 100% agreement, indicating a perfect concordance between the *T. cruzi*-GPI-AMP test and the condition of the chagasic patients following clinical, parasitological and serological criteria. Separation by electroelution revealed the presence of several *T. cruzi*-GPI-AMP fractions, ranging from 23 to 123 kDa, specifically recognized by anti-*T. cruzi* antibodies contained in sera from acute and chronic chagasic patients. These results lead us to conclude that *T. cruzi*-GPI-AMP antigen is an alternative diagnostic test sensitive and specific enough to warrant a reliable sero-diagnosis of Chagas disease.

1. Introduction

Chagas disease is basically diagnosed using variables such as clinical presumption, epidemiological data and laboratory tests (1,2). The latter is orientated according to the suspected phase of the infection. This includes parasitological tests for the acute phase to confirm the presence of *Trypanosoma cruzi* blood circulating trypomastigotes, and serological tests in chronic phase to detect specific anti-*T. cruzi* antibodies (1,3). For any assay to be established as a routine diagnostic test to detect Chagas disease, it is

mandatory to know its sensitivity and specificity values, which are useful tools to discriminate the phase of the infection suffered by the attended patient. Consequently, parasitological tests are highly specific while serological ones are especially useful during the chronic phase when parasitemia in the host is low and inconstant.

T. cruzi has been recognized as a parasite exhibiting a great surface antigenic diversity able to induce activation of distinct lymphocyte clones as well as production of antibodies with different specificities (4,5). For this reason the *T. cruzi* membrane proteins, including those anchored to it by glycosyl-phosphatidyl-inositol (GPI) bonds, have been considered as a potential specific antigenic component amenable to be used for developing alternative methods to serologically diagnose Chagas disease. This possibility sounds feasible, taking into consideration that GPI-anchored membrane proteins have been previously recognized and characterized in different forms of *T. cruzi* (6,7), being able to mediate in the expression of surface proteins(8,9).

It is well known that conventional tests for Chagas disease sero-diagnosis are frequently made up of intact unbroken parasites or semi-purified fractions of *T. cruzi* epimastigotes used as antigenic substrate. This way, the heterogenic antigenic mixtures may frequently generate false positive results due to cross reactions with antibodies produced upon infections caused by etiological agents other than *T. cruzi*. This circumstance encourages the production of better diagnosis for Chagas disease using highly purified antigens which, at the same time, must be homogeneous and of known composition in order to avoid diagnostic interference. As potential candidates to fulfill the above statement, emerged the *T. cruzi* surface antigens given their described immunogenic importance due to its capacity of being the first to be recognized by the host immune system (10). Consequently, knowing on one hand the presence of mucine-like proteins and glyco-inositol-phospholipids (GIPL) in the *T. cruzi* membrane, both anchored by GPI bonds and, on the other, the demonstrated potential of GPI-anchored membrane proteins as biochemical markers to diagnose *T. cruzi* infections (7), they may be considered as candidates to produce specific diagnostic tools. Therefore, in the present work we validated *T. cruzi*-GPI-anchored membrane proteins (*T. cruzi*-GPI-AMP*)* as a specific and sensitive antigen to be used in the sero-diagnosis of Chagas disease patients.

2. Materials and Methods

2.1. Parasites

Trypanosoma cruzi epimastigotes forms kept in culture medium at the Center for Parasitological Research, Faculty of Sciences, University of Los Andes, Merida, Venezuela, were selected to carry out the present work. *T. cruzi* isolates including MHOM/Ve/1992/2-92, MHOM/Ve/1991/1-91, MHOM/Ve/1994/4-94, MHOM/Ve/1994/9-94 and MHOM/Ve/1995/36-95, were obtained by hemoculture from acute chagasic patients from Barinas state, Venezuela, where Chagas disease is endemic. Details on the origin, isolates maintenance and clinical profile of each corresponding patient have previously been published (11). Prior to the experiments, identification and genetic characterization of the selected isolates were performed by specific PCR assays (12). In all cases parasites were kept at 25ºC until they reached a mass of flagellates at the exponential phase equivalent to $13X10^6$epimastigotes/mL. They were then collected and centrifuged at 3000Xg at 4ºC for 15 min, washed with phosphate buffered saline (PBS) pH 7.2, and maintained at -20ºC until use. Most experiments were carried out using the isolate MHOM/Ve/1992/2-92, which derived from an emblematic acute chagasic patient who has been followed-up during the last 23 years, while the other four previously described isolates were included in the study for comparison purposes.

2.2. Fractioning and Purification of *T. cruzi*-GPI-AMP

T. cruzi-GPI-AMP from the selected isolates were obtained using the partition Triton-114 method previously described (13), and later adapted to be used with *T. cruzi* isolates (7). This procedure allowed separating three different fractions according to their solubility in the used detergent. These included the hydrophilic proteins (HP), the integral membrane hydrophobic proteins (IMP-hp) and the GPI-anchored membrane proteins. Details for the *T. cruzi*-GPI-AMP purification procedure have been reported in a previous publication (7).

2.3. Protein Concentration Measurement

Protein fraction (HP, IMP-hp and GPI) concentrations were measured following the method reported by Lowry et al. (14). The estimation of the concentration of each of the purified protein fractions was determined extrapolating the absorbance values to a standardized curve using bovine serum albumin. This procedure also allowed us to estimate the amount of *T. cruzi*-GPI-AMP recovered from the total proteins of the parasite, to know the resultant yield of the method used.

2.4. Gel Electrophoresis of Samples Under Reducing Conditions

T. cruzi-GPI-AMP gel electrophoresis was carried out on 12% sodium dodecyl sulphate polyacrylamide (SDS-PAGE) on a 0.75 mm thick minigel in a Mini-Protean system (Biorad®) and stained with Coomassie blue R-250 as previously described (15). In all cases, 20 µg samples of the *T. cruzi-GPI-AMP* fraction re-suspended in sampling buffer were treated with 100mM DL-dithiotreitol (DTT) as reducing agent, and heated at 95ºC during 5 min. in order to avoid catenary-unions that may influence protein antigenicity.

2.5. Rabbit Immunization with *T. cruzi*-GPI-AMP and Assay of Protein Immunogenicity

Immunizations were carried out in rabbits using *T. cruzi*-GPI-AMP fractions following the schedule of three challenges, every 15 days. The first immunization was done by subcutaneously inoculating 100 µg of the protein extract, followed by a second and third challenge with 50 µg each of same extract. Prior to inoculation, proteins were re-suspended in PBS and mixed 1:1 with incomplete Freund´s adjuvant (v/v) to 1mL final volume. Fifteen days after last immunization, the rabbit serum anti-*T. cruzi*-GPI-AMP was collected, frozen and preserved at -20ºC until used. To assay the immunogenicity of the used proteins, the specific antibodies anti-*T. cruzi*-GPI-AMP were tittered using three different serological methods including direct agglutination test (DAT), indirect immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), as previously reported (16,17).

2.6. Assay of the Antigenicity of *T. cruzi*-GPI-AMP in an Experimental Model and Chagasic Patients

To demonstrate the presence of anti-*T.cruzi*-GPI-AMP specific polyclonal antibodies in immunized rabbits and chagasic patients previously diagnosed, the Western blot technique was used. Briefly, a 20µg in sampling buffer of purified fraction of *T. cruzi*-GPI-AMP in the presence of 100mM DTT as reducing agent was used as antigenic substrate, and separated by SDS-PAGE and electro-transferred to PVDF membranes (Immobilon P). In the experimental model, the immune-detection was carried out using as source of primary antibody a serum diluted 1:100 from a previously immunized rabbit, and as secondary antibody an IgG-peroxidase anti-rabbit conjugate, diluted 1:15000. For chagasic patients, the primary antibody consisted of anti-*T. cruzi* serum diluted 1:100, and as secondary antibody the following dilutions: 1:6000 human anti-IgM for acute cases, 1:8000 human anti-IgG for samples of chronic patients, and 1:15000 human polyvalent anti-IgM, IgG and IgA for individuals bearing inapparent or subclinical infections, in all cases associated to peroxidase. They were then incubated, washed with PBS pH7.2 and developed with 4-chloro-1-naphthol and H_2O_2. Positive samples showed stained bands indicating the presence of specific anti-*T. cruzi*-GPI-AMP antibodies in the serum of both the immunized rabbit and chagasic patients.

2.7. Selection of *T. cruzi*-GPI-AMP Optimal Concentration for Diagnostic Test

To select the optimal amount of antigen to be used in the proposed diagnostic assay, different amounts of the *T. cruzi-GPI-AMP* fractions from a stock of 2µg/µL were tested. Four distinct amounts of 5, 10, 15 and 20 µg were then run in a 12% SDS-PAGE and stained with Coomassie blue in order to visualize bands intensities. To select the optimal amount, a Western blot was performed taking a constant dilution (1:100) of sera samples from unquestionable acute and chronic chagasic patients.

2.8. Validation of *T. cruzi*-GPI-AMP for Specific Sero-Diagnosis of CHAGAS Disease

Validation of *T. cruzi*-GPI-AMP fractions selected as candidates for serological diagnosis of Chagas disease was carried out using sera samples from unquestionable chagasic patients previously diagnosed by clinical, parasitological, serological, and molecular methods, as well as sera from patients showing other etiologies. This design was adapted in order to estimate values of sensitivity and specificity of the here proposed test after being compared with conventional serological methods (DAT, IFAT and ELISA) previously used for diagnosis. To validate the sensitivity of *T. cruzi*-GPI-AMP fractions, 100 sera samples were selected: 38 from patients in acute phase of Chagas disease, and 62 from chronic patients at different time post primary infection. They came from different regions of western and central Venezuela, including the states of Barinas (56), Mérida (20), Trujillo (11), Portuguesa (6), Zulia (5), Táchira (1) and Carabobo (1). In addition, 12 *T. cruzi* seropositive asymptomatic individuals from endemic areas, recorded as patients bearing inapparent infection, were also included.

The sero-diagnostic criteria used to categorize the selected sera samples from *T. cruzi*-infected patients bearing acute, chronic or inapparent infections were established following a methodology previously reported (16,17,18,19). Briefly, samples were considered positive when showed specific anti-*T. cruzi* antibody titters≥1:64 for DAT and IFAT, and an absorbance >0.4 for ELISA, while sero-positive patients were those with reactivity in 2 of the 3 methods used. In addition, the patient clinical condition of acute, chronic and inapparent infection was corroborated after detecting specific anti-*T. cruzi* IgM high level (≥1:512), IgG high level (≥1:512) and IgM-IgG low level (≤1:256) by IFAT, respectively.

To validate the specificity of *T. cruzi*-GPI-AMP fractions as diagnostic test, 62 samples from non-chagasic seronegative individuals from different areas and clinical conditions were selected. These included 19 sero samples from individuals living in areas where Chagas disease is endemic, 7 from non endemic localities, and 36 sera from patients suffering infections caused by etiological agents other than *T. cruzi*. The latter made up a group of patients with toxoplasmosis (10), HIV-AIDS (2), schistosomiasis (2), hepatitis B (2), hepatitis C (2), amoebiasis (2), malaria (3), cutaneous leishmaniasis (3), visceral leishmaniasis (3), *T. rangeli* infection (4) and TBC (3).

2.9. Ethical Considerations

The present study was approved by the technical committee of the “Luis Razetti” General Hospital, Ministry of Health, Barinas, Venezuela, and by the Research Council

of University of Los Andes (CDCHTA-ULA), Merida, Venezuela. A written consent was obtained from each of the patients selected for the diagnostic comparison in order to comply with the criteria established by the Biomedical Committee of the National Research Council of Venezuela.

2.10. Statistical Analysis

To perform the statistical analysis, parameters such as sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were estimated, including their confidence intervals (95%). The confirmatory diagnosis was established by comparison with conventional serological tests and the *T. cruzi*-GPI-AMP antigen was considered as the test to be evaluated (20). In addition, the kappa agreement (*k)*, the critical value of the statistic z and its respective p-value, were also estimated (21).

3. Results

3.1. Purification, Characterization, Reproducibility and Yield Estimation of Epimastigote *T. cruzi*-GPI-AMP

Using the partition Triton-114 method for fractioning *T. cruzi* cultured epimastigotes, we were able to separate membrane protein fractions according to its solubility. Following this approach, fractions identified as hydrophilic proteins (HP), hydrophobic proteins (hp) which contain integral membrane proteins (IMP), and GPI-anchored membrane proteins (*T.cruzi*-GPI-AMP) were collected. These fractions showed different patterns and molecular masses ranging from 7 to 200 kDa. Details on the migration pattern observed in SDS-PAGE are shown in Fig. 1. The purification process allowed knowing the amount of the GPI fraction from the original cellular protein concentration, which revealed an estimated yield of about 7%. The used method also showed high level of pattern reproducibility of GPI-AMP when different *T. cruzi* isolates from distinct localities and patients were compared (Fig. 2). Observation of *T. cruzi*-GPI-AMP patterns revealed molecular masses ranging from 20 to 200kDa, distinguishing a group of proteins between 30 and 90 kDa, which were similar to those showed by the selected isolate to perform the study.

3.2. Demonstration of *T. cruzi*-GPI-AMP Immunogenicity and Antigenicity

Sera samples taken from rabbits experimentally immunized with *T. cruzi*-GPI-AMP, revealed high titters of specific anti-*T.cruzi*-GPI-AMP antibodies according to DAT (1:1024) and IFAT (1:4096) results. This fact demonstrates the immunogenicity of the studied protein fractions in the experimental model used. In addition, the use of Western blot assays, both in sera from immunized rabbits and chagasic patients at different phase of *T. cruzi*-infection, allowed revealing the presence of specific anti-*T. cruzi*-GPI-AMP antibodies evidenced by the detection of stained bands of distinct molecular masses. In all cases the Western blot was carried out using 20 µg *T. cruzi*-GPI-AMP as the selected amount. The antigenicity of *T. cruzi*-GPI-AMP tested by Western blot in control sera from chagasic patients suffering acute, chronic and inapparent infections revealed that from the total of glycoproteins presented as antigen, 7 of them showing a range between 7 and 95 kDa were recognized. Interestingly, some of the recognized fractions reacted with sera from patients at any phase of the chagasic infection. This finding also demonstrates the high probability of the anti-*T. cruzi* antibodies produced in the human host to specifically recognize some of the *T. cruzi*-GPI-AMP here proposed as antigen.

3.3. Validation of *T. cruzi*-GPI-AMP as an Alternative Test for the Specific Sero-Diagnosis of Chagas Disease

To validate the use of *T. cruzi*-GPI-AMP as antigen for an alternative reliable test to diagnose Chagas disease, 174 sera samples were considered in the present work. From these, 112 individuals considered as chagasic (38 acutes, 62 chronic and 12 with inapparent infection) previously diagnosed by conventional serological methods (DAT, IFAT, ELISA) were comparatively analyzed using *T. cruzi*-GPI-AMP in order to assay the sensitivity of the proposed test (Table 1). The characteristics of the included chagasic patients previously detected by serological analysis and the validation of sensitivity of *T. cruzi*-GPI-AMP are presented in Tables 2-4. It is interesting to note that the use of *T. cruzi*-GPI-AMP antigen in the Western blot assays revealed detection of anti-*T. cruzi* antibodies in 100% samples of the tested chagasic patients. Fig. 3 shows selected samples of positive results obtained in sera of patients during the estimation of the sensitivity of the proposed test. In addition, the remaining 62 sera samples, belonging to non-chagasic individuals, including those with other etiologies that showed no reactivity at all, demonstrated the high specificity of *T. cruzi*-GPI-AMP. Details are given in Table 5.

Statistical analysis revealed 100% sensitivity, specificity, positive predicted value and negative predictive value, with $P<0.005$. In addition, estimation of the Kappa index showed 100% agreement, indicating a perfect concordance between the *T. cruzi*-GPI-AMP test and the condition of the chagasic patients following clinical, parasitological and serological criteria.

3.4. Separation of *T. cruzi*-GPI-AMP Fractions and Selection by Its Antigenicity

Once the antigenicity of the total collected *T. cruzi*-GPI-AMP was demonstrated, and its sensitivity and specificity validated, the next step was to separate fractions to be considered as candidates for diagnosis after being recognized by the blood circulating antibodies of chagasic patients. To know how many fractions, with different molecular masses, could offer the antigenic spectrum to provoke a specific response, *T. cruzi*-GPI-AMPs were separated by electro-elution, individually collected and submitted to immuno-

detection using a pool of sera from chagasic patients. This way, a total of 30 protein fractions were electro-eluted and challenged to a pool of sera from chagasic patients previously diagnosed. From these, 30 fractions showed reactivity as evidenced by the presence of bands of variable molecular masses. The observation of the different bands allowed us selecting as candidates those fractions which revealed bands of 123, 104, 93, 38, 33, 29 and 23 kDa (Fig. 4). To corroborate these findings and select the *T. cruzi*-GPI-AMP fractions to be proposed as candidates for a diagnostic test, two more Western blot assays were performed using sera from unquestionable acute and chronic patients separately. Slight differences were detected: while using sera of acute patients, bands of 123, 104, 93, 38 and 33 kDa were observed, the sera of chronic chagasic patients showed those evidenced in acute patients and two more bands of 29 and 23 kDa (Fig. 5).

Table 1. *Selected patient categories used to validate T. cruzi-GPI-AMP as diagnostic test*

Patients	Number	Percentage	Groups	Group percentage
Acute	38	21,8		
Chronic	62	35,6	112	64,4
Inapparent	12	6,9		
Negative control (endemic areas)	19	14,9	62	35,6
Negative control (non-endemic areas)	7			
Negative control (other etiologies)	36	20,8		
Total	174	100		100

Table 2. *Characteristics of the acute chagasic patients diagnosed by conventional serological methods and validation of sensitivity of T. cruzi-GPI-AMP*

Code	Age	Gender	Origin	DAT	IFAT	ELISA	IgM	IgG	*T. cruzi*-GPI-AMP
08-95	30	M	Barinas	1: 2048	1:128	1:100	1:2048	1:128	Positive
34-95	47	M	Barinas	1: 512	1:4096	1:100	1:512	1:128	Positive
35-95	21	M	Barinas	1: 128	1:2048	1:200	1:1024	1:256	Positive
36-95	51	F	Barinas	1: 2048	1:4096	1:800	1:1024	1:128	Positive
37-95	12	M	Barinas	1: 1024	1:2048	1:200	1:1024	1:512	Positive
02-96	24	F	Barinas	1: 2048	1:2048	1:200	1:1024	1:512	Positive
09-96	8	M	Barinas	1: 512	1:1024	1:100	1:2048	1:128	Positive
10-96	6	M	Barinas	1: 2048	1:256	1:100	1:512	1:256	Positive
17-96	19	F	Barinas	1: 2048	1:2048	1:800	1:2048	1:256	Positive
20-96	21	F	Barinas	1: 1024	1:1024	1:200	1:1024	1:128	Positive
23-96	38	M	Barinas	1: 2048	1:1024	1:200	1:1024	1: 64	Positive
24-96	2	M	Barinas	1: 128	1:512	1:200	1:1024	1:256	Positive
25-96	12	M	Barinas	1: 256	1:512	1:200	1:2048	1:256	Positive
26-96	22	F	Barinas	1: 64	1:1024	1:200	1:2048	1:128	Positive
27-96	12	M	Barinas	1:1024	1:64	1:100	1:1024	1:128	Positive
30-96	46	M	Barinas	1:64	1:256	1:100	1:1024	1:256	Positive
04-97	15	M	Barinas	1:128	1:256	1:100	1:1024	1:16	Positive
07-97	46	M	Barinas	1:64	1:64	1:400	1:512	1:32	Positive
09-97	4	M	Barinas	1:2048	1:128	1:200	1:1024	1:128	Positive
27-97	8	M	Barinas	1:4096	1:128	1:100	1:2048	1:256	Positive
29-97	13	F	Barinas	1:64	1:64	1:100	1:2048	1:128	Positive
30-97	19	F	Zulia	1:64	1:1024	1:100	1:512	1:128	Positive
01-98	22	M	Barinas	1:2048	1:256	1:200	1:2048	1:32	Positive
07-98	27	M	Barinas	1:128	1:512	1:200	1:1024	1:256	Positive
19-98	11	F	Mérida	1:128	1:512	1:800	1:512	1:16	Positive
24-98	9	M	Barinas	1:2048	1:512	1:100	1:2048	1:128	Positive
26-98	19	M	Barinas	1:512	1:1024	1:200	1:512	1:64	Positive
34-98	17	M	Portuguesa	1:128	1:128	1:200	1:1024	1:64	Positive
01-99	6m	M	Barinas	1:128	1:512	1:200	1:1024	1:256	Positive
08-99	8	M	Barinas	1:2048	1:1024	1:400	1:1024	1:64	Positive
15-00	4	M	Barinas	1:128	1:64	1:100	1:1024	1:64	Positive
06-01	8	F	Barinas	1:128	1:128	1:400	1:512	1:16	Positive
12-02	8	M	Barinas	1:4096	1:1024	1:400	1:1024	1:256	Positive
05-07	23	F	Trujillo	1:512	1:64	1:400	1:1024	1:256	Positive
06-07	26	M	Trujillo	1:4096	1:512	1:400	1:512	1:128	Positive
06-10	27	F	Barinas	1:512	1:512	1:200	1:512	1:64	Positive
07-10	6	M	Barinas	1:512	1:256	1:100	1:512	1:32	Positive
09-10	11	M	Barinas	1:4096	1:512	1:100	1:512	1:64	Positive

Table 3. *Characteristics of the chronic chagasic patients diagnosed by conventional serological methods and validation of sensitivity of T. cruzi-GPI-AMP*

Code	Age	Gender	Origin	DAT	IFAT	ELISA	IgM	IgG	*T. cruzi*-GPI-AMP
07-94	21	M	Barinas	1:256	1:512	1:400	1:64	1:1024	Positive
10-94	42	F	Barinas	1:128	1:256	1: 200	1:32	1:2048	Positive
11-94	30	F	Barinas	1:256	1:128	1:200	1:64	1:1024	Positive
05-95	33	F	Trujillo	1:4096	1:64	1:200	1:128	1:1024	Positive
10-95	55	M	Barinas	1:256	1:512	1:200	1:256	1:512	Positive
11-95	43	F	Mérida	1:1024	1:2048	1:400	1:64	1:1024	Positive
20-95	42	F	Barinas	1:1024	1:2048	1:800	1:256	1:1024	Positive
22-95	35	M	Mérida	1:4096	1:1024	1:800	1:32	1:512	Positive
27-95	30	M	Barinas	1:512	1:512	1:400	1:128	1:128	Positive
29-95	51	M	Zulia	1:2048	1:2048	1:800	1:256	1:1024	Positive
30-95	40	M	Zulia	1:1024	1:2048	1:100	1:256	1:2048	Positive
04-96	33	F	Barinas	1:512	1:512	1:200	1:128	1:2048	Positive
07-96	33	M	Barinas	1:2048	1:1024	1:400	1:256	1:1024	Positive
08-96	25	F	Barinas	1:1024	1:512	1:200	1:256	1:1024	Positive
11-96	5	F	Barinas	1:2048	1:512	1:200	1:64	1:512	Positive
13-96	41	F	Barinas	1:512	1:512	1:400	1:128	1:1024	Positive
18-96	46	F	Mérida	1:2048	1:2048	1:400	1:64	1:2048	Positive
21-96	44	M	Mérida	1:128	1:128	1:200	1:128	1:1024	Positive
22-96	60	M	Barinas	1:1024	1: 128	1:200	1:256	1:2048	Positive
29-96	34	M	Barinas	1:2048	1:256	1:200	1:64	1:2048	Positive
31-96	35	M	Portuguesa	1:2048	1:2048	1:800	1:256	1:2048	Positive
01-97	47	F	Barinas	1:2048	1:1024	1:400	1:256	1:1024	Positive
05-97	59	M	Barinas	1:512	1:256	1:200	1:16	1:128	Positive
13-97	39	M	Mérida	1:2048	1:512	1:400	1:128	1:1024	Positive
19-97	35	F	Mérida	1:256	1:128	1:200	1:256	1:512	Positive
20-97	36	M	Barinas	1:512	1:256	1:200	1:64	1:1024	Positive
21-97	36	F	Táchira	1:128	1:256	1:200	1:128	1:1024	Positive
23-97	58	M	Carabobo	1:64	1:128	1:200	1:256	1:1024	Positive
24-97	78	F	Zulia	1:256	1:256	1:400	1:256	1:4096	Positive
25-97	39	F	Barinas	1:1024	1:128	1:400	1:128	1:1024	Positive
26-97	47	M	Zulia	1:4096	1:512	1:200	1:512	1:2048	Positive
31-97	59	M	Mérida	1:512	1:1024	1:200	1:128	1:1024	Positive
04-98	51	M	Trujillo	1:1024	1:128	1:200	1:256	1:2048	Positive
09-98	66	M	Mérida	1:512	1:256	1:200	1:128	1:1024	Positive
10-98	51	F	Trujillo	1:2048	1:256	1:1600	1:128	1:2048	Positive
13-98	45	M	Mérida	1:512	1:512	1:800	1:32	1:2048	Positive
14-98	58	F	Mérida	1:2048	1:128	1:800	1:128	1:1024	Positive
16-98	45	F	Mérida	1:128	1:512	1:200	1:256	1:512	Positive
20-98	28	F	Mérida	1:128	1:64	1:800	1:64	1:1024	Positive
21-98	33	F	Mérida	1:256	1:256	1:400	1:64	1:512	Positive
23-98	38	F	Barinas	1:1024	1:512	1:200	1:128	1:1024	Positive
25-98	40	F	Barinas	1:512	1:128	1:200	1:128	1:1024	Positive
29-98	53	M	Mérida	1:2048	1:256	1:200	1:256	1:2048	Positive
30-98	47	F	Mérida	1:2048	1:1024	1:400	1:64	1:2048	Positive
09-99	39	M	Mérida	1:2048	1:512	1:200	1:64	1:1024	Positive
10-99	35	M	Mérida	1:2048	1:256	1:400	1:256	1:2048	Positive
11-99	65	M	Trujillo	1:512	1:1024	1:400	1:128	1:2048	Positive
19-99	11	M	Trujillo	1:1024	1:128	1:800	1:64	1:1024	Positive
22-99	40	F	Trujillo	1:4096	1:128	1:800	1:128	1:2048	Positive
23-99	12	F	Trujillo	1:256	1:128	1:800	1:64	1:1024	Positive
09-00	41	M	Trujillo	1:4096	1:512	1:200	1:64	1:1024	Positive
13-00	20	M	Barinas	1:1024	1:512	1:400	1:32	1:1024	Positive
21-00	50	M	Portuguesa	1:512	1:128	1:800	1:32	1:512	Positive
22-00	39	M	Mérida	1:128	1:128	1:400	1:128	1:512	Positive
24-00	43	M	Barinas	1:128	1:128	1:200	1:64	1:512	Positive
26-00	23	M	Barinas	1:128	1:1024	1:400	1:32	1:512	Positive
01-02	44	M	Portuguesa	1:4096	1:512	1:400	1:32	1:1024	Positive
03-02	38	M	Portuguesa	1:4096	1:128	1:400	1:128	1:1024	Positive
04-02	39	F	Portuguesa	1:1024	1:128	1:400	1:64	1:1024	Positive
23-02	42	M	Barinas	1:4096	1:512	1:200	1:128	1:1024	Positive
02-03	52	M	Mérida	1:4096	1:256	1:400	1:32	1:1024	Positive
04-10	63	F	Trujillo	1:256	1:64	1:100	1:64	1:512	Positive

Table 4. *Characteristics of the patients diagnosed by conventional serological methods for inapparent infection and validation of sensitivity of T. cruzi-GPI-AMP*

Code	Age	Gender	Origin	DAT	IFAT	ELISA	IgM	IgG	*T. cruzi*-GPI-AMP
ER T	42	M	Barinas	1:128	1:256	1:200	1:64	1:32	Positive
JC SL	9	M	Barinas	1:64	1:128	1:200	1:16	1:64	Positive
59 EL	36	F	Falcón	1:4096	1:128	1:200	1:16	1:64	Positive
22T	6	M	Mérida	1:128	1:64	1:100	1:64	1:64	Positive
81T	73	M	Mérida	1:128	1:64	1:100	1:32	1:128	Positive
13 VR	36	F	Portuguesa	1:256	1:64	1:100	1:64	1:128	Positive
49VR	50	F	Portuguesa	1:128	1:64	1:100	1:32	1:32	Positive
10EZ	22	F	Anzoátegui	1:64	1:128	1:200	1:32	1:64	Positive
28LL	59	M	Monagas	1:2048	1:256	1:200	1:64	1:64	Positive
25 LS	78	M	Cojedes	1:2048	1:512	1:400	1:32	1:128	Positive
21A	42	M	Cojedes	1:4096	1:512	1:200	1:16	1:128	Positive
18B	86	M	Trujillo	1:512	1:1024	1:200	1:16	1:128	Positive

Table 5. *Validation of specificity of T. cruzi–GPI-AMP using negative control sera sample from non-chagasic individuals diagnosed by conventional tests. A. From areas where Chagas disease is endemic. B. From non-endemic areas. C. Patients with other etiologies (showing 19 from 36)*

A

CODE	AGE	GENDER	ORIGIN	DAT	IFAT	ELISA	Tc-GPI-AMP
Toromo-1	14	M	Zulia	Negative	Negative	Negative	Negative
Toromo-2	15	F	Zulia	Negative	Negative	Negative	Negative
Toromo-3	13	M	Zulia	Negative	Negative	Negative	Negative
Bitubú-7	17	M	Trujillo	Negative	Negative	Negative	Negative
Bitubú-8	22	M	Trujillo	Negative	Negative	Negative	Negative
Bitubú-9	6	M	Trujillo	Negative	Negative	Negative	Negative
P.C-3	27	M	Portuguesa	Negative	Negative	Negative	Negative
P.C-13	16	F	Portuguesa	Negative	Negative	Negative	Negative
P.C-23	18	M	Portuguesa	Negative	Negative	Negative	Negative
Erika-1	27	F	Barinas	Negative	Negative	Negative	Negative
Erika-2	36	M	Barinas	Negative	Negative	Negative	Negative
Erika-3	49	F	Barinas	Negative	Negative	Negative	Negative
Erika-4	63	M	Barinas	Negative	Negative	Negative	Negative
S.Mateo-1	47	M	Anzoátegui	Negative	Negative	Negative	Negative
S.Mateo-2	33	M	Anzoátegui	Negative	Negative	Negative	Negative
S.Mateo-3	32	M	Anzoátegui	Negative	Negative	Negative	Negative
R.G-1	73	M	Sucre	Negative	Negative	Negative	Negative
R.G-2	44	M	Sucre	Negative	Negative	Negative	Negative
R.G-3	86	M	Sucre	Negative	Negative	Negative	Negative

B

CODE	AGE	GENDER	ORIGIN	DAT	IFAT	ELISA	Tc-GPI-AMP
AMBS	36	F	Mérida	Negative	Negative	Negative	Negative
ADVRE	42	F	Mérida	Negative	Negative	Negative	Negative
MER	28	M	Mérida	Negative	Negative	Negative	Negative
GECR	42	F	Mérida	Negative	Negative	Negative	Negative
C	35	F	Mérida	Negative	Negative	Negative	Negative
R	25	F	Mérida	Negative	Negative	Negative	Negative
CJ	30	F	Mérida	Negative	Negative	Negative	Negative

C

CODE	ORIGIN	SEROPOSITIVE TO:	Tc-GPI-AMP
28 HMI	Barinas	Toxoplasmosis	Negative
106 HMI	Barinas	Toxoplasmosis	Negative
306224 IMT	Distrito Capital	HIV-AIDS	Negative
SH 22021 IMT	Distrito Capital	Schistosomiasis	Negative
SH 22191 IMT	Distrito Capital	Schistosomiasis	Negative
337623 IMT	Distrito Capital	Hepatitis C	Negative
337280 IMT	Distrito Capital	Hepatitis C	Negative
SH 23726 IMT	Distrito Capital	Amoebiasis	Negative
SH 23724 IMT	Distrito Capital	Amoebiasis	Negative
LEM 102 IMT	Distrito Capital	Malaria	Negative
LEM 105 IMT	Distrito Capital	Malaria	Negative
JFT- J.R	Mérida	Cutaneous leishmaniasis	Negative
JFT- J.D	Mérida	Cutaneous leishmaniasis	Negative
JFT-J.A.G	Falcón	Visceral leishmaniasis	Negative
JFT-EP	Falcón	Visceral leishmaniasis	Negative
JFT- M.O	Barinas	T. rangeli infection	Negative
JFT- G.M	Barinas	T. rangeli infection	Negative
HSJD-1	Barinas	Tuberculosis	Negative
HSJD-2	Barinas	Tuberculosis	Negative

Tc-GPI-AMP: Trypanosoma cruzi-GPI-AMP

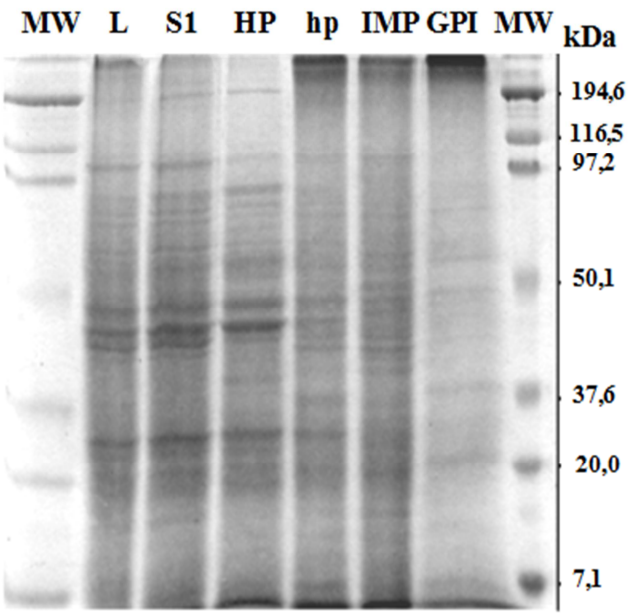

Figure 1. *Fractioned Trypanosoma cruzi cultured epimastigotes using the partition Triton-114 method to collect fractions identified as hydrophilic proteins (HP), hydrophobic proteins (hp) integral membrane proteins (IMP) and GPI-anchored membrane proteins (T.cruzi-GPI-AMP). L: Lysate of parasites, S1: Supernatant, MW: Molecular weight*

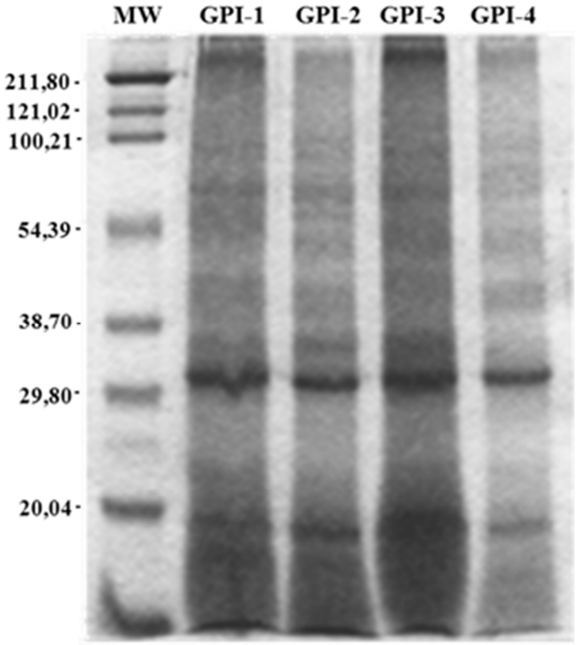

Figure 2. *SDS-PAGE of T. cruzi-GPI-AMP isolates: GPI-1: MHOM/Ve/1991/1-91, GPI-2: MHOM/Ve/1994/9-94, GPI-3: MHOM/Ve/1995/36-95, GPI4: MHOM/Ve/1994/494; MW: Molecular weight.*

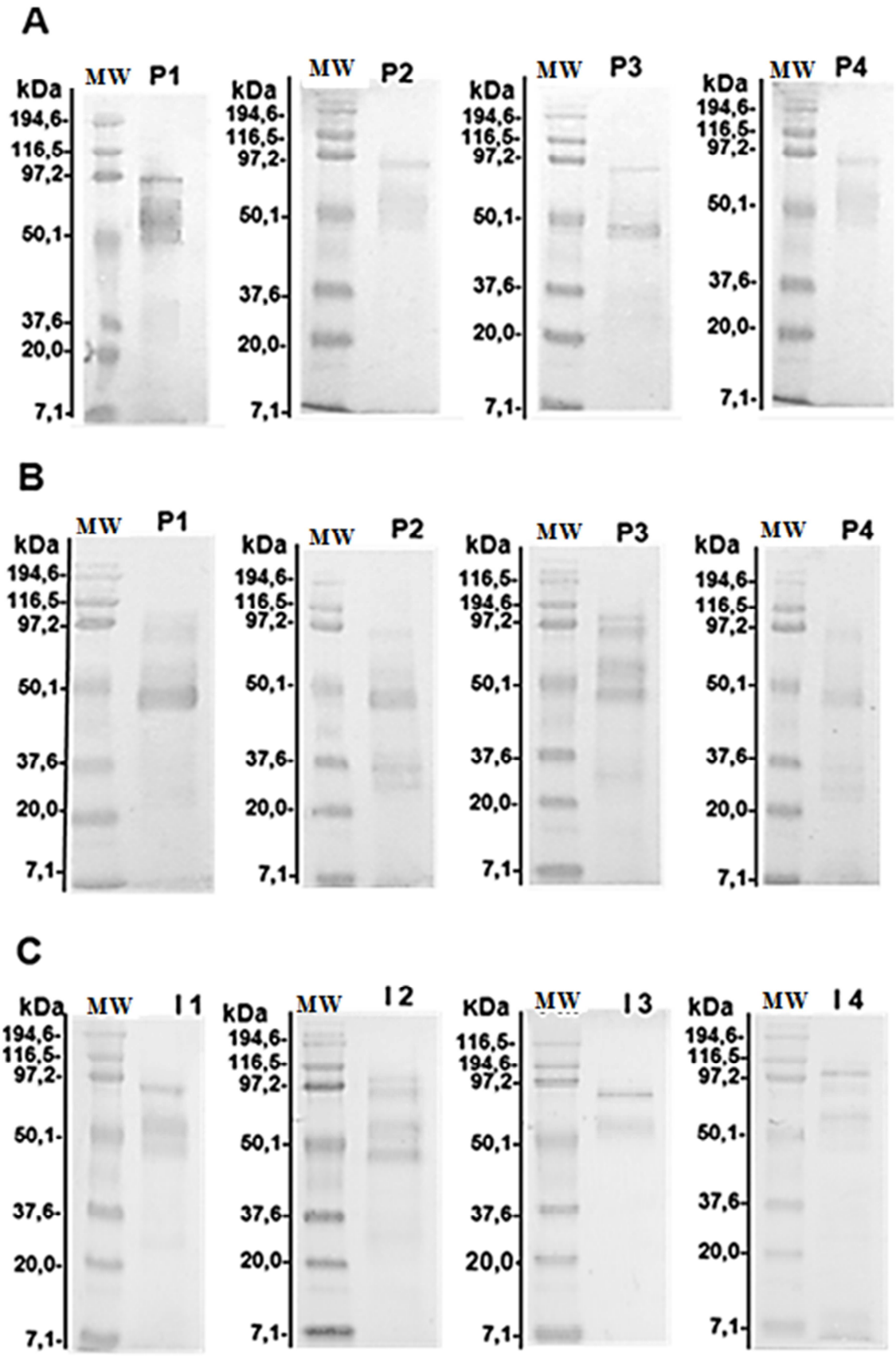

Figure 3. *Western blots using 20 µg total T. cruzi-GPI-AMP as antigen upon reaction with sera samples from selected chagasic patients: A. Acute; B. Chronic; C. inapparent infection. 1:100 dilution used in all cases. P1-P4, I1-I-4, indicates patient identification accordingly. MW: Molecular weight*

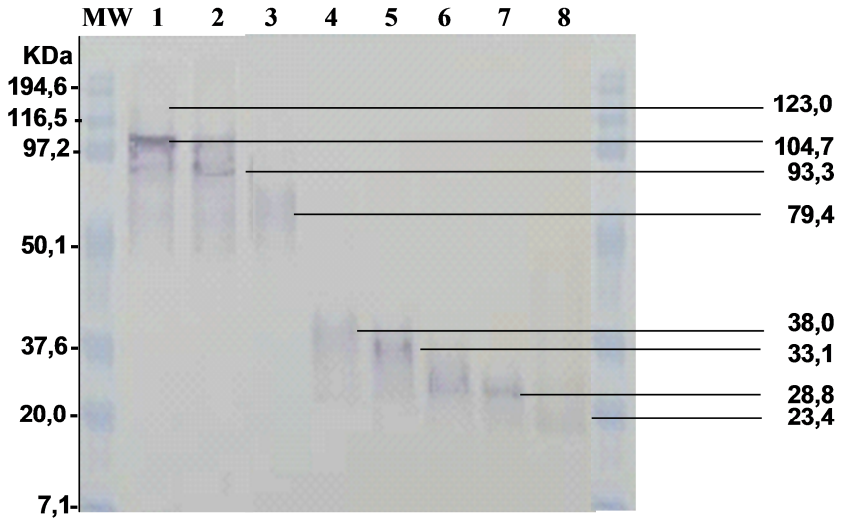

Figure 4. *Western blots of the selected T. cruzi-GPI-AMP fractions upon reaction with a pool of sera from chagasic patients. Fractions were detected in a 12% SDS-PAGE, and developed with 4-chloro-naphtol.*

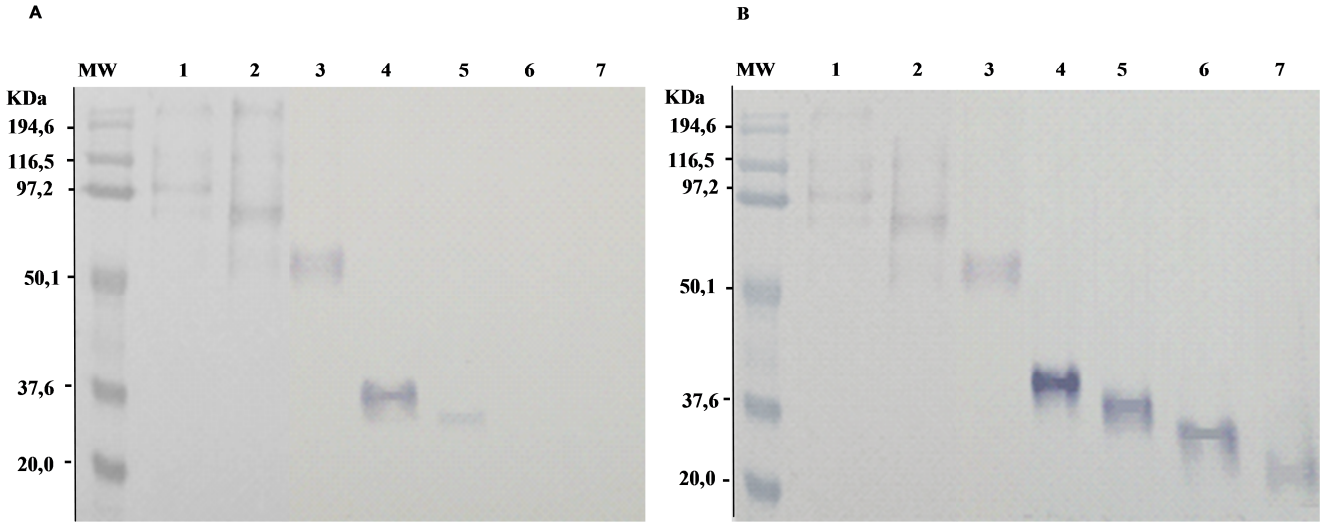

Figure 5. *Western blot showing the antigenicity response of selected T. cruzi-GPI-AMP fractions. A: With serum from an acute chagasic patient. B. With serum from a patient in chronic phase. Conjugates associated to IgM (A) and IgG (B) peroxidase, developed with 4-chloro-naphtol*

4. Discussion

In the present work *T. cruzi*-GPI-AMP fractions were evaluated as a sero-diagnostic tool for Chagas disease due to their capability to induce immunogenicity, along with its proven antigenicity demonstrated in previous parasitic systems using these kinds of glycoproteins (6,7,22). After identifying the selected *T. cruzi* isolates by PCR (12,23) the GPI anchored membrane proteins were purified by using the partition Triton-114 method, which showed to be efficient and reproducible enough to recover a 7% *T. cruzi*-GPI-AMP from the original proteins of the parasite. The molecular similarity of the isolates revealed by the constant similar patterns and the obtained purification yield demonstrated the reliability of the used method. These results were comparable with those previously reported using a similar methodology with *Trypanosoma rangeli* and *Leishmania braziliensis* (7, 22).

The immunogenicity of *T. cruzi*-GPI-AMP demonstrated in experimentally immunized rabbits allowed us to assaying the capability of these glycoproteins to activate the host immune system with the consequent making up of specific anti-*T.cruzi*-GPI-AMP antibodies. This fact was evidenced using conventional serological methods including DAT and IFAT to detect specific anti-*T. cruzi* antibodies. In addition, the antigenicity of *T. cruzi*-GPI-AMP was detected by Western blot using serum of an immunized rabbit, evidencing bands of different molecular masses from both pooled fractions and isolated fractions obtained by electro-elution, indicating that independently of the purification process the protein fractions were specifically recognized by the *T. cruzi*-GPI-AMP antibodies.

Regarding the antigenicity of the studied glycoprotein fractions, the use of sera from unquestionable chagasic patients revealed several antigenic proteins of distinct molecular masses, which may be related to the host

immunological condition. This finding revealed the potential of *T. cruzi*-GPI-AMP as a biochemical marker to be used as diagnostic tool to specifically detect Chagas disease. To demonstrate this potentiality we corroborated the results obtained with GPI-AMP fractions with three serological techniques (DAT, IFAT, ELISA), previously evaluated statistically (16,24). Interestingly, in all cases assays carried out with *T. cruzi*-GPI-AMP as antigen revealed reliable results, independently of using sera of chagasic patients bearing acute, chronic or inapparent infection. In addition, the circumstance that several *T. cruzi*-GPI-AMP fractions, with distinct molecular masses, were recognized as antigenic by sera of patients at any phase of chagasic infection, may suggest that the here proposed test show antigenic determinants, recognizable by specific anti-*T. cruzi* antibodies made up at any time after the primary antigenic stimuli.

The statistical validation of *T. cruzi*-GPI-AMP, as antigen for specific sero-diagnosis of Chagas disease, was carried out using a total of 174 sera samples. From these, 112 samples belonging to unquestionable chagasic patients previously diagnosed by clinical, parasitological and serological methods, were used to evaluate the sensitivity of the here proposed test. These included sera of 38 individuals in acute phase, 62 chronic and 12 *T. cruzi*-seropositive asymptomatic, bearing inapparent infections, according to previous established criteria (16). The remaining 62 sera samples were used to evaluate the specificity of *T. cruzi*-GPI-AMP as reliable antigen to be proposed as sero-diagnostic test. This group comprised 36 patients suffering from other etiologies, and 26 individuals which resulted seronegatives to *T. cruzi* infection: 19 of them from localities of western Venezuela where Chagas disease is endemic, and 7 from non-endemic areas. In all cases, the here proposed test was compared with three conventional serological methods (DAT, IFAT, ELISA) commonly used in different laboratories and research centers in Venezuela and other latitudes. The recorded estimation for sensitivity, specificity, predictive positive value and negative predictive value, was 100% ($p < 0.005$). In addition, the estimated value for kappa index was 1 (100% agreement), indicating a perfect concordance between the results of the assays using *T. cruzi*-GPI-AMP and the condition previously declared for the chagasic patients. The present results allow us concluding that the proposed methodology to diagnose Chagas disease seem to be a suitable alternative because of its high level of sensitivity and specificity.

The above results are supported by recent findings using *L. braziliensis*-GPI-AMP to sero-diagnose patients suffering active cutaneous leishmaniasis (25). In this case immunogenic protein bands of 50 and 28 kDa were revealed and the method showed statistical values for sensitivity and specificity of 100% and 99%, respectively. Taking into consideration the present results using *T. cruzi*-GPI-AMP and those with GPI-AMP as antigen for other etiologies, which together have shown evidence for reliable diagnosis, we strongly recommend this methodology as a new alternative to serologically diagnose Chagas disease.

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