An inexpensive antigen for serodiagnosis of Chagas’ disease.

Luis Briceño1, Eva Mary Rodríguez2, Mehudy Medina3, Yelitza Campos1, Walter Mosca1, Amanda Briceño4 and Graciela León5.

1Laboratory of Physiopathology, Institute of Biomedicine, Faculty of Medicine, Central University of Venezuela, 2Research Center in Public Health “Jacinto Convit”, Faculty of Medicine, Central University of Venezuela, Dirección General de Salud Ambiental, 3Ministerio de Salud, 4Università degli Studi di Milano, Italia and 5Municipal Blood Bank, Caracas, Venezuela.

Key words: Chagas’ disease, diagnostic, antigen, inexpensive.

Abstract. In this prospective study we evaluated the performance characteristics of a specific and sensitive antigen preparation (AgA) used in an enzyme-linked immunosorbent assay (ELISA) for the detection of anti-
Trypanosoma cruzi antibodies in serum samples, for Chagas’ disease diagnosis. The antigen production was achieved by combination of nutritional stress and autoclaving the parasites. Specificity and sensitivity were evaluated in two separate tests, using 152 sera from healthy individuals and 175 sera from Chagas’ patients (70 by xenodiagnosis). Cross-reactivity was tested using 289 sera from patients who had a parasitological diagnosis of a disease known to induce antigenic responses towards T. cruzi. All of these sera were tested with our AgA-ELISA and with 3 commercial diagnosis kits. To evaluate the agreement of results between our AgA-ELISA and a “gold standard” test for Chagas, we tested 566 sera from an endemic area. Results: sensitivity and specificity were 100%; cross-reactivity was the lowest compared with commercial kits. Overall agreement with the gold standard test was excellent (kappa=0.92). AgA-ELISA exhibits levels of sensitivity, specificity and cross-reactivity comparable or superior to those shown, obtained with the commercial kits used in our country, while being at least 10 times less expensive. This balance between diagnostic accuracy and cost makes AgA-ELISA useful for blood bank screening in poor regions of the world suffering from Chagas’ disease. Further validations of this antigenic formulation in other countries are necessary.
Un antígeno económico para el serodiagnóstico de la enfermedad de Chagas.
Invest Clin 2010; 51(1): 101 - 113

Palabras clave: Enfermedad de Chagas, serodiagnóstico, antígeno, económico.

Resumen. Este estudio fue realizado para evaluar las características de sensibilidad y especificidad de una formulación antigénica (AgA), producida a bajo costo, para detectar anticuerpos dirigidos a Trypanosoma cruzi, en muestras de suero de pacientes con enfermedad de Chagas. El AgA fue producido por el efecto combinado de estrés nutricional y autoclave de los parásitos. La especificidad y sensibilidad fueron evaluadas en dos estudios separados, con 152 sueros de individuos sanos y 175 de pacientes Chagásicos. La reactividad cruzada con 289 sueros de pacientes con diagnóstico parasitológico de enfermedades con anticuerpos que reaccionan con antígenos de T. cruzi. Estos sueros fueron evaluados con AgA-ELISA y con tres estuches comerciales. 566 muestras de suero provenientes de un área endémica, fueron empleadas para estudiar la concordancia entre nuestro diagnóstico y una prueba designada por nosotros como patrón oro estándar. Resultados: la sensibilidad y especificidad fue de 100%. El AgA presentó el más bajo porcentaje de reactividad cruzada, respecto a los estuches comerciales evaluados. La concordancia con la prueba patrón oro, en Venezuela, fue excelente (kappa=0,92). En conclusión, AgA-ELISA, presentó niveles de sensibilidad, especificidad y de reactividad cruzada, comparables o superiores a los obtenidos por los tres estuches comerciales mas empleados en el país, pero con costos de producción al menos 10 veces menor. Este balance conveniente, favorece su potencial uso para el despiste en los bancos de sangre de los países pobres y endémicos para la enfermedad de Chagas. Futuras validaciones de esta formulación en otros países es necesaria.

Received: 01-07-09. Accepted: 10-12-2009.

INTRODUCTION

Chagas' disease is an important public health problem; according to the World Health Organization (WHO) nearly 15 million people are infected and some 28 million are at risk of acquiring the disease (1). In Latin America, considering the number of disability-adjusted life years lost due to Chagas’ disease, it is the third largest tropical disease burden after malaria and schistosomiasis (1). Even though the Southern Cone Initiative to Control/Eliminate Chagas Disease (INCOSUR) has made important progress (2), a large proportion of Latin American population is exposed to the parasite. For this reason, strategy for Chagas’ disease eradication over the period 1996-2010 is based on interrupting vectorial transmission and on the systematic screening of blood donors (3).

Parasitological diagnosis during the chronic phase of the illness has levels of sensitivity that oscillate among 50-65%,
with time and effort consuming techniques (4, 5). Consequently, serological diagnosis is the most common way to determine the presence of anti-T. cruzi antibodies. The WHO recommends (6), and in some countries, like Brazil, it is mandatory (7), to carry out this screening with at least two different tests, because antigens usually present important problems due to false or discrepant results (8). In order to meet this objective, efforts have been made to substitute the whole parasite by purified parasite proteins (9), but their purification is arduous and the yield is very low due to proteases (10). Cloning and expressing the recombinant antigens have given excellent results with increasing specificity and sensitivity (11-15). However, although most of these purified proteins have been successfully tested in research laboratories, they are not commercially available or have not been included in blood banks screenings for cost or/and technical reasons (10,11). Due to these difficulties, together with financial reasons, in countries where this disease is endemic, the diagnosis is made using a single serological test.

Considering the above factors, the development of an economic kit in an Enzyme-Linked Immunosorbent Assay (ELISA) format has become necessary. As the components of the ELISA are relatively common, the goal should be to produce a low cost antigen. We have recently shown (16) that combined treatment (nutrient stress and autoclaving) of epimastigote forms enhance the sensitivity and reduce cross-reactivity. The results of this study allow us to obtain an antigen that could be used in the serological diagnosis.

The aim of the present investigation was to evaluate and validate a serological ELISA developed with an antigenic formulation with high sensitivity, specificity and low cost, so that it can be used as second diagnostic test for Chagas’ disease.

MATERIALS AND METHODS

Antigen

The antigen A (AgA) was prepared by autoclaving and nutrient stress of epimastigote forms in stationary phase Trypanosoma cruzi, Y strain, as previously described (16). Briefly, parasites were grown in liver infusion tryptose (LIT) medium (17), diluted 50:50 with RPMI medium (Gibco) and supplemented with 5% fetal bovine serum. Once the epimastigote forms culture reached the stationary phase of growth, the parasites were processed to eliminate the culture media by centrifugation at 2000 g for 10 min, in PBS, pH 7.2. The parasites were resuspended in DMEM supplemented with gentamicin (50 µg/mL) and incubated at 25°C. After 24 hours in the above conditions, the culture was centrifuged at 2000g for 10 min, the supernatant discarded and the parasites were resuspended in sterile PBS pH 7.2. The last step was repeated once more. After the last wash, they were resuspended at 30 × 10^6 parasites/mL, and autoclaved (15 lb, 15 min). After autoclaving, the suspension look cloudy (Proteins and remnants of the parasite) is named by us as AgA, and kept at 4°C until used.

A simple reversed-phase high-performance liquid chromatographic (VYDAC C18 column) method was developed and validated to evaluate the batches production variation. The batches evaluated for the antigen formulation was highly reproducible.

Western blot

The AgA suspension (one or three times concentrated) and epimastigote forms in stationary phase (30 × 10^6) was separated by SDS-PAGE, under denaturing and reducing condition, at the same parasite concentration, transferred to nitro-
cellulose sheet as previously described (18). The sheet was blocked with 150 mM NaCl, 50 mM tris-base (TBS), 5% low fat milk, for 1 h at room temperature. To study the effect of increasing temperature on the cross reactivity, the sheet was incubated with a pool of six mucocutaneous Leishmaniasis patients’ sera and membrane with AgA (3X) strips were incubated with a pool of Chagas patients’ sera, with low, medium or high titer to T. cruzi. For all test, the membranes were incubated with sera (1:2000) diluted in TBS, for 1 h, at room temperature, washed, and the bound antibodies were detected with biotinylated goat anti-human IgG (GIBCO Laboratories, Grand Island, NY, USA) for 30 min washed three times with TBS-T, and incubated with avidin-alkaline phosphatase. The presence of bound antibodies was detected with BCIP/NBT (GIBCO Laboratories, Grand Island, NY, USA) and analyzed using a Gel Doc 2000 System (BIO-RAD, USA).

Sera samples
All the sera samples used in this study were taken under conditions, procedures and use approved by the ethics committee of the Biomedicine Institute, UCV.

Pilot reactivity test
To demonstrate the capability of our AgA-ELISA to discriminate between healthy controls and Chagas’ disease patients, we used a total of 92 sera samples from healthy individuals and 106 sera samples from chagasic patients (positive to lymphoblastogenesis and in at least two serological assays), 37 of which were from chronic chagasic cardiomyopathy (CCC) and 68 from asymptomatic (ASY) patients. This would give us additional information about the ability of the test to differentiate between CCC and ASY patients.

Sensitivity and specificity test
To evaluate the sensitivity and specificity of the AgA-ELISA we used 60 sera from healthy individuals (with negative results in three different serological tests) and 70 previously positive sera from chagasic patients (positive by xenodiagnosis). Samples were assayed using a single blind model.

Cross reactivity test
In the areas where Chagas disease is endemic, there is often a superposition of parasitic diseases, the antibodies of which have shown to have cross reactivity with T. cruzi. To determine the cross reactivity of the AgA-ELISA, we used 289 sera from parasitologically diagnosed patients with other diseases known to induce antigenic response against T. cruzi: 89 with cutaneous Leishmaniasis (56 localized cutaneous, LCL; 12 mucocutaneous, MCL and 21 diffuse cutaneous, DCL), and 111 with visceral Leishmaniasis (VL), and sera from patients infected with Toxoplasmosis (15), Schistosomiasis (39), Malaria (30) and Giardiasis (5). All sera were also independently tested by the Caracas Municipal Blood Bank, using three of the most commonly commercial kits used in the country (Bioschile Ingenieria Genetica S.A Kit, BioKIT and Pharmatest (Laboratorios Pharmatest).

As we do not have an experimental tool to confirm mixed infections, to compare the serological cross-reactivity between different tests studied, we excluded all samples that came up positive in all the four Chagas tests used (3 commercial kits plus our AgA-ELISA).

Tests in sera from endemic areas.
Accuracy and reliability
To evaluate the agreement of results between our AgA-ELISA and a “gold standard” test for Chagas’ disease diagnosis, we
tested 566 serum samples from an endemic area. These samples were tested simultaneously and independently by our laboratory and by the laboratory of the epidemiological division of the Health and Social Development Ministry (MSDS, in Spanish). In Venezuela we do not have a real gold standard test but the epidemiology division of the MSDS is the oldest and the most experienced state laboratory for Chagas’ disease diagnosis. The MSDS diagnostic was obtained by the consensus results of three different methods: epimastigote immunofluorescence, indirect hemagglutination and ELISA.

**AgA-ELISA**

Chagas’ antibody detection was done through a previously described indirect method (19). A dilution (in buffer carbonate-bicarbonate) the AgA suspension was used to sensitized 96-well plates at a concentration 0.8 µg/well (protein on suspension). Briefly, the plates (Maxi-Sorp, Nunc, Denmark) were sensitized with the AgA, at 4°C, in sodium carbonate-bicarbonate buffer (0.05 M, pH 9.6). After overnight incubation the plate was blocked for one hour, with 1% fetal bovine serum and 4% skim milk (Nestlé) in Tris-buffered saline plus Tween 20 (10mM Tris pH 7.5, 100mM NaCl, 0.1% Tween 20). The plates were then incubated with human sera (1:100), for 30 min, at 37°C. As second antibody we used alkaline phosphatase conjugated goat anti-human IgG (GIBCO) at a 1: 2000 dilution, for 30 min. The immune complexes were developed by the addition of 100 µL/well of p-nitrophenyl phosphate as substrate, in diethanolamine buffer assay reagent kit (Pierce, Co) and the 405 nm absorbance was determined using Multiskan Ex, Labsystems.

**Assay cut off**

Cut off of the AgA-ELISA was applied as follows: 

**Cut off = Y + Z**

In each assay we included a sample from a serum pool from 46 healthy persons in four of the ELISA plate wells and we calculated the mean, which we called **Z**.

**Y** represents the individual dispersion value from the mean value obtained from the same 46 serums. We calculated the standard deviation (DESVest) for each serum of this pool. Then we calculated the percentage of deviation from the mean (P.DESVest). This value is used for all the determinations from this pool. Then,

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Y = (P . \text{DESVest} \times Z)/ 100 \times 2
\]

**Data analysis**

Data were analyzed with the GraphPad Prism 3.0 (Graphpad Software, Inc, San Diego, CA). To assess the validity under field conditions of the diagnostic test, we calculated the descriptive statistics (sensitivity, specificity, efficiency rate and likelihood ratios) with their 95% CIs. To evaluate the concordance between our test and the epidemiological division diagnosis, we calculated Cohen’s kappa statistics (20). To assess agreement beyond that expected by chance with their respective 95% confidence intervals, we also calculated prevalence and bias adjusted kappa (PABAK) (21). The agreement degree was defined as follows: low agreement ≤ 0; slight from 0.01 to 0.20; fair from 0.21-0.40; moderate from 0.41-0.60; substantial from 0.61 to 0.80 and almost perfect of 0.81-1.00 (22).

For the analysis we used the STATA Release 9.2® software (Stata, College Station, Tex), and DAGstat spreadsheet (23).

**RESULTS**

**AgA characterization**

Because T. cruzi shares antigenic determinants with other pathogens and par-
particularly with members of the *Leishmania* genus, many serodiagnosis tests for the Chagas disease have a persistent high number of false positive results. This cross reactivity may be lost through heat treatment, thus changing their conformation shape or losing the thermolabile antigens. In this sense, we have studied this effect in epimastigote forms of *Trypanosoma cruzi*, in stationary phase, by increasing the temperature or autoclaving. In Fig. 1, we found that the reactivity of pooled sera from patients with Leishmaniasis disease decreases with increasing temperature and the best result was obtained with autoclaving. All bands were not completely “destroyed” by the last treatment, because the three times concentrated sample was recognized by antibodies. Recently (16), we have observed (immunofluorescence) that parameters, like culture conditions (parasites in stationary phase of growth plus nutritional stress), significantly affect sensitivity, and induce changes in the distribution and density of antigens recognized by a pool of sera from experimentally infected mice. In the same study, we also tested whether whole parasites extract or sub fractions improve sensitivity or cross-reactivity when they are autoclaved. Surprisingly, the best sensitivity and lowest cross-reactivity were observed with whole parasite extract from cultures under nutrient stress and autoclaved.

As we identified the conditions for obtaining a low cost antigen, we studied by a Western Blot assay how the AgA formulation was recognized by a pool of Chagas patients’ sera, with low, medium or high titers to *T. cruzi* (Fig. 2). The AgA was recognized for all conditions evaluated. Once demonstrated by a Western Blot assay that the AgA formulation was recognized by antibodies from the chagasic patients, its feasibility as diagnostic test was studied with a group of sera from healthy subjects, ASY and CCC patients. Results are shown in Fig. 3. We found that the AgA-ELISA was capable of recognizing 100% of sera from infected patients. We did not find any differences in antibody levels between ASY patients and the ones with CCC.

**Sensitivity and specificity Test**

Using a blind assay to test 130 sera (70 positive by xenodiagnostic), we confirmed 100% of specificity and sensitivity of the AgA-ELISA.

**Estimation of cross reactivity**

When AgA-ELISA was tested using sera from patients diagnosed with other para-
sures, we found a low percentage of cross reactivity. There was 8.93% (5/56) of reactivity with sera from patients with LCL; 16.66% (2/12) with MCL and 4.76% (1/21) with sera of patients with DCL (Fig. 4). The 8 sera that showed cross reactivity for T. cruzi were re-tested using commercial kits which are used frequently in blood banks for diagnosis in Venezuela. Six sera were positive with all commercial kits, suggesting a mixed infection.

Fig. 5 shows results obtained with 111 serums from VL patients; twenty-four of them were from a presumably non-endemic area for Chagas disease (Nueva Esparta

Fig. 2. AgA, western blot analysis of antibodies to T. cruzi. SDS-PAGE of AgA (3X) proteins were separated by electrophoresis on a 10% gel and electrotransferred onto nitrocellulose membrane. AgA strips were incubated with a pool (six sera for each condition) of Chagas patients’ sera with low (L), medium (M) or high (H) titer to T. cruzi. For all test the membranes were incubated with sera (1:2000) diluted in TBS. MW: molecular weight mark.

Fig. 3. Pilot Reactivity Test: Distribution of individual OD₄₀₅ values obtained by AgA-ELISA (IgG antibodies against T. cruzi proteins), in 92 sera samples from healthy individuals and 106 sera samples from chagasic patients. Dotted line denotes cut-off. ASY: Chagasic asymptomatic patients (69 sera); CCC: chronic chagasic cardiomyopathy (37 sera).

Fig. 4. Cross reaction with sera from patients with cutaneous Leishmaniasis. Distribution of individual OD₄₀₅ values obtained by AgA-ELISA (IgG antibodies against T. cruzi proteins), in 89 sera from patients with cutaneous Leishmaniasis. Localized cutaneous Leishmaniasis (LCL, 56 sera). Mucocutaneous Leishmaniasis (MCL, 12 sera). Diffused cutaneous Leishmaniasis (DCL, 21 sera). Dotted line denotes cut-off.
We did not find cross reactivity in samples from the non-endemic area, but in the other 87 serum samples we observed a 9.20% (8/87) of positives. All 8 samples resulted positive with at least one of the commercial diagnostic kits for Chagas disease: Bioschile Ingenieria Genetica S.A., BioKIT and Pharmatest, Laboratorios Pharmatest.

Additionally, 89 sera from patients infected with Toxoplasmosis (15), Schistosomiasis (39), Malaria (30) and Giardiasis (5) were evaluated. Of these only one serum from a patient with Schistosomiasis gave a positive result (Fig. 6).

We compared our AgA-ELISA with three commercial kits for Chagas’ disease diagnosis (Table I). With our formulation we obtained the lowest cross reactivity results. The higher percentage of cross reactivity was observed in VL patients.

**Agreement evaluation**

Five hundred and sixty-six sera (89 positive sera and 477 negative sera diagnosed by the MSDS) were simultaneously and independently tested in our lab, using AgA-ELISA, and by the epidemiological division of MSDS, using 3 different methods (epimastigotes immunofluorescence, indirect hemagglutination and ELISA) for a single result. In this field evaluation, the MSDS results were considered as reference tests. Results are shown in Table II. After analyzing the results, we found good agreement for all parameters evaluated: 98.41% (95% CI: 97.60-99.21) for negative agreement and 92.15% (95% CI: 88.18-96.11) for positive agreement. Cohen’s kappa coefficient was 90.56% (95% CI: 85.87-95.26) and PABAK was 94.70%. The correct classification rate (Efficiency) was 97.35% (95% CI: 95.67-98.51). The sensitivity and specificity index were 98.88% and 97.06% respectively. The likelihood ratio of negative tests (86.39) was higher than the likelihood ratio of positive tests (33.69).
The ratio of positive test 33.69 (95% CI: 20.10-56.47) and Likelihood ratio of negative test 0.01 (95% CI: 0.00-0.08). There were 1.12% (1/89) false negative and 2.94% (14/477) false positive results.

**DISCUSSION**

The control strategy for the elimination of Chagas disease over the 1996-2010 period is based on interruption of vector transmission and the systematic screening of blood donors (3). The interruption of vectorial transmission has been obtained with great efficiency in vast areas of the south cone, but for financial reasons Chagas’ disease screening is not performed in all Latin-American blood banks, even by using only one test (24); so we are far from the goal of a more accurate diagnose using two tests. In consequence, there is an urgent need for an inexpensive test that can be used in countries with low budgets and endemic for this disease.

To minimize false Chagas disease diagnoses, purified, recombinant or synthetic antigens have been evaluated. Most of them have been found to be very specific but their sensitivity has not been good enough for their use in routine Chagas disease laboratory diagnosis. To improve this aspect, different combinations of them has been evaluated (10, 15, 25, 26). This approach has improved the sensitivity of the assays but has complicated their production and increased their cost. The cost of production of the ELISA test kits depends on numerous factors like antigen preparation, conjugate synthesis and purification, validation of reagents, assay optimization and validation. We performed a preliminary assessments taking into account our individual costs and compared them with the most economical Kit assay distributed in Venezuela. In this assessment our formulation is at least ten times less expensive.

In this study we present experimental evidence that allows us to suggest that we
can offer a very sensitive and specific test, that fulfills the financial requirements to be included in blood banks screening procedures as second diagnostic test for Chagas’ disease, with high sensitivity and specificity.

Our antigen formulation (AgA), resulted from a very long experience of autoclaving epimastigote forms in stationary phase of *T. cruzi*. The nutrient stress (16) increases the density of antigens recognized by the antiserum and/or induces the expression of different antigenic molecules of the parasite. This is particularly important because available kits are very effective at detecting blood donors presenting with high anti-*T. cruzi* antibody titers, but the results are often questionable when the kits are used for donors with low titers (27-29). Another factor that needs to be taken into consideration when one is using serological tests for Chagas’ disease is the cross-reactivity when Leishmaniasis cases are included, particularly visceral leishmaniasis (29). We present evidence that autoclaving preferentially eliminated cross-reactive antigens. Consistent with observations in Fig. 1, our formulation obtained the lowest cross reactivity results. It is remarkable that with the all tests evaluated the higher percentage of cross reactivity was observed in VL patients.

As an added benefit, the high temperature and pressure reached with the autoclave procedure eliminate, either by inactivation or by degradation, most of the proteolitic activity present in extracts of the parasite. It is important to highlight that the effect that we obtained with *T. cruzi*, cannot be obtained by the same treatment with subcellular fractions from the parasite, and that this effect could be species specific, given that the same treatment does not work with *Leishmania* spp. (16).

A possible explanation to the effect induced by the combined treatment could be that we obtain a preparation with high levels of specific antigens (suggesting thermostability) and low proportion of non-specific cross-reacting antigens (thermolabile). It is possible that the lability of these non-specific antigens is due to the fact that many of them are conformational antigens, while the specific ones are stable or become more exposed during the autoclaving process. Similar experiences have been observed in immunocytochemistry (30, 31) where paraffin sections (5 mm) of the human intestines and ovaries were cut onto silane-coated glass slides, dewaxed with toluene, and dehydrated with serial ethanol solutions. The sections were autoclaved at 120°C for 15 min in citrate buffer. The results showed that autoclaving is necessary for antigen retrieval (30), or for localization of alpha estrogen receptor in mouse intestine (31).

Regarding the 14 samples from endemic areas tested with the AgA-ELISA that did not match the results from the epidemiological division of MSDS, there are two possibilities: those samples are false positive with our test, or they are true positives that the MSDS test did not see. At this moment, in four patients from this group we were able to perform a polymerase chain reaction and the results demonstrated that they were true positives and infected with *T. cruzi*, and not detected by the MSDS test.

We were able to demonstrate that AgA-ELISA exhibits high sensitivity and specificity levels, and low cross reactivity, comparable or superior to those shown by commercial kits used in Venezuela. In addition, serological diagnosis with AgA-ELISA had almost a perfect agreement with the serological diagnosis of the epidemiological division of MSDS, suggesting that it could be used to perform blood bank diagnosis of *T. cruzi* infection and to perform epidemiological studies with large numbers of per-
sons, with good accuracy and low costs. Additional validations of this antigenic formulation in other countries and reassessments of commercial kits that are improving their antigens as Pharmatest (Pharmatest Laboratories) are necessary.

ACKNOWLEDGEMENT

We want to thank the following researchers for the generous donation of human sera: Dr. Marian Ulrich, Dr. Maria Diprisco, Dr. Belkisyole Noya and Dr. Oscar Noya.

This work was partially supported by FONACIT G2000001530, LANPIP 200001639, LOCTI P935015 to and CDCH PI 09-35-5286-2007.

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