The Type of Trypanosoma Cruzi Strain (Native or Non-Native) Used as Substrate for Immunoassays Influences the Ability of Screening Asymptomatic Blood Donors


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ABSTRACT

Background: The origin (native or non-native) of Trypanosoma cruzi strains used as substrate for immunoassays may influence their performance. Objective: To assess the performance of an immunoassay based on a native T. cruzi strain compared to another based on non-native T. cruzi strains, in asymptomatic blood donors from Mexico. Methods: Serum samples from a tertiary referral center were tested by both ELISA-INC9 (native) and Chagatest (non-native) assays. All reactive serum samples were further analyzed by indirect immunofluorescence. Results: Sera from 1,098 asymptomatic blood donors were tested. A 4.3 and 0.7% serum reactivity prevalence was observed using ELISA-INC9 and Chagatest, respectively (kappa = 0.13; −0.11 to 0.38). Subsequently, indirect immunofluorescence analyses showed higher positivity in serum samples reactive by ELISA-INC9 compared to those reactive by Chagatest (79 vs. 62.5%; p < 0.001). Furthermore, out of the 47 positive samples by both ELISA-INC9 and indirect immunofluorescence, only four (8.5%) were reactive in Chagatest assay. Meanwhile, four (80%) out of the five positive samples by both Chagatest and indirect immunofluorescence were reactive using ELISA-INC9. Conclusion: Immunoassays based on a native T. cruzi strain perform better than those based on non-native strains, highlighting the need to develop and validate screening assays in accordance to endemic T. cruzi strains. (REV INVES CLIN. 2016;68:286-91)

Key words: Chagas disease. Trypanosoma cruzi. Immunoassay.

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INTRODUCTION

Chagas disease is a chronic infection by the protozoan flagellate Trypanosoma cruzi. Vector-borne transmission to humans is the main route of infection followed by transfusion-associated (i.e. iatrogenic) transmission. Despite an estimated 1.1-2.0 million inhabitants being infected by Chagas disease, Mexico finally included mandatory serological screening of blood derivatives for *T. cruzi* as late as 2012. This is relevant since transfusion medicine continues to have a key role in medical care. For instance, in Mexico a total of 1,765,681 allogeneic units of blood were collected in 2011 from asymptomatic blood donors, and considering a nationwide seroprevalence ranging from 0.17 to 3.1% of blood donations, up to 54,736 of these hemoderivatives could have been potentially infectious. In addition, millions of at-risk individuals have migrated to cities in endemic regions as well as to countries outside the endemic range, thus urbanizing and globalizing the problem of Chagas disease and giving rise to new epidemiological and public health problems to places where trypanosomiasis is not endemic.

In 2001, the INC9 strain was isolated in a sample from a patient from the state of Guerrero presenting with chronic chagasic cardiomyopathy, who was treated at the Instituto Nacional de Cardiología in Mexico City, Mexico. This strain was further characterized as MHOM/MX/2001/INC9 according to the standard nomenclature. Analyses for *T. cruzi* mini-exon gene sequence (5’ GTGTCGCGACC TTCNTGCCCCG 3’) using the method proposed by Souto, et al., and confirmed by fluorescent DNA sequencing, allowed its identification as a discrete typing unit (DTU) I. This result agreed with previous reports showing *T. cruzi* DTU I as the prevalent type in Mexico, regardless of the geographic region of acquisition, in contrast to the prevalent DTU II strains observed in South America. The parasitic lysate from INC9 also has proved to be an adequate source of antigenic peptide substrates for the serologic identification of anti-*T. cruzi* antibodies, both in asymptomatic blood donors and in patients with chronic chagasic cardiomyopathy.

This study was aimed to evaluate the performance of an ELISA test based on whole protein extracts from epimastigotes of INC9 strain of *T. cruzi* compared to an ELISA test based on recombinant antigens from *T. cruzi* strains endemic to South America, in asymptomatic blood donors from Mexico.

MATERIALS AND METHODS

Subjects

This study included serum samples of asymptomatic blood donors from the Instituto Nacional de Cardiología in Mexico City. Although the local authorities collated clinical and demographic characteristics from blood donors, these data were not available for publication. The study protocol was approved by the institutional review board and ethics committee of the institute and was performed following the principles of the Declaration of Helsinki and local regulations. In addition, an informed consent was obtained from all the individuals.

*Trypanosoma cruzi* cultures and antigenic whole protein extract

Isolated epimastigotes of the INC9 strain of *T. cruzi* (native strain, TcI; see below) were cultured in an
axenic culture using liver infusion tryptose medium supplemented with 10% fetal bovine serum with prior inactivation at 56°C for 30 minutes and 25 mg/ml of hemin\textsuperscript{16}. Cultured parasites were processed at the exponential growth phase. For the antigenic whole protein extract elaboration, parasites were collected by centrifugation at 1000 x g for 30 minutes at 4°C, then washed and centrifuged two times using a phosphate-saline buffer (PBS), pH 7.2. The pellet was suspended in PBS, pH 7.2 (1-3 times packed volume of pellet) and a protease inhibitor cocktail was added. The parasite suspension underwent 8-10 rounds of sonication for periods of one minute. Finally, this mix was centrifuged at 10,000 x g for 30 minutes at 4°C and the supernatant (total extract) was collected. After determining the protein concentration, the extract was stored frozen at –20°C in aliquots of 0.5 ml each.

**Immmunoassay based on a native Trypanosoma cruzi (ELISA-INC9) strain**

A modified version of a previously validated ELISA method was used for serological screening of anti-\textit{T. cruzi} antibodies\textsuperscript{17}. Briefly, ELISA polystyrene microplate (Costar, Cambridge, MA, USA) wells were incubated with 1 μg of antigenic whole protein extract from \textit{T. cruzi} (INC9 strain) and 200 μl of carbonate-bicarbonate buffer, pH 9.6, 0.05 M (J.T. Baker, Phillipsburg, NJ, USA). After one hour incubation at 37°C, microplates were washed five times with 215 μl of PBS (J.T. Baker)-Tween 20 (Sigma-Aldrich, St Louis, MO, USA) 0.05%. Wells were incubated with blocking solution (PBS-Bovine serum albumin 0.5%) for 20 minutes at 37°C. Serum samples were diluted 1:200 in PBS and anti-human IgG conjugated to peroxidase (Invitrogen, Carlsbad, CA, USA) was diluted 1:10,000 in PBS. The developing process used O-phenylenediamine (OPD; Sigma-Aldrich) as substrate for peroxidase and a 10-minute incubation time at room temperature. A 5N sulfuric acid diluted solution (Sigma-Aldrich) was employed for stopping the reaction, followed by absorbance determination at 450 nm\textsuperscript{17}.

The cut-off value for positivity was set after analyzing serum samples from five healthy blood donors known to be non-reactive for anti-\textit{T. cruzi} antibodies by three different assay methods, Western blot, commercially available ELISA, and IIF assays (see below). The cut-off was set to the mean value plus three standard deviations, and it was calibrated in each ELISA plate.

**Immmunoassay based on non-native Trypanosoma cruzi strains**

The Chagatest ELISA test v.3.0 (Wiener Lab, Rosario, Argentina) was used for the qualitative serological screening of anti-\textit{T. cruzi} antibodies, following the instructions provided by the manufacturer. In this commercially available assay, antigens are obtained by DNA recombinant techniques starting from specific proteins from the epimastigote and trypomastigote stages of \textit{T. cruzi} strains endemic in South America.

**Indirect immunofluorescence assays**

Indirect immunofluorescence (IIF) assays were used to confirm the positivity of reactive sera by any of the ELISA assays. For analysis of fixed cells, \textit{T. cruzi} epimastigotes were allowed to settle onto a microscopy slide stored at –20°C until used. Prior to test, microscopy slides were thawed and hydrated with PBS. Patients’ serum samples and known positive controls were diluted 1:40 in PBS. Positive control samples were obtained from known patients with chronic chagasic cardiomyopathy reactive to anti-\textit{T. cruzi} antibodies by three different assay methods (Western blot, commercial ELISA, and IIF assays). The anti-human IgG antibody conjugated to FITC was diluted 1:150 in PBS. Indirect immunofluorescence assays

**Statistical analysis**

Proportions and percentages were used to describe categorical data and the differences were assessed using the chi-square or the Fisher’s exact tests, as appropriate. Meanwhile, the degree of consistency between the different tests was determined by the un-weighted Cohen’s kappa coefficient with 95% confidence intervals (95% CI). A p value < 0.05 was set for significance. The GraphPad Prism v 4.02 (GraphPad Software, La Jolla, CA, USA) software and the online calculator (http://www.red-caspe.org) from the CASPe (Critical Appraisal Skills Program Español) network were used for calculations.
RESULTS

A total of 1,098 serum samples of asymptomatic blood donors from the blood bank of the Instituto Nacional de Cardiología were tested using both the Chagatest and the ELISA-INC9 assays (Fig. 1). Reactivity was observed in 47 samples by ELISA-INC9; meanwhile, Chagatest displayed reactivity in eight samples (4.3% vs. 0.7%; \( p < 0.0001 \)). Further, only four reactive samples were common to both assays, resulting in a slight agreement as disclosed by a kappa index of 0.13 (95% CI: −0.11 to 0.38).

Serum reactivity was subsequently confirmed using IIF. Thirty-seven out of 47 reactive samples by ELISA-INC9 were confirmed to be positive by IIF; in contrast, only five out of eight reactive samples by Chagatest were confirmed to be positive by IIF (79% vs. 62%; \( p = 0.1 \)). Overall, only 4 (10.8%) out of the 37 positive samples by both ELISA-INC9 and IIF were reactive in the Chagatest assay, and four (80%) out of the five positive samples by both Chagatest and IIF were reactive in the ELISA-INC9 (Fig. 1).

Figure 2 shows the optical density (OD) values for each serum sample tested in the ELISA-INC9. Samples were grouped as follows: (A) ELISA-INC9 and IIF negative; (B) ELISA-INC9 positive but IIF negative; and (C) both tests shown to be positive. Notably, the mean OD was similar in groups B and C, and significantly higher when compared with the OD observed in group A.

DISCUSSION

Transfusion medicine has contributed to a decrease in mortality and improved quality of life of many individuals suffering from a variety of diseases, and thus it is considered as a great achievement of modern therapeutics\(^\text{18}^\). In spite of this, Chagas disease, along with other disorders, has been recognized as having transfusion-associated transmission, hampering the therapeutic purpose of transfusion medicine\(^\text{19}^\). Furthermore, Chagas disease and its severity seem to vary according to different geographic regions and hence may be considered as a major problem for transfusion medicine in endemic regions. In December 2002, WHO guidelines incorporated high sensitivity tests for the screening of anti-\(T.\) cruzi antibodies in blood and blood components to be used for transfusion as an important approach to address
In this regard, the confidence of serologic tests depends on diverse factors that include cross-reactivity with other related protozoa, low anti- \textit{T. cruzi} antibody count, or serum samples inadequately processed. In addition, a dimorphism in the 24S alpha rRNA target region has allowed the characterization of DTU I and II, which probably resulted from long-term clonal evolution of \textit{T. cruzi}. Interestingly, multi-locus markers have further showed a total of six DTUs, one corresponding to \textit{T. cruzi} I and the others to subdivisions within \textit{T. cruzi} II, Ila-e. However, by consensus, currently recognized nomenclature for \textit{T. cruzi} now classifies strains into six DTUs, \textit{T. cruzi} I-VI. In this vein, Tcl and Tcl are now used to name the previously known DTU I and DTU IIb, respectively. For our study, it is important to recognize that Mexican Tcl strains are a homogeneous group closely related to each other and considered as the primary agents of serum reactivity in Mexico. On the other hand, Mexican TclII are not closely related to TcII strains circulating in domestic cycles in Argentina, Brazil, Bolivia, and Chile. Thus, the use of antigenic substrate from a native strain (TcI) may have given an advantage to ELISA-INC9 over the commercial ELISA assay based on autochthonous South American strains (where non-TcI strains predominate) by potentially providing different antigenic determinants key for screening these two particular samples and for yielding a higher seroprevalence.

Concurrently, our ELISA-INC9 findings indicate that by using antigenic substrate from a native \textit{T. cruzi} strain, it is possible to achieve identification of a higher number of positive individuals and, thus, less infectious blood and blood derivatives available for transfusion therapy, contributing to decrease transfusion-associated transmission of Chagas disease. On the contrary, the screening based on non-TcI strains from South...
America, which exhibit little relationship with *T. cruzi* strains from Mexico, could be inadequate for sero-prevalence surveys in the Mexican population.

The present study has several limitations. First, parasite isolation by xenodiagnosis in those cases with serum reactivity in the ELISA-INC9 method would have further confirmed the performance of the test by determining true positives. Conversely, an alternative approach would include the use of well-characterized serum samples from subjects known to be infected with different *T. cruzi* strains. Second, in an attempt to support the previously validated immunologic screening tests employed, both the home-made ELISA and IIF assays were standardized employing the same *T. cruzi* strain (INC9). Finally, the possibility of cross-reaction with antibodies elicited by other pathogens (such as *Leishmania*) was not assessed.

In conclusion, our results suggest that each geographic region should develop and validate its own screening immunassays for anti-*T. cruzi* antibodies in accordance to endemic *T. cruzi* strains.

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