Contents available at **PubMed**
www.clinicalandtranslationalinvestigation.com

PERMANYER

Rev Inves Clin. 2016;68:286-91 BRIEF COMMUNICATION

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

Martha A. Ballinas-Verdugo[1,2*], Ana M. Mejía-Domínguez[3], Sergio A. Sánchez-Guerrero[4], Claudia Lerma[5], Mariana Martínez-Cruz[6], Elsa Álvarez-Manilla-Toquero[3], Xochiquetzali Jiménez-Díaz[4], Francelia Barrera-Trujillo[3], Marisela del R. Ticante-Cruz[4], Irving O. Estevez-Garcia[2], Luis M. Amezcua-Guerra[2,7] and Pedro A. Reyes-Lopez[8]

Departments of [1]Molecular Biology, [2]Immunology, and [5]Electromechanic Instrumentation, Instituto Nacional de Cardiología Ignacio Chávez; Blood Bank, [3]Instituto Nacional de Cardiología Ignacio Chávez, and [4]Instituto Nacional de Cancerología; [6]Perronee-Gatonee Veterinary Clinics; [7]Health Care Department, Universidad Autónoma Metropolitana; [8]Division of Research, Instituto Nacional de Cardiología Ignacio Chávez; Mexico City, Mexico

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.

Corresponding author:
*Martha A. Ballinas-Verdugo
Departamento de Immunología
Instituto Nacional de Cardiología Ignacio Chávez
Juan Badiano, 1
Col. Sección XVI, Del. Tlalpan
C.P. 14080, Ciudad de México, México
E-mail: maalbave_64@yahoo.com

Received for publication: 28-10-2016
Accepted for publication: 22-12-2016

INTRODUCTION

Chagas disease is a chronic infection by the protozoan flagellate *Trypanosoma cruzi*[1]. Vector-borne transmission to humans is the main route of infection followed by transfusion-associated (i.e. iatrogenic) transmission[2]. 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[3]. 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[4-6]. 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[7,8].

Currently, prevention for transfusion-associated transmission of Chagas disease is based on detecting antibodies specific to several parasite antigens using serological tests. Enzyme-linked immunosorbent assays (ELISA), indirect hemagglutination chemiluminescence (IHA), and immunofluorescence assays are the most commonly employed[9]. These tests use antigens either from parasite lysates or recombinant proteins, which, if coupled with adequate antigen processing, can achieve acceptable sensitivity. However, a single test is not sufficiently sensitive or specific for diagnosis, and therefore diagnostic accuracy is further improved when a second test (based on a different principle from the first used) is performed as currently recommended by the World Health Organization (WHO)[10]. Further, the correct identification of the endemic *T. cruzi* strains to the region of study is of importance due to strain heterogeneity, which may lead to biologic and genetic differences that may potentially influence serological identification of *T. cruzi* strain-specific antibodies[11]. This emphasizes on the importance of selecting the ideal strain for parasitic antigen preparation, particularly considering its potential effects on the performance of serologic diagnostic tests, especially when employed in a region with endemic strains different to that used for the standardization process of the test.

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' GTGTCCGCCACC TCCTTCGGGCC3') using the method proposed by Souto, et al.[12], and confirmed by fluorescent DNA sequencing, allowed its identification as a discrete typing unit (DTU) I[13]. This result agreed with previous reports showing *T. cruzi* DTU I as the prevalent type in Mexico, regardless of the geographic region of acquisition[14], in contrast to the prevalent DTU II strains observed in South America[15]. 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[16].

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[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.

Immunoassay 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-*T. cruzi* antibodies[17]. Briefly, ELISA polystyrene microplate (Costar, Cambridge, MA, USA) wells were incubated with 1 µg of antigenic whole protein extract from *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[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-*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.

Immunoassay 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-*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 *T. cruzi* strains endemic in South America.

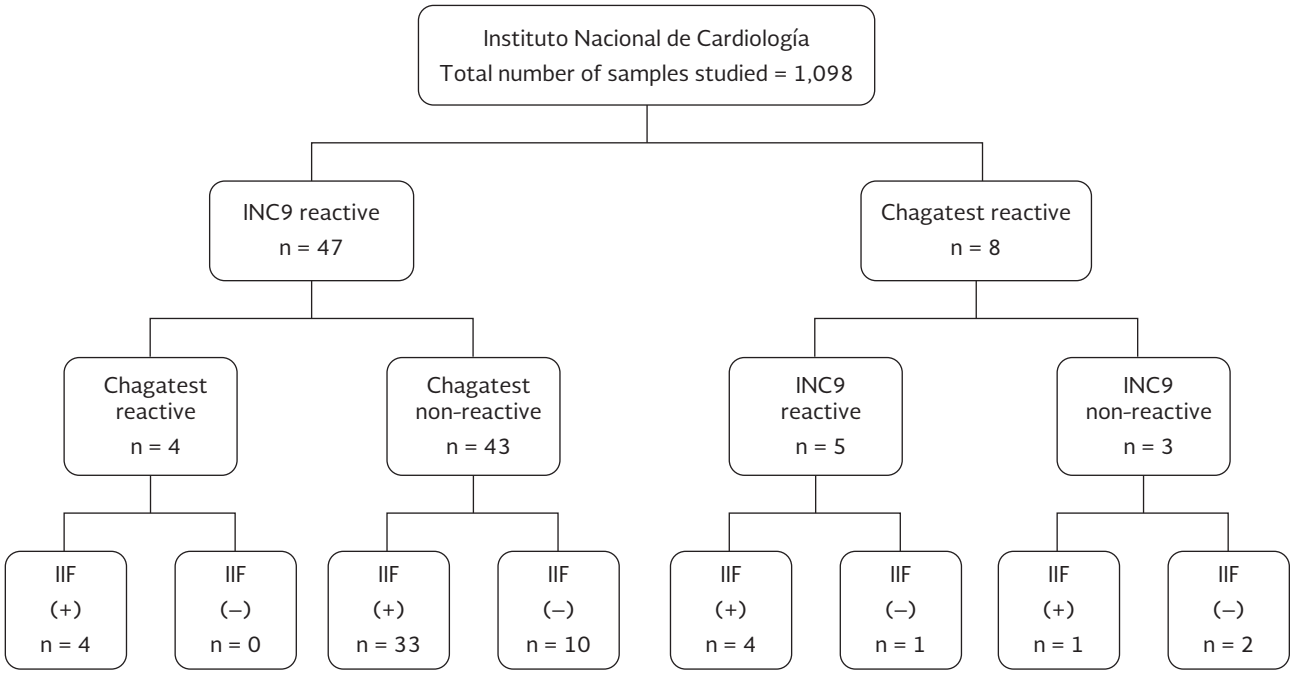
Indirect immunofluorescence assays

Indirect immunofluorescence (IIF) assays were used to confirm the positivity of reactive sera by any of the ELISA assays. For analysis of fixed cells, *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-*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. Microscopy slides were washed three times with PBS after an overnight incubation at 4°C, fixed with 100 µl 0.01 M (1X) PBS solution, and dried for 18 hours. Finally, indirect immunofluorescence analysis was done using epifluorescence microscopy.

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.redcaspe.org) from the CASPe (Critical Appraisal Skills Program Español) network were used for calculations.

Figure 1. Results of serum samples from asymptomatic blood donors. Serum reactivity by ELISA-INC9, Chagatest ELISA, and indirect immunofluorescence assays. IIF: indirect immunofluorescence.



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).

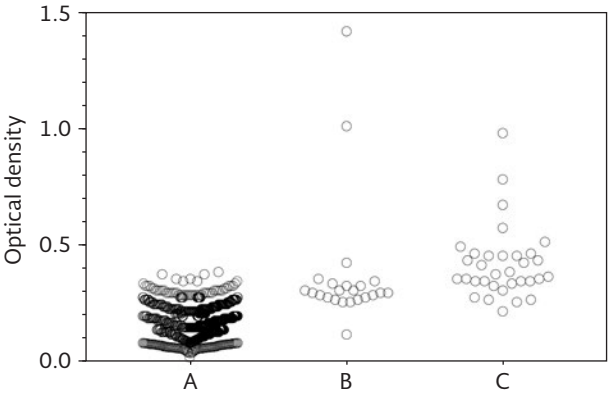
Serum reactivity was subsequently confirmed using IIF. Thirty seven out of 47 reactive samples by ELISA-INC9 were confirmed to be positive by IIF; in contrast, only 5 out of eight reactive samples by Chagatest were confirmed to be positive by IIF (79% vs. 62%; $p = 0.1$). Overall, only 4 (10.8%) out 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[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[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

Figure 2. Total values in optical densities for sera from blood donors tested in the ELISA-INC9. Serum samples are grouped as follows: **A:** ELISA-INC9 and IIF negative; **B:** ELISA-INC9 positive but IIF negative; **C:** both tests shown to be positive. The mean cut-off point was determined to be 0.30 ± 0.05 optical density. IIF: indirect immunofluorescence.



this problem[2]. However, currently there is no single widely accepted serologic reference standard for anti-*T. cruzi* antibody screening.

In the present study, we sought to determine whether the results of an immunoassay based on antigenic substrate from an autochthonous or native strain would resemble those of a commercially available immunoassay (based on non-native strains) and at the same time comply with WHO recommendations for anti-*T. cruzi* antibody screening in asymptomatic blood donors from Mexico. Overall, we found different seroprevalence when using either native or non-native based assays, and observed only a slight agreement between these two tests. In this vein, we found a higher seroprevalence using ELISA-INC9 in asymptomatic blood donors (overall seroprevalence of 8.8%) as compared to 0.7% for the Chagatest. It is important to note that 79% of serum samples reactive by ELISA-INC9 were subsequently confirmed to be positive by IIF. On the other hand, IIF assessment of reactive samples using the Chagatest assay resulted in a 62.5% confirmation. Though these figures were not statistically different, it is important to note that there were positive sera by both ELISA-INC9 and IIF that were non-reactive using the Chagatest assay. In fact, only four out of the 37 positive sera by ELISA-INC9 and IIF were reactive by the Chagatest ELISA as well, resulting in an alarming 89% non-detection rate of positive individuals by the Chagatest assay. In contrast, four out of the five positive sera by the Chagatest ELISA and IIF were also reactive using ELISA-INC9 (89% vs. 20% non-detection rate of positive sera; p= 0.002). A smaller study from Mexico has previously emphasized on this problem, where the use of antigens from Mexican *T. cruzi* strains in an ELISA assay were able to correctly identify as positive known sera from patients with trypanosomiasis in 100% of cases, whereas the use of antigens from Argentinian strains correctly identified only 18% of individuals[20].

In this regard, the confidence of serologic tests depends on diverse factors that include cross-reactivity with other related protozoa, low anti-*T. cruzi* antibody count, or serum samples inadequately processed[21-23]. 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 *T. cruzi*[24,25]. Interestingly, multi-locus markers have further showed a total of six DTUs, one corresponding to *T. Cruzi* I and the others to subdivisions within *T. cruzi* II, IIa-e[26-28]. However, by consensus, currently recognized nomenclature for *T. cruzi* now classifies strains into six DTUs, *T. cruzi* I-VI[29]. In this vein, TcI and TcII are now used to name the previously known DTU I and DTU IIb, respectively. For our study, it is important to recognize that Mexican TcI strains are a homogeneous group closely related to each other and considered as the primary agents of serum reactivity in Mexico[30]. On the other hand, Mexican TcII are not closely related to TcII strains circulating in domestic cycles in Argentina, Brazil, Bolivia, and Chile[14]. 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[16,20].

Concurrently, our ELISA-INC9 findings indicate that by using antigenic substrate from a native *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 seroprevalence 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 immunoassays for anti-*T. cruzi* antibodies in accordance to endemic *T. cruzi* strains.

REFERENCES

1. Jurberg C. Chagas: one hundred years later. Bull World Health Organ. 2009;87:491-2.
2. Control of Chagas disease. World Health Organ Tech Rep Ser. 2002;905:i-vi, 1-109.
3. Sanchez-Gonzalez G, Figueroa-Lara A, Elizondo-Cano M, et al. Cost-effectiveness of blood donation screening for Trypanosoma cruzi in Mexico. PLoS Neg Trop Dis. 2016;10:e0004528.
4. Finkelman J. Innovative community-based ecosystem management for dengue and Chagas disease prevention in low and middle income countries in Latin America and the Caribbean. Trans R Soc Trop Med Hyg. 2015;109:89-90.
5. Escamilla-Guerrero G, Martinez-Gordillo MN, Riveron-Negrete L, et al. Trypanosoma cruzi: seroprevalence detected in the blood bank of the Instituto Nacional de Pediatria, Mexico City, in the period 2004 through 2009. Transfusion. 2012;52:595-600.
6. Novelo-Garza BA, Benitez-Arvizu G, Pena-Benitez A, Galvan-Cervantes J, Morales-Rojas A. [Detection of Trypanosoma cruzi in blood donors]. Rev Med Inst Mex Seguro Soc. 2010;48:139-44.
7. Basile L, Jansa JM, Carlier Y, et al. Chagas disease in European countries: the challenge of a surveillance system. Euro Surveill. 2011;16.
8. Coura JR, Vinas PA, Junqueira AC. Ecoepidemiology, short history and control of Chagas disease in the endemic countries and the new challenge for non-endemic countries. Mem Inst Oswaldo Cruz. 2014;109:856-62.
9. Afonso AM, Ebell MH, Tarleton RL. A systematic review of high quality diagnostic tests for Chagas disease. PLoS Negl Trop Dis. 2012;6:e1881.
10. Control of Chagas disease. Report of a WHO Expert Committee. World Health Organ Tech Rep Ser. 1991;811:1-95.
11. Machado CA, Ayala FJ. Nucleotide sequences provide evidence of genetic exchange among distantly related lineages of Trypanosoma cruzi. Proc Nat Acad Sci USA. 2001;98:7396-401.
12. Souto RP, Fernandes O, Macedo AM, Campbell DA, Zingales B. DNA markers define two major phylogenetic lineages of Trypanosoma cruzi. Mol Biochem Parasitol. 1996;83:141-52.
13. Ruiz-Sanchez R, Leon MP, Matta V, et al. Trypanosoma cruzi isolates from Mexican and Guatemalan acute and chronic chagasic cardiopathy patients belong to Trypanosoma cruzi I. Mem Instit Oswaldo Cruz. 2005;100:281-3.
14. Bosseno MF, Barnabe C, Magallon Gastelum E, et al. Predominance of Trypanosoma cruzi lineage I in Mexico. J Clin Microbiol. 2002;40:627-32.
15. Fernandes O, Santos S, Junqueira A, et al. Populational heterogeneity of Brazilian Trypanosoma cruzi isolates revealed by the mini-exon and ribosomal spacers. Mem Instit Oswaldo Cruz. 1999;94(Suppl 1):195-7.
16. Ballinas-Verdugo M, Reyes PA, Mejia-Dominguez A, Lopez R, Matta V, Monteon VM. Enzyme-linked immunosorbent assay and polymerase chain reaction performance using Mexican and Guatemalan discrete typing unit I strains of Trypanosoma cruzi. Vector Borne Zoonotic Dis. 2011;11:1569-75.
17. Monteon VM, Guzman-Bracho C, Floriani-Verdugo J, Ramos-Echevarria A, Velasco-Castrejon O, Reyes PA. [Serological diagnosis of Chagas disease: self-sufficiency and interlaboratory concordance]. Salud Pub Mex. 1995;37:232-5.
18. Silver H, Tahhan HR, Anderson J, Lachman M. A non-computer-dependent prospective review of blood and blood component utilization. Transfusion. 1992;32:260-5.
19. Wendel S. Transfusion transmitted Chagas disease: is it really under control? Act Trop. 2010;115:28-34.
20. Sanchez B, Monteon V, Reyes PA, Espinoza B. Standardization of micro-enzyme-linked immunosorbent assay (ELISA) and Western blot for detection of Trypanosoma cruzi antibodies using extracts from Mexican strains as antigens. Arch Med Res. 2001; 32:382-8.
21. Remesar M, Sabino EC, Del Pozo A, Mayer A, Busch MP, Custer B. Bimodal distribution of Trypanosoma cruzi antibody levels in blood donors from a highly endemic area of Argentina: what is the significance of low-reactive samples? Transfusion. 2015;55: 2499-504.
22. Levin MJ, Franco da Silveira J, Frasch AC, et al. Recombinant Trypanosoma cruzi antigens and Chagas' disease diagnosis: analysis of a workshop. FEMS Microbiol Immunol. 1991;4:11-9.
23. Langhi DM, Bordin JO, Castelo A, Walter SD, Moraes-Souza H, Stumpf RJ. The application of latent class analysis for diagnostic test validation of chronic Trypanosoma cruzi infection in blood donors. Braz J Infect Dis. 2002;6:181-7.
24. Tibayrenc M. Genetic epidemiology of parasitic protozoa and other infectious agents: the need for an integrated approach. Int J Parasitol. 1998;28:85-104.
25. Souto RP, Zingales B. Sensitive detection and strain classification of Trypanosoma cruzi by amplification of a ribosomal RNA sequence. Mol Biochem Parasitol. 1993;62:45-52.
26. Brisse S, Barnabe C, Tibayrenc M. Identification of six Trypanosoma cruzi phylogenetic lineages by random amplified polymorphic DNA and multilocus enzyme electrophoresis. Int J Parasitol. 2000;30:35-44.
27. Brisse S, Verhoef J, Tibayrenc M. Characterisation of large and small subunit rRNA and mini-exon genes further supports the distinction of six Trypanosoma cruzi lineages. Int J Parasitol. 2001;31:1218-26.
28. Tibayrenc M. Genetic subdivisions within Trypanosoma cruzi (Discrete Typing Units) and their relevance for molecular epidemiology and experimental evolution. Kinetoplastid Biol Dis. 2003; 2:12.
29. Zingales B, Andrade SG, Briones MR, et al. A new consensus for Trypanosoma cruzi intraspecific nomenclature: second revision meeting recommends TcI to TcVI. Mem Inst Oswaldo Cruz. 2009; 104:1051-4.
30. Guzman Bracho C, Garcia Garcia L, Floriani Verdugo J, et al. [Risk of transmission of Trypanosoma cruzi by blood transfusion in Mexico]. Rev Panam Salud Publica. 1998;4:94-9.