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Multi-Antigen-Chemiluminescent-ELISA for the Diagnosis of Trypanosoma cruzi Infection in Chihuahua, Mexico.

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by

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Introduction

The genus *Trypanosoma* consists of flagellated protozoa parasites that cause infections in humans and animals. The most relevant infectious diseases to humans are African trypanosomiasis and Chagas disease caused by *Trypanosoma brucei*, *Trypanosoma cruzi*, respectively, which are considered as neglected tropical diseases (NTDs) [1]. Most affected by these parasitic diseases are low-income populations of countries located in tropical and subtropical areas of the world.

CHAGAS DISEASE

Chagas disease (ChD) or American Trypanosomiasis is caused by the protozoan parasite *Trypanosoma cruzi*. The parasite has a complex life cycle, which alternates between triatomine vectors and mammals, including humans. The disease can cause cardiac disorders, digestive or neurological alterations. *T. cruzi* is genetically diverse; it has been classified in six discrete typing units (DTU)s: TcI, TcII, TcIII, TcIV, TcV, and TcVI, which present different antigenicity, eco-epidemiological, clinical, and geographic associations [2]. It is considered a neglected tropical disease with endemic regions located in 21 Latin American countries, where it is mostly vector-borne when humans come across the feces or urine of the infected triatomine bug [3, 4].

Epidemiology

According to the World Health Organization (WHO), about 6 to 7 million people worldwide are estimated to be infected with ChD. Over the past half of the 20th century, there has been an increased migration of Latin Americans to non-endemic countries, greatly modifying the epidemiology of the disease. In the last decades, it has been detected in the U.S and Canada. Even in the Old World, *T. cruzi* has been detected in countries such as Japan, Australia and most of the European continent, where an approximate large number of immigrants infected with the protozoan live in Spain and Italy [3, 5]. Solely looking at the U.S., there are an estimated 300,000

infected individuals with *T. cruzi* in the states of New Mexico, Texas, Georgia, Louisiana and California [6, 7]. It is estimated that most individuals with ChD are immigrants that came from El Salvador, Guatemala, Honduras, and Mexico that acquired the infection in their countries of birth; the last accounting for around 174,388 infected individuals living in the U.S. [8-10]

Mexico is a country with immense climatic variety and vast biodiversity, providing the conditions for the development of etiological agents of all kinds, including *T. cruzi*. There are at least 30 species of triatomines reported in the country, all known to be potential vectors [11]. ChD remains as the most important parasitic disease in the country. As of 2010, it was estimated that 876,458 people was infected with *T. cruzi* and 70,117 people had chagasic cardiopathy [12]. The poverty in the country has forced people from endemic areas of the country to migrate to industrialized cities like Mexico City, in search of jobs. Carabarin-Lima et. al. report that the children under 5 years of age infected with ChD are distributed regularly in urban rather than rural areas, suggesting that ChD is becoming urbanized in Mexico [13].

In Mexico, there are 18 endemic areas located in the southeast comprising the states of Oaxaca, Jalisco, Yucatan, Chiapas, Veracruz, Puebla, Guerrero, Hidalgo and Morelos. A high prevalence is observed in the northeastern region of the country, which corresponds to the central area of a tropical region known as La Huasteca [13]. But the highest prevalence (1.94% - 1.68%) was observed in the US neighbor states of Nuevo Leon and Tamaulipas [13, 14].

In the southeast of the state of Chihuahua, near the limits of the states of Sonora and Sinaloa there is an area known as Barrancas de la Sierra Tarahumara, where there have been reports of triatomines infected with *T. cruzi*. In a study conducted by the Immunoparasitology Laboratory of the Medical School at the Autonomous University of Chihuahua, a sample of *Triatoma recurva* collected in the Chihuahuan municipality of Urique was found to be infected with *T. cruzi* [15].

Licon-Trillo et. al. (2007) conducted a study where they collected triatomine bugs for nearly one month from the municipalities of Ojinaga and Benavides in the state of Chihuahua. The results were 11 triatomine bugs collected (*T. gerstaeckeri* and *T. rubida*) with one of them being infected with *T. cruzi* [16].

Several species of triatomine insects have been reported in states bordering the southern United States with infections rates ranging from 0% to >90% [14]. A recent study conducted near Van Horn, Texas at The University of Texas at El Paso Indio Mountains Research Station has confirmed that 64.1% of the triatomines tested were infected with *T. cruzi* strains belonging to the DTU TcI [17].

Life cycle

The triatomine insect serves as the vector for *T. cruzi,* it ingests circulating trypomastigotes when it takes a blood meal from an infected host. The parasite transforms into epimastigote, which is the replicative stage, inside the midgut of the insect. Epimastigotes travel to the hindgut and differentiate into infective metacyclic trypomastigotes and are excreted in the feces of the vector. Metacyclic trypomastigotes enter through the bite wound or mucous membrane of the host and invade nucleated cells through a parasitophorous vacuole. The parasite escapes the from the lysosome vacuole; in the cytoplasm, it differentiates into the replicative form called amastigote. After several rounds of binary division, the amastigotes transform into trypomastigotes and burst out of the host cell. The trypomastigotes released into the blood stream can infect new cells and the cycle is repeated [6].

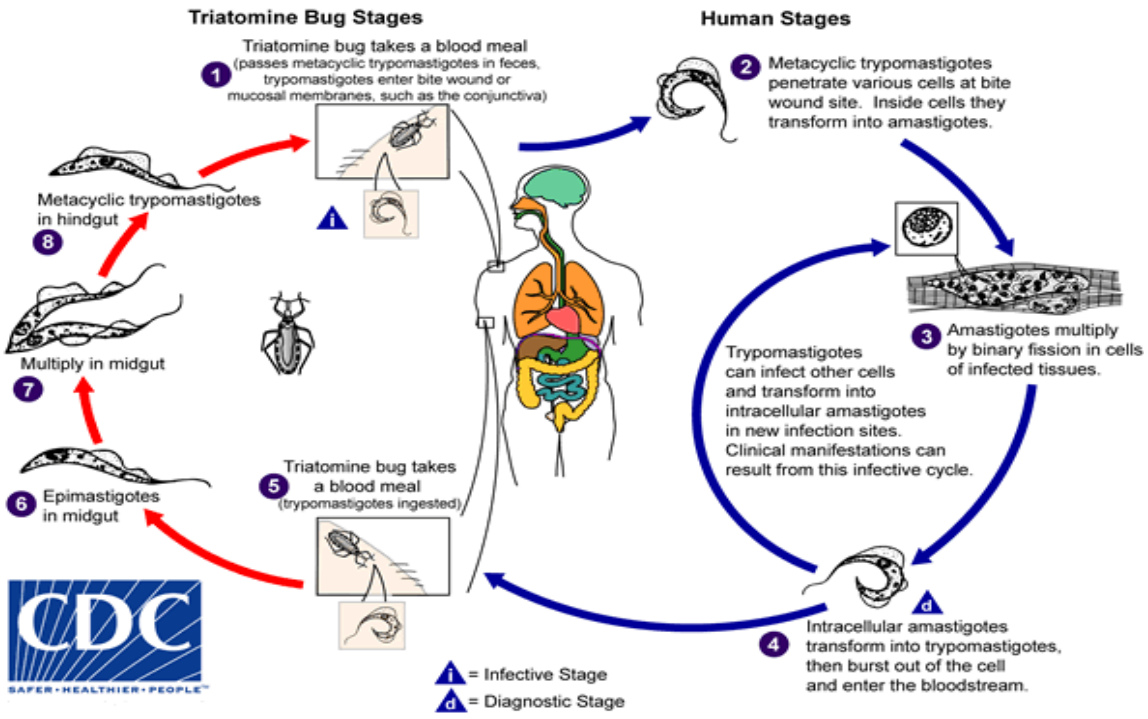

Figure 1. The life cycle of *Trypanosoma cruzi*. 1. Triatomine bug takes a blood meal releasing metacyclic trypomastigotes in the feces. 2. Metacyclic trypomastigoes enter the bite wound or mucosal membranes infecting nucleated cells. 3. Inside nucleated cells, the parasite transform into amastigotes and multiply by binary fission. 4. Amastigotes transform into trypomastigotes and burst out of the cell to infect new cells. 5. A triatomine bug takes a blood meal from infected mammalian host ingesting trypomastigotes. 6. Trypomastigotes transform into epimastigotes in the midgut. 7. Epimastigotes replicate by binary fission. 8. Epimastigotes travel to the hindgut where they transform into metacyclic trypomastigotes to be released in feces. (from Center for Disease Control and Prevention, 2015) [18]

Pathogenesis

T. cruzi is a hemoflagellate protozoan that can infect all nucleated cells: macrophages, fibroblasts, nerve cells, and muscle cells [19]. Infection with *T. cruzi* presents two clinical manifestations: the acute phase characterized by manifestations of generalized infection like fever followed by the chronic phase that can be divided into indeterminate, cardiac or, digestive form [20]. The acute phase it is characterized by trypomastigotes detectable by microscopy of fresh blood. Although patients are mostly asymptomatic, some develop swelling and inflammation at the site of infection, which is known as chagoma [6]. Tissue and organ damage is caused by both the parasite and the host's immune response. Several studies have suggested that a strong immune response with CD4+ and CD8+ cells and production of cytokines such as interferon γ, tumor necrosis factor α, and interleukin 12 are important in controlling parasitism [5].

In the absence of treatment, the individual enters the chronic phase, which can be asymptomatic, or it can lead to premature death. Up to 30% of individuals in the chronic stage develop cardiac alterations and up to 10% develop digestive, neurologic or a combination of alterations which require specific treatment [3]. Most patients that proceed to the chronic phase do not exhibit clinical disease despite presenting positive serology, abundant antibody production and a robust T cell response to parasite and host antigens [21]. These patients are in the indeterminate phase since they can carry the parasite for years and their life expectancy is comparable with individuals without ChD [20]. Studies in indeterminate patients have shown that they produce inflammatory cytokines such as TNF-α and IFN-γ. While regulatory T cells, monocytes and lymphocytes produce anti-inflammatory cytokines, especially IL-10. Most notably, in indeterminate patients the balance between inflammatory and anti-inflammatory cytokines leans towards the anti-inflammatory arm of the response [21].

Digestive ChD is seen mostly in the South of the Amazon basin, and rare in northern South America, Central America and Mexico and affects the esophagus and/or colon caused by the destruction of intramural neurons leading to severe megaesophagus or megacolon [5, 6]. According to Rassi et. al. (2010) megaoesophagus causes dysphagia with odynophagia, epigastric pain, regurgitation and in severe cases, malnutrition [5]. And megacolon often affects the sigmoid segment, rectum or descending colon, or a combination, and produces prolonged obstipation due to fecaloma or sigmoid volvulus [5].

Cardiac ChD is characterized by chronic inflammation of all chambers of the heart, damage to the conduction system and often an apical aneurism, bradyarrhythmia, tachyarrhythmia, cardiac failure, thromboembolism and sudden death [5, 6]. Dutra et. at. (2014) observes that, contrary to patients in the indeterminate phase, cardiac ChD present a predominance of inflammatory cytokines [21]. The author also mentions studies where, regardless of IL-10 production in cardiac patients, TNF-α producing cells were predominant in the damaged tissue. Additionally, T-cells in heart tissue from an infected individual displayed predominant expression of IFN-γ over other cytokines [21]. Dutra et. al. (2014) reported that the production of TNF-α and IFN-γ has been associated with clinical signs of cardiac pathology. Individuals with higher TNF-α production showed worse cardiac function and a worse prognosis of disease progression. In addition, a study displayed that patients with high production of IFN-γ presented a worse degree of cardiomyopathy [21].

Status in Mexico

Chagas disease has been largely considered neglected in Mexico. Blood screening of donors for *T. cruzi* was mandated in certain Latin America countries in 1990. But in Mexico, it was until 1994 when legislation was passed suggesting that donors who resided in endemic areas

should be tested serologically, but at that time no endemic areas defined by that legislation [22, 23]. ChD came to the government's attention again in 2000, when it passed new legislation mandating serological screening of blood donors, and later the Mexican Initiative for Surveillance and Control of Chagas Disease was convened in 2002 [24].

Despite the directive from the Ministry of Health to test donated blood, even in areas that self-identified as endemic, diagnostic testing is not being carried out in all blood banks. And one of the reasons is resource shortages, which is responsible for only 30% of the national blood supply being processed through initial screening and follow-up testing to confirm diagnosis [22, 24]. The health sector reports any cases and deaths due to ChD to the National Center for the Prevention and Control of Diseases, with most of the cases being reported by the Secretary of Health [25]. Although one problem remains in the current reporting system, and that is that it does not distinguish among acute, indeterminate and chronic phases of the disease. Even with implementation of surveillance methods, ChD continues to be understudied and poorly controlled in Mexico [24].

Health care and clinical personnel have deficient knowledge regarding ChD, its transmission, diagnosis or treatment due to neglect by healthcare system policies. Most individuals with ChD are asymptomatic or symptomatic without clinical manifestations, and the health care personnel are unaware of the potential risk in blood transfusions. In 2010, Trivedi M. and Sanghavi D. performed a study across 9 blood banks in Mexico City, where a total of 45 nurses who screen donors were given a survey to measure their knowledge about ChD. Their results showed that out of the three questions, 80% of nurses answered one question incorrectly or incompletely, and 13% had two incomplete or incorrect answers [22, 26]. Even the general population lacks an understanding of the disease. For example, in an endemic area such as state of Morelos, about 45%

of the general population is aware of the triatomine vectors, but only about 15% have an understanding of the disease, its clinical manifestations and how to prevent and treat it. This is mainly because is the responsibility of the state Secretaries of Health to implement education programs; as such there is serious heterogeneity in these programs in different states [25].

Contributing to the problem is the fact that there are no standard procedures for the isolation, study and keeping of *T. cruzi* strains in a "national" bank of stains. This could benefit research for the origin, virulence or other characteristics of the Mexican strains of *T. cruzi,* and possibly aid in the improvement of better diagnostic tools [11].

Another problem in Mexico is in regard to the treatment of confirmed cases of ChD. There are three major insurance schemes responsible for the access of treatment for ChD, the Mexican Social Security Institute (IMSS), Institute for Social Security and Services for State Workers (ISSSTE) and Seguro Popular (SP). These institutions diagnose patients and report confirmed cases to the State Secretaries of Health, who in turn report the cases to the national Program on Onchocerciasis, Leishmaniasis and Chagas Disease to provide the medicines to begin treatment of the disease [25]. As reported by Manne J. M., since 2009 the national program has requested nifurtimox from the WHO-Bayer Nifurtimox Donation Program to offer medicines for registered cases by the state Secretaries of Health, who then are in charge of the distribution to begin treatment. The only exception was the state of Morelos, where its state Program on Chagas Disease chose to purchase benznidazole directly from Masters Pharmaceutical, Ltd. in 2010. The Mexican Secretary of Health established guidelines that were updated in 2010 stating that nifurtimox is the first-line antitrypanosomal therapy in patients who have either acute or indeterminate ChD, even when benznidazole is widely considered the first-line treatment on basis of a better side-effect profile and a more extensive evidence base for efficacy [25, 27, 28].

Researchers have long recognized that studies in Mexico suffer from several limitations. Amongst those, they have reported that utilizing the official national surveillance data is difficult to interpret and can lead to a large underestimation of disease burden [24]. Data on prevalence of ChD is also reported to be limited both in Mexico and globally. Furthermore, underreporting is a serious issue because ChD has few acutely symptomatic cases and there is a limited pool of clinical experts in the country [25, 29].

Chapter 1: Multi-antigen Chemiluminescent-ELISA for diagnosis of *T. cruzi* infection.

The diagnosis of ChD has undergone great progress with the development and commercialization of assays based on different *T. cruzi* antigens. In recent years, utilizing recombinant antigen mixtures have shown better specificity, but their actual sensitivities were somewhat lower [30]. During the chronic stage, parasitemia is low and immunodiagnosis is the appropriate technique to determine the presence of *T. cruzi*. A diagnosis is made after testing with at least two serologic tests: indirect fluorescent antibody (IFA) and commercial enzyme-linked immunosorbent assay (ELISA) [31]. PCR is a useful tool when trying to confirm the diagnosis of inconclusive serology and as an auxiliary method in tracking treatment progression [5]. In Mexico, the Mexican Social Security Institute (IMSS) utilizes a wide variety of companies to provide serological tests for the diagnosis of ChD [32]. This lack of consistency within the assays and the lack of a gold standard test might contribute to unreliable reporting of the disease.

As mentioned previously, *T. cruzi* has been classified in several DTUs, and yet, the correlations between DTUs, geographical distribution, host species and pathogenicity remain controversial. It was considered that DTUs TcII, TcV, and TcVI were related to human infections and TcI, TcIII, and TcIV were related to the sylvatic cycle. However, reports in Colombia and Venezuela revealed that human infections were caused by TcI, TcIII, and TcIV [33]. The genetic variability in *T. cruzi* strains alters diagnostic results widely when testing sera from patients infected with parasites from different geographical regions or with different DTUs. This has led to the recommendation of favoring local strains to ensure the specificity and sensitivity of the test [30].

Serological tests using synthetic peptides have shown promising results. The combination of three *T. cruzi* recombinant proteins in an ELISA culminated in a test that is sensitive and specific

for the diagnosis of ChD [34]. Almeida I. C. et. al. (1997) developed a highly sensitive and highly specific chemiluminescent ELISA (CL-ELISA), that due to its increased sensitivity allows for the usage of a higher dilution of sera (1:2000), eliminating most indeterminate or inconclusive results [35]. He emphasizes the low sensitivity of current methodologies using low dilutions of human sera increasing background reactivity of natural antibodies. Also, the increase in low tittered polyclonal antibodies induced by various immunogens, such as anti-bacterial vaccines, can result in false-positives [35]. Previous studies have concluded that the combination of epidemiological data and high-performance testing can result in a more precise view on the status of donors [36].

HYPOTHESIS

We hypothesize that a multiantigen chemiluminescent-ELISA comprised of DTUs TcI, TcII and TcVI will detect the presence of anti-*T. cruzi* antibodies with higher sensitivity in sera samples from Mexico.

SPECIFIC AIM

Develop a chemiluminescent multiantigen ELISA assay using *T. cruzi* epimastigotes of five strains for detection of anti-*T. cruzi* antibodies.

MATERIALS AND METHODS

Epimastigote culture

T. cruzi epimastigote strains endemic in South America (Dm28c, Y-strain, and CL-Brener) and strains endemic in the Southern United States (Texas-Tulane and Houston) were obtained from the American Type Culture Collection (ATCC) (American Type Culture Collection, Manassas, VA, USA). Parasites were cultivated in 1L Liver Infusion Tryptose (LIT) medium supplemented with 10% inactivated fetal bovine serum (Corning Inc. Corning, NY, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) [37] for 6 days and incubated at 27°C.

Antigen preparation

The epimastigote multiantigen (EpM) was prepared using epimastigotes of five *T. cruzi* strains: Dm28c, Y-strain, CL-Brener, Texas-Tulane and Houston as previously described by Almeida et. al. (1997) with minor modifications [35]. Parasites were collected the sixth day after the last passage, centrifuged at 1000 xg for 15 minutes at 4°C, and washed three times in 1 X phosphate buffer saline (pH 7.4). The parasites pellet was resuspended in Tris-HCl 10 mM (pH 7.4) (Fisher Scientific, Hampton, NH, USA), EDTA 2mM (Fisher Scientific, Hampton, NH, USA), protease inhibitor cocktail EDTA-free (dilution 1:10) (Thermo Fisher Scientific, Waltham, MA, USA), and 1% nonanoyl-N-methylglucamide (Sigma-Aldrich, St, Louis, MO, USA). The suspension was then submitted to six cycles of freezing and thawing in liquid nitrogen and water bath at 37°C. Followed by sonication (35% amplitude for 8 seconds, five times) (VCX 750, Sonics & Materials Inc., Newton, CT, USA). The lysate suspension was centrifuged at 12,000 xg for 10 minutes at 4°C. Protein concentration was determined by the Bradford assay (Thermo Fisher Scientific, Waltham, MA, USA), and equal quantities of each (407 µg) were used to produce the EpM.

Sample collection

This experiment was approved by The University of Texas at El Paso Institutional Review Board, and performed under the IRBnet Exempt protocol [267936] Chagas disease screening in samples from State Blood Bank of Transfusion, Chihuahua-Mexico. All shipping in Mexico was done via a commercial courier with expertise in the transport of blood and blood components. Upon arrival to Ciudad Juarez, Mexico, the CDC transported the samples through the border to El Paso, Texas. It is important to highlight that Dr. Maldonado never had access to the patients' personal information.

As per Mexican law, all donors were between 18 and 65 years of age and weight at least 110 pounds (50 kg). Donors agreed to participate in the study by signing a consent form provided by the Pan American Health Organization (PAHO). Aliquots collected were maintained at the constant recommended temperature of -80°C during storage and throughout the shipping process. To minimize the frequency of shipping samples over the border during the period of study, samples were collected first throughout the State of Chihuahua and sent to the CETS in Chihuahua. Then, the CETS kept one aliquot and sent the other two to the University of Chihuahua in Chihuahua City (UACH) and to Ciudad Juárez, either once a month or when they were at least 100 samples collected, whichever was reached first. The one aliquot sent to UTEP (The University of Texas at El Paso), was used for examination with the CL-ELISA. If the donor is confirmed positive for Chagas disease the Mexican authorities will contact the donor to provide medical attention and follow up.

Sera

Serum samples were collected from the blood banks of the Mexican Social Security Institute (IMSS) No. 1, No. 35 and No. 66, the State Blood Transfusion Center (CETS) in Chihuahua City, and blood banks from the City of Juarez Hospital, Parral City General Hospital,

Delicias City Hospital and Cuauhtémoc City Hospital (Table 1). After the subjects have donated blood, each blood bank performed the routine screening for infectious agents (e.g. HIV, Syphilis, hepatitis C, etc), as well as the approved procedure for detection of ChD as outlined in Mexican norm NOM-032-SSA2-2002. The reactive sera were tagged and 4 aliquots of 1ml were frozen with glycerol, if non-reactive, one percent of samples were retained at random. The samples included in the study were assigned a coded serial number to protect the identity of the subjects and to facilitate operational research design. This serial number will be matched to correspond to a file at the blood donation center where the sample was originated from. Through this number, the following information is available: name of the donor, donation site, date and time of sampling procedure**,** and contact information (all registered as a bar code).

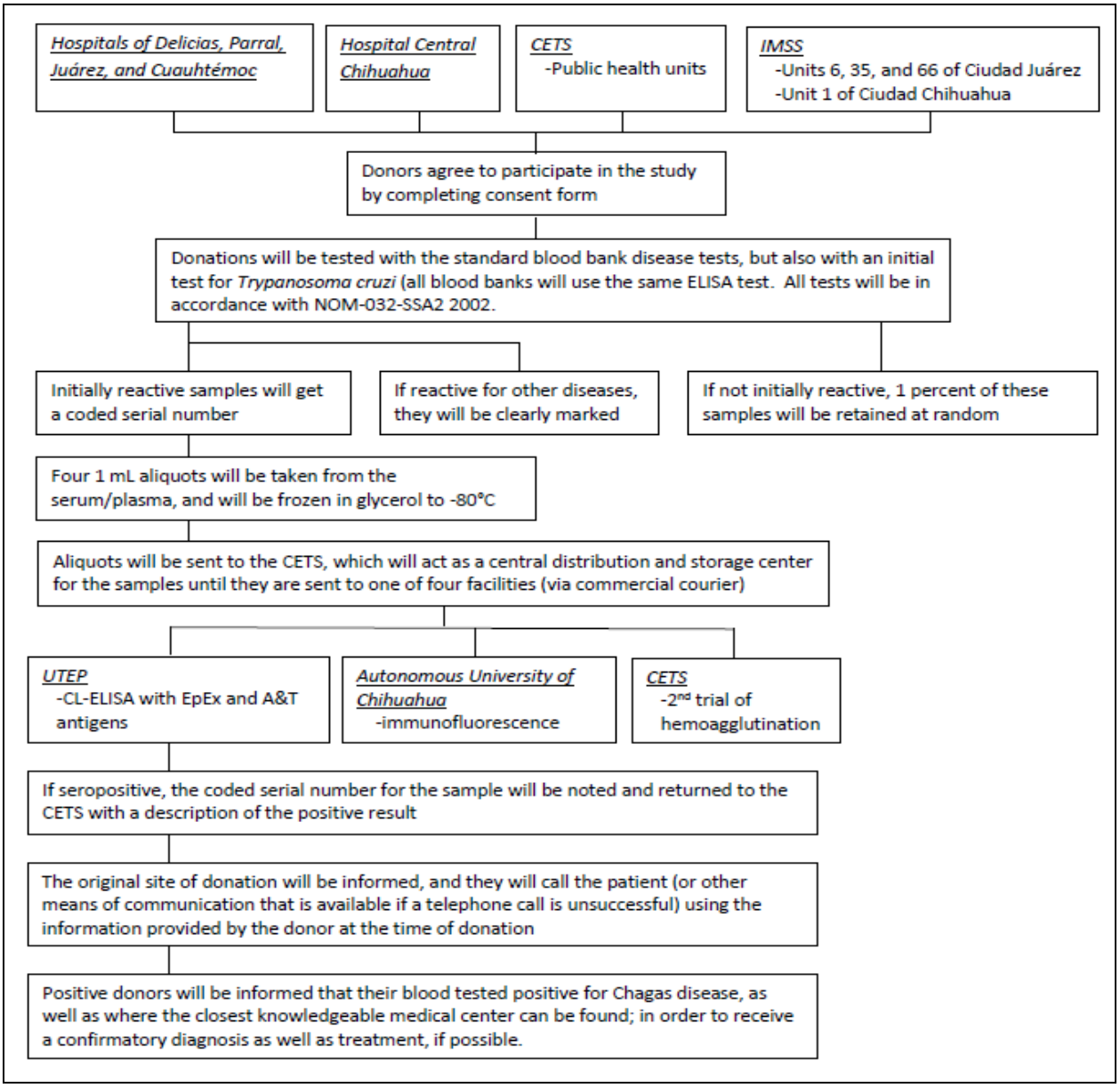

Table 1. Flow chart of the procedures that were followed for the collection, handling and analysis of the donors' samples.

Chemiluminescent Enzyme-Linked Immunosorbent Assay (CL-ELISA)

EpM antigens were diluted in carbonate-bicarbonate buffer (pH 9.6) and immobilized on 96-well white polystyrene microplates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA), by incubating 24 hr at 4°C. The plates were blocked with 5% bovine serum albumin in 1X phosphate buffered saline pH 7.4 (5% BSA-PBS). Followed by 1 h incubation with the patient sera diluted 1:500 in 1% BSA-PBS at 37°C. Plates were washed three times with PBS-Tween 0.05% (PBS-T) and then incubated for 1 h at 37°C with 50µl of anti-human IgG biotinylated (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) diluted 1:5000 in 1% BSA-PBS. Next, plates were washed three times with PBS-T, followed by 1 h incubation at 37°C with 50µl of neutravidin-horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:5000 in 1% BSA-PBS, then washed 4 times with PBS-T. Finally, 50µl of Supersignal ELISA Pico Stable Peroxide Solution, Supersignal ELISA Pico Luminol Enhancer (Thermo Fisher Scientific, Waltham, MA, USA) diluted in carbonate-bicarbonate buffer at a ratio of 1:1:8 were added to each well and the data was analyzed utilizing a microplate luminometer (Luminoskan, Thermo Fisher Scientific, Waltham, MA, USA). Each plate included a positive control (pool of Chagasic patients from Barcelona, Spain), a negative control (pool of healthy donors from Barcelona, Spain) and a background control (incubations done with no sera added). The assay was performed in duplicates, and both mean and standard deviation where calculated using GraphPad Prims v6.0. Positive and negative controls were pools of sera comprised of 10 patients with ChD and 10 healthy donors, respectively (kindly provided by Dr. I. C. Almeida, UTEP).

Cutoff value for the EpM CL-ELISA was calculated by first determining the reactivities, reported as relative light units (RLU), of the 10 healthy donor sera that comprised the pool used in the assay. The mean and the standard deviation of these reactivities were calculated. A value of 3 times the standard deviation was added to the mean for the cutoff value. The titer of the EpM CL-

ELISA is defined as the ratio of the serum's RLU to the cutoff value (titer = RLU/cutoff value). A serum sample with a titer equal or greater than 1.0 was considered positive. A serum sample that presented a titer lower than 0.9 was considered negative. And any serum sample with a titer in the range of 0.9 to 0.99 was considered as inconclusive.

RESULTS

Chemiluminescent Enzyme-Linked Immunosorbent Assay (CL-ELISA)

Several titrations were performed utilizing multiantigen concentrations ranging from a low 4 ng/well to a high concentration of 120 ng/well. Control sera were serially diluted pools (1:2) of Chagasic patients' sera and healthy donors from 1:250 to 1:2000. Titrations were performed in to determine the ideal concentration of antigen and sera dilution that would present a significant separation in the reactivities between both controls. We analyzed the results and determined that the greatest differential reactivity between control sera was observed at a 1:500 sera dilution and an EpM antigen concentration of 108 ng/ml (Fig. 2).

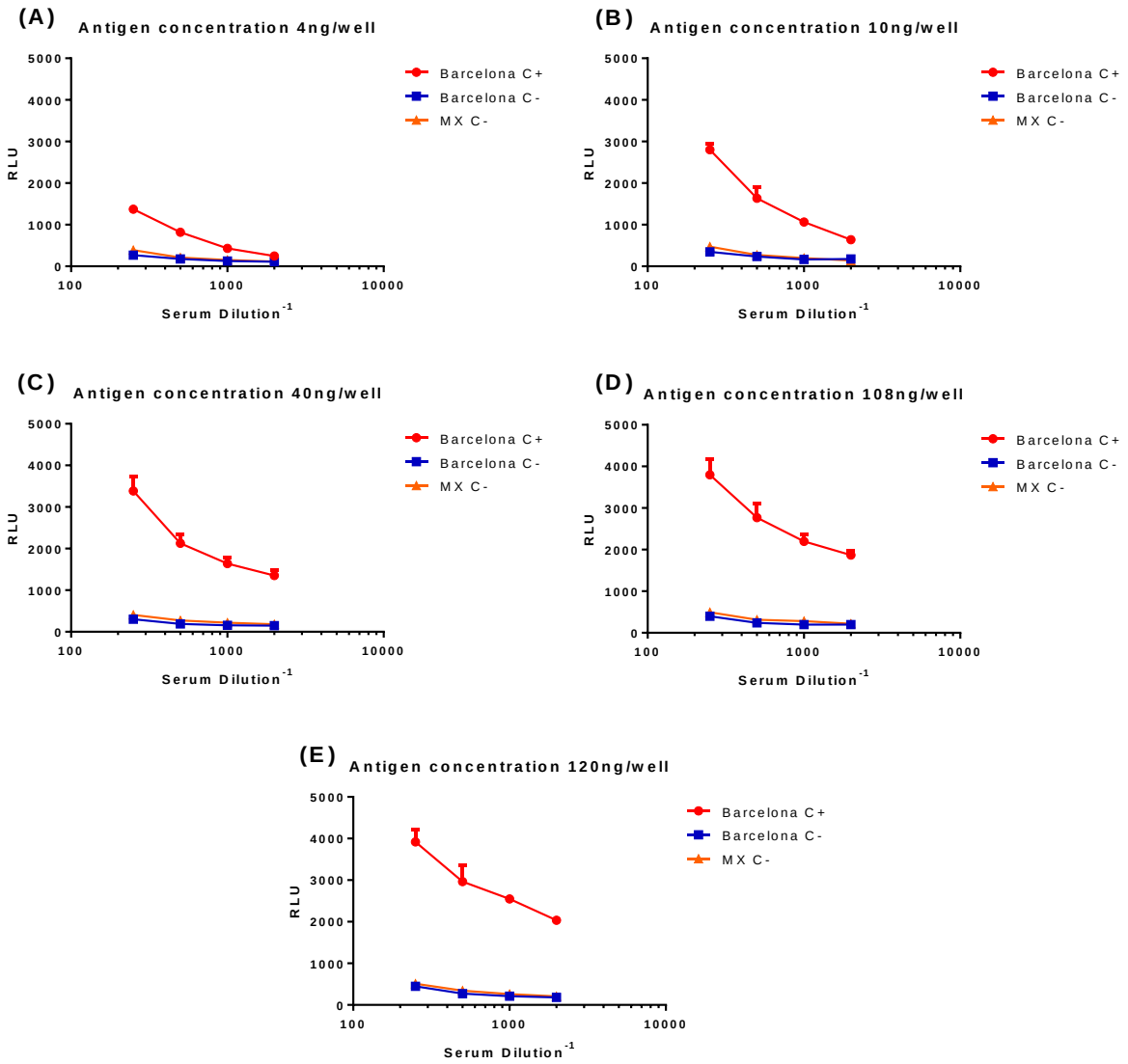

Figure 2. Titration curves for the EpM CL-ELISA. Sera were serially diluted (1:2) from 1:250 to 1:2000 in 1% BSA-PBS. (A) EpM CL-ELISA multiantigen at 4 ng/well. (B) EpM CL-ELISA multiantigen at 10 ng/well. (C) EpM CL-ELISA multiantigen at 40 ng/well. (D) EpM CL-ELISA multiantigen at 108 ng/well. (E) EpM CL-ELISA multiantigen at 120 ng/well. Positive control: pool of 10 Chagasic patients from Barcelona, -●-; negative control: pool of 10 healthy donors from Barcelona, -■-; serum sample of a known healthy donor -▲-. The highest differential reactivity between positive and negative sera was determined at (D) multiantigen concentration of 108 ng/well and sera dilution of 1:500. Titrations were performed in triplicates and the results represent the means of the replicates.

The sera samples were previously tested in Mexico utilizing the automated Chemiluminescent Microparticle Immunoassay (CMIA) with ARCHITECT® (Abbott, Chicago, IL, USA); reactive samples were later tested with serology, either IIF IHA or ELISA according to NOM-032-SSA2-2002, to confirm previous results. Concluding the second methodology, there were 54 samples that were deemed inconclusive. Using this multiantigen CL-ELISA (EpM CL-ELISA) as a reference, out of the 319 sera samples analyzed, 13 (4.1%) were positive and 306 (95.9%) were negative without any inconclusive samples (Fig. 3).

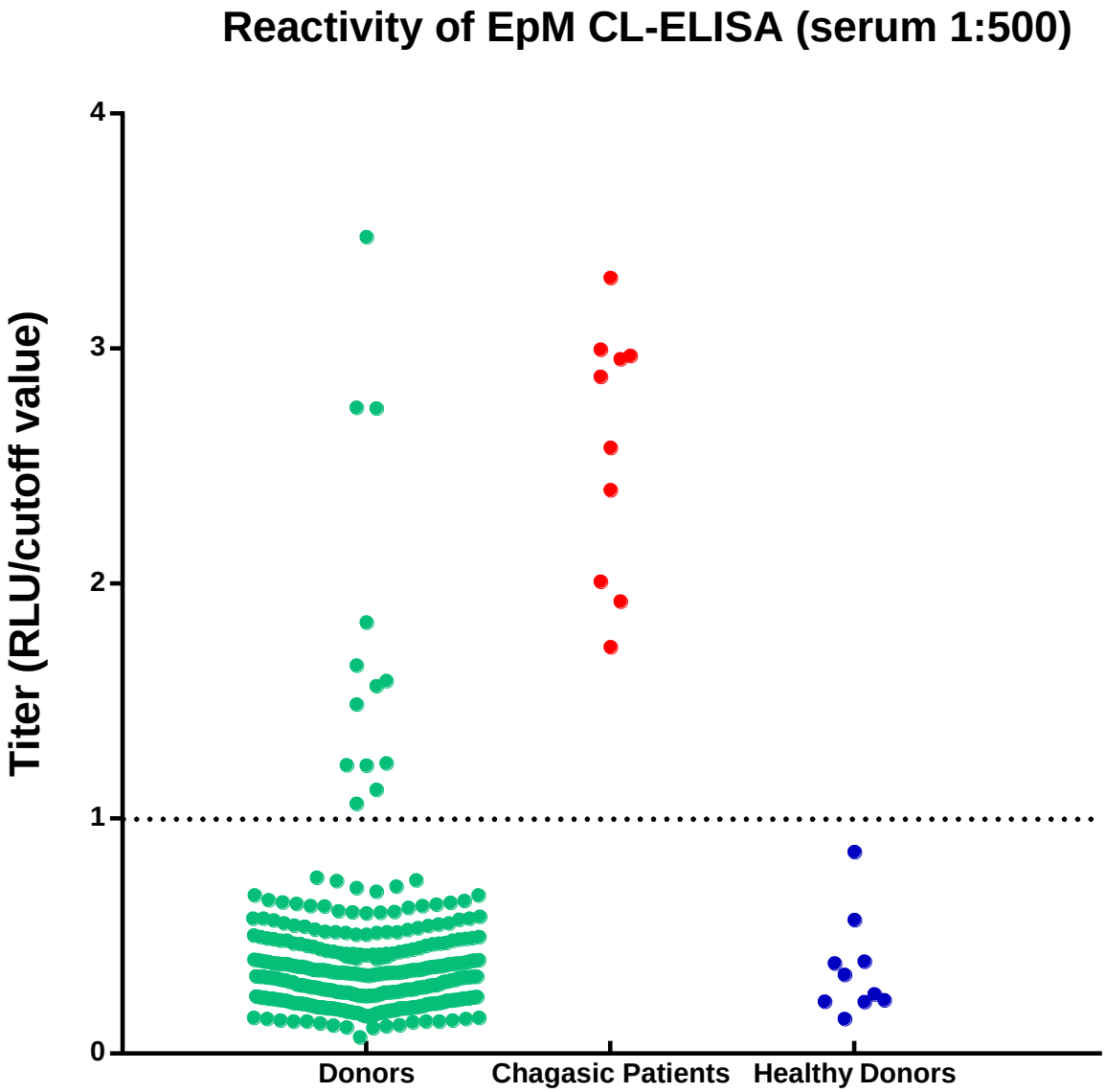

Figure 3. Reactivity of EpM CL-ELISA using the multiantigen of *T. cruzi* epimastigotes (108ng/well). Donor samples (n=319) were diluted 1:500 in 1% BSA-PBS and tested in duplicates. Individual sera comprising the pools of Chagasic patients (n=10) and healthy donors (n=10) respectively, were diluted 1:500 in 1% BSA-PBS and assayed in triplicates to determine the cutoff value. Dotted line, a cutoff value corresponding to titer = 1.0. A sample is considered positive when its titer is equal to or higher than 1.0 and negative when its titer is less than 0.9. Samples with titers ranging from 0.9-0.99 are considered as inconclusive. The reported results represent the means of the replicates.

Statistical analysis

The means and standard deviations were calculated utilizing GraphPad Prism 6, and the cutoff was established as the mean plus three times the standard deviation of the NHS (negative control). To assess the reproducibility of the assay, estimates of inter-assay and intra-assay for both controls were calculated in accordance with the methods previously described [38, 39]. The coefficients of variability (CV) for repeated assays was lower than 10 percent in all cases (Table 2); which indicates a good reproducibility of the assay.

Table 2. Reproducibility of the EpM CL-ELISA

Reproducibility of the EpM CL-ELISA					
Control Serum	Mean Relative Light Units (RLU)	Intra-assay		Inter-assay	
		Standard Deviation	Coefficient of Variability (%)	Standard Deviation	Coefficient of Variability (%)
Positive	3405.6	50.3	1.5	230.6	6.8
Negative	350.6	12.8	3.6	21.3	6.1

DISCUSSION

The protozoan parasite *T. cruzi* is the causative agent of Chagas disease; a neglected tropical disease affecting about 6 to 7 million people estimated to be infected with the parasite [3]. In the U.S., there are an estimated 300,000 infected individuals, with several reports of people infected with *T. cruzi* in the states of New Mexico, Texas, Georgia, Louisiana and California [6, 7]. In Mexico, the states of Tamaulipas and Coahuila have reported as endemic areas, this is important because those states are bordering with Texas [13, 14].

Based on the different characteristics of the different DTUs, we prepared the multiantigen with five *T. cruzi* strains to improve the specificity and sensitivity of the ELISA assay. *T. cruzi* Dm28c is classified as TcI [40], which is the most widely dispersed DTU in the American continent covering South, Central, and North America and is associated with both domestic and sylvatic cycles [41]. *T. cruzi* Y-strain corresponds to DTU TcII [40], which can be found in the south and central regions of South America and occasionally further north. It has been isolated mostly from domestic cycles [41]. *T. cruzi* CL Brener strain is classified in lineage TcVI [40]; together with lineage TcV both are hybrids associated with ChD in southern and central South America and are known mostly as domestic isolates [41]. Zingales *et al.* stated that the lineages TcI and TcIV are found in the Southern United States; both *T. cruzi* Houston and Texas-Tulane strains were found in the state of Texas [42, 43] and have been classified as TcI (Ramirez, Orozco and Maldonado, unpublished).

The chronic stage of the disease is when parasitemia is low and serological tests are the most suited option for the diagnosis of the disease [5]. A study by Umezawa et. al. (2003) has shown promising results when several *T. cruzi* recombinant proteins are included in a single ELISA [44]. Pereira et. al. (2012) reported that the combination of high performing testing along with epidemiological data can improve diagnostic capabilities [36]. Previously, Almeida et al

(1997) developed a CL-ELISA highly specific, able to confirm the cure of treated patients after two years of treatment [35]. Following these ideas, we developed the multiantigen of *T. cruzi* epimastigotes by selecting strains classified in different DTUs across the American continent to use in combination with a CL-ELISA. In our experiment, the EpM CL-ELISA analyzed 319 sera samples and concluded with 13 (4.1%) positive samples and 306 (95.9%) negative samples without any inconclusive. From the 13 seropositive samples in our EpM CL-ELISA, the conventional serological tests previously diagnosed 3 sera as inconclusive, 5 as positive and another 5 as negative. The results of this assay indicate that the EpM CL-ELISA is highly sensitive for the diagnosis of ChD compared to conventional serological tests.

Chapter 2: Immunoblot of seropositive samples to assess performance of EpM CL-ELISA as a diagnostic tool.

T. cruzi displays on its surface numerous glycoconjugates that are involved in the recognition and invasion of mammalian host cells, as well as establishing and sustaining the chronic infection of ChD [45]. Its genome is at least 50% repetitive sequence, consisting of large gene families of surface proteins, retrotransposons and subtelomeric repeats. The largest gene families encode surface proteins such as mucin-associated surface proteins (MASPs), members of the trans-sialidase (TS) super family, mucins and the surface glycoprotein gp63 protease, all accounting for ~18% of the total protein-coding genes [46]. Acting at the interface between the parasite and the vector or the mammalian host, these molecules are suited to fulfill two purposes: provide protection against the vector and/or host's defense mechanisms and to ensure the targeting and invasion of specific cells or tissues [47].

The surface of the different stages of *T. cruzi* is covered by a thick coat of glycoconjugates. The major component is a family of mucin-like proteins, which are glycoconjugates highly glycosylated rich in Thr, Ser and Pro that serve as acceptor sites for the addition of *O*-linked oligosaccharides. Their core polypeptides are only 50-200 amino acids in length and are anchored to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor [47-49]. The deduced polypeptide sequences of the native mucin polypeptide chain can be divided in two families. Family I polypeptides are composed of central domain rich in Thr, Lys and Pro organized in blocks with the repetitive motif $(Thr)_8Lys(Pro)_2$. These blocks are flanked by a highly variable and short N-terminal region and a more conserved C-terminal region. In Family II polypeptides, they too show N- and C-terminal regions similar to Family I polypeptides, but their Thr concentration is much lower and they lack the repetitive motif $(Thr)_8Lys(Pro)_2$. It has been observed that Family I

sequences are expressed in epimastigotes and metacyclic trypomastigotes, whereas Family II is expressed in trypomastigote mucins and probably in amastigote mucins [45].

One feature of the *T. cruzi* mucins is that they represent the major acceptors of sialic acid (SA) by the cell surface trans-sialidase. *T. cruzi* is unable to synthesize SA, but it expresses the enzyme TS, which transfers SA from the host to the parasite surface acceptors containing terminal β-galactosyl residues [49]. This reaction cleaves a SA residue linked in α(2-3) configuration to a terminal βGal*p* in the host macromolecule to produce the same linkage on the parasite surface mucins [50]. Sialylation of the cell surface takes place in all parasite stages except in the amastigote stage, which lacks TS activity [48]. It is the sialylation of surface molecules in *T. cruzi* that leads to the formation of the Ssp-3 epitope [51]. This epitope is found exclusively in the surface of the infective trypomastigote stage, and it has been demonstrated to be crucial for the recognition and invasion of mammalian cells as well as diverting the complement cascade [4, 47, 51].

Despite their complexity and heterogeneity, the mucins of *T. cruzi* can be divided in two major types: those present in the triatomine bug and those present in the mammalian host. Mucins in the epimastigote and metacyclic trypomastigote present a double or triple band in the range of 30-50 kDa when ran on a SDS-PAGE, they have an almost identical amino acid composition, carbohydrate content and structure [45, 47, 49]. These mucins are encoded by the *TcSMUG* gene family, which is composed of two groups named S and L that display an average of >80% identity. TcSMUG S products are the backbone of for the 30/50 kDa mucins, they bind to target cells and induce a bidireccional Ca^{2+} response which contributes to host cell invasion. *TcSMUG L* genes encode mucin-type glycoconjugates restricted to the epimastigote form and their products are not acceptors for SA residues. TcSMUG products have features of insect stage apo-mucins, including

the lack of major sequence variability and the presence of a region rich in Thr in the central domain [52].

A noticeable difference in the mucin structure between these two insect stages of the parasite is in the GPI anchor. When the epimastigote transforms into the metacyclic trypomastigote, the O-linked oligosaccharides and the GPI glycan core remain unchanged, but the lipid portion of the GPI anchor changes from alkylacylphophatidylinositol to mostly inositol phosphoceramides [45, 47, 53, 54].

Mucins from the cell-derived trypomastigote form run on SDS-PAGE as a smear with a wide variety of molecular masses ranging from 60-200 kDa and share the SA-containing epitope Ssp-3. Trypomastigote mucins (also known as tGPI-mucins) are encoded by the *TcMUC* gene family comprising ~800 members, their structure is made of a highly conserved N-terminal signal peptide, a cleavable C-terminal GPI signal and a Thr-rich region [47, 50, 55]. O-glycans in tGPI-mucins begin with the addition of αGlcNAc to Thr in the core polypeptide, which may remain unsubstituted or it can be elongated with carbohydrates, mainly Gal*p*. In the presence of SA donors, tGPI-mucins become rapidly sialylated by the parasite´s TS as previously mentioned, by linking SA to βGal*p*. The addition of SA will give a negative charge to the mucin coat providing protection to the parasite against human anti-α-galactopyranosyl antibodies [50].

Alternatively, the terminal βGal*p* displayed by the *O*-glycans of tGPI-mucins can be modified by αGal*p* in the parasite´s secretory pathway. This process provides diversification of the surface mucins and leads to the presentation of the αGal glycotope (Galα1-3Galβ1-4GlcNAcα), the main target for lytic antibodies in patients with acute and chronic ChD. Actually, the αGal units are the structural feature that distinguishes the glycoproteins of the trypomastigote stage [4, 47, 55].

Interestingly, it has been found that cell-derived trypomastigotes constantly shed extracellular vesicles from the cell body and the flagellum. These vesicles display a complex cargo of αGal-containing glycoconjugates along with gp85/TS and MASPs [4, 56]. It is postulated that they are involved in cell-cell communication or in the modulation of the host immune system in order to establish infection. In fact, these αGal reactive vesicles are internalized by epithelial cells and macrophages, the latter activating a TLR2-mediated pathway significantly enhancing cell invasion [47, 57].

A factor that must be taken into consideration when diagnosing ChD using serological tests is cross-reactivity. *T. cruzi* and *Leishmania* spp. belong to the Trypanosomatidae family and thus both share many antigens that can cause a strong cross-reaction in serological tests for ChD or leishmaniasis, reducing the accuracy of diagnosis for both infections [58, 59]. Another important factor is the geographical distribution of *T. cruzi* and *Leishmania* spp., which overlaps in several countries of South and Central America. Serological tests performed in the city of Oran, Argentina in patients with symptomatic cutaneous leishmaniasis, determined that 35% of the patients were also infected with *T. cruzi*. Additionally, in Brazil and some Central American countries, *Trypanosoma rangeli* has been found to infect the same vectors and vertebrate hosts as *T. cruzi* [60, 61].

Currently, there is not a single test that has 100% sensitive and 100% specificity, but the WHO and the Ministry of Health in Brazil agree that two or more tests must concur for a sample to be determined as positive or negative for ChD [62, 63]. It is possible to diagnose most cases serologically with a combination of a test with high sensitivity in parallel with a test with high specificity [64]. Our EpM CL-ELISA has shown to have a high sensitivity for anti-*T. cruzi* IgG antibodies utilizing epimastigote whole cell lysates. To assess specificity, and as a confirmatory

test for the EpM CL-ELISA, we decided to perform a western blot using the trypomastigote stage of the parasite which gives significantly less false-positive and cross-reactive results [35, 65, 66].

HYPOTHESIS

We hypothesize that the trypomastigote immunoblot will present protein bands indicating the presence of anti-*T. cruzi* IgG antibodies in the EpM CL-ELISA seropositive sera samples.

SPECIFIC AIM

To confirm the seropositive results of the EpM-CL-ELISA in the screening of sera samples from Chihuahua, Mexico.

MATERIALS AND METHODS

Parasite culture and antigen preparation

T. cruzi Y-strain Mammalian Tissue Culture-Derived Trypomastigotes (TCT) were obtained from the supernatant of 7 days old LLC-MK2 cells (American Type Culture Collection, Manassas, VA, USA) cultured with infected blood from C57black α-Gal knockout mice. LLC-MK2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Corning Inc. Corning, NY, USA) medium supplemented with 10% fetal bovine serum (Corning Inc. Corning, NY, USA) and 1% Penicillin/Streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), incubated at 37°C in a 5% CO_2 atmosphere (Napco Series 8000 WJ CO_2 Incubator, Thermo Fisher Scientific, Waltham, MA, USA). To collect the parasites the supernatant was removed from the tissue culture flaks and centrifuged for 10 minutes at 1500 xg. After centrifugation, the parasite containing medium was incubated at 37°C for 2 h to allow TCTs to separate from the pellet containing amastigotes and cell debris. Supernatant was then removed and centrifuged at 3000 xg for 10 minutes at 4°C, the TCT pellet was washed three times in 1 X phosphate buffer saline (pH 7.4). After the third wash, phosphate buffer saline was removed and the TCT pellet was lysed as previously described above. The lysate suspension was centrifuged at 12,000 xg for 10 minutes at 4°C and supernatant was stored at -80°C until used.

Electrophoresis and western blot

Proteins from the TCT lysate (1.5 µg/well) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 6% acrylamide concentration at 90V for 90 minutes. Western blot was carried out by transferring the proteins in the polyacrylamide gel into nitrocellulose membranes (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The blotted membranes were blocked with 10% bovine serum albumin in Tris buffered saline (20 mM Tris and 150 mM NaCl)/0.05% Tween 20 at pH 7.4 (10% BSA-TBST) for 16 h at 4°C with constant shaking. The

membranes were cut in strips and each was individually incubated with the serum to be evaluated; dilutions were 1:1000 for sera samples and the double negative control (non-infected mice sera), and 1:2000 for positive control (pool of sera from Barcelona patients being treated with fexinidazole at day zero, kindly provided by Dr. I. C. Almeida, UTEP) diluted in 10% BSA-TBST for 2 h at room temperature with constant shaking. Strips were washed three times with TBST for 10 min each, then incubated with anti-human IgG biotinylated (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) diluted 1:10,000 in 10% BSA-TBST at room temperature for 2 h with constant shaking. Three more washes were carried out and each strip was then incubated with streptavidin-horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:10,000 in 10% BSA-TBST at room temperature for 1 h with constant shaking. Another three washes were performed and the immunoblots were developed by chemiluminescence using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions.

RESULTS

TCT Y-strain immunoblot

In previous studies, immunoblots using TCT whole cell lysate antigens of *T. cruzi* Y-strain have shown a reactivity pattern where IgG of acute-phase rabbit sera identified mainly high-molecular-weight components, while the IgG of chronic-phase rabbit sera reacted mainly to low molecular weight components [67].

In this immunoblot, we assayed the reactivity of the 13 EpM CL-ELISA seropositive samples with antigens from a whole cell lysate of the trypomastigote form of *T. cruzi* Y-strain. We observed different patterns where 12 samples (Fig. 4, lanes 1-5 and 7-13) present reactivity to

mainly low molecular weight proteins; and 8 samples (Fig. 4, lanes 6-13) reacted mainly with low and some high molecular weight proteins, especially one ~260kDa.

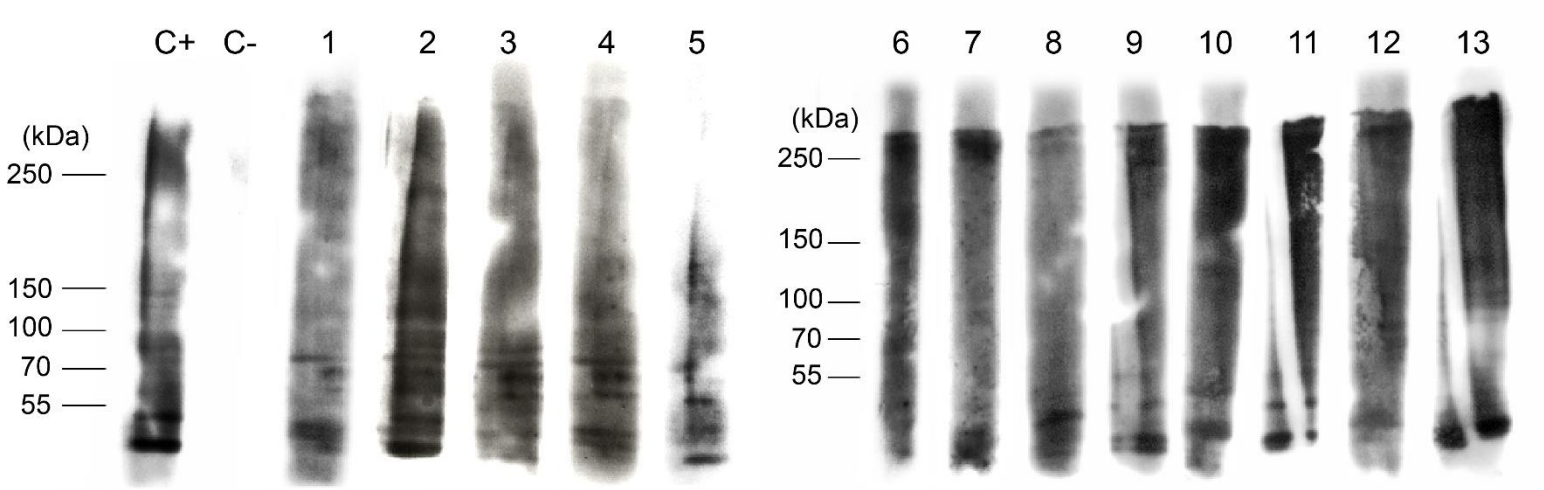

Figure 4. Western blot analysis of EpM CL-ELISA seropositive samples. TCT lysate was resolved in 6% polyacrylamide gel and then transferred to a nitrocellulose membrane for Western blot analysis of EpM CL-ELISA seropositive patients. Positive control: pool of Chagasic patients (1:2000), negative control: naïve mice sera (1:1000) and patient's sera (1:1000) used as primary antibodies. Secondary antibody anti-human IgG (1:10,000) and the horse-radish peroxidase conjugated streptavidin (1:10,000).

Data analysis

The results of the TCT Y-strain immunoblot were defined as positive by naked-eye observation of the bands.

DISCUSSION

There are two types of techniques for the detection of anti-*T. cruzi* antibodies: conventional tests which are the widely used IHA, IIF and the ELISA that employ the entire parasite or a complex of parasitic antigens. The likelihood of the infection being diagnosed is high even when there are low antibody levels. However, there is the chance of false-positive results due to cross-reactions between *T. cruzi* and *Leishmania* spp. which may lead to misdiagnosis, especially in areas where both parasites are endemic [57, 68]. In Mexico, it has been reported that leishmaniasis is endemic in three regions: Gulf of Mexico, Pacific of Mexico and Central Mexico, where *T. cruzi* [13] and different species of *Leishmania* coexist [69]. On the other hand, nonconventional tests such as PCR or western blot, are developed with the aim of increasing the specificity of serological diagnosis and to avoid cross-reactivity with other parasitic diseases [62].

Following the recommendations of the WHO for the diagnosis of ChD, we performed the EpM CL-ELISA which presented a high sensitivity, with 13 seropositive and 306 seronegative samples from a total of 314 sera. To confirm the results and to assess the specificity of our EpM CL-ELISA, we performed an immunoblot using a whole cell lysate of *T. cruzi* trypomastigotes, the infective stage in the human host, to reduce false positives and cross-reactivity with other parasites. All 13 samples tested showed several bands indicating the presence of IgG antibodies against *T. cruzi*, corroborating the previous results of the EpM CL-ELISA. It is important to notice the different patterns described by Umezawa E. S. et. al. [67], where reactivity to mainly low molecular weight proteins suggests ChD is in the chronic phase, and if reactivity is observed mainly with high molecular weight proteins, ChD is in the acute phase. In our assay, it is suggested

the 12 sera samples that reacted mainly with low molecular weight proteins samples (Fig. 4, lanes 1-5 and 7-13) represent infected patients in the chronic-phase of ChD; and one patient that reacted with a protein ~260 kDa represents a patient in the acute-phase of the disease.

The combination of our EpM CL-ELISA as a high sensitive assay and the TCT immunoblot as a high specificity and confirmatory assay, represent promising diagnostic tools for the diagnosis of ChD in South, Central and North America.

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Vita

Jose Andrei Orozco-Arroyo Jr was born in El Paso, Texas, the first born to Jose Alfredo Orozco Chavez and Martha Imelda Arroyo Cano. After graduating from high school in Mexico, he moved to El Paso to pursue a college education. He earned his Bachelor of Science in Microbiology from the University of Texas at El Paso (UTEP) in Spring 2014 and was accepted into the Master of Science program at UTEP that same year.

As a graduate student, he was trained in immunology and molecular biology focused in the study of parasites. His thesis entitled "Multi-Antigen-Chemiluminescent-Elisa for the Diagnosis of *Trypanosoma cruzi* Infection in Chihuahua, Mexico" was supervised by Dr. Rosa Maldonado. This work has been presented in various conferences; such as Annual Meeting of the Rio Grande Branch of the American Society for Microbiology (2014), Minority Health and Health Disparities Grantees Conference (2014), Annual Woods Hole Immunoparasitology Meeting (2015), Border Biomedical Research Center Symposium Health Disparities: From Molecules to Disease (2015 and 2017).

During his process in achieving his master's degree, he had the opportunity to interact with undergraduate students, mentoring and introducing them to trainings and laboratory techniques. As a teacher assistant he had the privilege to teach the laboratories of Prokaryotic Molecular Genetics, Medical Parasitology and Immunology.

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