Antigenicity and Diagnostic Potential of Vaccine Candidates in Human Chagas Disease

Shivali Gupta1, Xianxiu Wan1, Maria P. Zago2, Valena C. Martinez Sellers1, Trevor S. Silva1, Dadjah Assiah1, Monisha Dhiman1, Sonia Nuñez3, John R. Petersen4, Juan C. Vázquez-Chagoyán5, Jose G. Estrada-Franco5, Nisha Jain Garg1,4,6,+

1 Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas, United States of America, 2 Instituto de Patología Experimental, CONICET, Salta, Argentina, 3 Hospital Público de Gestión Descentralizada San Bernardo, Salta, Argentina, 4 Department of Pathology, University of Texas Medical Branch, Galveston, Texas, United States of America, 5 Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Estado de México, Toluca, México, 6 Faculty of the Center for Tropical Diseases, and the Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, Texas, United States of America

Abstract

Background: Chagas disease, caused by Trypanosoma cruzi, is endemic in Latin America and an emerging infectious disease in the US and Europe. We have shown TcG1, TcG2, and TcG4 antigens elicit protective immunity to T. cruzi in mice and dogs. Herein, we investigated antigenicity of the recombinant proteins in humans to determine their potential utility for the development of next generation diagnostics for screening of T. cruzi infection and Chagas disease.

Methods and Results: Sera samples from inhabitants of the endemic areas of Argentina-Bolivia and Mexico-Guatemala were analyzed in 1st-phase for anti-T. cruzi antibody response by traditional serology tests; and in 2nd-phase for antibody response to the recombinant antigens (individually or mixed) by an ELISA. We noted similar antibody response to candidate antigens in sera samples from inhabitants of Argentina and Mexico (n = 175). The IgG antibodies to TcG1, TcG2, and TcG4 (individually) and TcGmix were present in 62–71%, 65–78% and 72–82%, and 89–93% of the subjects, respectively, identified to be seropositive by traditional serological test. Recombinant TcG1- (93.6%), TcG2- (96%), TcG4- (94.6%) and TcGmix- (98%) based ELISA exhibited significantly higher specificity compared to that noted for T. cruzi trypomastigote-based ELISA (77.8%) in diagnosing T. cruzi-infection and avoiding cross-reactivity to Leishmania spp. No significant correlation was noted in the sera levels of antibody response and clinical severity of Chagas disease in seropositive subjects.

Conclusions: Three candidate antigens were recognized by antibody response in chagasic patients from two distinct study sites and expressed in diverse strains of the circulating parasites. A multiplex ELISA detecting antibody response to three antigens was highly sensitive and specific in diagnosing T. cruzi infection in humans, suggesting that a diagnostic kit based on TcG1, TcG2 and TcG4 recombinant proteins will be useful in diverse situations.

Citation: Gupta S, Wan X, Zago MP, Martinez Sellers VC, Silva TS, et al. (2013) Antigenicity and Diagnostic Potential of Vaccine Candidates in Human Chagas Disease. PloS Negl Trop Dis 7(1): e2018. doi:10.1371/journal.pntd.0002018

Editor: Mauricio Martins Rodrigues, Federal University of São Paulo, Brazil

Received July 30, 2012; Accepted December 4, 2012; Published January 17, 2013

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Funding: This work was supported by a grant from the National Heart, Lung and Blood Institute of the National Institutes of Health (HL094802) to NUG. VCM5 and TS2 are recipients of a summer internship from National Institute of Allergy and Infectious Diseases T35 training grant (AI078878, PI: Dr. Lynn Soong). DA was a recipient of a fellowship from the Summer Undergraduate Research Program at the UTMB. SG is a recipient of the Sealy Center for Vaccine Development (SCVD) post-doctoral fellowship. MPZ is a scientist from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

The protozoan parasite Trypanosoma cruzi, transmitted by bloodsucking triatomines, causes Chagas disease, which is a health threat for an estimated 10 million people, living mostly in Latin America. More than 25 million people are at risk of the disease [1]. Increasing travel and immigration has also brought T. cruzi infection into non-endemic countries, e.g., the U.S., Spain and Australia, where natural transmission is absent or very low. The congenital and transfusion- or organ transplantation-related transmissions are becoming recognized as significant threats in recent decades [2,3]. Diagnosis and treatment of T. cruzi infection has remained difficult and challenging after 100 years of its identification. This is because the acute infection, in general produces mild clinical symptoms, e.g., fever, dyspnea, local swelling at the site of infection, that are infrequently reported [4]. As a result, acute exposure when detection of blood parasitemia and treatment is possible, remain largely unnoticed. Only those who develop severe acute myocarditis or when an outbreak of T. cruzi infection occurs may receive early diagnosis and therapeutic treatment [5][6]. In >95% of human cases, T. cruzi infection remains undiagnosed until several years later when chronic evolution of progressive disease results in clinical symptoms associated with cardiac damage. A conclusive diagnosis of T. cruzi infection then often requires multiple serological tests, in combination with epidemiological data and clinical symptoms. Unfortunately, after complicated diagnosis, no vaccines or therapies are available to treat the chronically infected individuals.
Author Summary

Chagas disease is the most common cause of congestive heart failure related deaths among young adults in the endemic areas of South and Central America and Mexico. Diagnosis and treatment of *T. cruzi* infection has remained difficult and challenging after 100 years of its identification. In >95% of human cases, *T. cruzi* infection remains undiagnosed until several years later when chronic evolution of progressive disease results in clinical symptoms associated with cardiac damage. Diagnosis generally depends on the measurement of *T. cruzi*-specific antibodies that can result in false positives. A conclusive diagnosis of *T. cruzi* infection thus often requires multiple serological tests, in combination with epidemiological data and clinical symptoms. In this study, we investigated the antibody response to TcG1, TcG2, and TcG4 in clinically characterized chagasic patients. These antigens were identified as vaccine candidates and shown to elicit protective immunity to *T. cruzi* and Chagas disease in experimental animals. Our data show the serology test developed using the TcGmix (multiplex ELISA) is a significantly better alternative to epimastigote extracts currently used in *T. cruzi* serodiagnosis or the trypomastigote lysate used in this study for comparison purposes.

We have, previously, employed an unbiased computational/bioinformatics approach for screening the *T. cruzi* sequence database and identification of potential vaccine candidates [7]. A strategic analysis of the sequence database led to selection of 71 candidates that were unique to *T. cruzi*. Of these, eight candidates (TcG1, TcG2, TcG3, TcG4, TcG5, TcG6, TcG7, and TcG8) were selected for their ability to induce agglutinating antibody response in mice [7]. Further studies indicated TcG1, TcG2, and TcG4 were maximally immunogenic because these were recognized by IgGs in infected mice and dogs [8], by CD8+ T cells in infected mice [9], and elicited type I cytokines (e.g. IFN-γ) in mice and dogs [10]. Immunization with candidate antigens as a DNA vaccine provided a degree of protective immunity (TcG2 = TcG4 > TcG1) that substantially controlled acute parasitemia after challenge infection in mice and dogs [10,11]. The delivery of candidate antigens as heterologous prime/boost vaccines further enhanced the protective efficacy, evidenced by >80% control of acute parasitemia and tissue parasite burden and associated inflammation in heart and skeletal tissue [9,12].

In this study, we investigated the antibody response to the three candidate antigens (TcG1, TcG2, TcG4) in clinically characterized chagasic patients. Our objectives were to evaluate the antigenicity of the candidate antigens in humans and determine their utility in generation of improved diagnostics for screening of *T. cruzi* infection and disease. Our data demonstrate that the candidate antigens are recognized by antibody responses in chagasic patients from two distinct study sites where diverse strains of the circulating parasites were reported. Further, a multiplex assay consisting of the mixture of the three antigens was highly sensitive and specific in diagnosing *T. cruzi* infection in human patients.

Materials and Methods

Parasites

*T. cruzi* trypomastigotes (SylvioX10/4, TCI lineage) were maintained and propagated by continuous *in vitro* passage in monolayers of C2C12 cells. Amastigotes were obtained by incubation of the freshly harvested trypomastigotes in RPMI-10% FBS medium, pH 5.0 at 37°C, 5% CO₂ for 2 h.

Human subjects

Human sera samples used in this study were obtained from Salta Argentina (located at the border of Bolivia) and Chiapas Mexico (located on the border of Guatemala) that are known to be endemic for *T. cruzi* transmission and human infection. All procedures were approved by the Institutional Review Boards at the University of Texas Medical Branch (UTMB), Universidad Nacional de Salta (UNSa), Argentina, and the Universidad Autónoma de Chiapas, Mexico. All sera samples obtained for the study were analyzed for *T. cruzi*-specific antibodies by commercially available kits. Those positive by ≥ two tests were considered seropositive (+ve) and those negative for these tests were considered seronegative (−ve). All samples were decoded and de-identified before they were provided for research purposes. Written informed consent was obtained from all individuals.

In Argentina, all individuals were clinically characterized, and venous blood samples were collected to obtain plasma or serum. Clinical data included medical history, physical examination and subjective complaint of frequency and severity of exertional dyspnea, electrocardiography (12-lead at rest and 3-lead with exercise) to reveal cardiac rhythm and conduction abnormalities and transthoracic echocardiogram to obtain objective information regarding the left ventricular (LV) contractile function. Chest X-ray was used to assess cardiomegaly (cardio-thoracic ratio >0.5).

Seronegative and physically healthy subjects exhibiting no history or clinical symptoms of heart disease were used as controls. Seropositive chagasic patients were classified according to clinical exam as follows: Group I: negligible to minor EKG alterations, EF: 55–70%, no indication of heart involvement; Group II: a degree of heart involvement with systolic dysfunction (EF: 40–55%); and Group III: moderate to severe systolic dysfunction (EF≤40%), left ventricular dilatation (diameter ≥57 mm), and/or potential signs of congestive heart failure.

In Mexico, human sera samples were collected within the framework of a research project on emerging zoonotic diseases conducted jointly by several institutions, including Chiapas State University (UNACH), Mexican Social Security Institute (IMSS), Chiapas Health Institute (IEEECH) and UTMB at Galveston [13]. The seropositive subjects generally represented indeterminate/asymptomatic form of disease. Patients’ detailed information is presented in Table 1.

1st-phase screening

All sera samples from Salta Argentina were analyzed for *T. cruzi*-specific antibodies by the personnel of the Clinical laboratories at the San Bernardo Hospital using a Wiener Chagatest-ELISA recombinant v.4.0 kit (cut-off absorbance at 450 nm: average of seronegative samples (<0.1) +0.2, i.e. ≥0.3). Serological tests were also done following the specifications of the commercial IHA test kit (Wiener Chagast-RI, positive ≥1:16 dilution). Those positive by both tests were considered seropositive, and exposed to *T. cruzi* infection. All sera samples from Chiapas Mexico were screened by epimastigote antigenic lysate-based ELISA, trypomastigote-based flow cytometry, and Chagas Stat-Pak immuno-chromatographic test (Chembio Diagnostic Systems, Medford NY). Those positive by at least two tests were considered seropositive [13].
Table 1. Characterization of the subjects included for screening of TcG1-, TcG2-, and TcG4-specific antibody responses in this study.

| Clinical characterization | Enrolled subjects (numbers) | Age range in years | Sex Males (%) |
|---------------------------|-----------------------------|--------------------|---------------|
| **Salta, Argentina**b     |                             |                    |               |
| Seropositive for T. cruzi-specific antibodiesb |                         |                    |               |
| Chagasic 0 n = 54          | 19–75                       | 44 (40%)           |
| Chagasic I n = 30          |                             |                    |               |
| Chagasic II n = 17         |                             |                    |               |
| Chagasic III n = 9         |                             |                    |               |
| Seronegative for T. cruzi-specific antibodies |                     |                    |               |
| Healthy, no disease n = 40 | 18–55                       | 19 (48%)           |
| Leishmaniasis n = 35       | 18–62                       | 25 (71%)           |
| Autoimmune diseases n = 15 | NA                          | NA                 |
| **Chiapas, Mexico**c       |                             |                    |               |
| Seropositive for T. cruzi-specific antibodies |                  |                    |               |
| Chagasic 0-I n = 65        | 18–73                       | 29 (45%)           |
| Seronegative for T. cruzi-specific antibodies |                    |                    |               |
| Healthy, no disease n = 34 | 18–73                       | 15 (45%)           |
| **Non-endemic areas**d    |                             |                    |               |
| Seronegative, healthy, no disease n = 42 | 18–55                  | 16 (38%)           |
| Seronegative, other Cardiomyopathy n = 20 | 42–75                  | 12 (60%)           |

aSubjects in Argentina were screened for T. cruzi-specific antibodies by Wiener Chagatest-ELISA and Wiener Chagatest-HAI kits. Clinical exam included physical exam, electrocardiography and echocardiography. Confirmation of leishmaniasis was obtained by parasitological test, Montenegro reaction, clinical demonstration, and two PCR approaches. One of the seropositive patient presenting acute infection was referred for chemotherapy treatment with Benznidazole.
bFive of the seropositive/chagasic subjects from Argentina were positive for Leishmania infection, determined by two PCR approaches.
cSera samples from Chiapas Mexico were screened by epimastigote antigenic lysate-based ELISA, trypomastigote-based flow cytometry, and Chagas Stat-Pak immunochromatographic test.
dSeronegative subjects from non-endemic areas were screened for T. cruzi-specific antibody response using T. cruzi trypomastigote lysate as antigen source in ELISA assays. Seronegative/cardio-myopathy patients were identified based upon blood levels of NT-proBNP to be >2000 pg/ml (normal<450 pg/ml).
N/A: not available.
doi:10.1371/journal.pntd.0002018.t001

2nd-phase screening

Based upon the above analysis, we created sera pools for 2nd phase screening. Briefly, from Chiapas Mexico, a seropositive pool (n = 65) and a seronegative pool (n = 34) was included in the 2nd phase. From Salta Argentina, sera and plasma samples collected in year 2009 (seropositive, n = 65; seronegative, n = 20) and year 2010 (seropositive, n = 45; seronegative, n = 20) were included. All blood samples positive for T. cruzi-specific antibodies from Salta Argentina were analyzed for Leishmania infection by two PCR approaches. Briefly, total DNA was extracted from blood samples with a phenol-chloroform mixture, precipitated with ethanol and dissolved in 20 μl of TE buffer. A PCR reaction was performed using 1 μl isolated DNA with primer pairs (5'-GTGGGGGAGGCGGT-TCT-3' and 5'-ATTTTACAC-GAACCAGTT-3) that specifically amplify 120 base pair product from the conserved region of Leishmania kDNA [14]. The polymorphism-specific PCR (PS-PCR) allows the identification of Leishmania species from Argentina, and was performed using the primer pairs as previously described [15].

To evaluate the specificity of the TcG1-, TcG2-, and TcG4-based diagnostic ELISA, sera samples from volunteer donors from Galveston TX, Buenos Aires Argentina, and Toluca Mexico with no history of residence in the endemic areas, and were healthy (n = 42, true negative controls) or exhibited cardiomyopathy of other etiologies (n = 20) were included in the study. Seronegative/cardio-myopathy patients were identified based clinical exam, and blood levels of NT-proBNP to be >2000 pg/ml (normal<450 pg/ml). To examine the cross-reactivity of antigen-based assay, sera samples from subjects living in Salta Argentina, and diagnosed for Leishmania infection (n = 35) or certain autoimmune diseases (n = 15) were also included in the study. Patients were diagnosed for leishmaniasis (cutaneous, mucocutaneous or visceral) based upon three criteria (smears and lesions, Montenegro reaction, epidemiology and clinical demonstration) as described in [15], and further identified to be positive for Leishmania infection by kDNA-specific PCR and PS-PCR approaches, as above. Briefly, parasitological analysis was done on smears of dermal scrapings stained with May-Grünwald Giemsa and examined under a microscope. For Montenegro skin test, promastigote protein lysates (4 μg/100 μl) of Leishmania braziliensis were injected intradermally on the ventral forearm of the patients, and indurations ≥5 mm in diameter, observed 48 h after the injections were considered reactive. Clinical features included the presence of compatible regentmatory injuries with ulcerative, nodulous, papulous cutaneous or mucocutaneous lesions and a congruent epidemiological history. Patients' information is presented in Table 1.

Sera samples were analyzed for IgG antibody levels by using TcG1-, TcG2-, and TcG4-based ELISA. Recombinant TcG1, TcG2 and TcG4 proteins were purified from E. coli. The nucleotide sequences of TcG1, TcG2 and TcG4 antigens have previously been submitted to GenBank under accession numbers AY727914, AY727915 and AY727917, respectively [7]. The cDNAs encoding TcG1 (1–166 amino acids), TcG2 (1–220 amino acids), and TcG4 (1–92 amino acids) were cloned in pCR2.1 T/A vector, and then
sub-cloned in to pET-22b plasmid (Novagen, Gibbstown, NJ) such that the encoded proteins were in-frame with a C-terminal polyhistidine tag. All cloned sequences were confirmed by restriction digestion and sequencing at the Recombinant DNA Core Facility at UTMB. The pET22b plasmids containing TcG1, TcG2 or TcG4 were transformed in BL21 (DE3) pLYS competent cells (Invitrogen, Carlsbad CA) and recombinant proteins purified using the polyhistidine fusion peptide metal chelation chromatography system (Novagen) [9]. After purification, proteins were dialyzed to remove contaminant particles, and endotoxins, and stored at −80°C till further use. Culture-derived parasites (70% trypomastigotes/30% amastigotes) were lysed by repeated freeze-thaw in PBS (10 mM) and used as a source of T. cruzi total antigen for positive control [10].

Flat bottom, high-binding, 96-well plates (Greiner bio-one) were coated overnight at 37°C with 100-µl/well of recombinant antigens (0.5 µg each antigen/well, individually or in combination) or T. cruzi total lysate (5×10^5 parasite equivalents/well). Plates were blocked for 2 h at 37°C with 200-µl/well of 5% non-fat dry milk (NFDM) in PBS, washed with PBS-0.05%Tween-20 (PBST) twice, and PBS once, and then incubated for 2 h with test sera (100-µl/well) added in triplicate in 2-fold dilutions. Plates were then washed and incubated at room temperature for 30 min with 100-µl/well of horseradish peroxidase-labeled goat anti-human IgG (1:5000 dilution in PBST-1% NFDM), and color was developed for 5 min with 100 µl/well of Sure Blue TMB substrate (Kirkegaard & Perry Laboratories). The colorimetric reaction was stopped with 2N H_{2}SO_{4}, and absorbance measured at 450 nm using SpectraMax M5 Microplate Reader (Molecular Devices).

The sensitivity of the antigen-based ELISA (2nd phase) was determined by calculating the percentage of chagasic samples that exhibited reactivity with recombinant proteins out of the total samples previously categorized as seropositive based on 1st phase screening with commercially available kits. The data from antigen-based ELISA analysis of sera samples from healthy individuals, other cardiomyopathy and leishmaniasis patients was utilized to calculate the specificity of the assay as follows: [ number of sera samples analyzed – number of sera samples that exhibited false positive reaction or cross-reactivity with TcG1, TcG2 or TcG4/ number of sera samples analyzed] ×100.

Data analysis

All samples were analyzed in duplicate and assayed at least twice for all experiments. Box plots and dot plots were made using Sigma Plot 12.0 and GraphPad Prism 5 software, respectively, and statistical analysis was conducted using SPSS v.18 software. Results were analyzed using Student’s t test for statistical evaluation of mean values for experimental and control samples, and the level of significance was taken at α<0.05. Pearson’s correlation analysis was performed to determine the relationship between predictive efficacies of the antibody response to selected antigens in diagnosis of disease severity. Seropositivity rates for anti-T. cruzi antibodies in different tests, and their confidence intervals [CIs], were calculated using the mid-P 95% confidence interval (95% CI) using Epi Info (version 6.0) software.

Results

To proceed with sample analysis, we optimized ELISA components by cross-titration, using a pool of known positive and negative controls (1:20–1:1600 dilutions). The optimal sera and HRP-conjugated secondary antibody dilutions providing maximum signal-to-noise ratio were determined to be 1:50 and 1:5,000, respectively, and used in all further investigations. The variations in reactivity of negative and positive sera among different assays and plates of the same experiment ranged from 3–12%.

We, first, monitored the antigenicity of TcG1, TcG2, and TcG4 using sera samples collected from volunteers enrolled in the study in Argentina in year 2010. Samples were stored at −80°C just after collection, and thawed when utilized. The negative sera samples (n = 20) from the endemic area near Argentina-Bolivia border exhibited low reactivity for TcG1, TcG2, and TcG4, similar to that noted for confirmed negative controls (n = 42) from non-endemic areas (TcG1: 0.216±0.033 versus 0.211±0.048, TcG2: 0.240±0.04 versus 0.230±0.044, TcG4: 0.225±0.041 versus 0.252±0.038, expressed as mean absorbance ± SD). In comparison, a 4-fold, 2.75-fold, and 2.65-fold increase in sera levels of antibody response to TcG1, TcG2, and TcG4, respectively, was noted in previously characterized seropositive subjects (n = 45) from Argentina-Bolivia border (TcG1: 0.81±0.33, TcG2: 0.66±0.20, TcG4: 0.57±0.09, expressed as mean absorbance ±SD, p<0.001 for all, Fig. 1A–C). The sera levels of antibodies to TcG1, TcG2, and TcG4 were above the mean_{seropositive} level in 62.2%, 66.6% and 75.5% of the 1st-phase seropositive subjects. When analyzing plasma samples from the same individuals, we noted a 3.34-fold, 2.4-fold and 2.3-fold increase in plasma levels of antibodies to TcG1, TcG2 and TcG4 in seropositive subjects as compared to seronegative controls (TcG1: 0.77±0.21 versus 0.20±0.05, TcG2: 0.65±0.12 versus 0.21±0.057, TcG4: 0.67±0.09 versus 0.20±0.048, expressed as mean absorbance ± SD, p<0.001 for all, Fig. 1D–F). The plasma levels of antibodies to TcG1, TcG2, and TcG4 were above the mean_{seropositive} levels in 71.1%, 77.7% and 80% of the 1st-phase seropositive subjects. These data suggested that antibody response to TcG1, TcG2 and TcG4 are recognized by antibody responses elicited in human patients infected by T. cruzi, and b) both plasma and sera samples can be utilized to monitor the antibody response.

We then analyzed the plasma samples that were collected in 2009, and characterized as seropositive (n = 65) and seronegative (n = 20) by 1st-phase serology tests. These samples were subjected to two cycles of freezing/thawing during the two-year storage. Our data showed a 6.72-fold, 2.4-fold and 2.9-fold increase in plasma levels of antibodies to TcG1, TcG2 and TcG4 in seropositive subjects as compared to seronegative controls (TcG1: 1.66±0.55 versus 0.204±0.05, TcG2: 0.69±0.13 versus 0.239±0.039, TcG4: 0.75±0.20 versus 0.227±0.05, expressed as mean absorbance ± SD, p<0.001 for all, Fig. 1G–I). The plasma levels of antibodies to TcG1, TcG2, and TcG4 were above the mean_{seropositive} level in 61.5%, 64.6% and 81.5% of the seropositive subjects. These results suggest that antibody response to TcG1, TcG2, and TcG4 is stable, and field samples can be utilized to examine antigenicity of the selected candidates in large-scale population studies. Overall, the data presented in Fig. 1 also indicate that TcG1, TcG2 and TcG4 are expressed by T. cruzi isolates circulating in the endemic areas at the Argentina-Bolivia border.

It is important to know if antibody recognition of the three antigens can be expanded for diagnosis of T. cruzi infection in other countries where different isolates are suggested to be present in domestic and sylvatic cycle of parasite circulation. For example, in Argentina and neighboring countries in South America, TCI isolates are predominantly identified in peripheral blood of seropositive patients, though molecular studies have revealed the presence of TCI parasites also in human biopsies of chronic chagasic patients [16,17,18]. In Mexico and Guatemala, TCI is dominantly found in epidemiological evaluation of infected triatomines as well as in blood samples from acute and chronic chagasic cardiomyopathy patients [19,20]. We, therefore, monitored the antigenicity
of TcG1, TcG2, and TcG4 in human sera samples from Mexico-Guatemala border area that were characterized as seropositive (n = 65) and seronegative (n = 34) by 1st-phase serology tests. Our data showed 6.72-fold, 2.4-fold and 2.9-fold increase in sera levels of antibodies to TcG1, TcG2 and TcG4 in seropositive samples as compared to that noted in seronegative healthy controls (TcG1: 0.76 ± 0.25 versus 0.21 ± 0.047, TcG2: 0.64 ± 0.22 versus 0.25 ± 0.05, TcG4: 0.65 ± 0.20 versus 0.21 ± 0.048, expressed as mean absorbance ± SD, p < 0.001 for all, seropositive versus seronegative, Fig. 2). The sera levels of antibodies to TcG1, TcG2, and TcG4 were above the mean seronegative level in 69.2%, 76.9% and 72.3% of the seropositive subjects from Mexico. These data demonstrate that TcG1, TcG2 and TcG4 are antigenic, and recognized by antibody responses in chagasic patients from Mexico, and suggest that the three antigens are expressed by T. cruzi isolates circulating in Mexico-Guatemala border area.

Next, we determined if the three candidate antigens can be utilized together to improve the diagnosis of exposure to T. cruzi. For this, we coated the 96-well plates with either the mixture of TcG1, TcG2 and TcG4 (0.5 μg/well each) or T. cruzi trypomastigote lysate (TcTL, 2×10^5 parasite equivalent), and monitored the antibody response by ELISA under similar experimental conditions. When antibody response was captured using the TcGmix, the seronegative controls from the non-endemic areas exhibited a mean absorbance ± SD of 0.225 ± 0.039. Using the controls’ mean absorbance + 2SD, our data validated 40 of the 45 sera samples (88.8%) from Argentina, characterized as seropositive in 1st-phase screening in the year 2010, were seropositive for TcGmix-specific antibodies (mean absorbance ± SD: 0.73 ± 0.17, maximum OD: 1.2, Fig. 3A). One of the volunteer previously characterized as seronegative exhibited anti-TcGmix antibody response above the mean seronegative level. Similarly, the plasma detection of antibody response to TcG mix identified 102/110 of the seropositive subjects (92.7%) identified in 1st-phase screening in the years 2009 and 2010 (Fig. 3B,C). In comparison, when plates were coated with TcTL to capture anti-T. cruzi antibodies, the seronegative true controls from non-endemic areas, exhibited a mean absorbance ± SD value of 0.233 ± 0.044 (Fig. 3C,E). Using the mean absorbance for controls + 2SD as a cut off, our data validated 97.7–100% sera and plasma (year 2010) and 96.9% plasma (year 2009) samples characterized as seropositive in
1st-phase screening in Argentina were also positive for TcTL-specific antibodies; the mean absorbance ± SD for the positive population was 1.1 ± 0.6 (year 2009) and 0.73 ± 0.08 (year 2010) with the highest value being 2.5 and 0.98, respectively (Fig. 3B,D,F).

One of the volunteer previously characterized seronegative exhibited anti-TcTL antibody response.

To validate that diagnostic potential of the TcGmix based ELISA is not restricted to samples from Argentina, we monitored the antibody response using sera samples collected in Mexico. Of the 65 samples characterized as seropositive in 1st-phase screening, 58 (89.2%) and 63 (96.9%) exhibited reactivity when plates were coated with TcGmix and TcTL antigens, respectively (Fig. 4). The mean absorbance ± SD for the antibody response to TcGmix and TcTL in the positive population positive population was 0.75 ± 0.14 (max: 1.2) and 0.87 ± 0.4 (max: 2.6), respectively, the difference between the two values being observed non-significant.

No significant difference was observed when either plasma or sera were used as the source of antibodies.

Five of the 110 seropositive/chagasic subjects from Argentina exhibited reactivity for Leishmania-specific antibodies and were likely infected with both pathogens. To examine if the antigen-based ELISA is specific for T. cruzi detection, we performed recombinant antigens-specific ELISA using the sera samples from non-chagasic individuals (total 112), including leishmaniasis patients (n = 35), volunteer donors with cardiomyopathy (n = 20) and autoimmune diseases (n = 15) of other etiologies, and healthy donors from non-endemic areas (n = 42), and calculated the specificity of the antigen-based ELISA. Sera samples from

Figure 2. TcG1, TcG2 and TcG4 are recognized by antibody response in human subjects from Mexico. Sera samples obtained from volunteers living in the endemic areas of Chiapas Mexico were characterized as seropositive (+ve) and seronegative (−ve) by whole-parasite antigen based serology tests in the 1st phase. The TcG1 (A), TcG2 (B) and TcG4 (C) specific antibody response was measured by ELISA, and data are presented as box plot (details in Fig. 1).
doi:10.1371/journal.pntd.0002018.g002

Figure 3. TcGmix-based ELISA provides superior efficacy in identifying exposure to T. cruzi infection among inhabitants of Salta Argentina. Sera (A&B) and plasma (C–F) samples, characterized as seropositive (+ve) and seronegative (−ve) by conventional approaches, were submitted to multiplex ELISA using the mixture of recombinant TcG1, TcG2 and TcG4 proteins for capturing the antigen-specific antibody response (A,C,E). Shown are the antibody response captured using the T. cruzi trypomastigote lysate (TcTL) as a source of crude antigen (B,D,F) for comparison purpose.
doi:10.1371/journal.pntd.0002018.g003

Figure 4. TcGmix-based ELISA was effective in diagnosing exposure to T. cruzi infection among inhabitants of Chiapas Mexico. Sera samples obtained from volunteers living in the endemic areas of Chiapas Mexico were characterized as seropositive (+ve) and seronegative (−ve) by whole-parasite antigen based serology tests in the 1st phase. Shown are the antibody response captured by an ELISA using mixture (TcGmix) of recombinant TcG1, TcG2 and TcG4 proteins (A) or T. cruzi trypomastigote lysate (TcTL) (B) as a source of antigen.
doi:10.1371/journal.pntd.0002018.g004
leishmaniasis patients (n=35) exhibited very low reactivity for TcG1, TcG2, and TcG4, similar to that noted for sera samples from confirmed negative controls from non-endemic areas (TcG1: 0.18±0.04 versus 0.21±0.048, TcG2: 0.15±0.06 versus 0.230±0.044, TcG4: 0.232±0.05 versus 0.252±0.038, expressed as mean absorbance ± SD). Likewise, sera samples from patients exhibiting symptoms of cardiomyopathy of non-chagasic etiology or autoimmune diseases showed reactivity to TcG1, TcG2, and TcG4 below the cut-off threshold values derived from healthy/seronegative controls. The specificity for TcGmax was highest (98%), followed by TcG2 (96%), TcG4 (94.6%), TcG1 (93.6%) and TcTL (77.8%), determined by detection of false positive signal for 2, 4, 6, 7 and 25 of the samples, respectively, out of the total 112 samples from non-chagasic individuals that were submitted for antigen- and TcTL-based ELISA. These data indicated that the recombinant antigens were highly specific for the detection of anti-\(T. cruzi\) antibodies than the whole parasite (trypomastigote) lysate, and exhibited no cross-reactivity to \(L. Donovani\)-specific antibodies.

Pearson correlation analysis was employed to identify correlation between antigen-specific antibody response and disease state including the data derived from seronegative/healthy controls and seropositive/chagasic subjects. For this, seronegative/healthy subjects were labeled as 0, and patients classified as 0, I, II and III (Materials and Methods) were labeled as 1, 2, 3, and 4, respectively. Antibody response was titrated using 2-fold sera dilutions (1:50–1:1600). We observed no significant correlation between the anti-TcG2, anti-TcG4, anti-TcGmax and anti-TcTL antibody titration curves and clinical disease category in any of the patient population (data not shown). The representative correlation data from sera levels of anti-TcGmax and anti-TcTL antibody response (1:50 dilutions) and clinical disease category for the clinically characterized Argentine patients enrolled in the study is shown in Fig. 5. It is worth noting that TcGmax-specific antibodies exhibited a clear downward trend with patients’ disease severity, indicating that presence of antibodies for TcG1, TcG2, and TcG4 is protective during progressive Chagas disease (Fig. 5A). No clear trend or correlation was observed for TcTL specific antibody response and disease severity in any of the patient population (Fig. 5B).

**Figure 5. Pair-wise correlation analysis.** Shown is pair-wise correlation analysis of antibody response to TcGmax (A) and TcTL (B) with clinical disease category in patients enrolled in the study from Argentina-Bolivia border area. For this, seronegative/healthy subjects were labeled as 0, and patients classified as 0, I, II and III (Materials and Methods) were labeled as 1, 2, 3, and 4, respectively. Dots, individual subjects. doi:10.1371/journal.pntd.0002018.g005

### Discussion

Serological diagnosis of Chagas disease is frequently based on tests such as enzyme-linked immunoassays (EIAs), indirect immunofluorescence assays, and indirect haemagglutination assays (IHAs). Most of these serological tests, including one recently licensed by the United States Food and Drug Administration for use as a blood screening test in the U.S. [21], use crude or semi-purified \(T. cruzi\) epimastigote forms as the antigen, the stage that is present in insects but not in infected humans. Overall, the current diagnostics fail to provide a high degree of sensitivity and specificity, requiring use of multiple tests for diagnosis of \(T. cruzi\) infection [22]. Further, most of the currently available kits produce questionable results when used for donors with low titers [23]. The absence of a true gold standard makes it difficult for the medical practitioners to provide proper treatment as not all cases are properly identified and treatment response cannot be accurately monitored. Accordingly, World Health Organization has emphasized the need to employ defined antigens for improved serodiagnosis of \(T. cruzi\) infection [24]. However, before an antigen can be used for diagnostics, several criteria should be met: (i) the selected candidate antigen(s) should be expressed in isolates circulating in different areas of endemity, (ii) antigens should be highly immunogenic in populations with different genetic background, (iii) they should be absent from other pathogens to prevent cross-reactivity, and (iv) easily expressed and purified from traditional protein expression systems (e.g. \(E. coli\)) to ensure reproducibility and quality control [25]. Because of the complexity involved in antigen selection and testing, limited progress has been made towards the development of antigen-based kits for the diagnosis of \(T. cruzi\) infection.

Keeping the above guidelines in mind, we believe that TcG1, TcG2 and TcG4 are ideal candidates for the development of diagnostic assays. One, these antigens were previously shown to be expressed in infective and intracellular forms of clinically relevant multiple isolates of \(T. cruzi\) [7]. Two, the small size of the three antigens (TcG1: 18.4 kDa, TcG2: 24 kDa, TcG4: 10 kDa) allows reproducible high-yield purification of the proteins from \(E. coli\) (0.3–1.0 g/L) and is amenable to large-scale production. Three, the presence of multiple 12-mer B cell epitopes of high specificity (Table 2) suggests that these antigens will be immunogenic,
recognized by B cells of the immune system, and elicit antibody response. Indeed, we have found that TcG1, TcG2 and TcG4 are recognized by antibodies elicited in infected dogs and mice [9,11]. The diagnostic potential of the three antigens in humans was evident from our analysis of a panel of sera or plasma from chronic chagasic patients from Argentina-Bolivia and Mexico-Guatemala border areas. T. cruzi strains of lineages TCV (IId), TCII (IIb) and TCI were identified in seropositive chagasic patients from Argentina/Bolivia (Patricio Diosque and Monge Rumi, personal communication), and of lineage TCI in seropositive subjects from Mexico/Guatemala (unpublished results). Others have documented the predominance of TCI isolates in peripheral blood of seropositive patients from South America [16,17,18] and TCI in Mexico and Guatemala [19,20]. Thus, our data presented in Figs.1, 2, 3, 4 suggest the diagnostic kit developed using TcG1, TcG2 and TcG4 will be useful in identifying T. cruzi circulation and transmission in South, Central and North America.

It is important to note that TcG1, TcG2 and TcG4, when used individually, exhibited 93.6-96% specificity in detecting anti-T. cruzi antibody response that was in the range for other recombinant proteins (e.g. TSSA, CP1 and CP2) proposed for Chagas disease immunodiagnosis [26,27]. However, 98% specificity of the TcGmix-based ELISA was much higher than that observed for single antigens in this study or other recombinant antigens in other reports [26,28]. Further, TcGmix specificity at 98% was significantly better than that observed with T. cruzi trypomastigote-based ELISA (77.8%) that is not desirable because culturing of human-infective form of the pathogen requires special facilities, technology, and expertise.

Another major concern that is generally not taken into consideration when one is using serological tests for Chagas disease is the potential frequency of cross-reactivity. In some areas of endemicity in Central America and Brazil, where T. cruzi and the nonpathogenic protozoan Trypanosoma rangeli can be found infecting the same vectors and vertebrate hosts, cross-reactivity has been proposed to contribute to miscalculated higher percentage of T. cruzi transmission and misdiagnosis of patients may have severe health-related and economic consequences [29,30]. Others have documented the crude antigen-based serodiagnostic kits exhibit cross-reactivity between sera of patients infected with T. cruzi and Leishmania spp. [31,32]. In our study, homology searches suggested that only TcG1 is similar to a ribosomal protein of other trypanosomes (>90% identical) while TcG2 and TcG4 exhibited no clear paralog in the public databases (Fig.S1). Thus, to rule out the possibility of cross-reactivity with Leishmania, we have screened sera samples from confirmed leishmaniasis patients for antibody cross-reactivity to TcG1, TcG2 and TcG4. Our data clearly showed that the three antigens exhibited no cross-reactivity to antibodies generated in cutaneous, mucocutaneous and visceral leishmaniasis patients, providing evidence that the antibody response to TcG1, TcG2 and TcG4 was solely directed by exposure to T. cruzi infection.

Finally, TcGmix-ELISA performed at a higher potency in discriminating weakly positive samples from background, demonstrated statistically by calculating the quotient of measured optical density values divided by the cutoff values. Only 12 of all the seropositive samples had a quotient smaller than 2.0 in the TcGmix-ELISA whereas TcG1-, TcG2- TcG4 and TcTL-based ELISA resulted in 18, 16, 14 and 16 samples below the mean value. This potency will facilitate diagnosis because weakly positive results routinely need to be confirmed by alternative assays. Thus, based upon high sensitivity (93%) and specificity (98%) for sera from chagasic patients from different endemic countries, we surmise that TcGmix-based ELISA can serve as a single assay to determine the T. cruzi status of a given blood sample, and diagnose Chagas disease. We plan to further standardize the assay for large-scale screening and establishing the prototype assay for commercial use of the selected antigens for diagnostic kits.

Table 2. B cell epitopes in candidate antigens.

| Protein name (Acc# in Genbank) | Protein (aas) | Predicted B cell epitopes | Position | Epitope | Score |
|--------------------------------|---------------|--------------------------|----------|---------|-------|
| TcG1 (AAU47265.1) | 166 | | 14 | RIRGPRQDVRG | 1 |
| | | | 154 | VDSKPAAKKRS | 0.947 |
| | | | 66 | SRNCSTRLKNV | 0.904 |
| | | | 127 | VFDENDQKPV | 0.788 |
| | | | 109 | ERYLVRAKRSL | 0.699 |
| | | | 27 | VVDIDGNIRVL | 0.667 |
| TcG2 (AAU47266.1) | 221 | | 113 | FYATDGNAANYT | 0.99 |
| | | | 144 | EKETSTNRIRSK | 0.838 |
| | | | 161 | YDISGSNTNLCD | 0.815 |
| | | | 127 | AAVEDGVAHRSL | 0.793 |
| | | | 7 | ESGFVPSGDMRIR | 0.751 |
| | | | 190 | SVHDSKDVSPQK | 0.716 |
| | | | 210 | EAFRLRPPLLIG | 0.715 |
| | | | 91 | P DHFVAPLNSNS | 0.667 |
| TcG4 (AAU47268.1) | 91 | | 39 | MWVEHQRIRLQE | 0.973 |
| | | | 1 | MSAPKPTXLHQ | 0.958 |
| | | | 74 | IPTIVPKHELH | 0.542 |

Linear B cell epitopes (12 amino acid lengths) were predicted using a BCPred tool (http://ailab.cs.iastate.edu/bcpreds/).

doi:10.1371/journal.pntd.0002018.t002
The immune interactions necessary to eradicate *T. cruzi* parasites are extremely complex and require both humoral and cell-mediated components of the immune system. Previous experimental studies and a few studies in humans indicate that antibodies are able to kill *T. cruzi* in the presence of phagocytic cells, such as macrophages. Others have shown in B cell knock-out mice or mice depleted of B cell function, the pivotal role of antibody molecules in attaining resistance to *T. cruzi* infection and Chagas disease (reviewed in [34]). In this study, the mean antibody response to TcG1, TcG2 and TcG4 (individually or in combination) exhibited a downward trend in correlation to clinical disease severity suggesting that antibody response to the candidate antigens was protective, though further studies with larger patient population will be required to validate the significance of this observation. The experimental data indicating the up regulation of IgG1 antibodies specific to TcG1, TcG2 and TcG4 [9,11] suggest that isotypes related to up regulation of opsonization, cell dependent cytotoxicity and activation of classical complement pathway are elicited by the candidate antigens, and might be present in significant levels in seropositive/chagasic individuals. Considering that the three antigens have been shown to be located on plasma membrane of trypomastigote/amastigote stages of *T. cruzi* by flow cytometry [7], we predict that these will be available as a target to antibody-dependent cell cytotoxicity effector mechanisms mediated by IgG1. Indeed, TcG1, TcG2 and TcG4 have been shown to elicit potent trypanolytic antibodies in accordance with the intensity of the surface expression of these antigens in infective and intracellular stages (as compared to eight others tested in similar experiments) [7]. In addition to IgG1, candidate antigens elicited IgG2b known to drive the type 1 adaptive immunity in experimental mice and dogs [9,11]. High levels of candidate antigens-induced IgG2b in vaccinated mice were linked to complement-dependent trypanolytic activity [9]. Patients in the indeterminate phase display higher levels of lytic antibodies compared with patients with chagasic heart disease indicating an association between the presence of lytic antibodies and a protective response in chronic patients [33,36]. This was also evidenced by absence of lytic antibodies in patients treated with anti-parasite drugs that displayed negative hemocultures for over ten years [37]. Based upon these observations, we consider that activation of IgG2a (along with IgG1) will be an important attribute that contributes to host protection against *T. cruzi* infection and Chagas disease when TcG1, TcG2 and TcG4 will be tested as vaccine candidates in humans.

In summary, this is the first report identifying the antigenicity of TcG1, TcG2 and TcG4 in humans. We have concluded that the three candidate antigens are recognized by IgGs in sera samples of seropositive/chagasic patients. Further, the candidate antigens were represented in diverse strains of *T. cruzi* as the sera samples from two different endemic areas from the South, Central and North America recognized the antigens at the same rate. Our data show the serology test developed using the TcGmix is a significantly better alternative to epimastigate extracts currently used in *T. cruzi* serodiagnosis or the trypanomastigate lysate used in this study for comparison purposes. TcGmix ELISA was >98% specific and 93% sensitive, was not discriminatory of sex, age or geographic location of the individuals, performed at a higher potency in identifying weakly positive samples, and, thus, has a potential to serve as a single assay for the diagnosis of *T. cruzi* infection. Future studies with large cohorts of patients will be required to determine if immunological responsiveness to the three antigens detects with high confidence the chronicity of infection, severity of disease, and effectiveness of treatment etc.

**Supporting Information**

**Figure S1** TcG1, TcG2 and TcG4 homology to proteins in other trypanosomatids. Homology searches were performed using the NCBI BLASTP against nr database to identify homology for TcG1, TcG2 and TcG4 proteins of *T. cruzi* in other trypanosomatids (e.g. *Leishmania* spp, *T. brucei*, *T. congolense*, *T. rivax*). Shown are the results from maximum observed homology. (DOC)

**Acknowledgments**

We appreciate the consistent and long-standing support of Dr. Ines Vidal (Bionchim in charge of Central Laboratory at San Bernardo Hospital, Salta Argentina) and Ms Rosana Flores, Miriam Aramayo, Vanesa Ramos, and Federico Ramos (lab technicans) for collecting human chagasic patient samples. We are thankful to Drs. Jorge D. Marco, Alejandra Barrio and Fernanda Garcia Bustos at Instituto de Patología Experimental (IPE-CONECT, Salta, Argentina for providing Leishmania positive samples.

**Author Contributions**

Conceived and designed the experiments: SG NJG. Performed the experiments: SG XW VCMS DA TSS. Analyzed the data: SG NJG

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