NATURAL HISTORY OF DISEASE AND LABORATORY FINDINGS IN *TRYPANOSOMA CRUZI* ANTIBODY-POSITIVE BRAZILIAN BLOOD DONORS

Abstract

Chagas disease is caused by the parasite *Trypanosoma cruzi*, which infects up to 10 million people in Latin America causing approximately 50,000 deaths annually. There are many unanswered scientific questions regarding *T. cruzi* serological test performance, persistence of parasitemia and antibody, and rates and determinants of progression to clinical disease, that warrant investigation given implementation of U.S. donor screening at most blood centers. Dr. Sabino and colleagues have been engaged in a PAHO-coordinated effort to develop *T. cruzi* plasma panels for quality control, and consequently have access to 495 frozen plasma units from confirmed *T. cruzi* seropositive donors identified since 1996. These donors were infected in endemic areas many years earlier and later moved to Sao Paulo where they were detected by donor screening – a pattern similar to what is seen among US immigrants who test *T. cruzi* reactive. We plan to recall and enroll 250 of these donors with stored units into a retrospective cohort study. Paired plasma samples from the mid-1990s and 2007-2008 will be investigated to determine if *T. cruzi* antibody levels decrease over time (which may explain the high frequency of borderline reactivity in U.S. donors), and whether persistence of detectable parasitemia in blood by PCR correlates with high-titer antibody persistence. An additional 250 infected donors identified from 1998-2002 but for whom stored samples are not available will be enrolled in a small city in the State of Minas Gerais (Montes Claros) where there is a high *T. cruzi* seroprevalence and a high probability of successful follow-up. 250 demographically matched seronegative donors will also be enrolled in each city as comparison groups. We will evaluate seropositive donors (relative to uninfected donors) to define the rate and pattern of clinical Chagas disease, and correlate signs and symptoms with estimated duration of infection and demographic and laboratory parameters.

Specific Aims

Aim A. Define the natural history of Chagas disease in *T. cruzi* seropositive blood donors, according to donor demographic parameters, time since donation and exposure, and presence of persistent parasitemia.

> ***Hypothesis.*** *T. cruzi seropositive donors will have an overall 10% to 20% increased rate of cardiac and gastrointestinal symptoms and EKG or ECHO abnormalities relative to control donors, and the frequency of symptoms and signs will correlate with duration of infection and PCR positivity.*

Aim B. Characterize persistence of *T. cruzi* antibody reactivity over time, relative to detection of parasitemia by PCR.

> ***Hypothesis.*** *T. cruzi seropositive donors who are negative for parasitemia by PCR will demonstrate lower level antibody reactivity than PCR positive donors, and the reactivity levels will decline over time in the PCR-negative group.*

Aim C. Determine rate of seronegative *T. cruzi* infection by performing coded PCR on seronegative populations from endemic and non-endemic regions and seropositve controls.

> ***Hypothesis.*** *In contrast to T. cruzi seropositive donors in which T. cruzi PCR will be positive in greater than 70% of subjects, parasite DNA will not be detected in ELISA negative donors from either endemic or non-endemic regions.*

Background

<u>*Chagas disease epidemiology in Latin America*</u>

Chagas disease is a parasitic infection caused by *T. cruzi*, which is naturally transmitted through several species of haematophagous reduviid bugs (e.g., Triatoma infestans). Infections with *T.*

cruzi are also acquired congenitally, by organ transplant, and by blood transfusion.[1] The disease is highly prevalent in most Latin American countries. Vector-mediated transmission was aggressively addressed in many Latin American countries including Brazil during the 1980's, and transmission controlled to a large extent.[2,3] However, due to previous high rates of transmission and generally lifelong infection, there are currently an estimated 8 to 10 million people infected in Latin America, with approximately 50,000 Chagas-related deaths annually.

Chagas disease in the United States

During the past several decades, millions of people have emigrated to the United States and Canada from countries in which *T. cruzi* is endemic.[4] Estimates suggest that in the United States alone, 50,000 to 100,000 of these immigrants may harbor chronic, asymptomatic *T. cruzi* infections.[5] The children of these immigrants may also be infected, having acquired the infection congenitally.[6] Thus, these immigrants and their children represent a growing reservoir population for the potential transmission of *T. cruzi* by blood transfusion.

Nationwide estimates suggest that 1 in 25,000 US blood donors are infected with *T. cruzi*. Local infection rates can be much higher, depending on the proportion of at-risk donors in the population.[4] These have led to recommendations by BPAC in 1989 and 2002 for *T. cruzi* antibody screening of U.S. blood donations as soon as a test is approved by FDA. Many blood centers have now implemented *T. cruzi* screening following FDA approval of an antibody screening for the U.S. in December 2006.

Natural history of T. cruzi infection and Chagas disease

Once introduced into the host, the parasite disseminates hematogenously to muscle tissue, with a predilection for the heart. The subsequent acute phase is generally mild and of short duration, lasting only 1–2 months. Infected persons then enter a lifelong indeterminate phase of disease that is generally asymptomatic but characterized by detectable antibody and intermittent parasitemia. Later in life, it is believed that 20%–30% of infected persons will develop clinical Chagas disease, manifested by potentially fatal cardiac and gastrointestinal involvement.[1] Currently, there is no effective therapy for chronic infection, with high rates of side effects, toxicity and low cure rates using currently available drugs (Nifurtimox and Benznidazole).

Most studies addressing natural history of Chagas disease have relied on data obtained from follow-up of outpatients detected as a result of clinical findings.[7-9] To our knowledge, there is only one study in the literature that has evaluated the natural history of *T. cruzi* infection based on follow-up of subjects identified using a cross-sectional serological screening approach.[10] In this study, 238 people living in a rural Pacific coastal village in Mexico were tested for antibody and the overall seroprevalence for adults was 67%. Seropositive individuals were evaluated from 1971 and 1981. Seropositive individuals had a higher prevalence of suffering from chronic fatigue and difficult breathing while lying down in relation to seronegatives. Electrocardiogram (EKG) results showed a two-fold higher frequency of complete right bundle branch block and premature ventricular contractions among the seroreactive individuals. The rate of new EKG abnormalities among seroreactive persons was 3%/year. In a cross-sectional study performed in Brazil the prevalence of EKG abnormalities was 42.7% in seropositives relative to 19.7% in controls, consistent with the results from the Mexican study.[11]

Diagnosis of T. cruzi infection

Although parasitemia can be intermittently detected in asymptomatic individuals using PCR or hemoculture, these tests have variable sensitivity depending on sample volume and assay performance characteristics as well as stage of infection, and consequently donor screening and laboratory diagnosis are based on serological assays.[1] Based on concern over the inadequate

sensitivity of a single assay, PAHO has recommended that blood donations should be screened with two serological tests in parallel. Discordant results for a given donor are common when screening is performed using this algorithm.[12] Supplemental or confirmatory testing is optimally performed using radio-immunoprecipitation assay (RIPA) or immunoblot assays, although these assays are rarely performed in Latin America for logistic and cost reasons; "confirmation" instead relies on performance of serial EIAs or IFAs. Most assays use a lysate of the epimastigote form of the parasite that easily grow in culture as antigen source, although recent assays using recombinant antigens have been developed.[13-16]

One hypothesis to explain indeterminate and low level positive results is that these individuals may have resolved their infection and are losing antibody reactivity. Cases of treated and presumptively cured individuals who seroreverted have been reported in the literature.[17,18] Results from two small cohorts of treated Chagas patients have suggested that antibody may wane when parasitemia is controlled.[16,19] More recently, in a clinical trial to evaluate the usefulness of benznidazole for treatment of the indeterminate form of Chagas, Viotti and colleagues showed that 15% of treated patients became seronegative yet 6% of non-treated patients also became seronegative during the study.[27]

Performance of assays that detect the parasite itself in blood has been highly variable. There are significant discrepancies between PCR and hemoculture results and at least one study has suggested that parasetimia (measured by PCR) may occur in seronegative Chagas.[20] This concern needs to be addressed in order to evaluate studies that conclude that there are significant rates of seronegative infection in persons in endemic regions.

Approach and Methods

Rationale

This is a retrospective cohort study of *T. cruzi* seropositive donors and seronegative donors. We will enroll *T. cruzi* exposed donors from the seropositive donor registries that were established at Sao Paulo (Fundação Pró-Sangue/Hemocentro de São Paulo, FPS/HSP) and Montes Claros, Minas Gerais (Fundação Hemominas) blood centers beginning in 1996, in order to have extended follow-up periods to investigate rates of development and progression of disease and, for a subset of donors with stored index donation plasma, changes in serological reactivity over time. As with other infections, sustained antibody levels may require ongoing antigenic stimulation. If *T. cruzi*-infected individuals were able to clear infection we would expect antibody levels to decrease over time, because those individuals are no longer being exposed to active antigenic stimulation. Documentation of waning antibody in *T. cruzi* exposed donors years after infection could explain why low level positive and inconclusive results are obtained in large scale donor screening programs both in Brazil and the US. Correlations of initial and persistent antibody levels with time of exposure history and detection of parasitemia by PCR would also be of interest. Antibody levels will be evaluated through serial dilution using IHA and IFA assays and also by the ELISA approved in the US. This study will therefore seek to make use of plasma units from ~500 seropositive donors that were saved at FPS/HSP since 1994. Those donors will be recalled for a second sample and for a medical interview and other laboratory studies. We will supplement this group with a second population of *T. cruzi* seropositive donors identified in the late-1990s in Montes Claros blood centers to expand our power to examine natural history and laboratory correlates of disease. We will evaluate seropositive donors (relative to seronegative donors) for rates and pattern of development of clinical Chagas disease, EKG and echocardiogram abnormalities, and correlate signs and symptoms with demographic and laboratory parameters. We will also perform PCR on coded samples from seropositive donors and seronegative donors

from endemic and non-endemic regions to determine the validity of previous reports of high levels of immunosilent infection in endemic regions.

<u>*Selection of Study Subjects*</u>

Seropositive (infected) donors

Seropositive donors from Sao Paulo City: As part of standard operational procedures at blood centers in Brazil, donors who test reactive at screening are asked to return for collection of a second sample. When the testing of the second sample is completed each donor is counseled. The goal in our study is to recall 250 blood donors who confirmed positive and returned for counseling. Exposed donors will be selected from *T. cruzi* reactive donors who were identified by FPS/HSP from 1996 through 2002. Eligible donors will be prioritized for enrollment based on existence of a frozen stored plasma unit in order to allow evaluation of change in reactivity over time. The table to the right shows the plasma units that are available from FPS/HSP. Of these 495 donors 255 returned for counseling and will be the first group we recruit for this study. We anticipate successful recruitment of 100 donors from this group. To achieve the total sample size of 250, we plan to recruit 150 blood donors who returned, but who do not have plasma units available. From the list of approximately 1200 returning *T. cruzi* seropositive donors, we will prioritize recruitment of people who donated in the earlier years thus providing a longer follow-up period for Chagas disease symptoms to develop.

Table 1.
Plasma Units Available for FPS/HSP

Year	No. of Available Units
1996	5
1997	65
1998	121
1999	75
2000	92
2001	5
2002	132
Total	495

Seropositive donors in Sao Paulo who did not return: Because the stored plasma samples represent a valuable resource for investigating Chagas disease progression in blood donors, in a secondary study we plan to recall the donors whose stored plasma units are available but who did not return for counseling when they originally donated. From this group we believe we will be able to recruit an extra 100 donors for phlebotomy, risk factor assessment and EKG. These donors will not be part of the matched study as we will not attempt to recruit seronegative comparators, but will provide information on the natural history of *T. cruzi* infection. These donors and samples will allow us to obtain additional data for disease marker progression and to confirm current *T. cruzi* antibody and parasitemia status in seropositive donors, and particularly donors who are unlikely to have sought any medical care for *T. cruzi* infection.

Seropositive donors from Montes Claros City: Montes Claros is a city of 300,000 inhabitants in the State of Minas Gerais. The Montes Claros blood center collects 11,000 units/year and is part of Hemominas (One of the blood collection agencies participating in REDS II International Brazil). From 1998 to 2003 there were 63,636 units collected of which1180 were confirmed positive at the time of return. Recruitment will be restricted to the time period of 1998 – 2002 in order to parallel the eligibility time period for donors in Sao Paulo. Because Montes Claros is a small city, we believe we will be able to achieve a return rate of 40-50%, with the goal of 250 enrollees.

Seronegative (uninfected) donors

In both sites we will select and enroll seronegative donors who donated contemporaneously with the seropositive donors. Seronegative donors will be strata matched to enrolled seropositive donors based on city of blood donation, gender, and age within 5 years. During this time period

up to 80% of donors were first time donors. Repeat donors will not be excluded from being selected as seronegative comparators. However each repeat donor will only be represented once in the list of potential seronegatives to be recruited using their earliest donation record within the 1996 – 2002 time period. These control donors are intended to serve two purposes: 1) the primary goal is to ascertain background rates of symptoms and signs related to Chagas disease for comparison with the findings from the *T.cruzi* seropositive donors in each city; 2) the secondary goal is to obtain specimens from ELISA-negative donors from high and low endemic regions; PCR for *T. cruzi* on coded samples from these seronegative donors and in order to address the controversial issue of antibody negative ("immunosilent") infections. In light of previous reports indicating that up to 40% of seronegative persons from endemic areas are PCR positive[20] we believe that 500 seronegative controls, 250 from a high endemic and 250 from a low endemic blood center, will be adequate to rule out significant rates of PCR positive, seronegative infection, and hence support the sensitivity of serological screening.

Recruitment Procedures
In Sao Paulo all donors who had confirmed positive tests for *T. cruzi* antibodies were referred to the University Hospital Infectious Disease clinics for follow up. We have checked the patient rolls and found that there are only 10 current patients that were former blood donors. Therefore this route of case identification is not likely to be successful. Our intended recruitment procedures are described below:

A letter informing the donor of the study will be sent to the address in our blood center records (see Appendix 5 for the two types of recruitment letters, one for seropositive and one for seronegative donors). We will request that the Post Office system verify if the letter was received or not and the name of the person who signed the receipt. If the certified mail is returned to us because the recipient was no longer at the address, we will attempt to contact the former donor by telephone using the phone numbers present in our blood center records and searching for new telephone numbers in the public lists for that address. We will also try to find phone numbers using the donor name. In a previous attempt to recall Chagas donors using this approach we were able to contact by phone 35% of the donors in Sao Paulo.

In the city of Montes Claros we will follow the same procedures, except for when the letters are returned to us because they could not be delivered. In this circumstance we will make an in-person visit to the former address and ask the people living in the house and neighbors whether they have information about the new address of the former donor. In small Brazilian cities it is common to have family and relatives living in the neighborhood and because of this we believe will we be able to successfully track up to 70% of the donors and enroll 40-50% of the seropositive donors into this study.

We will identify initial pools of seropositive and seronegative donors using a study management system developed by the data-coordinating center with roughly a 1:10 ratio of positives to negatives. Once a seropositive is enrolled, a group of three potential strata-matched seronegatives will be “released” for recruitment. All three will be approached at the same time and enrolled if they agree to participate. If none of the three agree, then a second batch of three potential seronegatives will be released. The maximum number of potential seronegatives to be contacted per enrolled seropositive will be ten

Seropositive and seronegative consent forms are provided in Appendix 4a and 4b, respectively. Appendix 4c provides the consent for the secondary project focused on seropositive donors in Sao Paulo with existing plasma specimens who did not return for counseling.

Control for non enrolled people:
For individuals (cases and controls) that we could not locate we will submit their names to the Brazilian mortality database to verify their status.
For those who were located but did not enroll, we will ask them to answer through telephone a short questionnaire about their clinical status (appendix 1a).

Phlebotomy and Sample Processing in Brazil:
At the time of enrollment 4 EDTA tubes (10 mL each) of blood will be collected by venipuncture from each study subject. Two tubes will be used to separate, aliquot and freeze (at -20C) for serological assays. The remaining 2 tubes will be combined and 20mL of blood transferred by pipet to a 50-ml polypropylene centrifuge tube containing 20mL of a 6 M guanindine-HCl/0.2 M EDTA solution. Samples will be heated for 15 minutes in boiling water to shear the DNA molecules. After cooling, 3mL will be aliquoted into 5 cryovials and stored at –80C prior to shipping to BSRI for RIPA and PCR.

Interview and Clinical Examination:
In addition to the collection of 40mL of blood, all cases and controls will be interviewed using a questionnaire designed to elicit risk factors for and symptoms associated with Chagas disease (Appendix 1). The questionnaire content covers geographic risk factors, symptoms that may be indicative of Chagas and medications the former donor may currently be taking. A blood center physician will perform a brief physical examination and each patient will be referred to a local cardiologist in order to obtain a more formal clinical examination including electrocardiogram (EKG) and echocardiogram. In so much as it is feasible the cardiologist will not be informed as to whether the donor is a case or control. However, we cannot prevent communication of case or control status by the donor, so we will not attempt to actively blind the cardiologist to the study subjects' *T. cruzi* status. A single physician in each study location (Sao Paulo and Montes Claros) with expertise in cardiac and GI signs and symptoms of Chagas disease will perform the physical examination. The physicians will be trained to perform the clinical examination as consistently as possible, and the results will be recorded on a special form developed for this protocol (Appendix 2) that covers signs, symptoms, EKG and echocardiogram parameters. Results will be used to counsel the donor with regard to finding and any evidence requiring further clinical follow-up.

Electrocardiography (EKG): Standard criteria will be used for EKG diagnosis of Chagas disease.[25] A core reading laboratory (EKG Core Lab) will review all EKGs. The following EKG abnormalities will be considered related to Chagas heart disease whereas other abnormalities will be considered nonspecific.[26]

- Complete left and right bundle-branch block,
- Left anterior fascicular block,
- Sinus bradycardia less than 50 beats/min,
- Electric inactivation areas,
- Types 2 and 3 atrio-ventricular block,
- Sustained supraventricular arrhythmias,
- Non sustained and sustained ventricular tachycardia,
- Pacemaker implantation

EKG Core Lab
The EPICARE center at Wake Forest University has agreed to serve as the core reading laboratory (EKG Core Lab) for this study. Electronic images will be captured and transmitted to Westat the data coordinating center (DCC) for the study. Westat will forward the EKG images to

EPICARE under code so that they will be blinded to the study subject *T. cruzi* status. The EKG Core Lab will review the materials and assign a diagnosis of the type and extent of evidence of cardiac disease for each study subject.

Echocardiography: Bidimensional echocardiography will be performed according to the guidelines from the American Society of Echocardiography [24] on all patients (excluding those Sao Paulo seropositives who did not return for confirmatory testing.) A core reading laboratory (Echo Core Lab) will review all echocardiograms.

Data to be acquired

Digital acquisition of echo data will be performed with simultaneous EKG tracings. Record 3-5 beats for two-dimensional images using harmonic imaging. Doppler should be performed at 100mm/second sweep speed with low filter. All Doppler parameters are to be recorded at end-expiration and at least 3-5 sequential complexes should be included. Standard echocardiography views as listed below should be performed with special attention to acquisition of specific measurements described.

- ***Patient demographics***
  Height
  Weight
  Heart rate at start of examination
  Race
  Sex
  Age

- ***Parasternal long axis view***
  Left ventricle
  Aortic valve
  Mitral valve
  Left atrium
  Right ventricle
  Pericardium
  Color flow Doppler of aortic and mitral valves

- ***Parasternal RV inflow view***
  Tricuspid valve structure and function
  Color flow and conventional Doppler assessment of the tricuspid valve

- ***Parasternal short axis view***
  Aortic valve leaflet morphology and color Doppler
  RVOT pulse wave Doppler and continuous wave Doppler across pulmonic valve
  Assessment of TR
  Short axis of LV at 3 levels: chordal (base), papillary muscle (mid) and apical portions.

Before the beginning of the study, we will organize meetings in Sao Paulo for the purpose of allowing the cardiologists in Brazil to establish criteria for interpreting the exams. In addition,

the cardiologists may consult on the findings of each exam when they elect to do so. The goal is to obtain as consistent readings between the two physicians.

Echo Core Lab

The Echocardiography Laboratory of the U.S. National Heart, Lung, and Blood Institute (NHLBI) has agreed to serve as a core reading laboratory (Echo Core Lab) for this study. Video and electronic images will be captured and transmitted to Westat the data coordinating center (DCC) for the study. Westat will forward the images to NHLBI under code so that they will be blinded to the study subject *T. cruzi* status. The NHLBI Echo Core Lab will review the materials and assign a diagnosis of the type and extent of evidence of cardiac disease for each study subject.

An outline of the activities of the Echo Core Lab is provided below:

1. **Purpose of the echocardiography analysis**
   Describe in detail the structure and function of *T. cruzi* seropositive and seronegative comparators in order to permit comparison of the two; describe the natural history of the disease; provide phenotypic data for correlation with other variables including biomarkers.

2. **Flow of data and data archiving**
   Data will be acquired by the clinical sites and transmitted on computer disc (CD) to the DCC. The DCC will assure that the data are anonymous and then transmit them to the Echo Core Lab where the images will be analyzed and the echocardiographic interpretation generated. These data will be recorded on a form to be designed for transfer to the DCC who will be responsible for entering them into a database (Appendix 3).

3. **Equipment requirements**
   Echocardiography equipment that is capable of 1D, 2D, conventional and color flow Doppler, tissue Doppler will be used at both clinical sites in Brazil.

4. **Training of personnel**
   Echo Core Lab personnel will work with the Brazilian cardiologists and expert sonographers from the clinical sites to assure that they are trained to properly acquire the echocardiography data. The location of training (in Bethesda or Brazil) will be decided later.

Laboratory and Biomarker Analyses

Serological Tests

IFA titers will be performed at FPS/HSP as detailed in previous publications. An aliquot of plasma from the index donation of enrolled subjects and from all follow-up samples will be sent to BSRI for Ortho ELISA testing. Any case that tests negative for *T. cruzi* antibody on the stored index plasma and/or follow-up samples or shows evidence of a declining antibody level (>25% drop in IFA titer or ELISA s/c) will be further studied by additional biomarkers such as RIPA to confirm infection and identify specific antibody patterns associated with seroreversion.

Ortho T. cruzi ELISA Test System: The ELISA for antibody to *T.cruzi* developed by Ortho Clinical Diagnostics uses an indirect ELISA procedure carried out in 96-well micro titer plates.

Briefly, 20uL of serum or plasma are added, along with 200uL of specimen diluents, to micro wells coated with *T. cruzi* antigens prepared from lysed epimastigotes. After incubation of the specimen with the micro wells for 60 minutes, the wells are washed and human antibodies bound to *T. cruzi* antigens on the solid-phase are detected by addition of anti-human IgG (murine monoclonal) conjugated to horseradish peroxidase (HRP). Following 30-minute incubation with 200uL of conjugate, the micro wells are washed and bound HRP conjugate is detected by incubating with 200ul of *o*-phenylenediamine (OPD) substrate for 30 minutes. Absorbance is measured at 492nm. The cutoff value for each micro plate is calculated by the mean absorbance of the Positive Calibrator multiplied by a constant. The entire assay procedure can be performed using automated systems currently existing for high throughput screening of blood donations, or can be carried out using manual (semi-automated) methods. Specimens giving absorbance readings greater than the cutoff are considered initially reactive and are repeated in duplicate. If one or both of the repeat tests are reactive, the specimen is considered repeatedly reactive and referred for supplemental testing.

<u>*Radio-Immunoprecipitation Assay (RIPA):*</u> *T. cruzi* epimastigotes produced in axenic culture are radio labeled with ^{125}I and lysed in a detergent solution. A volume of the parasite lysate having a specific activity of 500,000 counts per minute (cpm) is mixed with 10 uL of a test specimen, and three well-characterized positive and negative controls are included in each run. After incubation, protein A Sepharose (PAS) is added to precipitate the antigen antibody complexes that will have formed in the reaction tubes containing positive sera. After several washes to remove unbound radio labeled parasite antigens, the immunoprecipitated radio labeled antigens are released from the PAS by brief boiling in the presence of a reducing agent. The resulting solution of released antigens is subjected to polyacrylamide gel electrophoresis, and after drying of the gel, autoradiography is performed with a several day exposure. The pattern in the test specimen lane is viewed in the context of the patterns in the lanes of the six control specimens. The presence of 72 and 90 kDa bands is the criterion for classifying specimens as positive for the presence of antibodies against *T. cruzi*. If both bands are present the specimen is considered positive. If both bands are absent, the result is classified as negative. If is not possible to determine whether both bands are present or absent, the sample is repeated in duplicate and the same interpretative algorithm is applied. If it is still not possible after repeat testing to determine whether the bands are present or absent, the sample is classified as indeterminate. Similarly, the presence of only one band will result in the sample being classified as indeterminate.

PCR Testing: PCR will be used to amplify kinetoplast DNA (kDNA) minicircle sequences of *T. cruzi*[21-23]. Initially, parasite kDNA present in the stored GEB lysate was cleaved by boiling 1 ml of lysate for 5 min. Thereafter, kDNA will be extracted by standard phenol/chloroform procedures or by using an Isoquick nucleic acid extraction kit (MicroProbe Corp., Bothell, WA) as per the manufacturer's instructions. PCR amplification of minicircle kDNA will be performed as described previously using S35 (5'AAATAATGTACGGGKGAGATGCATGA3') and S36 (5'GGGTTCGATTGGGGTTGGTGT3') primers to yield 330-bp kDNA minicircle fragments from the variable region. The 330 bp products are visualized on a 2% agarose gel stained with ethidium bromide in 1X TAE buffer. Appropriate positive, negative and extraction controls will be included.

<u>*Lipid Profile:*</u> An important potential confounder of any cardiology findings that may be interpreted as indicative of Chagas heart disease is heart disease resulting from family history or life style. We will also have a lipid panel performed (total cholesterol, triglycerides, LDL, and HDL) in addition to blood pressure, smoking history and family history of heart disease, which will be collected on the physical and clinical evaluation forms. The analysis of lipid profiles will be done on fresh samples obtained during the clinical evaluation at local institutions.

Expert Review Panel.
An expert review panel will be assembled to characterize the Chagas Disease status of each study subject. This panel will be comprised of three Brazilian physicians, selected for their knowledge and experience with diagnosing and treating Chagas disease. The panel will be blinded to the subject's *T. cruzi* status and will review the EKG and echocardiogram findings from the core reading centers as well as the physical exam findings. They will come to a consensus regarding each subject's Chagas Disease status and report this classification back to the DCC. The classifications will label study subjects as having Chagas-related symptoms versus Non-Chagas related symptoms (or no symptoms.)

Data Analysis and Statistical Considerations.
We plan to enroll 500 *T. cruzi* antibody positive cases who returned to blood centers in Sao Paulo and Montes Claros. If 65% are PCR positive we will have 324 PCR-positive and 176 PCR-negative pairs of samples. The analysis will examine the rate of clinical findings relevant to Chagas disease in the 500 *T. cruzi* seropositive donors relative to the 500 controls. Due to possible reporting bias we will focus the analysis on objective measures of disease such as echocardiogram and EKG findings[10,11]. The frequency of disease symptoms will be analyzed for cases relative to time since their seropositive donation and estimated time since last exposure to *T. cruzi*. Presence of symptoms will also be compared relative to detectable parasitemia by PCR, with anticipated sample sizes of 324 PCR positives and 176 PCR negatives in the seropositive donors. We also plan to compare the titers of antibody levels between symptomatic and asymptomatic groups defined by EKG and medical interview/questionnaire findings using Anova methods. We also compare signs and symptoms between the 500 seropositive and 500 seronegative donors. Finally in the secondary study, we will examine changes in antibody levels between the index and follow-up specimens for the subset of seropositive donors with paired specimens (expected n=200; 100 counseled at time of donation and 100 not counseled at time of donation), and correlate continued seroreactivity with PCR results (persistent parasitemia).

Human Subjects Considerations
An example of the informed consents is provided in Appendix 4a and 4b. The protocol was submitted and approved by the IRB in Brazil.

Risks to subjects: The primary risk to participants beyond the usual blood donation process will be the potential for psychological distress if there is evidence of cardiac injury. Another risk is the discomfort subjects may feel in answering some of the survey questions. We will instruct subjects that participation is voluntary, inform them of the nature of the questions and allow them to stop the survey and blood sample collection at any time. Finally, as the echocardiogram and EKG usually take between 20–30 minutes each, we intend to perform all of the evaluations on the same day of the physical examination in order to minimize the time required to participate in the study. Subjects will be allowed to schedule an appointment that best fits his or her schedule.

Benefits to subjects: Donors may benefit from knowing their cardiac status, and by counseling about treatment options. We will provide referrals to local physicians who will be able to treat and further counsel subjects on their options. For the majority of the subjects, this represents an important opportunity to have a physical and cardiac check-up performed at an appropriate clinical institute with no out-of-pocket expense.

Study subjects will be reimbursed only for the cost of travel to the blood center and to the clinical evaluation as this is the only reimbursement allowed in Brazil.

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