BEYOND VECTOR CONTROL: CONGENITAL AND IMPORTED CHAGAS DISEASE

by

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Abstract

Globalization has made Chagas disease an important public health problem in non-endemic areas with high numbers of Latin American immigrants. The U.S. has the highest number of imported cases among all non-endemic countries and is second highest in terms of the economic impact due to Chagas disease-related costs. Congenital transmission represents more than 22% of new cases of Chagas disease worldwide. Although early detection provides an opportunity for effective and safe treatment, diagnostic tests for early detection suffer from low sensitivity.

This dissertation aims to: 1) Determine the burden of *Trypanosoma cruzi* infection and cardiac abnormalities in Latin American individuals living in the Washington Metropolitan area, 2) Evaluate the accuracy of current FDA-approved diagnostic tests of Chagas disease in this immigrant population, and 3) Evaluate a newly developed test “IgM Shed Acute Phase Antigen (SAPA) ELISA” for the early diagnosis of congenital Chagas disease in an endemic population, Santa Cruz, Bolivia.

We found a high prevalence of Chagas disease in a community-based study of 1571 Latin American immigrants living in the Washington Metropolitan (WMA) area (4.3%). Having seen the vector in an endemic area was an important risk factor for infection in individuals from different geographic areas. Other factors previously described in endemic areas (having ever lived in a rural area, heard of Chagas disease, or had a family member with the disease) were mainly important among southern South American immigrants. Infected participants from Mexico and Central America where *T. cruzi* Discrete Typing Unit I (TcI) is prevalent have a higher percentage of cardiac conduction system abnormalities than do participants from southern South America where TcII/TcV/TcVI are prevalent (Chapter 4).

In a subset of 1093 samples from Latin American immigrants in the WMA, an evaluation of current FDA-approved tests to diagnose Chagas disease in the U.S. was conducted. Low sensitivity of the recombinant-based ELISA test (Chagatest recombinant v.3.0, Wiener Laboratories, Argentina) was observed, and a large percentage of indeterminate results were
obtained using the lysate-based ELISA (Hemagen Chagas EIA, Hemagen, U.S.). Low levels of antibodies to recombinant and lysate antigens was noted in infected participants from TcI areas compared to participants from TcII/TcV/TcVI areas (Chapter 5).

In the third study (chapter 6), we found that socioeconomic factors were determinants of low maternal adherence to the 9-month follow-up screening program for detection of congenital infection. The IgM SAPA ELISA test was validated in two prospective cohort studies of infants born from mothers with *T. cruzi* infection in Santa Cruz, Bolivia. The IgM SAPA ELISA provided similar sensitivity and specificity to the more sophisticated qPCR assay in samples obtained at birth.

In conclusion, Chagas disease and associated cardiac abnormalities are important public health problems in Latin American immigrants living in the WMA. Access to more sensitive and robust diagnostic tests is required to provide accurate diagnosis in this highly diverse group of immigrants at-risk of infection. Finally, the IgM SAPA ELISA is a high accurate and simple platform that can be implemented in endemic countries to improve early detection of congenital Chagas disease.
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To God,

Science has allowed me to see the majesty that you have given to humans, but there are things that go beyond the predictions of rigorous statistical models, which we often dare to call miracles or blessings.

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and that his plans are always better than ours.
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<tr>
<td>AABB</td>
<td>American Association of Blood Banks.</td>
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<td>ACS</td>
<td>American Community Survey.</td>
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<tr>
<td>adOR</td>
<td>Adjusted odds ratio.</td>
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<tr>
<td>AIC</td>
<td>Akaike Information Criteria.</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool.</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin.</td>
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<td>CDC</td>
<td>Centers for Disease Control and Prevention. (U.S.).</td>
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<tr>
<td>CChD</td>
<td>Congenital Chagas Disease.</td>
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<tr>
<td>ChCD</td>
<td>Chagas cardiac disease.</td>
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<tr>
<td>CI</td>
<td>Confidence Intervals.</td>
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<tr>
<td>FP</td>
<td>Follow-up Program.</td>
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<tr>
<td>DTU</td>
<td>Discrete Typing Units.</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay.</td>
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<td>FDA</td>
<td>Food and Drug Administration.</td>
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<td>HR</td>
<td>Heart rate</td>
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<tr>
<td>IQR</td>
<td>Interquartile range</td>
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<tr>
<td>JHSPH</td>
<td>Johns Hopkins Bloomberg School of Public Health.</td>
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<tr>
<td>LAFB</td>
<td>Left anterior fascicular block.</td>
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<tr>
<td>LPFB</td>
<td>Left posterior fascicular block.</td>
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<tr>
<td>LVH</td>
<td>Left ventricular hypertropia.</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>OD</td>
<td>Optical density.</td>
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<tr>
<td>OR</td>
<td>Unadjusted odds ratio</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline.</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate Buffered Saline 0.05% Tween20.</td>
</tr>
<tr>
<td>PVC</td>
<td>Premature ventricular contractions.</td>
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<tr>
<td>RBBB</td>
<td>Right bundle branch block</td>
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<tr>
<td>RDT</td>
<td>Rapid Diagnostics Test.</td>
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<tr>
<td>SAPA</td>
<td>Shed Acute Phase Antigen.</td>
</tr>
<tr>
<td>TESA</td>
<td>Trypomastigote Excretory-Secretory Antigen.</td>
</tr>
<tr>
<td>TESA-blot</td>
<td>Western Blot based with TESA.</td>
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<tr>
<td>WHO</td>
<td>World Health Organization.</td>
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<tr>
<td>WMA</td>
<td>Washington Metropolitan Area.</td>
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Chapter 1. **Introduction**

Public health interventions to control Chagas disease have been focused on vector control and blood/organ donor screening; these interventions have dramatically decreased the number of infected individuals from 16-18 million in the 1980s to 5.7 million in 2016.\(^1\) However, worldwide there are still more than five million infected individuals. Without anti-parasitic treatment, infection is lifelong and around 20-30% of infected individuals will progress to develop gastrointestinal dysfunction or cardiac disease including heart failure, strokes, and sudden death after 10-30 years of infection.\(^2\)

Globalization has made this infection an important public health problem in non-endemic areas with high numbers of Latin American immigrants. In the U.S., Chagas disease is one of the five most common neglected parasitic infections:\(^3\) the U.S. has the highest number of imported cases among all non-endemic countries,\(^4\) and is the country with the second-highest economic impact after Brazil in Chagas disease-related costs with an annual healthcare cost of $2,162 ($1,158–3,628) per individual.\(^5\)

Chagas disease has been noted to be an important public health problem mainly in the immigrant population living in the U.S. A total of 1908 cases were identified between 2007-2013 after screening of blood donors was implemented in 2007,\(^6\) and sporadic cases of congenital transmission have been reported in Miami, Virginia and Washington among immigrant women.\(^7,8\) Although many states in the U.S. with high numbers of Latin American immigrants are expected to be affected, the disease is only formally reported in Texas, Arkansas, Arizona, Massachusetts and Tennessee.\(^6\) A recent updated calculation by the CDC estimated a prevalence of 1.04% among Latin American immigrants; this was calculated using national prevalence of infection in each endemic country reported by the WHO multiplied by the number of immigrants from each country living in the U.S.\(^9,10\) This estimate does not consider clustering of Chagas in endemic countries, does not account for undocumented immigrants and differences in age and sex distributions, and socioeconomic background between immigrants and the average individual in endemic countries.\(^11\)
Reaching Latin American immigrants through the health system is a challenge due to legal status, low-socioeconomic status, and language barriers. Community research projects are needed to better estimate the health problems that occur in this population. The diagnosis, clinical manifestations, and treatment of Chagas disease varies by geographic area. Although the reasons for these differences are not yet clear, it is attributed to the differences in the distributions of *T. cruzi* genotypes, with Mexico and Central America having mainly *T. cruzi* DTU I (Discrete Typing Unit, Tcl), Brazil with Tcl, and Argentina and Bolivia having genotypes TcV and TcVI.12 The great diversity of individuals from different endemic countries in the U.S. creates challenges in diagnosis and clinical manifestations.6

Congenital Chagas affects 21 endemic countries in Latin America as well as several non-endemic countries with high numbers of Latin American immigrants in the U.S. and Europe. This disease is still an important cause of neonatal morbidity, causing low birthweight and/or respiratory distress in 29% of infected infants.13 Currently, diagnosis of congenital Chagas in National Control Programs still relies on the observation of circulating parasites by microscopy (micromethod) or detection of IgG antibodies by serology at 9-months.14 This diagnostic process is inefficient; causing a 9-month delay in diagnosis and missed diagnosis of more than 58% of infants at-risk of infection in Bolivia.14 Studies done by our research group in Bolivia showed that microscopy, the only technique that is used at birth, has a sensitivity of 20-40%, and only 42% of infants complete the 9-month follow-up programs.13,14 Molecular techniques, such as qPCR, are only used in research studies and have limited use in reference laboratories of some countries. Detection of congenital Chagas disease represents a great opportunity for administration of anti-parasitic treatment since the two anti-parasitic drugs, benznidazole and nifurtimox, are highly efficacious and produce lower rates of adverse events in infants than the observed 23% in adults.15,16 Early anti-parasitic treatment also prevents establishment of chronic infection in the infected children, thereby preventing the 30% probability of developing the serious sequelae of chronic disease, such as cardiomyopathy, in the future.15
1.1 Specific aims
This dissertation has the following specific aims evaluated in three research studies:

1. To determine the seroprevalence of Chagas disease in Latin American immigrants living in the Washington Metropolitan Area, as well as the risk factors of infection and associated cardiac abnormalities.
2. To evaluate the accuracy of FDA-approved diagnostic tests of Chagas disease in the Latin American immigrants.
3. To validate an IgM SAPA ELISA test for early detection of congenital Chagas disease in a prospective cohort study of infants born from seropositive women in Santa Cruz, Bolivia.

1.2 Organization of the dissertation
The dissertation is presented in seven chapters. Chapter 1 provides an introduction and specific aims of the study. Chapter 2 reviews the current literature on Chagas disease in the immigrant population and challenges in the diagnosis of chronic and congenital Chagas disease. Chapter 3 provides an overview of the methods for each specific aim corresponding to the three research studies for submission to peer-reviewed journals. Chapters 4, 5, and 6 present each of the manuscript. Chapter 4 describes the burden of Chagas disease and associated cardiac abnormalities in the Latin American population living in the WMA. Chapter 5 shows the limitations of current FDA-approved tests in the diagnosis of Chagas disease in the U.S. Chapter 6 provides the results of the evaluation of the IgM SAPA ELISA in the early diagnosis of congenital Chagas disease as well as an evaluation of main factors associated with maternal adherence to the 9-month follow-up screening programs in Santa Cruz, Bolivia. Finally, the conclusions, public health implications, and suggestions for future research are provided in chapter 7.
Chapter 2. **Theoretical Framework**

### 2.1 Chagas disease

Chagas disease or American trypanosomiasis is caused by the protozoan parasite *Trypanosoma cruzi* and is mainly transmitted by Reduviidae insects of the subfamily triatomines, but it can also be transmitted through vertical transmission, contaminated food and drinks, blood transfusion and organ transplantation. An individual infected with *T. cruzi* can develop two phases of the disease if not treated. The acute phase occurs from 1-2 months after infection and is characterized by high levels of parasitemia and the presence of specific anti-IgM and anti-IgG antibodies after seven days of infection. In most cases, this phase is unnoticed but there could be the presence of fever, rash, headache, hepatomegaly, splenomegaly or myocarditis among other general manifestations. After the acute phase, individuals will enter into the chronic phase of infection; in this phase the host immune response is able to maintain low levels of parasitemia without parasite clearance, parasites are found in small numbers in different tissues. Almost 70% of infected individuals in this phase will never develop clinical manifestations related to Chagas disease in their life. However, 20-30% will develop cardiac or gastrointestinal manifestations associated with the infection. Cardiac disease is the most frequent and detrimental sequelae associated with Chagas disease and is characterized by arrhythmias, heart failure and sudden death during early adulthood, usually after 10-30 years of infection. Individuals under immunosuppression during the chronic phase may develop reactivation of Chagas disease that, if not treated, results in serious clinical manifestations such as severe myocarditis and/or meningoencephalitis with a high risk of mortality (80-90%).

### 2.2 Epidemiology of Chagas disease

Chagas disease is one of the neglected tropical diseases, affecting 21 Latin American countries including: Argentina, Belize, Bolivia, Brazil, Chile, Colombia, Costa Rica, Ecuador, El Salvador, French Guiana, Guatemala, Guyana, Honduras, Mexico, Nicaragua, Panama, Paraguay, Peru, Suriname, Uruguay, and Venezuela. Although vector transmission of the disease occurs in different animals and in rare occasion in humans in the South of the U.S., the disease is not considered endemic in this country.
Due to improvements in socioeconomic status and efforts made by multinational control programs such as the Southern Cone, Central American, Andean Pact and Amazonian Initiatives many countries have decreased or interrupted transmission through vector forms, blood transfusion and organ transplantation; this has resulted in the decrease of the number of cases from 18 million in 1991 to 5.7 million in 2010. Currently, 62.4% of Chagas cases are in South America. Countries with the highest prevalence are Bolivia (6.1%), Argentina (3.6%), Paraguay (2.1%), Ecuador (1.4%), El Salvador (1.3%) and Guatemala (1.2%). These numbers represent national prevalence of infection; however, the prevalence of Chagas disease is usually highly variable in each endemic country with extremely high prevalence in regions such as El Gran Chaco (51.7%) in Bolivia and Queretaro (18.9%) in Mexico.

Chagas disease had been an exclusive public health problem of Latin American countries, but this situation has changed due to globalization and it is currently considered a significant public health problem in areas with high numbers of Latin Americans such as the U.S., Canada, Spain, Germany, Switzerland, Italy, France and Japan. The U.S. is considered to have the highest numbers of imported Chagas cases (238,091 estimated cases not including undocumented individuals and congenital infections) (Figure 1). A recent meta-analysis calculated a prevalence of 4.2% (95% CI: 2.2%-6.7%) among Latin American immigrants in the five European countries where Chagas disease has been reported (Spain, Germany, Switzerland, Italy, France). This analysis showed that prevalence of Chagas disease by country of origin among immigrants significantly differs from the national prevalence reported in endemic areas. Approximately 44% of studies in Europe reported a higher prevalence than estimates for each endemic country. Chagas prevalence in Europe was higher in immigrants from countries such as Bolivia (18%, 95% CI: 13.9%-22.7%), El Salvador (3.7%, 95% CI: 1.62%-11.7%), and Honduras (4.2%, 95% CI: 1.27%-7.36%). This phenomenon was also observed in a recent study in Los Angeles county in the U.S. where Chagas prevalence was higher in immigrants from El Salvador (3.45%, 95% CI: 2.19-4.71%, versus the national prevalence of 1.3% reported for this...
country). The higher prevalence by country of origin among immigrants as compared to the national prevalence could be explained by differences in the characteristics of immigrants that may come from hyperendemic areas, differences in age and sex distribution of immigrants as compared with the average of individuals living in endemic areas, and bias in study design.11

Figure 1. Prevalence of imported cases of Chagas disease in non-endemic countries. The U.S. has the highest number of cases with 238,091 cases without considering undocumented individuals and congenital infections. Source: Chagas disease. Rassi A, et al. Lancet 2010; 375: 1388–402.
2.3 Latin American immigrants living in the U.S.

The U.S. Office of Management and Budget's defines as Latin American or Latino a person of Mexican, Cuban, Puerto Rican, South or Central American or individuals from Spain independent of race.\textsuperscript{22}

The Latin American population is the largest minority group in the U.S. with 50,477,594 individuals representing 16.3\% of the total population, the majority from Mexico (10.3\%), Puerto Rico (1.5\%) and Cuba (0.6\%).\textsuperscript{22} Different from other minority groups, Latin American immigrants are clustered in specific geographic areas with the metropolitan areas of Los Angeles-Long Beach-Anaheim, New York-Newark-Jersey City and Miami-Fort Lauderdale-West Palm Beach being the ones with the largest numbers of Latin American immigrants.\textsuperscript{23}

Clustering in Latin Americans immigrants is explained by network and herding effects. Network effects occur when the first immigrants provide guidance and assistant to recent immigrants, while herding effects occur when new immigrants tend to go to areas where other immigrants go without having a relationship with them. Clustering mainly occurs among undocumented and less educated immigrants.\textsuperscript{23,24} At the national level, Mexicans are the majority group among Latinos (64.6\%), and this characteristic persists around the Southwest border; however, Mexican immigrants are less predominant in the East Coast where immigrants from the Caribbean and Central America are most predominant.\textsuperscript{23,24}

Heart disease represent the primary cause of morbidity and the second most-frequent cause of mortality in the Latin American population living in the U.S.\textsuperscript{25,26} Latin Americans tend to live longer than the non-Latin American white population, and aging rates are expected to be higher in the Latino group (328\% VS 83\%) between 2000-2030, a characteristic that is called the “Latino paradox”. However, this longevity in the Latino population is also associated with the Latino population living a greater number of years with chronic health problems than their non-Latino counterparts.\textsuperscript{26} Experts are still trying to solve the puzzle, but the evidence suggests a strong link between poverty, lack of education, and the loss of the immigrant advantage through selective health risk behaviors, such as smoking and fast-
food diets, increasing the risk of mortality by heart disease. The most studied risk factors of heart disease in this minority group are the common risk factors found in non-Latin American whites. However, Latin Americans have specific risk factors of heart diseases that are rarely studied in the U.S.

2.4 Chagas disease in the U.S.
Although the U.S. is not listed as one of the 21 endemic countries of Chagas disease, there are eleven species of triatomines that have been detected in the southern half of the country with nine species of vectors and 23 species of mammalian reservoirs that are infected with T. cruzi. Vector transmission has been reported but occurs in lower frequency than in Latin America due to better housing infrastructure, less opportunity for humans to have contact with the vector and low efficacy of circulating vectors in the transmission of the parasite.

In the U.S., Chagas disease has been noted to be an important public health problem mainly in the Latin American population, a total of 1908 cases were identified between 2007-2013 after screening for blood donors was implemented in 2007, and sporadic cases of congenital transmission has been reported in Miami, Virginia and Washington among immigrant women from Bolivia. Although many states in the U.S. with high numbers of Latin American immigrants are expected to be affected, the disease is only formally reported in Texas, Arkansas, Arizona, Massachusetts, and Tennessee. A recent updated calculation by the CDC estimated a countrywide prevalence of 1.04% among Latin American immigrants; this was calculated using national prevalence of infection in each endemic country reported by the WHO multiplied by the number of immigrants from each country living in the U.S. This estimate does not consider clustering of Chagas in endemic countries, does not account for undocumented immigrants or for differences in age and sex distributions between immigrants and country distributions. Using the same approach for estimation of cases, the CDC showed differences in estimated cases by state with California (70,860 cases), Texas (36,977), Florida (18,096), New York (17,403), Illinois (9,316), New Jersey (8,868) and Virginia (7,346) being the seven states with the highest number of estimated cases. These estimates are slightly different from the number of cases reported by blood donor screening with the states of California (707 cases), Florida (260), Texas (176), New York (160),
Virginia (103), North Carolina (41) and New Jersey (32) being the ones with the highest numbers of cases. There is only one recent U.S. community-based study; investigators used a convenience sample to determine the burden of infection in Los Angeles county where most immigrants are from Mexico; this study found a total prevalence of 1.24% (59/4755) with the highest prevalence among immigrants from El Salvador (3.45%, 28/811) followed by Mexico (0.79%, 25/3182). Among the risk factors of infection that this study found were knowledge of the disease and having lived in a house that favors domiciliary infestation with the vector.

Studies conducted in New York and Los Angeles showed that Chagas disease is responsible for 13-19% of cardiomyopathy cases among Latin Americans. In the hospital-based study done in Los Angeles, patients with Chagas cardiomyopathy have mortality rates or require heart transplantations in higher proportions than patients with non-Chagas cardiomyopathy (hazard ratio [HR]=4.46; 96% CI: 1.8-10.8).

Chagas disease also has an important economic impact in the U.S.; an individual with chronic Chagas disease will produce an annual health-care cost of $2,162 ($1,158–3,628), making the U.S. the second country after Brazil in Chagas-disease-related health care costs, and the second region after Latin America in DALY burden (27,590 DALYs suffered).

2.5 The Washington Metropolitan Area and evidences of high burden of infection
The Washington Metropolitan area (WMA) comprises 22 jurisdictions distributed in three zones (the city of Washington, District of Columbia and the states of Virginia and Maryland); those 22 jurisdictions include: The District of Columbia, Calvert County, Charles County, Frederick County, Montgomery County, and Prince George’s County in Maryland, Arlington County, Clarke County, Fairfax County, Fauquier County, Loudoun County, Prince William County, Spotsylvania County, Stafford County, Warren County, Alexandria City, Fairfax City,
Falls Church City, Fredericksburg City, Manassas City, Manassas Park City, and Jefferson County in Virginia.\textsuperscript{24}

The WMA is on the list of the 15 areas with the largest number of Latin American immigrants, accounting for 13.8\% (770, 795) of its population.\textsuperscript{29,30} A stunning characteristic of the WMA is the diversity of country of origin of Latin American immigrants that differs from other zones with even higher numbers of Latin Americans; this characteristic suggests that there may be a higher prevalence of Chagas disease than in other areas in the U.S. Immigrants from El Salvador are the largest immigrant group in the area representing 12.5\% of the total foreign-born population and 33.3\% of the Latin American population. The Salvadorian group are followed by Mexicans (14.6\%), Guatemalans (7.6\%), Hondurans (2.8\%) and Bolivians (2.8\%). These numbers represent the highest numbers of immigrants from Bolivia and the second largest number from El Salvador in the U.S.; both of these are endemic countries with high prevalence of Chagas disease (Figure 2).\textsuperscript{31}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Percentage of Latin Americans immigrants by country of origin in Metropolitan Areas of the U.S. with high numbers of Latin American individuals.}
\end{figure}

The Washington Metropolitan area has remarkable diversity of immigrants and is mainly composed of Salvadorians, Central Americans and South Americans.  
### 2.6 Chagas cardiac disease

Chagas cardiac disease is the most important clinical consequence of *T. cruzi* infection and occurs in 20-30% of infected individuals. Between 30,000 and 45,000 cases of Chagas related cardiomyopathy cases are expected to occur each year in the U.S. The disease is characterized by a chronic inflammatory process, damage to the conduction system, and often an apical aneurysm. The earliest signs are usually conduction system abnormalities observed by electrocardiogram (right bundle branch block and/or left anterior fascicular block and segmental left ventricular wall motion abnormalities). Later, patients develop complex ventricular extrasystoles, ventricular tachycardia, sinus node dysfunction and severe bradycardia, high degree atrioventricular block, thromboembolic, and progressive dilated cardiomyopathy with congestive heart failure.

The Centers for Disease Control and Prevention (CDC) recommends an annual clinical evaluation of patients with Chagas disease that includes ECG analysis to detect cardiac abnormalities early and to provide early medical care to patients at risk of developing Chagas cardiomyopathy (CC). This medical examination could be extended to patients that have already received anti-parasitic treatment because treatment effectively clears parasites in only 65-88% of chronic cases, and diagnostic tools are not available to demonstrate treatment efficacy. Early diagnosis and treatment is even more important after the results of the Benznidazole Evaluation for Interrupting Trypanosomiasis (BENEFIT) trial were released in 2015 that showed no effect of anti-parasitic treatment in patients with established CC.

The 12-lead electrocardiogram (ECG) is the most common non-invasive technique to evaluate cardiac abnormalities in clinical settings and epidemiological studies. The ECG is widely used in endemic areas because of its low cost, its availability in the main hospital of rural areas, and the ability of physicians and health technicians to perform the test. However, it lacks sensitivity, and 25% of infected patients without ECG alterations have left ventricular abnormalities detected by more sensitive techniques such as echocardiogram. Although
the clinical significance of these early abnormalities is still not clear, longitudinal studies showed that 33% of patients with normal ECG but with early left ventricular segmental abnormalities detected by echocardiogram may develop cardiac disease after 4.9 years.\textsuperscript{34,35}

During the early stages of Chagas cardiac disease, abnormalities in the conduction system are seen in the ventricular area and are reflected in alterations of the QRS complex in a 12-lead electrocardiogram. The most common early ventricular conduction defects in Chagas patients correspond to right bundle-branch block and/or left anterior fascicular block. Later markers of cardiac dysfunction are complex ventricular extrasystoles, nonsustained or sustained ventricular tachycardia, sinus bradycardia and high degree heart block.\textsuperscript{35–37}

Since Chagas disease affects populations of low income in endemic and non-endemic areas, the echocardiogram, ambulatory ECG monitoring, exercise test, and other more sensitive techniques are usually recommended only if ECG abnormalities are present. Approximately 2-3% of infected patients without ECG alterations or in the so-called indeterminate form will progress to cardiac disease each year.\textsuperscript{2} For this reason, the CDC recommends performing an annual ECG evaluation for Chagasic patients without baseline ECG alterations; if cardiac abnormalities are detected, an intense evaluation is performed using echocardiography.\textsuperscript{2} However, when changes in the ECG are observed, cardiac damage has already developed and maybe irreversible.\textsuperscript{33}

Early markers of ventricular systolic dysfunction that can be measured in a regular ECG with or without the support of circulating biomarkers of cardiac progression may facilitate the detection of cardiac disease in infected patients.\textsuperscript{33} The QRS duration is positively and negatively associated with left ventricular size and ejection fraction, respectively. However, its used as an independent predictive factor needs to be validated.\textsuperscript{33} A longitudinal study of Chagasic patients from Brazil followed for 58 months showed that QT-interval dispersion could be used as predictor of mortality (hazard ratio, 1.45; 95% CI: 1.29 to 1.63 for 10-ms increments).\textsuperscript{35} The QRS scar score is a quantitative parameter that determines ventricular abnormalities detected on Q-, R- and S-wave durations, amplitudes and morphologies. In Chagasic patients the QRS score has been associated with the degree of fibrosis as measured
by cardiac magnetic resonance. A QRS scar score of more than 2-3 was used as a cut-off to predict the presence of more than 6% myocardial fibrosis (sensitivity and specificity of 95% and 83%, respectively) and a QRS score higher than 7 was highly predictive of left ventricular dysfunction (sensitivity and specificity of 92% and 89%, respectively). These estimates originate in two different studies of infected individuals living in Mexico and Brazil with consistent results between the two studies. However, these evaluations were done with small samples sizes.

### 2.7 Congenital Chagas disease

Congenital Chagas affects 21 endemic countries in Latin America as well as several non-endemic countries, such as the U.S. with large numbers of Latin American immigrants, and is still an important cause of neonatal morbidity, causing low birthweight and/or respiratory distress in 29% of infected infants. Vertical transmission of *Trypanosoma cruzi* is considered the second largest contributor to new infections with this parasite after vector transmission and is responsible for 30% (9,000/30000) of incident cases of *T. cruzi* yearly.

Congenital Chagas disease represents a great opportunity for anti-parasitic treatment since the two anti-parasitic drugs, benznidazole and nifurtimox, are highly efficacious in the acute phase and do not produce adverse events in infants (compared to an adverse event rate of 23% in adults). Early treatment prevents establishment of chronic infection in the infected children, thereby preventing the 30% probability of developing serious sequelae of chronic disease, such as cardiomyopathy in the future.

### 2.8 Diagnosis of Chagas disease

Diagnosis of Chagas disease depends on the phase of the infection. Diagnosis of the acute phase is based on the detection of circulating parasites by microcopy, DNA of the parasite by PCR and/or IgM antibodies to *T. cruzi* by serology. Presumably, most cases of Chagas disease in Latin American adult immigrants are in the chronic phase of infection; during this phase, diagnosis is based on the detection of IgG antibodies to *T. cruzi* by at least two different serological tests using different parasitic antigens (recombinant or crude parasite antigens) or immunologic techniques (Chemiluminescence, Enzyme-Linked Immunosorbent
2.8.1 Diagnosis of Chronic Chagas disease

The detection of IgG antibodies to *T. cruzi* by two serological tests is considered diagnostic for *T. cruzi* infection if the patient has not received anti-parasitic treatment. Negative results by two different serological tests rule out the infection. Samples that are positives by at least one of the two diagnostic tests are considered discordant and they must be evaluated by a third assay or by a confirmatory test. Most of the ELISA tests can sometimes provide indeterminate results, which means that the optical density of the sample is near the cut-off of the test (usually ± 10% of the cut-off). These samples are called indeterminate and should be treated in the same way as discordant samples.

Various serological tests to detect IgG antibodies to *T. cruzi* exist depending on the clinical or epidemiological aim: screening, diagnostic and confirmatory tests.

Screening tests are used to detect the infection in asymptomatic individuals. These tests need to have high sensitivity and can be used for screening in blood banks or epidemiological studies. In the U.S., the American Association of Blood Banks (AABB) screens for Chagas disease in all their facilities, a policy that has been implemented since 2007. Two FDA-approved tests are available for the screening and diagnosis of blood donors: the ORTHO *T. cruzi* ELISA Test System (Ortho-Clinical Diagnostics, Raritan, New Jersey) and the ABBOTT PRISM Chagas assay (Abbott Diagnosis, Illinois). The AABB recommends that family members of suspected cases be screened for Chagas disease.

The ORTHO *T. cruzi* ELISA test uses epimastigote lysate antigens and has a sensitivity and specificity of 98.9% (95% CI: 94.2%-100.0%) and 99.9% (95% CI: 99.9%-100.0%), respectively. The ABBOTT PRISM Chagas assay is a chemiluminescence test and uses recombinant proteins (FP3, FP6, FP10, and TcF) that are present in different stages of the parasite, this test has also good sensitivity and specificity (sensitivity of 100% with 95% CI:...
93.2% - 100% and specificity of 98.21% with 95% CI: 90.45% 99.95%). Individuals with positive results for any of these tests are considered suspicious for \textit{T. cruzi} infection and cannot donate blood and/or blood-products, since no single serological test is enough for clinical diagnosis, suspect individuals must receive another test to confirm or discard the infection.\textsuperscript{42}

There are different ELISA tests for the clinical diagnosis of Chagas disease in Latin America with sensitivities determined in cohort studies of 90\% (95\% CI: 89\% to 91\%) and specificity of 98\% (95\% CI: 98\% to 98\%). Only four tests are FDA-cleared in the U.S.; evaluation of the performance of these tests has been done in South American countries. These tests are:

Chagatest EIA Recombinant v.3.0 (Wiener Laboratories SAIC, Argentina): an ELISA test with recombinant antigens (SAPA, 1, 2, 13, 30 and 36) with 99.3\% sensitivity and 98.7\% sensitivity as provided by the manufacture.\textsuperscript{45}

Hemagen Chagas kit (Hemagen Diagnostics, Inc, U.S): an ELISA test with lysate antigen of the epimastigote and amastigote forms of \textit{T. cruzi} strains Y (DTU II) and CL (DTU VI) with 100\% sensitivity 98.7\% specificity as provided by the manufacture. \textsuperscript{46}

Chagas Detect Plus (InBios International, Inc, U.S): A Lateral-flow test that uses multiepitope proteins and provides results in 20 minutes using capillary blood and serum samples. The manufacturer states that this test has 95.1\% sensitivity and 98.7\% specificity.\textsuperscript{47}

ABBOTT ESA Chagas (Abbott Laboratories): an enzyme strip assay with recombinant antigens (FP10, FP6, FP3, and TcF) and a sensitivity of 100\% and specificity of 93.2\% as provided by the manufacturer.\textsuperscript{48}

Six discrete typing units (DTUs, TcI-TcVI) have been recognized in \textit{T. cruzi} with differences in geographical distributions, clinical and epidemiological characteristics. TcI is the principal lineage north of the Amazon. TcII, TcV and TcVI are the three principal linages in the Southern Cone region of South America. TcIII is mainly found in animals. TcIV is an uncommon lineage in Venezuela (Figure 3).\textsuperscript{12}
Diagnosis, clinical manifestations, and treatment of Chagas disease varies according to geographic area and the variation could be attributed to differences in the distributions of *T. cruzi* genotypes. The great diversity of individuals from different Chagas-endemic countries living in the U.S leaves challenges in diagnosis, clinical manifestations, and treatment. Evaluation of the diagnostic tests in a population that represents the immigrant population in the U.S is needed.
Figure 3. Evaluation of FDA-cleared diagnostic tests of Chagas disease was done in endemic areas with different genotypes of *T. cruzi.*

Participants from Mexico, Central America and northern South America were denotated as participants from TcI areas, while participants from Southern South America were grouped in TcII/TcV/TcVI areas.

2.8.2 Diagnosis of congenital Chagas disease

Most vertical transmission of *T. cruzi* occurs during the last trimester of pregnancy. Infected neonates are born in the acute phase of the infection which is characterized by presence of IgM antibodies to *T. cruzi* antigens and detectable parasitemia by microscopy and/or PCR. The algorithm for diagnosis in national control programs in endemic areas depends on the observation of circulating parasites by microscopy (micromethod) at birth and the detection of IgG antibodies to *T. cruzi* antigens at 9-months of age; however, this algorithm detects only 64% of congenital cases because of the low sensitivity of microscopy (40%) and the low adherence (58%) to the 9-month follow-up program required for IgG serology.\textsuperscript{13,14}

Other techniques such as PCR and the Western blot based on the use of the trypomastigote excretory-secretory antigen (TESA-blot) for detection of IgM antibodies are used only by research groups and some reference laboratories in some endemic countries to detect the infection early.\textsuperscript{13,14} Although PCR provides the highest sensitivity early in life, it is difficult to implement in endemic areas because requires the use of a thermocycler, a well-trained technician, and the use of different laboratory rooms to prevent DNA cross-contamination. Implementation of PCR, even only in reference laboratories, for screening proposes is labor intensive and costly.\textsuperscript{13} If implemented only in reference laboratories, it can also delay diagnosis. Similarly, since there is not a commercial IgM TESA blot, this test is only used by some research laboratories that are able to perform all the steps of the Western blot technique including antigen production, cell culture of the parasite, and horizontal and vertical electrophoresis.

Detection of IgM antibodies to *T. cruzi* antigens has been suggested before. Some previous studies have showed low sensitivity and specificity for the detection of IgM antibodies in congenital Chagas disease.\textsuperscript{49,50} The low accuracy in these studies can be explained by the use of crude antigens and/or polyclonal conjugates to IgM antibodies that can have cross-reaction with IgG antibodies.
2.9 The Shed Acute Phase Antigen (SAPA)

SAPA is part of the SAPA/trans-sialidase protein and is a major surface protein of the trypomastigote stage and is also released in the circulation.\textsuperscript{51,52} In the TESA-blot, SAPA corresponds to a group of 6 bands of 130-200 kDa\textsuperscript{53}, detection of IgM antibodies to the six SAPA bands have a cumulative sensitivity of 73.4\% compared to qPCR and the 9-month IgG.\textsuperscript{13}

The carboxyl terminal group of SAPA contains a sequence of 12 tandemly repeated amino acids that induce a strong immune response.\textsuperscript{54,55} During the acute phase of Chagas, SAPA activates the B-cell immune response and most IgM and IgG antibodies are produced in response to SAPA. The trans-sialidase protein does not strongly activate the immune response, which could be an important mechanism of immune evasion since the SAPA region of the SAPA/Trans-sialidase does not have as much functional activity as the trans-sialidase region, which has the enzymatic activity needed for transference of sialic acid and parasite invasion.\textsuperscript{51}

There is only one report of the use of a recombinant SAPA for detection of IgM antibodies in congenital cases, reporting a sensitivity of 75\% (9/12) and specificity of 100\% (12/12) in a single serum sample from umbilical cord blood. However, this study has been done with a small number of samples.\textsuperscript{50}

The presence of IgM antibodies to other multiepitope antigens: 1, 2, 13 and 36 has been also detected in congenital infections.\textsuperscript{50} Purified human antibodies against antigens 2 and 13 recognize the trypomastigote stage of the parasite, while antigens 1 and 36 are not detected in the epimastigote or trypomastigotes state.\textsuperscript{50} Antigens 1 and 2 can detect antibodies in chronically infected individuals, while antigens 13 and 36 recognize antibodies in adults with acute infections.\textsuperscript{50}
Chapter 3.  **Summary of the Methodology**

*Specific Aims*

**Study 1: “The burden of Chagas disease and cardiac abnormalities in the Washington Metropolitan area”**.

**Primary aim:**  *To determine the seroprevalence of Chagas disease in Latin American immigrants living in the Washington Metropolitan Area, risk factors for infection and associated cardiac abnormalities.*

**Sub aims:**
1. *Determine the seroprevalence of Chagas disease in Latin American immigrants living in the Washington Metropolitan Area:* A community-based study was conducted to enroll Latin American immigrants. At least two FDA-approved diagnostic tests of Chagas disease were used in parallel to make the diagnosis. To calculate the adjusted prevalence of Chagas disease, a weight was administered to individuals from each endemic country based on the distribution of Latin American immigrants reported by the American Community Survey.

2. *Determine the risk factors of highest importance for T. cruzi infection among Latin Americans at risk for Chagas disease in the Washington Metropolitan Area:* Studies in endemic areas have identified specific risk factors for Chagas disease including location of birth and residence, infected family members, vector exposure, and housing conditions. Participants (n=1571) answered a questionnaire similar to those utilized in endemic areas to document the extent to which those same risks factors reflect disease epidemiology in non-endemic areas.

3. *Determine the extent that people with T. cruzi infection in a non-endemic setting manifest Chagas disease-associated cardiac conduction abnormalities:* In a nested cross-sectional evaluation, we determined differences in the distribution of cardiac conduction system abnormalities detected by electrocardiogram (sinus bradycardia, right bundle branch block,
left anterior fascicular block, premature ventricular contractions, left ventricular hypertropia, and atrial fibrillation) between infected (n=89) and non-infected participants (n=184).

Study 2: “Challenges in the diagnosis of Chagas disease in the U.S.: Experience from a community-based study in the Washington Metropolitan area”.

Primary aim: To evaluate the accuracy of FDA-approved diagnostic tests of Chagas disease in Latin American immigrants living in the Washington Metropolitan Area.

Sub aims:
1. Evaluate the performance of FDA-approved diagnostic tests of Chagas disease in two different groups of Latin American immigrants living in the Washington Metropolitan Area: Participants (n=1099) were grouped based on their country of origin. Immigrants from Mexico, Central America and northern South America were referred as TcI areas, and individuals from southern South America were referred as TcII/TcV/TcVI areas. Three FDA-approved tests were compared to two confirmatory assays including a lysate-based ELISA test and the Western Blot based on the Trypomastigote Excretory-Secretory Antigen (TESA-blot).

2. Determine differences in levels of antibodies and band patterns observed in diagnostic tests of Chagas disease between immigrants from TcI and TcII/TcV/TcVI areas. Levels of antibodies and band intensity detected in FDA-approved tests, as well as patterns observed in the TESA-blot were compared among infected individuals without anti-parasitic treatment from the two geographic areas.
**Study 3:** “Addressing early diagnosis of congenital Chagas disease in the time of the goal of mother-to-child transmission elimination in the Americas”.

**Primary aim:** To determine factors for low maternal adherence to the 6- to 9-month screening program of congenital Chagas disease and to validate an IgM SAPA ELISA test for early detection of the infection in a prospective cohort study of infants born to seropositive women in Santa Cruz, Bolivia.

**Sub aims:**
1. To determine the main differences between mothers that completed and did not complete the 6- to 9-month follow-up screening programs in three different hospital-based prospective cohort studies of congenital Chagas disease in Santa Cruz, Bolivia: Factors associated with *T. cruzi* infection and socioeconomic factors were evaluated in 1356 seropositive mothers.

To determine the accuracy of the IgM SAPA-ELISA test based on the use of a recombinant SAPA and camelid antibodies in the early detection of congenital Chagas disease at birth: The IgM SAPA-ELISA was validated in 38 and 217 congenitally infected and non-infected infants, respectively enrolled at birth in Percy Boland Hospital in Santa Cruz, Bolivia. The specificity of the test was also validated in 186 serum samples of newborns from Piura, Peru, a non-endemic area of Chagas disease.

**Study design**

**Study 1 and 2 (Chapter 4 and 5)**
This study was a cross-sectional evaluation of the risk factors for and seroprevalence of *T. cruzi* infection among Latin Americans older than 18 years old who reside in the Washington Metropolitan area (n=1571). A cross-sectional analysis of cardiac abnormalities detected by ECG was also conducted in seropositive individuals (n=89) and in randomly selected seronegative participants (n=184).
Chagas infection was determined using two FDA-approved ELISA tests that were run in parallel. The TESA-blot and a lysate-based ELISA test were used for confirmation of the infection.

**Study 3 (Chapter 6)**

A prospective birth cohort study on congenital Chagas disease was conducted in three Hospitals in Santa Cruz, Bolivia to evaluate the performance of the IgM SAPA ELISA. The initial evaluation of the test was done in two hospitals (a training cohort): Hospital Universitario Japones and the Municipal Hospital of Camiri. The validation of the assay was done in the Municipal Women’s Hospital Dr. Percy Boland Rodriguez (validation cohort). The IgM SAPA ELISA was compared to microscopy, qPCR, IgM TESA-blot and 9 months IgG serology. Infants from Chagas seropositive mothers were enrolled in this study and were followed-up for 9-months to confirm the diagnosis of congenital Chagas disease at 9-months by IgG serology. Samples from infants were also collected at birth to determine the diagnosis using microscopy and qPCR.

A cross-sectional evaluation of factors associated with *T. cruzi* infection and socioeconomic status of mothers that completed or did not complete the 9-month follow-up screening program was also performed in the three hospitals.

The specificity of the test was also evaluated in serum samples of neonates from women from Cayetano Heredia and Santa Rosa hospitals in Piura, Peru, a non-endemic region for Chagas.

**Participants and enrollment**

**Study 1 and 2 (Chapter 4 and 5)**

The study enrolled individuals 18 years old and older that were born in any of the 21 Chagas-endemic countries. Counties and cities of the Washington Metropolitan Area with percentages of Latin Americans greater than 15% were considered for recruitment and enrollment. These areas included: in Virginia (Prince Willian County, Fairfax County, Arlington County, and the Cities of Manassas Park, Manassas, Fairfax, Falls Church, and Alexandria), in the state of Maryland (Montgomery County and Prince George’s County) and Washington, District of Columbia.
Latino individuals were enrolled via recruitment in churches, community centers, consulate events and health fairs. Education about the disease was provided to participants before enrollment to increase participation of individuals who lack knowledge about the disease. We provided education to the community by performing workshops prior to and/or during the day of enrollment. Education about the disease in the area was also provided using Latino media such as journals (El Tiempo Latino from the Washington Post), television (Telemundo) and radio (radio Poder, radio America). Educational brochures were also provided to potential participants (Figures 4-7).

After obtaining informed consent, blood samples from fingersticks were taken to rapidly screen patients for Chagas disease using the FDA-cleared rapid test (Chagas Detect Plus rapid test, InBios, U.S). A questionnaire was used to identify risk factors for infection while the participants waited for the results of the rapid tests. To describe the cardiac morbidity in this population, an electrocardiogram was performed on patients with positive results by the rapid test and on the next two participants with negative results. The use of the rapid test allowed us to perform the cardiac analysis on the same day as enrollment to avoid second visits and loss of follow-up.
Workshops were conducted in Spanish during community events that increased awareness about different health issues that affect Latin Americans including high blood pressure, diabetes, sexually transmitted infections and other vector-borne diseases such as Lyme disease. Community workshops about Chagas disease used culturally appropriate language to explain general aspects about the disease, diagnosis, mechanisms of transmission, clinical manifestations and treatment and were provided in churches, health fairs and consulates.
Figure 5. Educational brochure and flyer. IRB-approved educational brochures and flyers with more detailed information about the disease and our study activities were distributed to potential participants during community-events and community centers.
Figure 6. Interview and appearance in “El Tiempo Latino” from the Washington Post and Telemundo.
The journal “El Tiempo Latino” is one of the main journals in the area for Latin American individuals.
Figure 7. Interview with “Radio Poder” to increase awareness about Chagas disease in the Latin American community.

**Study 3 (Chapter 6)**
For the third study, women in labor or within three days of delivery in Santa Cruz, Bolivia were invited to participate. Venous blood samples were collected from individuals that provided consent to determine the presence of antibodies to *T. cruzi* antigens by two rapid tests. An epidemiological interview was conducted by a nurse from the hospital to evaluate socioeconomic factors, family history and vector exposure. Clinical information about the mother and neonate were obtained from medical records. Venous blood samples were collected from neonates and infants born from seropositive mothers at ages of 0-3 days, 1 month and 6-9 months.
Chapter 4. Study 1: “The burden of Chagas disease and cardiac abnormalities in the Washington Metropolitan area”

Abstract

Background: Although clinical reports suggest the public health importance of Chagas disease in the Washington Metropolitan Area (WMA), there have not been studies conducted to determine the burden of the infection.

Methods: A cross-sectional study of the seroprevalence and risk factors for infection was conducted in 1571 Latin American immigrants enrolled via community centers. Immigrants from Mexico, Central America and northern South America were designated as being from as Tcl areas (n=1014), and participants from southern South America as from TcII/TcV/TcVI areas (n=557). T. cruzi infection was diagnosed using the Hemagen Chagas EIA (Hemagen Diagnostics, U.S) and the Chagatest recombinant v.3.0 (Wiener Laboratories, Argentina). The Western Blot assay with the Trypomastigote Excretory-Secretory Antigen (TESA-blot) was used for confirmation of results. Cardiac conduction system abnormalities in infected (n=89) and non-infected (n=184) participants were evaluated using the electrocardiogram. The prevalence of the infection was adjusted by the percentages of Latin Americans from each specific country among the total number of immigrants from endemic countries living in the WMA based on the reports of the American Community Survey.

Findings: A weighted prevalence adjusted by the reported distribution of Latin American immigrants in the American Community Survey was calculated as 4.3% (95% CI: 3.3%-5.6%). Having seen the vector in an endemic area was the only risk factor associated with the infection in the two geographic groups (Tcl: adjusted OR: 3.6, p=0.004, TcII/TcV/TcVI: adOR: 1.9, p=0.041). Conduction system abnormalities were higher in infected versus noninfected individuals from the two areas but was not statistically significant for participants from TcII/TcV/TcVI areas. Infected participants from Tcl areas had higher percentages of at least one cardiac abnormality associated with Chagas disease (Tcl: 5/11, 45.5%; TcII/TcV/TcVI: 14/78, 17.9%, p=0.0371) than participants from TcII/TcV/TcVI areas.

Interpretation: The prevalence of T. cruzi infection found in this study was higher than our estimate using national prevalence in endemic countries and the number of individuals from each country in the WMA, suggesting a greater number of immigrants from areas where Chagas disease is endemic. Higher prevalence of cardiac abnormalities in participants from Tcl areas could be the result of more severe cardiac disease associated with T. cruzi Discrete Typing Unit I.
Introduction

Latin American immigrants are the largest minority group in the U.S. representing 17.8% (57.5 million) of the total population. Individuals from Mexico (80.5% of Latin Americans), El Salvador (4.8%), Guatemala (3.1%), Colombia (2.5%), and Honduras (2.0%) are the five most predominant groups at the country level; all are also endemic Chagas disease countries.

The burden of Trypanosoma cruzi infection in the U.S is difficult to determine because of the vulnerability of the Latin American population, composed of both documented and undocumented individuals, the scant attention given to the disease worldwide, the lack of awareness about the disease by the medical community in the U.S, of systematic reporting of cases, and of active screening even in high risk groups.

Conservative estimates made by the CDC suggest that 238,091 infected individuals (estimated prevalence 1.04%) live in the U.S. These estimates were calculated using specific country prevalence and the number of immigrants from each country. Using this approach, the states with the highest estimated cases were: California (70,860 cases), Texas (36,977), Florida (18,096), New York (17,403), Illinois (9,316), New Jersey (8,868) and Virginia (7,346). These estimates do not take into consideration the clustering of Chagas disease in endemic countries, clustering of immigrants in the U.S., and undocumented individuals. In addition, characteristics of immigrants may not be the same as the average non-immigrant from their home countries, so national prevalence in an endemic country cannot be translated to its immigrants.

Other attempts to determine the burden of the infection constitute reports from blood donor screening that provided similar results to the estimates described above with the states of California (707 cases), Florida (260), Texas (176), New York (160), Virginia (103), North Carolina (41) and New Jersey (32) reporting the most Chagasic cases. However, because of legal status, cultural beliefs, education and language barriers, Latin American individuals with low socioeconomic status maybe less likely to be blood donors. Furthermore,
Chagasic individuals and immigrants with travel history to malaria endemic countries that are usually also endemic for Chagas must wait at least 12 months for future blood donation. There has only been one large community-based study, of 4577 Latin American-born individuals conducted in Los Angeles county, California where the majority of at-risk individuals are of Mexican (82%) and Salvadoran (8%) origin. The authors reported a seroprevalence of 1.24%. Since the distribution and clustering of Latin Americans differ by states due to network and herding effects, community-based studies in other areas are needed to determine the concentration of infected cases for appropriate allocation of resources.

Heart disease represent the primary cause of morbidity and the second most frequent cause of mortality in Latin American immigrants in the U.S. and they also have higher risk of obesity and diabetes that may increase their risk of developing Chagas associated-cardiomyopathy. No studies to determine the burden of Chagas cardiac disease (ChCD) at the community-level have been conducted in the U.S. Based on CDC estimations, 30,000 to 45,000 related cardiomyopathy cases occur each year, and in New York and Los Angeles the infection is responsible for 13-19% of cases of cardiomyopathy in Latin Americans.

Reaching Latin American immigrants through the health system is a challenge due to legal status, lack of health insurance and language barriers. Community research projects are needed in order to better estimate the health problems that occur in this population. This is the first community-based study that determines the prevalence of Chagas disease in Latin American immigrants in the Washington Metropolitan area, and the first study in the U.S to determine the extent of Chagas cardiac disease in an asymptomatic population.
**Methods**

**Study area**

Enrollment was conducted in counties and cities of the WMA with percentages of Latin Americans higher than 15%. These included areas in the state of Virginia (Manassas Park City, Fairfax City, Fairfax County, Arlington County, Falls Church City, and Alexandria City), the state of Maryland (Montgomery County and Prince George’s County) and Washington, District of Columbia (Figure 8).

![Map of Washington Metropolitan Area](image)

Figure 8. Places in the Washington Metropolitan Area were enrollment of Latin American immigrants was performed.

Areas of enrollment are inside the red contour and correspond to places where the Latin American population represents more than 15% of their population.  

22, 24, 29
Study design, enrollment and participants

A cross-sectional study was conducted from February 2016 to April 2018 in the WMA. Latin American individuals 18 years of age and older from any of the 21 Chagas endemic countries were enrolled via recruitment in churches, community centers, consulate events and health fairs. As requested by community leaders, Chagas testing was provided together with glucose testing and blood pressure measurement using the HemoCue Glucose 201 DM System (HemoCue America) and the automated Omron® BP760 7 series Automatic Blood Pressure cuff (Omron Healthcare, Inc). Education about the disease was performed before enrollment to increase participation of individuals who lack knowledge about the disease. Education consisted of workshops, distribution of educational brochures, and interviews in media outlets (journals, television and radio programs) directed to the Latin American community. After consenting, blood samples from fingersticks were tested in the field using the FDA-cleared rapid test (Chagas Detect Plus rapid test, CDP, InBios, U.S., sensitivity of 95.1% and specificity of 98.7%).

A cross-sectional evaluation of electrocardiogram (ECG) findings in Chagas positive and negative individuals (ratio 1:2) was nested within the seroprevalence study. Participants with positive results to the CDP and the next two consecutive negative participants were evaluated using ECG. Both the CDP and the ECG were done in the community setting during the day of enrollment to avoid loss to follow-up. Participants with false negative results to the CDP and randomly selected negative participants from the same site were contacted to undergo an ECG in a second visit (Figure 9).

The institutional review board of the JHSPH approved the protocols (IRB 6325, 6713, and 6854). All participants provided written informed consent for their participation.
Serological tests

Two FDA-approved ELISAs were performed in parallel in all participants following the instructions of the manufacture by a trained microbiologist with a master’s degree. These tests included (sensitivity and specificity provided based on the manufacture’s information): The Chagatest ELISA Recombinant v.3.0 (Wiener Laboratories SAIC, Argentina, sensitivity of 99.3% and specificity of 98.7%) and the Hemagen Chagas kit (Hemagen Diagnostics, Inc, U.S., sensitivity of 100% and specificity of 98.7%). Samples with indeterminate and discordant results were evaluated by Western Blot using the Trypomastigote Excretory-Secretory Antigen (TESA-blot).

The TESA-blot using the TESA from *T. cruzi* Y strain was developed following published procedures. Reactions to the band of 150-160 kDa and ladder-like bands of 130-200 kDa were considered specific for *T. cruzi* infection. The test was performed by a microbiologist with master’s degree and was read by an experienced clinical microbiologist in parasitic infections.

Samples were considered truly positive or negative when congruent negative and positive results were obtained by at least two of the three tests described above. Results of the rapid test CDP were not considered for final determination of serological status.
Epidemiological data

A validated questionnaire standardized in Santa Cruz, Bolivia was used to obtain epidemiological data related to risk factors of *T. cruzi* infection, clinical factors associated with Chagas disease, and socioeconomic factors. The questionnaire was mainly self-administered by the participants and mainly in Spanish (Figure 10). In cases participants being illiterate in both in English and Spanish, a study volunteer administered the questionnaire. Questions related to the identification of the vector were accompanied by a colorful picture of the insect to help with its identification because the vector goes by different names in various endemic areas. Individuals that have been previously tested for Chagas disease were removed from the calculation of seroprevalence and the risk factor analysis. Participants were classified in two groups based on their country of origin. Participants from Mexico, El Salvador, Guatemala, Honduras and other Central America countries (Belize, Nicaragua, Panama, Costa Rica) and northern South American countries (Colombia and Venezuela) were classified as TcI areas, and participants from Peru, Bolivia and other southern countries (Argentina, Brazil, Chile, and Ecuador) were classified as TcII, TcV and TcVI areas.

![Figure 10. Medical volunteers assisting participants with epidemiological interviews.](image)
**Electrocardiogram (ECG)**

The ECG was obtained and interpreted by a certified attending cardiologist using a PC-Based Resting Electrocardiogram (Welch Allyn, U.S). The presence of the following abnormalities was consistent with Chagas cardiomyopathy: complete right bundle branch block, left anterior fascicular block, premature ventricular contractions, sinus bradycardia (heart rate lower than 50 bpm), atrial fibrillation, junctional rhythm, first-degree atrioventricular block, left ventricular hypertrophy or Q-waves. Isolated incomplete right bundle branch block did not meet the criteria because it can be a normal variant in young individuals.

**Sample size**

We estimated a seroprevalence of Chagas disease in the WMA of 1.4%, this calculation was done based on the CDC approach of multiplying the number of Latin American immigrants from any of the 21 endemic countries by the national prevalence of Chagas disease in each country of origin. Numbers of immigrants and national prevalence of Chagas disease in each endemic country were taken from the American Community Survey (ACS) - U.S. Census Bureau, 2010 and the report of WHO on Chagas seroprevalence in 2010, respectively (Table 1). Assuming a prevalence of 1.4%, allowing for 0.7% error in either direction, a normal population distribution, and 95% confidence in the sample size estimate, this study would require a sample size of 1082 participants. Adding an additional allowance of 30% to compensate for inter-test variability with respect to prevalence estimates, the total sample size required for this study was 1546.

We assumed that differences in ECG abnormalities between seropositives and seronegatives will be the same as previously reported in endemic areas in population-based studies. To obtain an OR of 5.31 (95% CI: 1.23–22.86) for the presence of right bundle branch block (RBBB) and left anterior fascicular block (LAFB), we calculated that performing the ECG in at least 59 Chagas seropositive and 117 seronegative participants will provide an statistical power of 80%-90% to detect these differences.
Statistical analysis

To determine the prevalence of Chagas disease, a weight was assigned to individuals from each country based on the distribution of Latin American immigrants using the reports of the ACS-US Census Bureau 2010 (Table 1). Individuals who knew their results of Chagas disease before their participation in the study were excluded for the calculation of seroprevalence and risk factors analysis. The chi square-test or Fisher exact test was used to determine associations with categorical variables between groups.

The median age of the participants (43 years old) was used to dichotomize age as individuals younger and older than 43 years old. The 75th percentile for time lived in an endemic area (36 years) was used to dichotomize this variable as less or more than 36 years.

Unadjusted and adjusted logistic regression models were constructed in a stepwise backward selection process and the selection of predictors was evaluated using the Akaike's information criteria. Variance inflation factors was used to determine collinearity. The Wilcoxon rank-sum (Mann-Whitney) and the Kruskal Wallis test were used to compare variables that were non-normally distributed. Percentages of categorical or binary variables were obtained using the chi square test and 95% CI were calculated using the Clopper-Pearson interval. A two-sided p-value of less than 0.05 was considered significant. All statistical analyses and graphs were done using the Stata statistical software package version 15 (Stata Corp, College Station, TX).
Table 1. Estimated seroprevalence, number of infected individuals and population at risk of infection in the study area of Washington Metropolitan Area.

<table>
<thead>
<tr>
<th>Country</th>
<th>Virginia</th>
<th>Maryland</th>
<th>Washington District of Columbia</th>
<th>Individuals at risk (a)</th>
<th>Chagas Prevalence (b)</th>
<th>Estimated Number of Infected individuals (c)</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mexico</td>
<td>14,908</td>
<td>14,819</td>
<td>8,507</td>
<td>88,366</td>
<td>0.78</td>
<td>688</td>
<td>0.096</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>251</td>
<td>858</td>
<td>258</td>
<td>2,284</td>
<td>0.17</td>
<td>4</td>
<td>0.002</td>
</tr>
<tr>
<td>Guatemala</td>
<td>4,495</td>
<td>10,596</td>
<td>2,635</td>
<td>42,160</td>
<td>1.12</td>
<td>473</td>
<td>0.046</td>
</tr>
<tr>
<td>Honduras</td>
<td>3,721</td>
<td>7,998</td>
<td>2,139</td>
<td>29,574</td>
<td>0.92</td>
<td>271</td>
<td>0.032</td>
</tr>
<tr>
<td>Nicaragua</td>
<td>1,301</td>
<td>4,743</td>
<td>859</td>
<td>11,352</td>
<td>0.52</td>
<td>59</td>
<td>0.012</td>
</tr>
<tr>
<td>Panama</td>
<td>866</td>
<td>1,268</td>
<td>742</td>
<td>5,218</td>
<td>0.52</td>
<td>27</td>
<td>0.006</td>
</tr>
<tr>
<td>El Salvador</td>
<td>27,269</td>
<td>52,615</td>
<td>16,611</td>
<td>173,849</td>
<td>1.30</td>
<td>2,255</td>
<td>0.188</td>
</tr>
<tr>
<td>Argentina</td>
<td>490</td>
<td>2,841</td>
<td>1,134</td>
<td>8,177</td>
<td>3.64</td>
<td>298</td>
<td>0.009</td>
</tr>
<tr>
<td>Bolivia</td>
<td>2,747</td>
<td>5,356</td>
<td>591</td>
<td>31,218</td>
<td>6.10</td>
<td>1,906</td>
<td>0.034</td>
</tr>
<tr>
<td>Chile</td>
<td>365</td>
<td>2,407</td>
<td>697</td>
<td>5,713</td>
<td>0.70</td>
<td>40</td>
<td>0.006</td>
</tr>
<tr>
<td>Colombia</td>
<td>1,452</td>
<td>7,228</td>
<td>1,982</td>
<td>17,122</td>
<td>0.96</td>
<td>164</td>
<td>0.019</td>
</tr>
<tr>
<td>Ecuador</td>
<td>744</td>
<td>3,492</td>
<td>707</td>
<td>7,941</td>
<td>1.38</td>
<td>110</td>
<td>0.009</td>
</tr>
<tr>
<td>Paraguay</td>
<td>58</td>
<td>828</td>
<td>161</td>
<td>1,597</td>
<td>2.13</td>
<td>34</td>
<td>*</td>
</tr>
<tr>
<td>Peru</td>
<td>4,068</td>
<td>12,005</td>
<td>1,482</td>
<td>30,872</td>
<td>0.44</td>
<td>136</td>
<td>0.033</td>
</tr>
<tr>
<td>Uruguay</td>
<td>219</td>
<td>727</td>
<td>216</td>
<td>1,755</td>
<td>0.24</td>
<td>4</td>
<td>0.002</td>
</tr>
<tr>
<td>Venezuela</td>
<td>471</td>
<td>1,704</td>
<td>596</td>
<td>4,620</td>
<td>0.71</td>
<td>33</td>
<td>0.005</td>
</tr>
<tr>
<td>Total</td>
<td>14,908</td>
<td>129,485</td>
<td>99,866</td>
<td>461,818</td>
<td>6,501</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Prevalence (%) 1.41

a) Numbers of individuals at-risk of infection was based on the report of the American Community Survey (ACS) - U.S. Census Bureau, 2010\textsuperscript{22}.

b) Chagas prevalence in each endemic country was from the recent report of the WHO of Chagas prevalence in each endemic area in 2010\textsuperscript{59}.

c) Estimated number of infected individuals was calculated using the CDC approach by multiplying number of immigrants at-risk with prevalence of Chagas disease in each country of origin.\textsuperscript{9} * No participants from Paraguay were enrolled.
Results

A total of 1624 participants were enrolled; 46 were excluded because of missing data in more than two explanatory variables and 7 for having inconclusive serological results. Of the 1571 remaining participants, 1014 were from Tcl areas and 557 from TcII/TcV/TcVI areas (Figure 11). Among the 1571 individuals, 116 had previously been tested for Chagas disease and were excluded from further analysis. Including the ones with a previous Chagas diagnosis, a total of 183 cases of Chagas disease were detected. The unweighted prevalence of Chagas disease was 7.35% (107/1455), 79.44% (n=85) and 20.56% (n=22) were from TcII/TcV/TcVI and Tcl areas, respectively. The weighted seroprevalence following the distribution of the ACS was 4.3% (95% CI: 3.3%-5.6%).

Differences in demographic characteristics in their country of origin and socioeconomic status were observed between individuals from Tcl areas and TcII/TcV/TcVI areas. Participants from Tcl areas were significantly younger, had lower socioeconomic status (education lower than secondary school and speak only Spanish) in higher proportions than individuals from TcII/TcV/TcVI areas. Furthermore, individuals from Tcl areas were more likely to have lived in rural areas but less likely to have lived in a mudbrick/palm house, have seen the vector in an endemic area and have been tested for Chagas disease before (Table 2).

Because of the differences between participants from Tcl and TcII/TcV/TcVI areas, factors of infection were also evaluated by geographic area. Infection was more common among male participants in TcII/TcV/TcVI areas in the unadjusted and adjusted logistic regression model. Individuals that have lived in a rural area, have heard of Chagas disease or have had a family member with the disease before were more likely to have seropositive Chagas results but the difference was only statistically significant for individuals from TcII/TcV/TcVI areas. Social factors such as less than high school education or speaking only Spanish were only associated with infection in the unadjusted model but not in the adjusted logistic models. Only two factors: having lived for >36 years in an endemic area and having
seen the vector were associated with infection in the two geographic areas in the unadjusted and adjusted regression models (Table 3).

Differences in sex and number of years lived in an endemic area were found between infected and non-infected individuals that were evaluated by ECG. Years lived in an endemic area were significantly different between infected and non-infected participants from Tcl and TcII/TcV/TcVI areas. In addition, numbers of female were also different between infected and non-infected participants in the TcII/TcV/TcVI group. Odd ratios of cardiac abnormalities were adjusted by years lived in endemic areas in participants from Tcl areas, and by sex and years lived in endemic areas in participants from TcII/TcV/TcVI areas. Cardiac abnormalities that have been previously described in Chagasic patients were more common in infected than non-infected participants, but no statistical significance was detected after individual evaluation of each cardiac abnormality. Infected participants from Tcl areas had higher percentages of at least one cardiac abnormality associated with Chagas disease than participants from TcII/TcV/TcVI areas (Tcl: 5/11, 45.5%; TcII/TcV/TcVI: 14/78, 17.9%, p=0.0371). In addition, having at least one Chagas-like cardiac abnormality was significant higher in infected than non-infected individuals from Tcl areas (OR: 5.1, p-value: 0.014, adOR: 4.3, p-value: 0.048). Although the OR of having at least one cardiac abnormality was also higher in participants from TcII/TcV/TcVI areas, the difference was not statistically significant (Table 4) (Figure 12).
Figure 11. Flow chart of Study 1: Chagas disease and cardiac abnormalities in the Washington Metropolitan area.
Table 2. Characteristics of Latin American immigrants living in the Washington Metropolitan area by area of origin.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TcI areas (n=1014)</th>
<th>TcII/TcV/TcVI areas (n=557)</th>
<th>Total (n=1571)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Older than 43 years old</td>
<td>411 (40.5)</td>
<td>385 (69.1)</td>
<td>796 (50.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female sex</td>
<td>580 (57.2)</td>
<td>347 (62.3)</td>
<td>927 (59.0)</td>
<td>0.049</td>
</tr>
<tr>
<td>Lived more than 36 years in an endemic country</td>
<td>241 (23.8)</td>
<td>233 (41.8)</td>
<td>474 (30.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ever lived in rural area</td>
<td>479 (47.2)</td>
<td>155 (27.8)</td>
<td>634 (40.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ever lived in mud house</td>
<td>352 (34.7)</td>
<td>256 (46.0)</td>
<td>608 (38.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ever seen vector in endemic area</td>
<td>202 (20.0)</td>
<td>282 (50.6)</td>
<td>484 (30.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ever bitten by vector in endemic area</td>
<td>115 (11.3)</td>
<td>154 (27.7)</td>
<td>269 (17.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ever lived in an infested house</td>
<td>181 (17.9)</td>
<td>225 (40.4)</td>
<td>406 (25.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ever heard of Chagas disease</td>
<td>181 (17.9)</td>
<td>395 (70.9)</td>
<td>576 (36.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>If heard of Chagas before, family member with Chagas(^a)</td>
<td>15 (8.3)</td>
<td>143 (36.2)</td>
<td>158 (27.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>If heard of Chagas, ever tested for Chagas</td>
<td>13 (7.2)</td>
<td>103 (26.1)</td>
<td>116 (20.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>If tested, ever informed to be Chagas positive</td>
<td>5 (38.5)</td>
<td>59 (57.3)</td>
<td>64 (55.2)</td>
<td>0.199</td>
</tr>
<tr>
<td>If tested, previous false positive and negative results compared to conventional diagnosis</td>
<td>3 (23.0)</td>
<td>19 (18.4)</td>
<td>22 (19.0)</td>
<td>0.887</td>
</tr>
<tr>
<td>Less than secondary school(^b)</td>
<td>457 (45.7)</td>
<td>76 (13.9)</td>
<td>533 (34.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Speak only Spanish(^c)</td>
<td>547 (54.8)</td>
<td>149 (28.7)</td>
<td>696 (45.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Do not have health insurance(^d)</td>
<td>733 (72.3)</td>
<td>336 (60.3)</td>
<td>1069 (68.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Discordant results(^e)</td>
<td>3 (0.3)</td>
<td>4 (0.7)</td>
<td>7 (0.4)</td>
<td>0.225</td>
</tr>
<tr>
<td>Country of origin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peru</td>
<td>-</td>
<td>76 (13.6)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Bolivia</td>
<td>-</td>
<td>452 (81.2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Other South American countries(^f)</td>
<td>-</td>
<td>29 (5.2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>136 (13.4)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>El Salvador</td>
<td>535 (52.8)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Guatemala</td>
<td>182 (17.8)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Honduras</td>
<td>126 (12.4)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Other Central American and northern South American countries(^g)</td>
<td>35 (3.5)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Missing data for 46 participants. \(^b\) Missing data for 42 participants. \(^c\) Missing data for 54 participants.
\(^d\) Missing data for 77 participants. \(^e\) Discordant results between TESA-blot and Chagatest Wiener lysate among individuals without history of anti-parasitic treatment. \(^f\) Other Southern countries included: Argentina, Brazil, Chile, Ecuador and Peru. \(^g\) Other Central America and northern South American countries: Belize, Colombia, Costa Rica, Nicaragua, Panama and Venezuela.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TcI areas (Infected=22, Non-infected=979)</th>
<th></th>
<th>TcII/TcV/TcVI (Infected=85, Non-Infected=369)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted</td>
<td>Adjusted*</td>
<td>Unadjusted</td>
<td>Adjusted**</td>
</tr>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>p value</td>
<td>OR</td>
</tr>
<tr>
<td>Older than 43 years old</td>
<td>2.2</td>
<td>0.91-5.09</td>
<td>0.080</td>
<td>1.0</td>
</tr>
<tr>
<td>Female sex</td>
<td>1.1</td>
<td>0.452-2.54</td>
<td>0.882</td>
<td>1.1</td>
</tr>
<tr>
<td>Lived more than 36 years in an endemic country</td>
<td>3.4</td>
<td>1.43-7.83</td>
<td><strong>0.005</strong></td>
<td>3.3</td>
</tr>
<tr>
<td>Ever lived in rural area</td>
<td>2.0</td>
<td>0.83-4.79</td>
<td>0.124</td>
<td>1.6</td>
</tr>
<tr>
<td>Ever lived in mud house</td>
<td>2.3</td>
<td>0.90-5.35</td>
<td>0.056</td>
<td>2.1</td>
</tr>
<tr>
<td>Ever seen vector in endemic area</td>
<td>4.3</td>
<td>1.82-9.98</td>
<td><strong>0.001</strong></td>
<td>3.6</td>
</tr>
<tr>
<td>Ever bitten by vector in endemic area</td>
<td>1.2</td>
<td>0.79-1.95</td>
<td>0.359</td>
<td>1.1</td>
</tr>
<tr>
<td>Ever lived in an infested house</td>
<td>1.5</td>
<td>0.86-2.53</td>
<td>0.157</td>
<td>1.1</td>
</tr>
<tr>
<td>Ever heard of Chagas disease</td>
<td>1.9</td>
<td>0.73-4.90</td>
<td>0.190</td>
<td>1.4</td>
</tr>
<tr>
<td>If heard of Chagas before, family member with Chagas</td>
<td>1.0</td>
<td>0.63-1.49</td>
<td>0.879</td>
<td>0.9</td>
</tr>
<tr>
<td>Less than secondary school</td>
<td>2.8</td>
<td>1.07-7.36</td>
<td><strong>0.036</strong></td>
<td>2.2</td>
</tr>
<tr>
<td>Speak only Spanish</td>
<td>3.3</td>
<td>1.11-10.0</td>
<td><strong>0.032</strong></td>
<td>2.4</td>
</tr>
<tr>
<td>Do not have health insurance</td>
<td>1.1</td>
<td>0.87-1.37</td>
<td>0.446</td>
<td>1.1</td>
</tr>
</tbody>
</table>

95% CI: 95% confidence interval. OR: Unadjusted odds ratio. adOR: Adjusted odds ratio.

* Adjusted by time lived in endemic area and ever seen the vector. ** Adjusted by time lived in endemic area, sex, ever lived in rural area, ever seen the vector, and ever heard of Chagas.
Table 4. Characteristics and cardiac abnormalities associated with Chagas disease in infected individuals without previous diagnosis.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>TcI areas</th>
<th>TcII, TcV/TcVI areas</th>
<th>p-value</th>
<th>adOR</th>
<th>p-value</th>
<th>TcI, TcII, TcV/TcVI areas</th>
<th>p-value</th>
<th>adOR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected (n=65)</td>
<td>Infected (n=11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Non-infected (n=119)</td>
<td>Infected (n=78)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median Age (IQR)</td>
<td>39.9 (33.1-48.9)</td>
<td>43.2 (35.9-47.4)</td>
<td>0.118</td>
<td>1.2*</td>
<td>0.673</td>
<td>48.2 (39.2-57.5)</td>
<td>52.0 (44.4-58.1)</td>
<td>0.005</td>
<td>1.5</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>42 (64.6)</td>
<td>4 (36.4)</td>
<td>0.076</td>
<td>1.1</td>
<td>0.810</td>
<td>79 (68.1)</td>
<td>38 (49.3)</td>
<td>0.009</td>
<td>0.5</td>
</tr>
<tr>
<td>Median years lived in endemic area, (IQR)</td>
<td>25.0 (20.0-33.0)</td>
<td>35.0 (22.0-38.0)</td>
<td><strong>0.039</strong></td>
<td>3.1**</td>
<td><strong>0.008</strong></td>
<td>31.0 (24.5-39.5)</td>
<td>36.0 (27.0-42.5)</td>
<td>0.166</td>
<td>1.8</td>
</tr>
<tr>
<td>Median Square Root HR (IQR)</td>
<td>8.1 (7.8-8.4)</td>
<td>8.2 (7.4-8.5)</td>
<td>0.690</td>
<td>0.9</td>
<td>0.915</td>
<td>8.0 (7.5-8.5)</td>
<td>7.9 (7.5-8.3)</td>
<td>0.146</td>
<td>0.8</td>
</tr>
<tr>
<td>RBBB, n (%)</td>
<td>2 (3.1)</td>
<td>1 (9.1)</td>
<td>0.343</td>
<td>3.2</td>
<td>0.389</td>
<td>5 (4.2)</td>
<td>4 (5.1)</td>
<td>0.761</td>
<td>1.0</td>
</tr>
<tr>
<td>LAFB, n (%)</td>
<td>2 (3.1)</td>
<td>2 (18.2)</td>
<td><strong>0.038</strong></td>
<td>3.3</td>
<td>0.306</td>
<td>6 (5.0)</td>
<td>3 (3.9)</td>
<td>0.694</td>
<td>0.7</td>
</tr>
<tr>
<td>PVC, n (%)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>3 (2.6)</td>
<td>4 (5.2)</td>
<td>0.336</td>
<td>1.5</td>
</tr>
<tr>
<td>Bradycardia, n (%)</td>
<td>3 (4.6)</td>
<td>1 (9.1)</td>
<td>0.529</td>
<td>2.3</td>
<td>0.509</td>
<td>4 (3.5)</td>
<td>4 (5.2)</td>
<td>0.551</td>
<td>1.0</td>
</tr>
<tr>
<td>Atrial ectopic, n (%)</td>
<td>1 (1.5)</td>
<td>1 (9.1)</td>
<td>0.148</td>
<td>12.25</td>
<td>0.096</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>1° atrioventricular block, n (%)</td>
<td>0 (0.0)</td>
<td>2 (18.2)</td>
<td>&lt;0.001</td>
<td>***</td>
<td>***</td>
<td>4 (3.4)</td>
<td>2 (2.6)</td>
<td>0.750</td>
<td>****</td>
</tr>
<tr>
<td>Q-waves, n (%)</td>
<td>1 (1.5)</td>
<td>0 (0.0)</td>
<td>0.679</td>
<td>***</td>
<td>***</td>
<td>0 (0.0)</td>
<td>1 (1.3)</td>
<td>0.216</td>
<td>***</td>
</tr>
<tr>
<td>LVH, n (%)</td>
<td>0 (0.0)</td>
<td>1 (9.1)</td>
<td>0.014</td>
<td>***</td>
<td>***</td>
<td>1 (0.8)</td>
<td>2 (2.6)</td>
<td>0.334</td>
<td>1.7</td>
</tr>
<tr>
<td>RBBB+LAFB, n (%)</td>
<td>0 (0.0)</td>
<td>1 (9.1)</td>
<td>0.014</td>
<td>***</td>
<td>***</td>
<td>2 (1.7)</td>
<td>2 (2.6)</td>
<td>0.667</td>
<td>1.1</td>
</tr>
<tr>
<td>One or more abnormalities, n (%)</td>
<td>9 (14.1)</td>
<td>5 (45.5)</td>
<td>0.014</td>
<td>4.3</td>
<td>0.048</td>
<td>16 (13.8)</td>
<td>14 (18.2)</td>
<td>0.410</td>
<td>1.0</td>
</tr>
<tr>
<td>Two or more abnormalities, n (%)</td>
<td>0 (0.0)</td>
<td>3 (27.3)</td>
<td>&lt;0.001</td>
<td>***</td>
<td>***</td>
<td>5 (4.3)</td>
<td>5 (6.5)</td>
<td>0.503</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Age was used as a binary variable (older and younger than 43 years old) in the logistic model.
** Years lived in an endemic area was used as a binary variable (less or more than 36 years) in the logistic regression model.
*** Cannot be determined. **** Omitted because of collinearity.

a) p-value of a Pearson’s chi-squared test. b) Adjusted by time lived in endemic area. c) Adjusted by age and sex.
IQR: Interquartile range, OR: Unadjusted odds ratio, adOR: Adjusted odds ratio, HR: Heart rate in bpm, RBBB: Right bundle branch block, LAFB: Left anterior fascicular block (LAFB), PVC: Premature ventricular contractions, LVH: Left ventricular hypertropia.
Discussion
This study shows a higher prevalence of Chagas disease in the Washington Metropolitan Area than was estimated using the CDC approached (4.3 vs 1.4%), and it was also higher than the prevalence reported in Los Angeles county (1.3%). Differences in demographics and socioeconomic background of immigrants living in the WMA may explain these results. A large percentage of participants in this study were from rural areas (47.2% of those from TcI countries and 27.8% from TcII/TcV/TcVI countries), greater than the study in Los Angeles county (20.0%). Furthermore, most participants in this study were from El Salvador and other Central American countries which are the predominant groups in the WMA and where Chagas disease is more prevalent. Conversely, participants in Los Angeles were mainly Mexicans which is the predominant Latin American group in that area and where Chagas disease is less prevalent.

This is the first study that used two ELISA tests (lysate-based and recombinant-based) run in parallel for initial screening, followed by the TESA-blot as a confirmatory test. The study conducted in Los Angeles was done using a recombinant-based enzyme-linked immunosorbent assays for initial testing and positive results were later confirmed by RIPA (radioimmune precipitation assay) or IFI (immunofluorescence antibody assay). The use of recombinant-based assays as a first tool of screening may lead to an underestimation of the infection in individuals from Mexico and Central America since previous publications have shown lower levels of antibodies to recombinant-based ELISAs in these populations.60

Having seen the vector in an endemic area was the only risk factor that reached statistical significance in the two endemic groups. In our experience, specifying where participants had seen the vector was an important question to determine if they are referring to triatomines. Most participants described having seen the vector in the U.S. because of the similarities with abundant bugs in the area including bed bugs and stink bugs. Like the study in Los Angeles county, participants from TcI areas that had lived in a rural area or in a mud/adobe house were more likely to be infected, but the difference did not reach statistical significance. This
could be attributed to multiple potential reasons: the small number of infected participants, the fact that most participants in TcI areas are from rural areas, and not all rural areas are endemic for Chagas disease. Different from the study in Los Angeles, risks factors related to Chagas knowledge were only significant in participants from TcI/TcV/TcVI areas. In our study, we removed participants that have been diagnosed with Chagas disease before because these participants may have received information about the disease at the time of previous diagnosis and may be more likely to recall other risk factors such as house infestation and family members with the disease. In our study, most participants in the TcII/TcV/TcVI group were from Bolivia, where Chagas disease is more common and education and screening for the disease occurs more often than in other endemic countries.

ECG abnormalities that are known to be associated with Chagas disease were predominant in the infected group, but only left anterior fascicular block (LAFB), first degree atrioventricular block and left ventricular hypertrophy reached statically significance when evaluated individually in participants from TcI. Participants from TcI areas were also younger and had higher percentages of cardiac abnormalities than the ones from TcII/TcV/TcVI group. This could be attributed to the TcI genotype that has been associated with more severe forms of cardiac disease in Central America and Colombia. In the hospital-based study done in Los Angeles, patients with Chagas cardiomyopathy have mortality rates or require heart transplantsations in higher proportions than patients with non-Chagas cardiomyopathy (hazard ratio [HR]=4.46; 96% CI: 1.8-10.8). Early detection of the infection and annual ECG analysis is recommended by the CDC in the U.S. However, due to legal and socioeconomic factors and lack of awareness about the disease in the healthcare systems, individuals are usually not screened, and when they are diagnosed, they are commonly informed by health professionals that because of the lack of effective treatment no further follow-up is needed since the disease is “silent”.

46
This study has some limitations. A convenience sample was used due to the difficulties of accessing this population in the U.S. To decrease the potential for participation bias, education was provided before enrollment. Furthermore, as requested by community leaders, Chagas testing was performed together with other better-known screening tests including glucose testing and blood pressure measurement, and enrollment was conducted in different community events including daily consulate activities where healthy Latin Americans from different socioeconomic backgrounds attend for procedures related to legal paperwork. Other limitations is that the prevalence of the infection was weighted using the distribution of country of origin reported by the ACS. However, undocumented individuals are usually underrepresented in census surveys and different consulates reported a higher number of specific groups than the ones reported by the ACS.

In conclusion, this study shows high percentages of T. cruzi infection in Latin Americans leaving in the WMA. We showed for the first time that having seen the vector in an endemic area is an important factor associated with infection in immigrants from both geographic groups. Interestingly, this risk factor has not been evaluated in previous studies in the U.S. Early cardiac abnormalities in asymptomatic infected individuals were 4.3 higher than non-infected individuals from Tcl areas (adOR: 4.3, p-value: 0.048) and may be present more often in asymptomatic Central American and Mexican immigrants due to the higher prevalence of Tcl genotype in this group.

Abstract
Background: The high variability of individuals at-risk of Trypanosoma cruzi infection in the U.S brings challenges in the diagnosis. We evaluated the accuracy of FDA-approved assays when they are used alone and in combination with other tests in a community-based study in the Washington Metropolitan area (WMA).

Methods: A cross-sectional study of 1093 individuals living in the WMA and born in Chagas endemic countries was conducted in community centers; 745 were from areas where T. cruzi Tcl is predominant (Mexico, Central America and northern South America) and 354 from TclI/TcV/TcVI areas (southern South America). The combination of the Western Blot using the Trypomastigote Excretory- Secretory Antigen (TESA-blot) and the Chagatest Wiener lysate ELISA was used as gold standard. Three FDA-approved tests were evaluated: the lysate-based ELISA Hemagen Chagas, and two recombinant based assays: Chagatest Wiener recombinant v.3.0 ELISA and the rapid test Chagas Detect Plus (CDP).

Findings: Recombinant-based tests provided low sensitivity (Wiener recombinant v.3.0: Tcl areas: 80.95%, non-Tcl areas: 89.47%; CDP: Tcl areas: 90.00%, non-Tcl areas: 92.31%). The Hemagen provided high sensitivity (100% in the two areas) but lower specificity (Tcl areas: 88.67%, non-Tcl areas: 87.64%) with high percentages of indeterminate results (Tcl areas: 5.64%, non-Tcl areas: 1.98%). The use of the Hemagen and the Wiener recombinant v.3.0 in parallel provided the best sensitivity (Tcl areas: 80.95%, non-Tcl areas: 92.31%) and specificity (Tcl areas: 99.73%, non-Tcl areas: 100.00%) but 10.85% and 12.06% of samples required further confirmation by a third test in non-Tcl and Tcl areas, respectively. Lower antibody levels and band intensity in the ELISAs and the rapid test were observed in infected individuals from Tcl areas compared to non-Tcl areas (p<0.001). Individuals with low antibody levels were more likely to react to the 150-160 kDa band only in the TESA-blot (p<0.001).

Interpretation: Better diagnostic tests are needed to improve the diagnosis of Chagas disease in the U.S. More robust and specific lysate-based tests are required to decrease indeterminate and false positive results. Recombinant-based tests need to be adapted to the antigenic repertoire and low levels of antibodies in patients from Tcl areas.
Introduction

The Latin American population represents 17.8% of the U.S population, at an estimated 57.5 million people, 78.3% (45.0 million) of whom are from areas where Chagas Disease is endemic. Recent calculations reported 238,091 estimated cases (estimated prevalence 1.03%) of T. cruzi infection in the U.S, resulting in 30,000-45,000 cases of cardiomyopathy yearly; though difficulties in assessment of the undocumented likely lead to an underestimation of the disease burden.

Chagas disease is one of the five most important neglected parasitic infections targeted by the Centers for Disease Control and Prevention (CDC) in the U.S, where it causes a total annual and lifetime health-care cost of $15,762 ($13,249-17,442) and $91,531 ($42,992-149,333), respectively, and a total Disability-Adjusted Life Years (DALYs) lost per infected individual of 3.57 (1.18-5.85) mainly from impaired productivity due to cardiovascular disease-induced early death.

Trypanosoma cruzi is comprised of a highly diverse population of seven Discrete Typing Units (DTUs) whose prevalence varies by geographic location. TcI predominates from the South of the U.S to northern South America (including Mexico, Central America, Colombia and Venezuela), while TcII/TcV/TcVI prevail in southern South America. Differences in the immune response by geographic area have also been reported. Lower levels of antibodies to TcII lysate antigens and recombinant proteins have been observed in individuals from Central America as compared to South Americans. Differences in the immune response of human peripheral blood mononuclear cells under stimulation with TcI and TcII antigens were observed. TcI produced higher monocyte activation, and anti-inflammatory cytokines (IL-10 and IL-17), while TcII induced inflammation through the production of TNF-alpha and granzyme A. A study conducted by our research group in Arequipa, Peru and Santa Cruz, Bolivia showed lower antibody response associated with lower sensitivity to recombinant-based assays and lower secretion of interferon-γ in the Peruvian group as compared to Bolivians.
The World Health Organization (WHO) recommends the use of at least two different serological tests based on different antigens or platforms to determine the diagnosis; discordant results require further evaluation by a confirmatory test. Different FDA-approved tests are available in the U.S. with sensitivities and specificities higher than 95% based on the information provided by the manufacturer. A western blot using the Trypomastigote Excretory-Secretory Antigen (TESA-Blot) is one of the confirmatory tests; however, due to the absence of a commercial TESA-blot, the test is only performed by the CDC and research laboratories. FDA-approved diagnostic tests have been mainly evaluated in South America. Evaluations in Mexico and Central America have mainly been done using repository samples that may provide inaccurate results and inadequate representation of at-risk populations.

The Washington Metropolitan Area (WMA) is one of the 15 metropolitan areas in the U.S. with the largest number of Latin American immigrants, accounting for 13.8% (770, 795) of the total population not including undocumented individuals. The Latin American population in the WMA is remarkable for the diversity of countries of origin; mainly composed of Salvadoran (5.2%), Mexican (2.3%), other Central Americans (2.3%) and South Americans (2.9%), including the largest number of immigrants from Bolivia (0.7%) in the U.S. This is significant, as Bolivia is the country with the highest prevalence of Chagas disease in the world. The great diversity of individuals leaves challenges in diagnosis, clinical manifestations and treatment of the infection. In the U.S., community-based studies are needed to have a better representation of the Latin American group since reaching this population through the health system is a challenge due to legal status, low access to health insurance, socioeconomic barriers, cultural beliefs, and language barriers.

To our knowledge this is the first study evaluating available diagnostic tests in a non-endemic country using samples obtained from a community-based study of Latin American immigrants. In this population, we evaluated the accuracy of FDA-approved diagnostic tests and provide advantages and limitations when they are used as the only tool for screening.
and in combination with other tests. Differences in levels of antibodies to each ELISA (Enzyme-Linked Immunosorbent Assay) and pattern of antigenic bands observed in the TESA-blot by geographic area are presented.
Methods

Study design, enrollment and participants

A cross-sectional study was conducted from February 2016 to April 2018 in the WMA. Latin American individuals older than 18 years old from any of the 21 Chagas endemic countries were enrolled via recruitment in churches, community centers, consulate events and health fairs. Education about the disease was performed before enrollment to increase participation of individuals who lack knowledge about the disease. Education consisted of workshops, distribution of educational brochures, and interviews in the media (journals, television and radio programs) directed to the Latin American community (Supplementary material 1). After consenting, blood samples from fingersticks were taken in the field using the FDA-cleared rapid test (Chagas Detect Plus rapid test, InBios, U.S.) (Figure 13).

To determine specificity and assay validation, capillary and venous blood samples of (n=200) U.S residents older than 18 years old without Latin American origin and travel history to an endemic area were obtained. Healthy volunteers attending the Center for Immunization Research for participation in vaccine trials and students/staff members from the Johns Hopkins Bloomberg School of Public Health (JHSPH) were enrolled. In addition, positive serum samples (n=49) of infected individuals detected in the U.S were provided by the CDC, 40 were from TcI areas and 9 were from TcII/TcV/TcVI areas.

The institutional review board of the JHSPH approved the protocols (IRB 6325, 6713, and 6854). All participants provided written informed consent for their participation.
Figure 12. Performing the rapid test Chagas Detect Plus in the community.

Serum samples

Venous blood samples were drawn from all individuals. Within 4 to 6 hours of collection, blood samples were transported to the laboratory at JHSPH in ice pads. Serum was separated by centrifugation at 3500 g, 4 °C for 15 minutes. Four aliquots of serum were obtained and frozen immediately at -20 °C. Serological assays were performed within 7-15 days of sample preservation at -20 °C. Samples were tested by all assays at the same time to prevent differences in results due to freeze-thaw cycles; samples were maintained at 4 °C until all tests were completed and for a maximum of 7 days. When a repeat was needed, a new aliquot of serum was used.
Serological tests

Three FDA-approved tests were evaluated against the results of the two confirmatory tests, all the assays were run in parallel.

Two FDA-approved ELISAs were performed following the instructions of the manufacture by a trained microbiologist with master’s degree. These tests included (sensitivity and specificity are provided based on the manufacture’s information):

1) The Chagatest ELISA Recombinante v.3.0 (Wiener Laboratories SAIC, Argentina). An ELISA test with 1, 2, 13, 30, 36 and SAPA recombinant antigens. Sensitivity of 99.3% and specificity of 98.7%.

2) The Hemagen Chagas kit (Hemagen Diagnostics, Inc, U.S). An ELISA test with lysate antigen of the epimastigote and amastigote forms of *T. cruzi* strains Y, TcII and CL, Tc VI. Sensitivity of 100% and specificity of 98.7%.

The cutoffs for positive, indeterminate and negative results were calculated following the instructions of the manufacturer. Samples were categorized as positives (OD values higher than the cutoff of positivity), indeterminates (OD values lower or equal than the cutoff of positivity but higher than the cutoff of negativity) or negatives (OD values lower than the cutoff of negativity).

The rapid test, Chagas Detect Plus (CDP) (Inbios International, Inc, U.S), a lateral flow immunochromatographic assay was evaluated using capillary and/or serum blood samples. This assay is based on the ITC8.2 multiepitope fusion antigen. Its sensitivity is 95.1% and specificity is 98.7%.
When serum samples were used, the rapid test was read by two different trained scientists with medical degrees but no laboratory experience. Band intensity in positive samples was determined by the two observers using a home-made intensity card with 6 categories (Figure 14). The mean value of band intensity obtained by the two scientists was used for subsequent analysis. Samples with any reactions in the test line were considered positive as described by the manufacturer.

![Home-made intensity card to determine degree of reaction in the test line in both rapid tests.](image)

The TESA-blot and the commercial Chagatest Wiener ELISA with lysate antigen (Wiener Laboratories SAIC, Argentina) were used as confirmatory tests. Samples were considered truly positive or negative when congruent negative and positive results were obtained using both confirmatory tests, respectively. Samples with discordant results between the confirmatory tests were removed from the analysis.

The TESA-blot using the TESA from *T. cruzi* Y strain was developed following published procedures. Reactions to the band of 150-160 kDa and ladder-like bands of 130-200 kDa were considered specific for *T. cruzi* infection. The test was performed by a microbiologist with master’s degree and was read by an experienced clinical microbiologist in parasitic infections.
**Epidemiological data**

A validated questionnaire standardized in Santa Cruz, Bolivia was used to obtain epidemiological data including place of origin, previous diagnosis and treatment of Chagas disease, and time living in an endemic area. The questionnaire was mainly self-administered by the participants and mainly in Spanish. In cases of illiterate participants, a study volunteer administered the questionnaire. Individuals with a history of anti-parasitic treatment were removed from the analysis since treatment may affect the levels of antibody response and accuracy of serological tests. Participants were classified in two groups based on their country of origin. Participants from Mexico, El Salvador, Guatemala, Honduras and other Central America countries (Belize, Nicaragua, Panama, Costa Rica) and northern South American countries (Colombia and Venezuela) were classified as TcI areas, and participants from Peru, Bolivia and other southern countries (Argentina, Brazil, Chile, and Ecuador) were classified as TcII/TcV/TcVI areas.

**Statistical analysis**

The chi square-test or Fisher exact test was used to determine associations with categorical variables between groups. The Wilcoxon rank-sum (Mann-Whitney) and the Kruskal Wallis test were used to compare variables that were non-normally distributed. Percentages of sensitivity and specificity were obtained using the chi square test and 95% CI were calculated using the Clopper-Pearson interval. The agreement between the two readers of the rapid test was evaluated using the Kappa coefficient following Landis and Koch interpretation: Excellent (1.00–0.81), good (0.80–0.61), moderate (0.60–0.41), weak (0.40–0.21) and negligible agreement (0.20-0.00). A two-sided p-value of less than 0.05 was considered significant. All statistical analyses and graphs were done using the Stata statistical software package version 15 (Stata Corp, College Station, TX).
Results

An internal validation of serological assays was done using 200 serum samples of American residents without risk of exposure to *T. cruzi*. All tests (Hemagen, Wiener recombinant v3.0, CDP in whole blood and serum, and Wiener lysate) had a specificity of 100% (95% CI: 98.17%-100.00%). Three participants had reactions to the six ladder-like bands of 130-200 kDa in the TESA-blot giving a specificity of 98.50% (95% CI: 95.68%- 99.69%). The internal sensitivity of the tests was evaluated using 49 positive serum samples provided by the CDC. The Hemagen, Wiener lysate, Wiener recombinant v3.0 and CDP in serum had similar sensitivities of 97.96% (95% CI: 89.15%-99.94%); the sensitivity of the TESA-blot was 100.00% (95% CI: 92.75%-100%).

A total of 1124 serum samples from individuals from endemic areas (66.46%, n=747 from TcI areas and 33.54%, n=377 from TcII/TcV/TcVI areas) were used in this evaluation (Figure 15). Individuals from TcI areas were significantly younger and had spent less time in endemic areas compared to those from TcII/TcV/TcVI areas. More females participated from TcII/TcV/TcVI areas; no differences in participation by gender was seen in individuals from TcI areas. No significant differences were observed in the percentage of discordant results in the two confirmatory tests among individuals without previous anti-*T. cruzi* treatment in the two areas, discordant results were excluded for further evaluations (Table 5).

We detected 22 and 115 seropositive participants by the two confirmatory tests from TcI and TcII/TcV/TcVI areas, respectively. Of those, one participant from TcI regions and 20 from TcII/TcV/TcVI areas had previously received anti-*T. cruzi* treatment and were excluded from further evaluations since treatment may affect antibody levels. Table 6 shows the performance of each test compared to the results of the confirmatory tests. Lower sensitivity and specificity were observed in TcI areas using recombinant-based tests and the lysate-based Hemagen ELISA as compared to TcII/TcV/TcVI areas, but these differences did not reach statistical significance. The specificity of the Hemagen improved after samples with initial indeterminate results were re-tested at least two more times as recommended by the
manufacturer (from 87.64%-88.67% to 92.27%-92.40%) without affecting the sensitivity of the test.

Excellent agreement (kappa index 0.93, p<0.001) was obtained between the two readers of the CDP performed on serum samples by two different analysts in a laboratory setting. Less agreement was found between the results in serum and capillary blood (0.80, p<0.001). We observed higher specificity in both areas (88.93%-90.17% VS 78.06%-80.85%) and sensitivity (100% VS 90.00%-92.31%) when the CDP was used in capillary blood versus serum, respectively.

Infected individuals from Tcl areas had significantly lower OD values in the three types of ELISA tests as well as lower band intensity in the CDP (Figure 16). In the TESA-blot, a higher percentage of individuals from Tcl areas showed antigenic reaction to the 150-160 kDa band only (40.00%, 8/20, 95% CI: 18.53-61.47) compared to individuals from TcII/TcV/TcVI areas (14.89%, 14/94, 95% CI: 7.70%-22.09%, p-value: 0.009). Lower OD values in the three ELISA tests were significantly associated with the presence of the 150-160 kDa band only (Figure 17).

Table 7 shows the frequency of indeterminate and discordant results using each of the tests individually and in combination with another assay. If the Wiener recombinant v.3.0 is used on its own, it missed the diagnosis in 8.70%-18.19% of truly infected individuals. Using the Chagas Hemagen ELISA only, all infected individuals were diagnosed, but 5.36%-6.67% of non-infected individuals were initially classified as positive and an additional 1.87%-5.63% had indeterminate results. The combination of the Hemagen ELISA and the Wiener recombinant v.3.0 diagnosed all of the truly infected individuals and decreased the percentage of false positive results, however, 12.06%-12.27% of samples required confirmation by a third test. The greatest percentage of samples that required confirmation by a third test was when the Hemagen and the CDP were used together (20.29%) but none of the true positives were missed using this combination. In addition, the combination of the
Wiener recombinant v3.0 and the CDP missed the diagnosis in 3.67%-9.52% of infected individuals, the majority of whom were individuals from Tcl areas due to the lower level of antibodies to recombinant antigens in this area.
Figure 14. Flow chart of the study 2: “Challenges in the diagnosis of Chagas disease in the U.S: Experience from a community-based study in the Washington Metropolitan area”.

CDP: Chagas Detect Plus.
Figure 15. Distribution and median of values of ELISA optical density (OD minus cut-off value) and band intensity of confirmed positive patients without previous anti-parasitic treatment by geographic area.

Black bars represent interquartile range, median is represented by a white line. p-values were obtained using the Kruskal Wallis test. OD values obtained using: A). Chagatest Wiener lysate ELISA, B). Hemagen Chagas EIA based on lysate antigen, and C). Chagatest Wiener recombinant v.3.0 and D.) Band intensities observed in the rapid test Chagas Detect Plus.
Figure 16. Band patterns observed in the Trypomastigote Excretory-Secretory Antigen Western Blot (TESA-blot).

A1. Infected individuals with low levels of antibodies were more likely to show reactions to the 150-160 kDa band only (40.00%, 8/20, 95% CI: 18.53-61.47, p-value: 0.009). A2. Individuals with higher levels of antibodies showed reactions to the 150-160 kDa band and the 130 kDa-200 kDa ladder-like bands and only 14.89%, 14/94, 95% CI: 7.70%-22.09% had reactions to the 150-160 kDa band only.

B. Distribution and median of ELISA optical density (OD minus cut-off value) of confirmed positive patients without previous anti-parasitic treatment by type of band in the TESA-blot. Bars represent interquartile range, median is represented by a gray line. p-values were obtained using the Kruskal Wallis test.
### Table 5. Characteristics of Latin American immigrants living in the Washington Metropolitan area by area of origin.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Tcl areas (n=747)</th>
<th>TcII, TcV, TcVI areas (n=377)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, (IQR)</td>
<td>39.6 (32.3-48.0)</td>
<td>49.1 (41.1-57.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>% Female sex, (95% CI)</td>
<td>56.8 (53.1-60.4)</td>
<td>64.6 (59.5-69.4)</td>
<td>0.012</td>
</tr>
<tr>
<td>Median years lived in endemic area,</td>
<td>25.0 (20.0-34.0)</td>
<td>32.0 (25.0-40.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have been tested for Chagas before,</td>
<td>1.9 (1.0-3.2)</td>
<td>23.81 (19.6-28.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>% (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>If have been tested, have received a</td>
<td>23.08 (5.0-53.8)</td>
<td>57.7 (46.4-68.3)</td>
<td>0.034</td>
</tr>
<tr>
<td>positive result, % (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>If tested positive, have received anti-</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>T. cruzi treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Discordant results, % (95% CI) *</td>
<td>0.8 (0.2—1.4)</td>
<td>2.0 (0.5-3.4)</td>
<td>0.095</td>
</tr>
<tr>
<td>% Country, (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peru</td>
<td></td>
<td>11.9 (8.8-15.6)</td>
<td>-</td>
</tr>
<tr>
<td>Bolivia</td>
<td></td>
<td>83.9 (79.8-87.4)</td>
<td>-</td>
</tr>
<tr>
<td>Other Southern countries**</td>
<td></td>
<td>4.2 (2.4-6.8)</td>
<td>-</td>
</tr>
<tr>
<td>Mexico</td>
<td>13.9 (11.5-16.6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>El Salvador</td>
<td>54.1 (50.4-57.7)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Guatemala</td>
<td>20.9 (18.0-24.0)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Honduras</td>
<td>8.0 (6.2-10.2)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Other Central America and northern</td>
<td>3.1 (2.0-4.6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>South American countries***</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IQR: Interquartile range. 95% CI: 95% confidence interval.

* Discordant results between TESA-blot and Chagatest Wiener lysate among individuals without history of anti-parasitic treatment.

** Other Southern countries included: Argentina, Brazil, Chile, Ecuador and Peru.

*** Other Central America and northern South American countries: Belize, Colombia, Costa Rica, Nicaragua, Panama and Venezuela.

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TcI areas</td>
<td>TcI, TcV, TcVI areas</td>
</tr>
<tr>
<td></td>
<td>Positive (n/N) % (95% CI)</td>
<td>Positive (n/N) % (95% CI)</td>
</tr>
<tr>
<td>Chagatest Wiener recombinant v.3.0</td>
<td>17/21 (81.0 (64.2-97.8))</td>
<td>85/95 (89.5 (83.3-95.6))</td>
</tr>
<tr>
<td>Hemagen Chagas 1st time sample evaluated*</td>
<td>21/21 (100.0 (83.9-100.0**))</td>
<td>95/95 (100.0 (96.2-100.0**))</td>
</tr>
<tr>
<td>Hemagen Chagas Conclusive results after repetitions****</td>
<td>21/21 (100.0 (83.9-100.0**))</td>
<td>94/95 (99.0 (96.8-100.0))</td>
</tr>
<tr>
<td>CDP in whole blood</td>
<td>18/20 (90.0 (76.9-100.0))</td>
<td>84/91 (92.3 (86.8-97.8))</td>
</tr>
<tr>
<td>CDP in serum</td>
<td>19/19 (100.0 (82.4-100.0))</td>
<td>39/39 (100.0 (91.0-100.0))</td>
</tr>
</tbody>
</table>

95% CI: 95% confidence interval.

* Results obtained for the first time the sample was evaluated by the test.

** One-sided, 97.5% confidence interval.

*** No difference was observed to test for statistical significance.

**** Results obtained after samples with indeterminate results were evaluated for at least two more times by the same test as recommended by the manufacture.
Table 7. Indeterminate and/or discordant results using FDA-approved tests by geographic area

<table>
<thead>
<tr>
<th>Test</th>
<th>Truly positives detected</th>
<th>Indeterminate or discordant</th>
<th>False positives</th>
<th>Required confirmation by a confirmatory test</th>
<th>Missed diagnosis in truly positives*</th>
<th>Truly positives detected</th>
<th>Indeterminate or discordant</th>
<th>False positives</th>
<th>Required confirmation</th>
<th>Missed diagnosis in truly positives*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wiener recombinant v.3.0</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
</tr>
<tr>
<td>81.0</td>
<td>0.3</td>
<td>0.5</td>
<td>3.1</td>
<td>19.1</td>
<td>89.4</td>
<td>1.1</td>
<td>0.0</td>
<td>24.9</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>Hemagen Chagas 1st time sample evaluated**</td>
<td>100.0</td>
<td>5.6</td>
<td>5.4</td>
<td>13.8</td>
<td>0.0</td>
<td>100.0</td>
<td>2.0</td>
<td>7.1</td>
<td>35.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Wiener recombinant v.3.0 and Hemagen Chagas 1st time sample evaluated**</td>
<td>81.0</td>
<td>11.8</td>
<td>0.3</td>
<td>12.1</td>
<td>0.0</td>
<td>92.3</td>
<td>10.9</td>
<td>0.0</td>
<td>10.9</td>
<td>0.0</td>
</tr>
<tr>
<td>(17/21)</td>
<td>(88/745)</td>
<td>(2/745)</td>
<td>(90/745)</td>
<td>(0/21)</td>
<td>(84/91)</td>
<td>(38/350)</td>
<td>(0/350)</td>
<td>(38/350)</td>
<td>(0/91)</td>
<td></td>
</tr>
<tr>
<td>Wiener recombinant v.3.0 and CDP in whole blood</td>
<td>80.0</td>
<td>11.6</td>
<td>0.3</td>
<td>11.9</td>
<td>10.0</td>
<td>85.6</td>
<td>9.9</td>
<td>0.0</td>
<td>9.9</td>
<td>4.4</td>
</tr>
<tr>
<td>(16/20)</td>
<td>(84/725)</td>
<td>(2/725)</td>
<td>(86/725)</td>
<td>(2/20)</td>
<td>(77/90)</td>
<td>(33/334)</td>
<td>(0/334)</td>
<td>(33/334)</td>
<td>(4/90)</td>
<td></td>
</tr>
<tr>
<td>Hemagen Chagas 1st time sample evaluated and CDP in whole blood</td>
<td>90.0</td>
<td>19.9</td>
<td>1.0</td>
<td>20.8</td>
<td>0.0</td>
<td>92.2</td>
<td>14.7</td>
<td>1.5</td>
<td>16.2</td>
<td>0.0</td>
</tr>
<tr>
<td>(18/20)</td>
<td>(144/725)</td>
<td>(7/725)</td>
<td>(151/725)</td>
<td>(0/20)</td>
<td>(83/90)</td>
<td>(49/334)</td>
<td>(5/334)</td>
<td>(54/334)</td>
<td>(0/90)</td>
<td></td>
</tr>
</tbody>
</table>

* Truly positive samples that were classified as negative.
** Results obtained for the first time the sample was evaluated by the test.
Discussion

This is the first study to demonstrate the limitations of some of the FDA-approved Chagas tests in a community-based study of at-risk individuals living in the U.S. We observed deficiencies in both recombinant- and lysate-based assays in terms of sensitivity and specificity. The diagnosis of Chagas disease in the U.S is initially done in clinical laboratories using the Hemagen Chagas EIA which is the most accessible FDA-approved test in this country. Although the test is highly sensitive, is based on lysate antigen, and based on the experience of different clinicians and on the results of this study, a large proportion of initial results were indeterminate or false positives. In the U.S., conclusive diagnosis of Chagas is usually provided only by the CDC. This creates logistical problems in the management of this population that lacks health insurance and are usually cared by community health centers or non-profit organizations with limited resources. In addition, positive or indeterminate results may create anxiety in immigrants that suffer from untreated mental health disorders at higher rates than the average population.64

Accurate diagnosis of Chagas disease is important because individuals younger than 50 years old are recommended to received anti-T. cruzi treatment in the U.S. to decrease the likelihood of developing cardiac disease, prevent congenital transmission and reactivation in immunosuppressed individuals.65,66 However, the treatment is not benign: 51.6% of adults receiving anti-T. cruzi drugs develop adverse events and 14.2% of them require treatment discontinuation to prevent serious adverse events including death.16,67 Benznidazole, one of the two drugs uses for anti-parasitic treatment, was approved for 2–12 years old children by the FDA in 2017, and can be prescribed and ordered off-label for other age groups using a central distributor.68 Rapid access to benznidazole may facilitate early treatment when needed but can also generate exposure to adverse events in individuals inaccurately believed to be infected. Having the CDC as the only source of confirmatory diagnosis is safer and manageable when only few cases are suspected but can lead to delays or complicate treatment.
The ORTHO T. cruzi ELISA Test System (Ortho-Clinical Diagnostics, Raritan, New Jersey) is an FDA-approved lysate-based assay used to test blood donors in the U.S. and provides robust results that are highly sensitive and specific.\textsuperscript{23,24} However, it can only be purchased as large number of kits and has an expiration time of 6 months which prevents its use in clinical and research laboratories.

Having a secondary test based on a different antigen or platform to increase the accuracy in the diagnosis is the recommendation of the WHO.\textsuperscript{41} However, the FDA-approved recombinant-based tests provide inaccurate diagnosis in this high variable population, probably because most recombinant antigens have been selected and evaluated in non-TcI DTUs samples, and the immune response to these antigens is lower in TcI areas. Another explanation is that the assays may be initially optimized using samples from non-TcI areas where high levels of antibodies to these antigens are presented and low antigen concentration is needed to discriminate between positive and negative samples providing high specificity in the two geographic areas but only high sensitivity in samples with greater antibody levels. The increase in antigen concentration especially using the Wiener recombinant v.3.0 could improve sensitivity without affecting specificity since the specificity with this test was high in both areas. The use of the Wiener recombinant v.3.0 combined with the Hemagen reduced the percentage of samples that require confirmation by a third test, but the Wiener recombinant v.3.0 is difficult to acquire in the U.S and shipping costs are higher since the test is produced and distributed by an Argentinian manufacturer.\textsuperscript{69}

The recently approved rapid test CDP only offers moderate accuracy with large numbers of false positives in the two geographic areas. This is different than what has been previously reported in the literature by different research groups working primarily in Bolivia.\textsuperscript{70,71} The test had 100\% sensitivity in individuals with low risk of exposure (such as in American residents without travel history and in Chile where there is no vector transmission), and high sensitivity in Bolivia\textsuperscript{47}, however, it has not been evaluated in other geographic areas. Further evaluation of these tests in different geographic areas such as in Mexico and Central America
where other diseases are endemic and other *T. cruzi* DTUs are prevalent should be required before FDA approval. CDP also has high cross-reactivity with toxoplasmosis (20%) and syphilis (40%).

The epidemiology of these diseases is not well determined and may have a different distribution in Latin America.

When the CDP was combined with the Hemagen a greater percentage of samples required further confirmation, but no positive individuals were missed in the diagnosis. Despite its limitations, the CDP has the advantage that it is a point-of-contact assay, can be performed in a primary care or community setting, can use whole blood or serum and provides the results within 20 minutes so individuals can receive their initial diagnosis during the first visit. Based on our community-based experience, individuals that are notified that they have a positive CDP are more likely to provide accurate contact information and are easier to contact by phone when confirmatory testing and cardiology evaluation is needed. However, to decrease anxiety, an appropriate explanation of the result of the rapid test and the probability of inaccurate diagnosis of the test must be provided to potential patients. Higher specificity and sensitivity of the CDP was obtained using capillary whole blood rather than serum, which has been previously reported in other studies.

The main advantage of this study is that we used samples obtained from a community-based study where 1124 at-risk individuals were tested in parallel by all the diagnostic tests. Most studies have been done using repository samples where the characteristics of the samples will depend on the initial tests used for screening. Our recruitment in different settings including churches, community centers, consulates and health fairs increases the diversity of our sample and education before enrollment decreased participant bias.
The limitations of the study are the small sample of positive individuals from TcI areas, since the infection is lower in prevalence and affects mainly rural sectors in endemic countries. Larger community-based studies are required to have greater number of Chagas cases. The use of samples collected by blood banks could also be an alternative to efficiently detect positive cases since blood banks in the U.S. used a more robust and highly sensitive lysate-based ELISA for screening. However, this procedure may also introduce participation bias because of cultural beliefs and fear of deportation in the Latin American population. Individuals that have recently traveled or lived in any malaria endemic country where Chagas disease is also endemic are also excluded from donating blood for at least 1 to 3 years.\textsuperscript{56} Furthermore, individuals that have been previously been diagnosed with \textit{T. cruzi} infection are informed that they cannot donate blood in the future. Another limitation is that other FDA-approved tests have not been included in the study because of logistical and economical resources. These tests included: ABBOTT ESA Chagas (Abbott Laboratories, an enzyme strip assay with FP10, FP6, FP3, and TcF recombinant antigens) and The ABBOTT PRISM Chagas assay (Abbott Laboratories, a chemiluminescence test that uses FP3, FP6, FP10, and TcF recombinant proteins. Future studies may include these tests for evaluation.

In conclusion, better diagnostic tests are needed to improve the diagnosis of Chagas disease in the U.S. More robust and specific lysate-based tests are required if only one test is to be used as a primary screening tool to decrease the number of indeterminate and false positive results. Furthermore, recombinant-based tests need to be adapted to the antigenic repertoire that patients from TcI areas are exposed or to the low levels of antibodies that they develop to non-TcI antigens. Although attempts have been done to determine immunodominant TcI antigens, this must be a long-term goal.\textsuperscript{72} In the short-term, there is a need for improved logistics and decreased cost of robust diagnostic tests that are currently approved by the FDA so that they can be used by clinical and research laboratories. A commercially available TESA-blot is also needed to increase early treatment.
Chapter 6. Study 3: Addressing early diagnosis of congenital Chagas disease in the time of the goal of mother-to-child transmission elimination in the Americas

Abstract

Background: Early diagnosis of congenital Chagas disease (CChD) represents a good opportunity for early treatment. We evaluated factors of maternal adherence to the 6- or 9-month follow-up program (FP) and the utility of the IgM Shed Acute Phase Antigen (SAPA) ELISA in the early diagnosis of CChaD).

Methods: Two prospective studies (consisting of a training and a validation cohort) were conducted in three hospitals in Santa Cruz, Bolivia. Pregnant women were screen for Chagas disease, and infants from seropositive mothers were examined to determine vertical transmission at birth and 1-month by microscopy, qPCR, and IgM TESA-blot (trypomastigote excreted-secreted antigens), and at 6- or 9-months for anti-Trypanosoma cruzi IgG antibodies. An IgM SAPA ELISA was optimized using a recombinant SAPA and camelid antibodies for IgM detection.

Findings: A total of 5318 women were evaluated, overall maternal seroprevalence was 25.49% (95% CI: 24.33%-26.69%) and adherence to FP was 37.24% (95% CI: 34.66%-39.88%), respectively. Lower maternal education (adjusted OR: 0.75, p=0.049), owning a computer (adOR: 1.97, p=0.002), and living in a house with more than three persons per room (adjOR: 0.73, p=0.022) impacted the odds of maternal adherence. When only one infant sample obtained at birth was evaluated in the validation cohort, the qPCR and the IgM SAPA ELISA have similar accuracy and were both higher than microscopy (sensitivity: 86.49%, 32/37; 81.58%, 31/38; 57.58%, 19/33, respectively, and specificity: 99.53%, 212/213; 96.15%, 209/217; 99.47%, 189/190, respectively).

Interpretation: Socioeconomic factors were mainly determinants of maternal adherence. The IgM SAPA ELISA performed similar to qPCR in the early diagnosis of congenital Chagas, however none of the early diagnostic tests achieved sensitivities higher than 90%, likely because of the biology of the infection. The IgM SAPA ELISA has the potential to be implemented in the short-term as an early diagnostic tool.
**Introduction**

Vertical transmission of *Trypanosoma cruzi*, the pathogen that causes Chagas disease, occurs with an incidence of 8668 cases per year and is considered a leading contributor to new infections with this parasite after vector transmission.\(^1\) Congenital Chagas disease is an important cause of neonatal morbidity, causing low birthweight and/or respiratory distress in 29% of infected infants; asymptomatic infants have 20%-30% of probability of developing Chagas cardiomyopathy later in life.\(^{13}\)

The Pan American Health Organization (PAHO) has launched the Framework for the elimination of mother-to-child transmission of HIV, syphilis, hepatitis B and Chagas (EMTCT-PLUS) as a public health problem in the Americas.\(^{73}\) This initiative’s goal is to increase testing of Chagas disease of pregnant women and neonates of seropositive mothers to more than 90% and provide treatment to more than 90% of infected infants by 2020.\(^{73}\) Detection of congenital Chagas represents a unique opportunity for anti-parasitic treatment since treatment is highly efficacious during the acute phase and does not produce the same adverse events in infants that are seen in adults.\(^{13}\)

The diagnosis of congenital Chagas in National Control Programs still relies on the observation of circulating parasites by microscopy (micromethod) and/or the detection of IgG antibodies by serology at 9 months of age\(^{13,14,74}\). Because of the low sensitivity of microscopy, a negative micromethod can only be confirmed by a negative 9-month IgG. Serological tests for IgG detection have good accuracy in most endemic areas with percentages of sensitivity and specificity greater than 95% when two tests are used in parallel, but they can only be used once maternal IgG antibodies are cleared.\(^{13,14,74}\) This diagnostic algorithm is inefficient, leading to missed diagnoses in more than 58% of infants at-risk for infection who are lost to follow-up even during research studies,\(^{13,14}\) and microscopy detects only 34.2% of cases during the first month of life.\(^{13,14}\) Nine months of follow-up is an unrealistic challenge for health systems in high endemicity areas such as Bolivia where 21% of pregnant women have Chagas disease.\(^{13,14}\) Two techniques, PCR and
the IgM-TESA-blot assay (Trypomastigote excretory-secretory antigen Western Blot) for detection of anti-*T. cruzi* IgM antibodies, have been extensively evaluated in large cohort studies,\textsuperscript{13,14} and have shown superior sensitivity over microscopy when two blood samples obtained at 0- and 1-month age are evaluated in parallel (PCR: 84.2\% and IgM-TESA blot for detection of six shed acute phase antigen [SAPA] bands: 73.7\%) The specificities of both techniques are greater than 97\%.\textsuperscript{13} However, both the PCR and IgM TESA-blot are only used in research studies because they are laborious, requiring special equipment and well-trained technicians. In addition, commercially available tests have not been validated. New diagnostic strategies are needed.

Even though diagnosis and treatment of congenital Chagas disease is recommended in Bolivia, many hospitals in endemic areas do not perform even microscopy at birth as a clinical practice due to lack of resources. Since 2010, our group has been working together with local hospitals in Santa Cruz, Bolivia. From 2010 to 2015, screening of maternal and infant Chagas disease in these hospitals was mainly supported by the research study, which involves the presence of a study nurse for sample collection, performing microscopy and 9-month follow-up. Awareness about early diagnosis and treatment of the infection was provided to mothers at birth, and phone calls were used as a reminder to increase adherence at 6- to 9-months.

This study evaluates the main differences between mothers that completed or did not complete the follow-up program in three different hospital-based cohort studies of congenital Chagas disease in Santa Cruz, Bolivia. The use of the IgM SAPA-ELISA test based on the use of a recombinant SAPA and camelid antibodies is proposed to increase early detection of congenital Chagas disease at birth in areas where the qPCR will be difficult to be implemented.
**Methods**

**Study design and participants**

Two prospective cohort studies of congenital Chagas disease were conducted in three hospitals in Santa Cruz, Bolivia. First, the IgM SAPA-ELISA was optimized and evaluated in the training cohort: The Japanese-Camiri study was performed from June 2010 to December 2014 in Hospital Universitario Japones (HUJ) and the Municipal Hospital of Camiri (HMC). After appropriate cutoffs were determined, the sensitivity and specificity of the assay was tested in the validation cohort: The Percy Boland study was carried out from May 2016 to June 2018 in The Municipal Women's Hospital Dr. Percy Boland Rodriguez (HPB).

The three hospitals are in urban Santa Cruz, but they also received women from rural areas. The HMC is located a few miles from rural areas where the seroprevalence of the infection is higher than 50% in the adult population. All mothers attending those hospitals for childbirth were invited to participate. As part of the National Control Program of Chagas disease in Bolivia, all pregnant women without exception received testing for Chagas disease. Neonates and infants less than 1 year old born to mothers with Chagas disease were also enrolled in the study. Neonates and infants were excluded if the family was planning to move to another city during the first year of life, they were lost to follow-up at 6 or 9-months of life and were not able to obtain a signed permission form.

The accuracy of the IgM SAPA-ELISA test was evaluated using microscopy, qPCR in blood, and IgM-TESA blot at birth and 1-month and IgG serology at 6- or 9-months to determine infection. Optimization of the assay and determination of different cut-offs using Receiver operating characteristic (ROC) was done initially in the training cohort. Potential cut-offs were later evaluated in the validation cohort (Figure 18).

To determine specificity of the IgM SAPA-ELISA, we also evaluated serum samples of neonates (n=92) from the training cohort that were born to seronegative mothers and serum
samples of neonates (n=186) from the Nutritional cohort study in the first two years of life in Santa Rosa Hospital, in Piura, Peru, a Chagas disease non-endemic area (Figure 18).

The Institutional Review Boards of the Hospital Universitario Japones; Universidad Catolica Boliviana Municipal Women’s Hospital Dr. Percy Boland Rodriguez, Hospital Santa Rosa, Universidad Peruana Cayetano Heredia, Asociacion Benefica PRISMA and Johns Hopkins Bloomberg School of Public Health approved the protocol. All women provided written informed consent for themselves and their infants.

Enrollment and follow-up

After consenting, capillary blood samples and an epidemiological interview were obtained from all women in labor attending the abovementioned hospitals for delivery. A rapid screening in the hospital was done using two tests in parallel: Chagas Detect Plus (CDP, InBios International, Seattle, Washington) and the PolyChaco indirect hemagglutination assay (IHA) at a dilution of 1:16 (Lemos Laboratories, Argentina). A venous blood sample was obtained from all women to determine the confirmatory diagnosis by conventional tests.

Infants born to women with positive results by the rapid test were followed-up for 9-months to determine vertical transmission as determined by the routine diagnostic procedure in each hospital. Blood samples from infants were obtained at 0-, 1-, and 6 or 9- months of age. Blood samples from umbilical cords were obtained in the training cohort while venous samples were obtained in the validation cohort from infants at birth. Venous blood samples were obtained in both cohorts at 1- and 6 or 9- months of age. To increase participants’ adherence, education about the importance of early diagnosis and treatment of congenital Chagas disease was provided to mothers.

The study in HMC was developed under complete research funding from June 2010 to April 2014, while the study in HUJ was always under complete research funding. Maternal and infant screening by microscopy at birth and IgG serology at 6 or 9 months is public health
policy in Bolivia, but due to the lack of financial resources, it is not always implemented. Complete research funding consisted of the incorporation of study nurses for obtaining samples and interviews, carrying out microscopy and monitoring infants without depending on the hospital’s resources. Complete research funding also included telephone calls, reimbursement to mothers for payment of transportation, and, in cases of suspected *T. cruzi* infection, home visits were made. From May 2014 to December 2014, the training cohort in HMC and for the entire duration of validation cohort at HPB, the studies were only under partial research funding: during this time, enrollment, sample and data collection was done using hospital's resources. The study was responsible for data organization, sample processing and transportation, and testing using qPCR, IgM TESA-blot and IgG serology.

**Diagnosis of Chagas disease in mothers and infants**

Maternal diagnosis: Conclusive results for chronic Chagas disease required congruent results using 2 or more IgG serological tests.\(^{41}\)

All maternal samples in the training cohort were tested by the rapid test CDP, the PolyChaco indirect hemagglutination assay (IHA) at a dilution of 1:16 (Lemos Laboratories, Argentina), and two commercial ELISA (enzyme-linked immunosorbent assay) tests: the Chagatest lysate, ELISA and Chagatest ELISA recombinant 3.0 (both by Wiener laboratories, Argentina). As previously published, we found that the use of the CDP in maternal screening in this population provides a sensitivity 95.1% (95% CI: 89.8%-97.7%) and specificity of 98.3% (95% CI: 94.1%-99.5%).\(^{70}\)

The validation cohort used the IHA at a dilution of 1:16 as a secondary test after the use of the lateral flow assay (CDP) for maternal diagnosis. Conclusive diagnosis was made based on the use of these two tests. Serodiscordant samples to the IHA and the CDP were tested by the Chagatest ELISA, recombinant 3.0.
Congenital diagnosis: Early diagnosis during the first month of life was done by microscopy (micromethod), qPCR and/or the IgM-TESA blot test. The procedures of the micromethod, qPCR and IgM-TESA-blot have been extensively described before. An improved version of the qPCR was used in the validation cohort. Diagnosis of chronic Chagas disease at 9-months in infants was done using the IHA and the Chagatest ELISA tests.

Cases of congenital Chagas were divided in two groups: Group A, the early diagnosed group that were detected during the first month of life. An infant was considered infected if she/he was positive by microscopy in at least one sample obtained at 0 or 1-month, positive by qPCR in two samples obtained at birth and 1 month, positive by at least one qPCR (at 0 or 1-month) and one IgM-TESA blot (at 0 or 1-month). Group B includes all the infants diagnosed with congenital Chagas: the cases detected in group A and the ones that were diagnosed at 6- or 9-months by two ELISA tests.

Infants were treated with anti-\textit{T.cruzi} drugs by the attending pediatrician in each hospital following the guidelines of the National Control Program of Chagas disease in Bolivia.

The IgM SAPA ELISA and evolutionary conservation across genotypes

A previously published SAPA sequence (GenBank J03985) was used to construct a short recombinant SAPA (sh-SAPA) for the IgM SAPA ELISA and to examine the evolutionary conservation of the region across \textit{T. cruzi} genotypes. The Basic Local Alignment Search Tool (BLAST) algorithm was used to assess alignment across genotypes using publicly available \textit{T. cruzi} genomes. Alignments were manually inspected and statistical similarity bit scores (S) and E-values (measures of significance representing the number of different alignments with scores equivalent to or better than S expected to occur by chance) were evaluated. The SAPA sequence was found to align with all \textit{T. cruzi} genomes irrespective of genotype (all E < 2x10^{-3}), demonstrating that the region is well conserved across strains and an ideal candidate for diagnostics.
IgM detection by ELISA was conducted as follows: sh-SAPA (6.4 ng/μl) was incubated overnight in carbonate/bicarbonate buffer (pH 9.0) in Immulon 4HBX ELISA plates. After five washing steps with PBS Tween 0.05% (PBST), nonspecific binding was blocked with 5% bovine serum albumin (BSA) in PBST at 37°C for one hour. After four washing steps with PBST, serum samples were diluted 1:50 in Inbios diluent buffer for serum (InBios International, Seattle, Washington) and added to specific wells and incubated at 37°C for one hour. After another five washing steps with PBST, wells were incubated with biotin anti-IgM conjugate (Thermo Scientific) at a dilution of 1/10000 in InBios diluent buffer for conjugate for 1 hour at 37°C. This conjugate consists of a 14 kDa llama antibody fragment (affinity ligand) that specifically binds to the μ chain of human antibodies. After five washing steps with PBST, an ultra-streptavidin-HRP conjugate was added at a dilution of 1/5000 in InBios diluent buffer for conjugate and incubated for 30 min at 37°C. After five washing steps, wells were incubated with 1 mg/ml of o-Phenylenediamine dihydrochloride (OPD, Sigma) and 0.3% of hydrogen peroxide for 30 minutes and stopped by addition of 50 μl of 2N H₂SO₄. OD values were measured at 492 nm using the EMax Plus Microplate Reader.

**Epidemiological and clinical data**

A standardized and validated epidemiological interview was conducted in Spanish, the main language in the city of Santa Cruz, and was administered by study/hospital staff to obtain information about socioeconomic factors, family history and maternal vector exposure.

Clinical information about the delivery and neonates was obtained from medical records. Based on the results of the training cohort, the most important clinical signs in infected infants are birth weight lower than 2500 g, respiratory distress and being premature. In this study, infected infants were classified as having clinical signs if any one of the three signs described before were present.
**Statistical analysis**

The chi square-test or Fisher exact test was used to determine associations with categorical variables between groups. The Wilcoxon rank-sum (Mann-Whitney) and the Kruskal Wallis test were used to compare variables that were non-normally distributed. Unadjusted and adjusted logistic regression models were constructed in a stepwise backward selection process and selection of predictors was evaluated using the Akaike's information criteria. Variance inflation factors was used to determine collinearity. Each potential explanatory variable of adherence to the 9-month follow-up program was evaluated individually and adjusted by maternal age, education, number of people per bedroom, and presence of a computer at home. ROC curves in the training and validation cohort were constructed to determine the appropriate OD value of the IgM SAPA ELISA to classify infected and non-infected infants. Percentages of sensitivity and specificity were obtained using the chi square test and 95% CI were calculated using the Clopper-Pearson interval. The interquartile range (IQR) was used to create categorical variables of levels of parasitemia at birth using the results of qPCR and determine associations with levels of IgM to SAPA antigen. Three categorical variables were created corresponding to the three quartiles of the distribution of logarithm levels of parasitemia. A two-sided p-value of less than 0.05 was considered significant. All statistical analyses and graphs were done using the Stata statistical software package version 14 (Stata Corp, College Station, TX).
Results

A total of 5505 women were enrolled; of them, 51/1286 (3.97%) and 136/4219 (3.22%) were excluded from the training and validation cohort, respectively. Reasons for exclusion were inconclusive results of Chagas serology or inability to make a diagnosis of Chagas disease because of the lack of blood sample (n=92, 1.67%), and missing information for more than two variables (n=98, 1.81%) (Figure 18). Of the ones with missing data, 79.59% (78/98) and 20.41% (20/98) were seronegative and seropositive women, respectively (p<0.001).

Seroprevalence of maternal Chagas disease was different in the three hospitals (p<0.001), with HMC having the highest prevalence (50.87%), followed by HPB (21.43%) and HUJ (18.70%). History of spontaneous abortion or stillbirth was significantly higher in the HPB (6.44%) and HUJ (6.23%) as compared to the HMC (2.54%) (p=0.003), suggesting that HPB and HUJ receive more complicated pregnancies in Santa Cruz. The percentage of mothers that have not completed secondary school was also higher in HPB (60.62%) and HUJ (64.21%) (p=0.002). Measures of economic status (more than three person per room, lives in a house with electricity, television or refrigerator) and past and recent vector exposure indicated that mothers in HMC have lower economic status and higher risk factors for vector exposure as compared to the other hospitals (p<0.001) (Table 8).

Complete research funding had a significant impact on maternal adherence; the lowest adherence to the 6- or 9-month follow-up period was observed in HPB (43.76%) (p<0.001) which had only partial funding. In HMC, 73.97% (108/146) of seropositive mothers returned at 6- or 9-months for infant diagnosis during the research funding period as opposed to 36.54% (38/104) during partial research funding (p value<0.001) (Table 9). Among the characteristics evaluated in this study, indicators of socioeconomic status were statistically different between mothers who were completers vs. non-completers during the follow-up period. Maternal education lower than secondary school and living in a house with more than three persons per bedroom significant decreased the probability of adherence in the
unadjusted and adjusted model. Conversely, living in a house with a computer and having received a previous positive diagnosis of Chagas disease increased the odds of maternal adherence in both regression models. No differences were observed among factors associated with vector exposure such as living in a rural area or having lived in an infested house (Table 9). Other variables were associated with maternal adherence only at specific hospitals. In the HPB, having a positive result in microscopy at 0- or 1-month (OR: 4.48, p-value=0.002 and adOR: 5.24, p-value 0.002) increases maternal adherence in the unadjusted (OR) and adjusted (adOR) model (data not shown).

A total of 72 congenital cases were evaluated, 34 and 38 were from the training and validation cohorts, respectively. In both cohorts, most cases (training cohort: 73.53%, 25/34, 95% CI 55.64%-87.12%; validation cohort: 83.78%, 31/37, 95% CI 67.99%-93.90%) were diagnosed during the first month of life (group A). Among mothers of infants that were diagnosed at 6- or 9-months, 64.29% (9/14, 95% CI: 35.14%) and 92.86% (13/14, 95% CI: 66.13%-99.82%) had not been living in a rural area and had not seen the vector during the year of their pregnancy, suggesting low risk of vector transmission, and 80% (12/15, 95% CI: 51.91%-95.67%) had caesarean-sections to deliver their neonates suggesting low risk of transmission at the moment of delivery.

Table 10 shows the performance of each test used for early diagnosis; the analysis was done separately for each cohort; differences were found between the two cohorts. In the training cohort, the IgM SAPA ELISA was conducted with samples that were preserved for three years. In some of these samples protein degradation was observed: when the IgM TESA blot results were repeated, they had converted to negative even though they were previously positive when initially tested after collection (data not showed). The qPCR technique was improved during the training cohort for the validation cohort, the modification consisted of better digestion of clotted blood samples using the FastPrep-24 5G Homogenizer, improving DNA recovering.77 As a result of these differences, the sensitivity of the qPCR and the IgM SAPA ELISA were lower in the training cohort.
Based on the results of the ROC curve, a cut-off of OD value equal to or greater than 0.23 provided the best balance of sensitivity and specificity with an area of 0.87 (95% CI: 0.78-0.96). qPCR at birth had greater sensitivity than IgM SAPA ELISA or microscopy, but any of the tests achieved a sensitivity greater than 90% when all cases of congenital Chagas (group B) were included (Table 10). The specificity of the IgM SAPA ELISA in infants with no risk of congenital transmission was 97.83% (90/92, 95% CI: 92.37%-99.74%) in infants born to seronegative mothers in Santa Cruz, Bolivia, and 98.92% (184/186, 95% CI: 96.17%-99.87%) in neonates born to mothers in Piura, Peru a non-endemic area for Chagas disease.

The Kruskal-Wallis and logistic regression analysis showed a positive association between levels of IgM antibodies and parasitemia (p<0.001) (Figure 19). IgM (p=0.007) and parasitemia levels (p<0.001) were both positively associated with the presence of clinical signs using the Kruskal-Wallis test and univariate logistic regression analysis (OR parasitemia levels: 4.18, 95% CI: 1.87-9.37, p=0.001; OR IgM levels: 3.33, 95% CI: 1.31-8.49, p=0.012). After using both IgM and parasitemia levels in the logistic regression model, only levels of parasitemia were positively associated with presence of clinical signs (adOR: 4.50, 95% CI: 1.61-12.59, p=0.004), no significant association of IgM levels and presence of clinical signs was observed in the adjusted model (adOR: 0.86, 95% CI: 0.23-3.22, p value: 0.820).
Figure 17. Flow chart of the study.
Table 8. Characteristics of women who were received for their delivery in Hospital Municipal Camiri, Camiri and Hospital Japones and Percy Boland in Santa Cruz and, Bolivia.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hospital Camiri &quot;HMC-1&quot;</th>
<th>Hospital Camiri &quot;HMC-2&quot;</th>
<th>Hospital Camiri Total &quot;HMC-1 and HMC-2&quot;</th>
<th>p-value (HMC-1 vs HMC-2)</th>
<th>Hospital Japones &quot;HUJ&quot;</th>
<th>p-value (HMC-1 and 2 vs HUJ)</th>
<th>Hospital Percy Boland &quot;HPB&quot;</th>
<th>p-value (HMC 1 and 2 vs HPB)</th>
<th>p-value (HUJ vs HPB)</th>
<th>p-value all comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total presenting, N</td>
<td>287</td>
<td>228</td>
<td>515</td>
<td>-</td>
<td>1286</td>
<td>-</td>
<td>4219</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Excluded Serodiscordant or no sample, % (n)</td>
<td>0.00</td>
<td>1.3</td>
<td>0.6</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>1.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Missing more than two variables, % (n)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>3.9</td>
<td>-</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total Included</td>
<td>287</td>
<td>225</td>
<td>512</td>
<td>-</td>
<td>1,235</td>
<td>-</td>
<td>4,083</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T. cruzi seroprevalence,%</td>
<td>50.9</td>
<td>46.2</td>
<td>48.8</td>
<td>0.296</td>
<td>18.7</td>
<td>&lt;0.001</td>
<td>21.4</td>
<td>&lt;0.001</td>
<td>0.039</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(n/N)</td>
<td>(146/287)</td>
<td>(104/225)</td>
<td>(250/512)</td>
<td>(231/1235)</td>
<td>(875/4083)</td>
<td></td>
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<tr>
<td>Seropositive mothers with complete follow-up, %</td>
<td>74.0</td>
<td>36.5</td>
<td>58.4</td>
<td>&lt;0.001</td>
<td>59.7</td>
<td>0.765</td>
<td>43.8</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(n/N)</td>
<td>(108/146)</td>
<td>(38/104)</td>
<td>(146/250)</td>
<td>(138/231)</td>
<td>(221/875)</td>
<td></td>
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<tr>
<td>Mean age (years)</td>
<td>24.9</td>
<td>24.4</td>
<td>24.7</td>
<td>0.314</td>
<td>25.4</td>
<td>0.06</td>
<td>24.9</td>
<td>0.873</td>
<td>0.021</td>
<td>0.052</td>
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<td></td>
<td>(SE)</td>
<td>(0.4)</td>
<td>(0.4)</td>
<td>(0.3)</td>
<td>(0.2)</td>
<td>(0.1)</td>
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<tr>
<td>History of spontaneous abortion or stillbirth, %&lt;br&gt; (n/N)</td>
<td>2.4</td>
<td>2.6</td>
<td>2.5</td>
<td>0.817</td>
<td>6.2</td>
<td><strong>0.001</strong></td>
<td></td>
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<tr>
<td></td>
<td>(7/287)</td>
<td>(6/225)</td>
<td>(13/512)</td>
<td>(77/1235)</td>
<td>(263/4083)</td>
<td>0.795</td>
<td><strong>0.003</strong></td>
<td></td>
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<tr>
<td>Mother did not complete secondary school, %&lt;br&gt; (n/N)</td>
<td>52.5</td>
<td>57.6</td>
<td>54.7</td>
<td>0.247</td>
<td>64.2</td>
<td><strong>&lt;0.001</strong></td>
<td></td>
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<tr>
<td></td>
<td>(150/286)</td>
<td>(129/224)</td>
<td>(279/510)</td>
<td>(793/1235)</td>
<td>(2469/4073)</td>
<td>0.032</td>
<td><strong>0.023</strong></td>
<td><strong>0.002</strong></td>
<td></td>
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<tr>
<td>Work outside home, %&lt;br&gt; (n/N)</td>
<td>22.3</td>
<td>27.4</td>
<td>24.5</td>
<td>0.213</td>
<td>25.1</td>
<td>0.808</td>
<td></td>
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<td></td>
<td>0.000</td>
<td>0.000</td>
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<tr>
<td>More than three person per bedroom, %&lt;br&gt; (n/N)</td>
<td>46.3</td>
<td>43.7</td>
<td>45.1</td>
<td>0.592</td>
<td>37.7</td>
<td><strong>&lt;0.004</strong></td>
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<tr>
<td></td>
<td>(133/287)</td>
<td>(99/227)</td>
<td>(232/514)</td>
<td>(580/1233)</td>
<td>(1632/4081)</td>
<td>0.059</td>
<td>0.153</td>
<td><strong>0.033</strong></td>
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<tr>
<td>Lives in house with electricity, %&lt;br&gt; (n/N)</td>
<td>85.7</td>
<td>80.4</td>
<td>83.4</td>
<td>0.121</td>
<td>98.5</td>
<td><strong>&lt;0.001</strong></td>
<td></td>
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<tr>
<td></td>
<td>(246/287)</td>
<td>(181/225)</td>
<td>(427/512)</td>
<td>(1216/1235)</td>
<td>(4053/4083)</td>
<td>0.016</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Lives in house with television, %&lt;br&gt; (n/N)</td>
<td>77.4</td>
<td>74.7</td>
<td>76.2</td>
<td>0.531</td>
<td>95.5</td>
<td><strong>&lt;0.001</strong></td>
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<tr>
<td></td>
<td>(222/287)</td>
<td>(168/225)</td>
<td>(390/512)</td>
<td>(1179/1235)</td>
<td>(3956/4083)</td>
<td>0.020</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Lives in house with refrigerator, %&lt;br&gt; (n/N)</td>
<td>50.2</td>
<td>55.1</td>
<td>52.3</td>
<td>0.285</td>
<td>67.0</td>
<td><strong>&lt;0.001</strong></td>
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<td></td>
<td>(144/287)</td>
<td>(124/225)</td>
<td>(268/512)</td>
<td>(826/1233)</td>
<td>(3281/4083)</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Lives in house with computer, %&lt;br&gt; (n/N)</td>
<td>15.0</td>
<td>20.4</td>
<td>17.4</td>
<td>0.126</td>
<td>6.5</td>
<td><strong>&lt;0.001</strong></td>
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<td></td>
<td>(43/287)</td>
<td>(46/225)</td>
<td>(89/512)</td>
<td>(80/1233)</td>
<td>(644/4083)</td>
<td>1.000</td>
<td><strong>&lt;0.001</strong></td>
<td><strong>&lt;0.001</strong></td>
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<tr>
<td>Has a motor vehicle, %&lt;br&gt; (n/N)</td>
<td>15.7</td>
<td>15.6</td>
<td>15.6</td>
<td>1.000</td>
<td>13.4</td>
<td>0.226</td>
<td></td>
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<tr>
<td></td>
<td>(45/287)</td>
<td>(35/225)</td>
<td>(80/512)</td>
<td>(165/1233)</td>
<td>(578/4083)</td>
<td>0.462</td>
<td>0.512</td>
<td>0.652</td>
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<td></td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
<td>% (n/N)</td>
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<tr>
<td>Had received a Chagas test before, % (n/N)</td>
<td>74.2 (213/287)</td>
<td>68.9 (157/228)</td>
<td>71.8 (370/515)</td>
<td>&lt;0.001 (794/1235)</td>
<td>64.3 (3571/4083)</td>
<td>0.001 (1572/287)</td>
<td>87.5 (213/287)</td>
<td>&lt;0.001 (1572/287)</td>
<td></td>
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<tr>
<td>Had a positive result of Chagas before, % (n/N)</td>
<td>31.7 (91/287)</td>
<td>21.5 (49/228)</td>
<td>27.2 (140/515)</td>
<td>0.075 (169/1235)</td>
<td>13.7 (443/4083)</td>
<td>&lt;0.001 (91/287)</td>
<td>10.9 (443/4083)</td>
<td>&lt;0.001 (91/287)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever lived in a rural area, % (n/N)</td>
<td>54.9 (157/286)</td>
<td>47.1 (106/225)</td>
<td>51.5 (263/511)</td>
<td>0.090 (639/1232)</td>
<td>51.9 (1897/4083)</td>
<td>0.090 (157/286)</td>
<td>46.5 (1897/4083)</td>
<td>0.013 (157/286)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lives is a rural area now, % (n/N)</td>
<td>35.5 (102/287)</td>
<td>30.9 (30/97)</td>
<td>34.4 (132/384)</td>
<td>0.459 (324/1232)</td>
<td>26.3 (843/4083)</td>
<td>0.003 (102/287)</td>
<td>20.7 (843/4083)</td>
<td>&lt;0.001 (102/287)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever lived in an infested house, % (n/N)</td>
<td>38.0 (109/287)</td>
<td>30.2 (68/225)</td>
<td>34.6 (177/512)</td>
<td>0.075 (293/1234)</td>
<td>23.7 (1083/4079)</td>
<td>&lt;0.001 (109/287)</td>
<td>26.6 (1083/4079)</td>
<td>&lt;0.001 (109/287)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lives is an infested house now, % (n/N)</td>
<td>24.7 (71/287)</td>
<td>20.2 (46/228)</td>
<td>22.7 (117/515)</td>
<td>0.245 (411/1235)</td>
<td>5.7 (278/4063)</td>
<td>&lt;0.001 (71/287)</td>
<td>6.8 (278/4063)</td>
<td>0.150 (71/287)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever lived in mud-wall house, % (n/N)</td>
<td>51.6 (148/287)</td>
<td>33.9 (76/225)</td>
<td>43.8 (224/512)</td>
<td>&lt;0.001 (411/1235)</td>
<td>33.3 (1100/4080)</td>
<td>&lt;0.001 (148/287)</td>
<td>27.0 (1100/4080)</td>
<td>&lt;0.001 (148/287)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever lived in house with earth floor, % (n/N)</td>
<td>48.4 (139/287)</td>
<td>38.7 (87/138)</td>
<td>44.1 (226/512)</td>
<td>0.031 (405/1235)</td>
<td>32.8 (1004/4078)</td>
<td>&lt;0.001 (139/287)</td>
<td>24.6 (1004/4078)</td>
<td>&lt;0.001 (139/287)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 9. Maternal characteristics associated with the adherence to the follow-up period in the diagnosis of congenital Chagas disease among seropositive mothers in Santa Cruz, Bolivia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Completed follow-up period</th>
<th>Non-completers of follow-up period</th>
<th>Unadjusted comparison</th>
<th>Adjusted comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Completed follow-up period</td>
<td>Non-completers of follow-up period</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>505/1356 (37.24%)</td>
<td>851/2160 (62.76%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per increase of years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.441</td>
<td>26.757</td>
<td>1.03</td>
<td>1.01-1.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.01-1.05</td>
<td>0.000</td>
</tr>
<tr>
<td>History of spontaneous abortion or stillbirth, n (%)</td>
<td>169/ 505 (33.47%)</td>
<td>240/851 (28.20%)</td>
<td>1.25</td>
<td>0.97-1.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.080</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.81-1.51</td>
<td>0.531</td>
</tr>
<tr>
<td>Infant positive microscopy result at 0- or 1-month age, n (%)</td>
<td>14/505 (2.77%)</td>
<td>20/851 (2.35%)</td>
<td>1.14</td>
<td>0.37-3.51</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.823</td>
<td>1.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.55-4.75</td>
<td>0.385</td>
</tr>
<tr>
<td>Mother did not complete secondary school, n (%)</td>
<td>321/504 (63.69%)</td>
<td>610/847 (72.02%)</td>
<td>0.64</td>
<td>0.51-0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.56-0.88</td>
<td>0.049</td>
</tr>
<tr>
<td>Work outside home, n (%)</td>
<td>133/297 (44.70%)</td>
<td>372/1059 (35.13)</td>
<td>1.37</td>
<td>1.04-1.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.025</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.88-1.66</td>
<td>0.232</td>
</tr>
<tr>
<td>More than three person per bedroom, n (%)</td>
<td>208/505 (41.19%)</td>
<td>406/851 (47.71%)</td>
<td>0.72</td>
<td>0.57-0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.005</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.56-0.92</td>
<td>0.022</td>
</tr>
<tr>
<td>Lives in house with television, n (%)</td>
<td>450/505 (89.11%)</td>
<td>771/851 (90.60%)</td>
<td>0.85</td>
<td>0.59-1.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.376</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.49-1.28</td>
<td>0.353</td>
</tr>
<tr>
<td>Lives in house with refrigerator, n (%)</td>
<td>337/504 (66.87%)</td>
<td>583/851 (68.51%)</td>
<td>0.93</td>
<td>0.73-1.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.531</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.68-126</td>
<td>0.633</td>
</tr>
<tr>
<td>Study</td>
<td>n (%)</td>
<td>Control n (%)</td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
<td>---------------</td>
<td>------</td>
<td>----------</td>
</tr>
<tr>
<td>Lives in house with computer, n (%), (Yes/No)</td>
<td>71/505 (14.06%)</td>
<td>69/851 (8.11%)</td>
<td>1.85</td>
<td>1.30-2.63</td>
</tr>
<tr>
<td>Ever lived in a rural area, n (%)</td>
<td>328/505 (64.95%)</td>
<td>543/851 (63.81%)</td>
<td>1.05</td>
<td>0.83-1.23</td>
</tr>
<tr>
<td>Ever lived in an infested house, n (%)</td>
<td>288/505 (57.03%)</td>
<td>478/850 (56.24%)</td>
<td>1.03</td>
<td>0.83-1.29</td>
</tr>
</tbody>
</table>
Table 10. Performance of early diagnostic tests of congenital Chagas disease in neonates at risk of infection in the training and validation cohort.

<table>
<thead>
<tr>
<th>Test</th>
<th>Training cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity, %</td>
<td>Specificity, %</td>
</tr>
<tr>
<td></td>
<td>95% CI n/N</td>
<td>95% CI n/N</td>
</tr>
<tr>
<td>qPCR at birth</td>
<td>84.0 (21/25)</td>
<td>100.0 (29/29) **</td>
</tr>
<tr>
<td>Microscopy at birth</td>
<td>24.0 (6/25)</td>
<td>63.3 (19/30)</td>
</tr>
<tr>
<td>IgM SAPA ELISA at birth</td>
<td>84.0 (21/25)</td>
<td>93.3 (28/30)</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>95% CI</td>
</tr>
<tr>
<td></td>
<td>63.9-95.5 *</td>
<td>43.9-80.1 *</td>
</tr>
<tr>
<td></td>
<td>91.1-98.1 *</td>
<td>97.4-99.2 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All congenital cases</td>
<td>66.7 (22/33) **</td>
<td>99.5 (32/37) **</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>95% CI</td>
</tr>
<tr>
<td></td>
<td>48.2-82.0 99.5</td>
<td>71.2-95.5 99.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97.4-99.9</td>
</tr>
<tr>
<td></td>
<td>95% CI n/N</td>
<td>95% CI n/N</td>
</tr>
<tr>
<td></td>
<td>(216/217) **</td>
<td>(212/213) **</td>
</tr>
<tr>
<td></td>
<td>48.2-82.0 99.5</td>
<td>71.2-95.5 99.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97.4-99.9</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>95% CI</td>
</tr>
<tr>
<td></td>
<td>69-35.5 100.0</td>
<td>39.2-74.5 99.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97.8-100.0</td>
</tr>
<tr>
<td></td>
<td>95% CI n/N</td>
<td>95% CI n/N</td>
</tr>
<tr>
<td></td>
<td>(236/236)</td>
<td>(189/190) **</td>
</tr>
<tr>
<td></td>
<td>69-35.5 100.0</td>
<td>39.2-74.5 99.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>97.8-100.0</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>95% CI</td>
</tr>
<tr>
<td></td>
<td>43.6-77.8 99.6</td>
<td>65.7-92.3 96.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>92.9-98.4</td>
</tr>
<tr>
<td></td>
<td>95% CI n/N</td>
<td>95% CI n/N</td>
</tr>
<tr>
<td></td>
<td>(236/237)</td>
<td>(209/217)</td>
</tr>
<tr>
<td></td>
<td>43.6-77.8 99.6</td>
<td>65.7-92.3 96.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>92.9-98.4</td>
</tr>
</tbody>
</table>

95% CI: 95% confidence interval
* Specificity was not determined because negative results by microscopy, qPCR or IgM TESA blot at 0-0 or 1-month life does not rule out the infection.
** Denominators in these cells are lower because the test was not performed due to sample unavailability.
Figure 18. Relationship between levels of parasitemia, IgM levels and clinical signs in infected infants. a) Square root of OD values of levels of IgM antibodies and relationship with levels of parasitemia at birth. b) Square root of OD values of levels of IgM antibodies at birth and relationship with presence of clinical signs and c) Relationship of levels of parasitemia at birth and presence of clinical signs.

Presence of clinical signs corresponds to the presence of birth weight lower than 2500 g, respiratory distress or being premature. Black bars represent interquartile range, median is represented by a white line. p-values were obtained using the Kruskal Wallis test.
Discussion
This study shows that the IgM SAPA ELISA can be used for the early diagnosis of congenital Chagas disease and has similar performance in the diagnosis of congenital Chagas disease to the improved version of qPCR. This test has the potential to be implemented in endemic areas in the short-term since ELISA tests are relatively easy to do and are performed by many laboratories. Early diagnosis of congenital Chagas disease is important because of the low maternal adherence to the 6- or 9- months of follow-up and it is an opportunity to provide safe treatment. Factors that affect maternal adherence were mainly socioeconomic factors that are difficult to address in the short-term. In addition, providing a positive result at birth by microscopy increased maternal adherence in the HPB hospital that was covered only partially by research funding. However, microscopy only detects 18.8%-57.58% of cases.

IgM detection is used in the diagnosis of congenital or acute infections in cases of syphilis, cytomegalovirus, toxoplasmosis and other infections.78–80 In Chagas disease, previous publications have showed contradictory results.49,50,81 Differences in the performance of IgM tests could be attributed to the source of antigen and the test used as a gold standard. When a lysate antigen of the trypomastigote stage was used the sensitivity and specificity was 82.9% and 29.4%, respectively.49 Higher specificity was obtained with a recombinant SAPA and using xenodiagnosis at birth as a gold standard (sensitivity and specificity: 83.33%, 10/12 and 100%, 12/12, respectively).50

The IgM TESA blot is difficult to implement in endemic areas because is based on a crude excretory-secretory antigen and electrophoretic separation is needed to discriminate specific SAPA reactions53, and because of the lack of a commercially available TESA-blot. The IgM SAPA ELISA used in this study was based on an improved sequence of a previously published recombinant SAPA82 and the use of single-domain llama antibodies for IgM detection.
SAPA constitutes an immunodominant and tandemly repeated amino acid motif that is expressed by the parasite mainly during the acute phase and has been suggested to modulate the catalytic function of the trans-sialidase protein. Single-domain llama antibodies offer high specificity and sensitivity, but because of their low molecular weight (12-15 kDa compared to 150-160 kDa for regular antibodies) provide the advantage of higher stability, reproducibility and adaptability to multiple tests platforms. During the optimization of our IgM ELISA test, different anti-IgM antibody conjugates were evaluated including polyclonal antibodies produced in rabbit, and goat and mouse monoclonal antibodies (data not shown). In this evaluation, polyclonal antibodies showed high levels of nonspecific reactions, while monoclonal antibodies decreased sensitivity. The use of a 14 kDa antibody that also lacks of the Fc region of regular antibodies may reduce the number of epitopes, preventing the nonspecific binding of Fc-receptors that are present in human samples. The low molecular weight also improves flexibility and recognition of epitopes that may be hidden in complex protein structures such as the pentameric form of IgM antibodies.

Previous studies have evaluated the use of the qPCR for early diagnosis of congenital Chagas, but difficulties in the implementation of this technique in the health system of endemic areas may delay diagnosis. Furthermore, detection of DNA by ultrasensitive PCR in cord blood has not been associated with infection and the analysis of consecutive blood samples taken at different times may be needed because of the transient detection of T. cruzi DNA from dead parasites.

This study confirms previously published results in our training cohort with smaller number of cases that parasitemia levels were associated with clinical signs. Although causation cannot be determined in this study, we hypothesize that the clinical status of those infants is related to a higher multiplication of the parasites as has been described in cases of HIV infection. Levels of IgM were highly associated with parasitemia levels but not with clinical signs after adjusting by parasitemia levels, suggesting that IgM production is the result of the stimulation of the immune response led by the burden of circulating parasites. The OR of the
association of parasitemia levels and clinical status did not change in the unadjusted and adjusted model with IgM levels suggesting no mediation effect in the relationship of parasitemia levels and clinical status.

Using only samples obtained at birth, none of the tests evaluated in this study have sensitivity higher than 90% when all cases of congenital Chagas disease were included. This could be attributed to the time that vertical transmission occurs, parasite strain, host response, and transmission during breastfeeding. Although, vector transmission cannot be excluded, most infected infants that were detected at 6- or 9-months were not living in a rural area and were not not exposed to the vector.

This study constitutes the first evaluation of the IgM-SAPA diagnostic test in a large number of cases of congenital Chagas disease in Bolivia, the country with the highest prevalence of Chagas disease in the world and where discrete typing units (DTUs) II, V and VI of T. cruzi are predominant. Since the immune response to recombinant antigens is different between geographic areas with different T. cruzi DTUs, future studies will be needed to determine the utility of the IgM test in those geographic areas. However, bioinformatic analysis of the SAPA sequence used in this study and previous literature of this antigen suggests that this is a conserved protein express by all T. cruzi DTUs.

Implementation of the IgM SAPA ELISA will facilitate early diagnosis of congenital Chagas disease and could provide the opportunity to administer anti-parasitic treatment during the stage in which treatment is highly effective and safe.
Chapter 7. **Implications and Recommendations**

### 7.1 **Implications for health policy and practice**

Results of these studies provide scientific evidence of the burden of Chagas disease in the Washington Metropolitan Area. Previous clinical observations in the media as well as some publications have provided evidence of the public health impact of this disease in the area\(^8,^9,^87\), but no systematic evaluations have been done. Our study demonstrates the necessity of increasing education and testing for Chagas disease in individuals at-risk of infection. These individuals can then qualify for early anti-parasitic treatment before cardiac abnormalities develop, since the results of the BENEFIT trial showed that anti-parasitic treatment in patients with early Chagas cardiomyopathy may not decrease disease progression.\(^16\)

Screening and early diagnosis of Chagas disease is important since the CDC recommends an annual clinical and cardiac evaluation using ECG to monitor for cardiac progression whether or not the patient has received treatment for Chagas.\(^2\) If patients are not eligible for anti-parasite treatment, but develop cardiac abnormalities they will be able to receive appropriate care using less invasive and inexpensive cardiac treatments, decreasing costs to the health system, improving the quality of life of patients, and extending their life expectancy.

In the U.S. pregnant women or women of reproductive age with risk factors for infection are not screened for Chagas disease. Screening is important to monitor the neonate because there is a 5-13% risk of vertical transmission.\(^15\) Furthermore treatment of women in reproductive age may decrease congenital transmission in future pregnancies though the decrease of circulating levels of parasitemia.\(^66\)
Due to the limited funding for testing a high number of Latin American individuals without or with limited health coverage, the results of this study also provided the identification of risk factors and suggests that the appropriate use of combination of available diagnostics tests can help efficiently allocate economic resources to high risk-groups.

This study showed the limitations of the most available FDA-approved diagnostic tests in the U.S. Is necessary to facilitate access of clinical and research laboratories to more robust diagnostic tests that are currently approved by the FDA in order to reduce cost and to prevent incorrect or indeterminate results.

In endemic countries, early detection of congenital Chagas disease provides the best opportunity for an effective and safe treatment and may prevent the development of cardiac disease later in life. Adherence to the 9-month follow-up screening program is unrealistic and low in endemic areas. This study shows that in a high-risk area, factors that contribute to low maternal adherence are mainly socioeconomic factors that are difficult to address in the short-term; thus, the implementation of shorter diagnostic algorithms is needed.

More than 80% of congenital infections can be detected during the first month of life. Our diagnostic test has the potential to be implemented in the current congenital Chagas diagnostic algorithm in the near-term. Different sophisticated platforms have been suggested before for early detection including PCR, nanoparticle-based tests, as well as the IgM-TESA blot. While these techniques have showed promising results, the potential to implement them in the short-term is unlikely. We believe that while waiting for these tools to be adapted in endemic areas, friendly platforms should be used. Implementation of the IgM SAPA ELISA will increase the sensitivity of early detection of the infection in neonates in areas that currently rely only on microscopy.
7.2 Future research

The study in the Washington Metropolitan Area provides baseline information that could be used to develop a local cohort for early prediction and biomarker discovery of cardiac disease using a longitudinal study as well as to better understand the barriers for diagnosis and treatment of the disease in the U.S.

Although we hypothesized that low levels of antibodies in individuals from TcI areas are due to differences in *T. cruzi* genotypes, future studies can evaluate this hypothesis using genotyping and/or serotyping analysis in blood samples. However, because of the low sensitivity of molecular techniques, a greater number of individuals should be evaluated.

Future studies can include the evaluation of other FDA-approved diagnostic tests that were not included in this study such as the ABBOTT ESA Chagas (Abbott Laboratories, an enzyme strip assay with FP10, FP6, FP3, and TcF recombinant antigens) and The ABBOTT PRISM Chagas assay (Abbott Laboratories, a chemiluminescence test that uses FP3, FP6, FP10, and TcF recombinant proteins).

The IgM SAPA ELISA was evaluated in Bolivia where TcII/TcV/TcVI DTUs are predominant. Future studies can evaluate the accuracy of the test in geographic areas where TcI is the most important DTU including Mexico and Central America.

Other acute phase antigens such as multiepitope antigens 1, 2, 13 and 36 that have been also detected in congenital infections can be added to the IgM ELISA test to increase sensitivity.
A point-of-care version of the IgM SAPA ELISA such as lateral-flow test can be developed to facilitate its use in endemic areas.

The IgM SAPA ELISA can be also evaluated in serum samples obtained at 1-month of life to determine if performing the diagnosis during the first month of life (at birth and 1-month) can increase sensitivity.

Future studies with well-validated samples can also evaluate cross-reaction with other vertically transmitted pathogens such as cytomegalovirus, syphilis, and toxoplasmosis. Although these pathogens are different from *T. cruzi*, some recombinant-based tests for this parasite have reported cross-reaction with syphilis (40%) and toxoplasmosis (20%).

In summary, the results of these studies help identify the tools necessary to detect cases of Chagas disease both in a diverse immigrant population of a nonendemic country, and in infants congenitally infected in highly endemic countries, hopefully leading to a decrease in the morbidity from this disease.
References


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Yagahira Castro-Sesquen
May 2019

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Consejo Nacional de Ciencia, Tecnología e Innovación Tecnológica, CONCYTEC
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2006-2008: MS/ National University of Trujillo, Peru. Clinical Microbiology.

PROFESSIONAL EXPERIENCE (in chronological order)

2012 - 2013: Visiting researcher. Center for Applied Proteomics and Molecular Medicine. George Mason University, Virginia-USA.
2017-present: External Peer Reviewer of grant applications. Consejo Nacional de Ciencia, Tecnología e Innovación Tecnológica, CONCYTEC, Peru.
RESEARCH ACTIVITIES

Publications:


Completed Research Support:
2011-2012: Hydrogel Microparticles as a toll to increase the sensitivity of *Trypanosoma cruzi* antigen detection tests in urine using *Cavia porcellus* as animal model. PROCYT. Role: Co-Investigator.

**EDUCATIONAL ACTIVITIES**

**Teaching Assistant:**

**External mentor:**

**Thesis:** Caracterization of *Taenia solium* circulant antigen detected by magnetic beads coupled to monoclonal antibodies for the diagnosis of human neurocysticercosis"
- Student: Luz Toribio Salazar
- University: Universidad Nacional Federico Villarreal, Lima-Peru.
- Degree: Bachelor in Biological Sciences
- Year degree completed: 2019.

**Thesis:** “Development of a lateral flow immunoassay for the diagnosis of human neurocysticercosis”
- Student: Luz Agueda Perez
- University: Universidad Nacional Mayor de San Marcos, Lima-Peru.
- Degree: Master’s in molecular biology.
- Year degree completed: To be completed.
HONORS

- 2010: Sanofi Pasteur Travel Grant for Latin American Women Physicians and Scientists. 14th International Congress on Infectious Diseases. Miami, USA.
- 2012: Sanofi Pasteur Travel Grant for Latin American Women Physicians and Scientists. 15th International Congress on Infectious Diseases. Bangkok, Thailand.

ORAL PRESENTATIONS

- 2013: “Novel nanotechnology to concentrate and preserve Trypanosoma cruzi antigens in urine for early diagnosis of reactivation of Chagas disease in patients co-infected with HIV virus”. In: 62th ASTMH. Washington DC -USA.