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## The Incidence of Chagas Coinfections Amongst Acute Dengue Patients in Machala, Ecuador

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# **The Incidence of Chagas Coinfections Amongst Acute Dengue Patients in Machala, Ecuador**

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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## Abstract

Dengue fever is a febrile illness found throughout the tropics that, in severe cases, can be deadly. The most rapidly spreading of any mosquito-borne disease, dengue is re-emerging as an illness of great concern in Latin America and around the globe. The CDC estimates that as many as 400 million cases of dengue occur each year. The pathogenesis of dengue virus is complicated and acts through modulation of the host immune system. Dengue polarizes the immune system balance of T helper 1 (Th1) and T helper 2 (Th2) cells towards a Th1 inflammatory response. Parasitic infections have also been shown to affect the Th1/Th2 balance of the immune response, although how these immunological changes alter the severity of dengue during possible coinfections has yet to be explored. One of the most common parasitic infections in Latin America is *Trypanosoma cruzi*, the etiological agent of Chagas disease. Like dengue, *T. cruzi* polarizes the Th1/Th2 balance of its host. By triggering a Th2 response, Chagas disease may counteract the Th1 response caused by dengue thereby masking dengue symptoms, increasing the frequency of asymptomatic infections and leading to underreporting of dengue in regions where Chagas is common. In order to begin to examine the effect of parasites on dengue pathogenesis, this study examined the incidence of Chagas disease and *T. cruzi*/DENV coinfections in Machala, Ecuador. The sample set used for this study was collected as part of a larger dengue study in Machala by SUNY Upstate Medical University. The incidence of Chagas was 3.1% (n=360) and that of *T. cruzi*/DENV coinfections was 0.6%. The average age of Chagas positive individuals was 50 and 81.8% were female. As this study was a preliminary screening, a larger study should be undertaken in order to better assess the *T. cruzi*/dengue situation in Machala and to further the knowledge base of the immune response to dengue via analysis of the effects of coinfections on disease progression.

## Executive Summary

Dengue fever is a viral disease transmitted via the *Aedes aegypti* mosquito that is common to the tropics, especially urban, coastal regions. The most rapidly spreading of any mosquito-borne disease, dengue is re-emerging as an illness of great concern in Latin America and around the globe. Although it is classified as a neglected tropical disease, according to the CDC over 400 million cases of dengue occur throughout the world each year. Many cases of dengue go unreported, as the majority of dengue infections are asymptomatic. When clinical symptoms do appear they include mild to severe fever, behind the eye pain, severe headache and muscle/joint pain. In its more serious forms, dengue can progress to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), which may lead to plasma leakage, severe hemorrhage, organ failure and even death. There is currently no accepted vaccine or specialized treatment for dengue.

One way that dengue affects infected patients is through polarization of the body's balance between T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 cells cause an inflammatory response, while Th2 cells are anti-inflammatory and are involved in the antibody-mediated response. Numerous factors affect the severity of dengue illness, including the presence of underlying infections. Many chronic underlying diseases in tropical environments are due to parasites. It is possible that a single person could be infected with both a parasitic and dengue at the same time. Parasitic infections have also been shown to affect the Th1/Th2 balance of the immune response, although few studies have examined how these changes alter the severity of dengue during possible coinfections.

*Trypanosoma cruzi*, a human parasite that causes Chagas disease, is a likely dengue coinfection. Also known as American trypanosomiasis, Chagas is found mostly in Latin

America, where it accounts for approximately 23,000 deaths per year. Chagas infection occurs through contact with the feces of triatomine bugs, commonly referred to as “kissing bugs,” or through contaminated blood. Like dengue, Chagas disease can be asymptomatic. Acute symptoms can include unilateral facial edema (known as Romaña’s sign), fever, muscle pain, difficulty breathing, abdominal pain and/or skin lesions. During chronic infections, parasites are often confined to the heart and digestive tracts and patients may develop cardiac, digestive, neurological or mixed complications. *T. cruzi* is a single celled organism that is known to cause a Th2 response and severe immune system disturbances. By promoting a Th2 response, Chagas disease may mediate the strong Th1 immune system polarization caused by dengue, thus masking dengue symptoms, increasing the frequency of asymptomatic infection and leading to underreporting of dengue in regions where Chagas is common.

In order to begin to examine the effect of parasites on dengue disease progression, this study examined the incidence of Chagas disease and *T. cruzi*/dengue virus coinfections in Machala, Ecuador. Machala is the capital of El Oro province and currently serves as a primary research site for the Center for Global Health and Translational Research at SUNY Upstate Medical University. The overarching goal of the Upstate study is to examine the incidence of dengue as well as the burden of clinical disease in the region. Since its beginning in 2012, over 500 samples have been collected for the Upstate study through referrals of hospitalized patients clinically diagnosed with dengue as well as cluster investigations. When a referred patient was confirmed to have dengue via a positive NS1 rapid test (which tests a patient’s blood for the presence of the dengue virus protein NS1), study field teams performed a cluster investigation by visiting said patient’s house and recruiting family members and neighbors to join the study.

Volunteers who partook in the study provided blood samples and answered demographic as well as housing surveys. These blood samples were then tested for dengue and Chagas disease.

360 blood samples were screened for Chagas disease via the ORTHO *T. cruzi* ELISA test system, which consists of three stages. First, samples and controls were added to microwells pre-coated with *T. cruzi* antigens (a part of the parasite that triggers an immune response). If antibodies against *T. cruzi* were present in the blood samples, they attached to the microwells. Unbound antibodies were washed away. During the second stage, conjugate (mouse antibodies that can attach to human antibodies and were connected to an enzyme – Horseradish Peroxidase) were added to the microwells. The conjugate specifically attached to anti-*T. cruzi* antibodies that bound to the microwells during the first stage. Subsequent washes removed unbound conjugate. The final stage involved an enzyme detection mechanism in which the compound *o*-phenylenediamine (OPD) was added and oxidized by the Horseradish Peroxidase, causing a measurable color change only in the microwells that were exposed to antibodies against *T. cruzi* from Chagas positive samples.

The incidence of Chagas in Machala was determined to be 3.1% and the incidence of *T. cruzi*/dengue coinfections was 0.6%. The average age of Chagas positive individuals was 50 and 81.8% were female. While this sample set only contained 360 volunteers, the fact that Chagas and *T. cruzi*/dengue coinfections were identified in such a relatively small sample is promising for future studies. These findings also confirm that it is worthwhile to continue testing the incoming samples from the larger SUNY Upstate dengue study for Chagas and *T. cruzi*/dengue coinfections. While this study was a promising preliminary screen for Chagas, a larger study must now be undertaken in order to better assess the *T. cruzi*/dengue burden of disease in

Machala and to further the knowledge base of the immune response to dengue by examining the effects of coinfections on disease severity.

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I thank Christine King for her mentorship and the use of her lab. I would also like to give a special thanks to Arturo Barbachano, who was always there to answer my questions and teach me something new. The streams of questions he would ask forced me to think critically on the spot and encouraged me to ask questions of my own. Arturo made going to lab educational, yet incredibly fun.

I also give my thanks to Tina Lupone who was instrumental in organizing my project and my work in Ecuador. If ever I needed something, I could always count on Tina to make it happen. Additionally, I would like to give warm thanks to Katty Cueva and the Ecuadorian field team, who made my time in Ecuador both meaningful and fun. I look forward to working with them, and the rest of the CGHAST family, again in the future.

I would like to thank the Renee Crown Honors Program for their support both with advising and through Crown funding. Finally, I would like to thank my reader Sandra Lane for her support through what has been a chaotic process.

## **Advice to Future Students**

Your Capstone will likely be the most exciting and daunting endeavor you undertake while at Syracuse University. I hope you view this massive project not as an obstacle, but as an opportunity. Capstone projects are a chance for you to explore a topic that genuinely interests you and Crown-Wise funding provides an incredible opportunity to take your research interests and ambitions to the next level. With that in mind, I leave this advice:

**1. Start early.** And don't let anyone tell you otherwise. As a freshman, I already had a general idea of what I wanted my Capstone project to be. After returning from a Global Medical Brigade to Honduras, I decided that I wanted to perform some sort of public health research in Latin America. I started reaching out to faculty members and found that field research, especially related to human health, was not common in the SU Biology department. When I would mention to upperclassmen that I was trying to develop my Capstone project, they would tell me to relax and not give it much thought until at least junior year. Looking back now, I wish I had disregarded this advice and worked harder to find a project that interested me. My Capstone has certainly taught me the value of patience. Institutional Review Board decisions take time, experiments go awry and unforeseen setbacks are commonplace in research. If I had solidified my Capstone plans sooner than my second semester junior year, such obstacles would have been less stressful.

**2. Take the initiative and develop a project that truly interests you.** When I was abroad, I took a class on tropical medicine and disease and became interested in the study of dengue fever. After I returned to Syracuse, I searched for people in the area who work with dengue or other neglected tropical diseases. One of the best decisions I made throughout my entire college career was reaching out to Dr. Tim Endy,

who leads dengue studies at Upstate. A simple email sparked my involvement with Upstate's Center for Global Health and Translational Medicine and led me not only to the Capstone I desired, but a trip to Ecuador that was one of the most incredible and educational experiences I have had to date. Don't be afraid to send that one email. Reach out to faculty, community members or anyone else who is doing something that you find interesting. You never know where it might lead.

**3. Dare to be different. Take a risk.** Most biology Capstones come from bench research in a lab, but I wanted to work in the field. I decided to take a risk and find my own project outside of the norm. Being different means different stresses and projects that are less relatable to peers. None of my friends had trouble with mentors being in other countries or not having someone to meet with face-to-face. It was hectic working in a large study with many people as I didn't have one advisor, but many and each served a different role. That being said, no one had the experiences I did including living and working in Ecuador, collaborating with lots of great people from different backgrounds and cultures and participating in numerous parts of a large research study from sample collection to data analysis. While my Capstone experience was one of the most stressful, it was also one of the most rewarding. With all this in mind, the most important advice I can give to an incoming Honors student is also the simplest – relax, follow your heart and enjoy the ride!

## Introduction

Dengue virus (DENV) is a pathogenic flavivirus with four serotypes that is known to cause febrile illness. The most rapidly spreading of any mosquito-borne disease, dengue is re-emerging as an illness of great concern in Latin America and around the globe. Despite being a neglected tropical disease, the World Health Organization estimates that 96 million clinical cases of dengue occur each year and that almost half of the world's population lives in countries where dengue is endemic (WHO - Dengue, 2015). The CDC reports the suspected number of dengue cases per year to be as high as 400 million (CDC, 2015).

Dengue is transmitted via the *Aedes aegypti* mosquito and is found throughout the tropics, primarily in urban, coastal regions. While the majority of DENV infections are asymptomatic, clinical symptoms include mild to severe fever, retro orbital pain, severe headache and muscle/joint pain (Chastel, 2012). In its more serious manifestations, DENV can cause dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), which may lead to plasma leakage, severe hemorrhage, organ failure and even death (Tsai, 2013). About 500,000 people are hospitalized each year due to DHF, with 2.5% of these cases resulting in mortality (CDC, 1989). Due to its lack of distinguishing clinical symptoms, however, the incidence of dengue in many regions of the world goes undocumented. There is currently no accepted vaccine or specialized treatment for dengue.

As in many Latin American countries, DHF is reemerging in Ecuador after an extended period relatively free of dengue transmission from 1948 to 1958. Dengue was reintroduced to Ecuador in 1988 when DENV-1 (serotype 1) caused an outbreak of classic dengue that affected 420,000 people (CDC, 1989). DENV-1, DENV-2 and DENV-4 all circulated throughout Ecuador from 1993 to 1999. In 2000, DENV-3 was introduced to the region and the first cases of

DHF were reported (Alava, 2005). El Oro province recorded its worst dengue epidemic in 2010, which involved approximately 3,900 cases of dengue fever and 108 cases of DHF (Stewart-Ibarra, 2013). Machala, a port city on the southern coast of Ecuador, is the capital of El Oro province and home to 41% of the province's population. During the 2010 epidemic, 48% of dengue cases from El Oro were reported from Machala (Stewart-Ibarra, 2013).

The pathogenesis of DENV is complicated and acts through modulation of the host immune system. One way that DENV affects the host is through polarization of the balance of T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 cells are responsible for the production of inflammatory cytokines, while Th2 cells have an anti-inflammatory function and are involved in the antibody-mediated response (Basso, 2013). DENV is known to cause a Th1 response and a polarization of the Th1/Th2 immune system has been shown to determine the severity of clinical dengue (Chaturvedi, 2000). Dengue pathogenesis has been linked to a variety of factors including: viral cytotoxicity, viral load, immunopathogenesis, cytokines, autoimmunity, host genetics, antibody-dependent enhancement (ADE), viral genotypic nucleotide variation and underlying host diseases (Tsai, 2013). Many chronic underlying diseases in tropical environments are due to parasites. It is possible that parasitic disease occurs concurrently with dengue infection in a single host. Parasites and humans have coevolved with parasites continuously developing new ways to exploit hosts for their own reproductive gain and humans responding with new adaptations to prevent initial infection and proliferation. Evolutionary pressures have made it so that many parasites cause underlying, chronic infections in humans (Sheldon, 1996). Parasitic infections have also been shown to affect the Th1/Th2 balance of the immune response, although few studies have examined how these immunological changes alter the severity of dengue during possible coinfections (Chaturvedi, 2000). A study in French

Guiana found that dengue/malaria coinfections are biologically and clinically distinct from independent infections. Specifically, coinfections were more severe in terms of hematological manifestations, length of fever and frequency of hospitalization (Epelboin, 2012).

*Trypanosoma cruzi*, a human parasite that causes Chagas disease, is a likely dengue coinfection in Latin America. Despite also being classified as a neglected tropical illness, approximately seven to eight million people are infected worldwide, making Chagas the second most important arthropod-borne disease in the world (Walter Reed, 1998; WHO - Chagas, 2015). Also known as American trypanosomiasis, Chagas is found mostly in Latin America, where the disease is endemic. The World Bank estimates that Chagas disease accounts for approximately 23,000 deaths per year in Latin America, making it the fourth largest socioeconomic burden for the region after acute respiratory infections, diarrheal diseases and AIDS (Prata, 2001).

Chagas infection occurs through contact with the feces of triatomine bugs, commonly referred to as “kissing bugs,” or through contaminated blood. Triatomine bugs live in poorly kept homes and Chagas is thus often associated with impoverish housing in low-income urban or rural areas. At night, triatomine bugs feed on human blood, breaking skin, usually on the neck or face, and providing an open wound for fecal matter to enter. The eyes, nose and mouth are also possible points of contaminated fecal matter entry (Walter Reed, 1998).

Like dengue, Chagas disease can be asymptomatic. Clinical manifestations of acute infection (within two months) usually appear 5-14 days post-exposure and include unilateral facial edema (known as Romana’s sign), fever, muscle pain, difficulty breathing, abdominal pain and/or skin lesions. During chronic infections, parasites are often confined to the heart and digestive tracts. 30% of patients with chronic Chagas disease develop cardiac disorders and as many as 10% acquire digestive, neurological or mixed alterations. 20% of cases that progress to

cardiac, digestive and/or neurological disorders result in fatalities (Walter Reed, 1998; WHO - Chagas, 2015).

In terms of its pathogenesis, *T. cruzi* is a flagellated intracellular protozoan that is known to cause a Th2 response and severe immunological disturbances, including progressive inflammation (DosReis 1997; Lopes, 1999; Walter Reed, 1998). It is possible that a *T. cruzi* coinfection with DENV may impact the pathogenesis and clinical severity of dengue. By promoting a Th2 response, Chagas disease may mediate the strong Th1 immune system polarization caused by dengue, thus masking dengue symptoms, increasing the frequency of asymptomatic infection and leading to underreporting of dengue in regions where Chagas is common.

The World Health Organization has recognized a need for additional research regarding dengue. Although there is a perceived increase in disease burden, dengue epidemiology of the Americas region has not been well documented (San Martín, 2010). This, coupled with the lack of data regarding disease incidence and the frequency of coinfections in Ecuador, reveals a need for dengue research in the region. Machala, Ecuador currently serves as a primary research site for the Center for Global Health and Translational Research at SUNY Upstate. Ecuador was chosen as the study site in order to promote international collaboration and because of the infrastructure already in place. Since 2012, the dengue study team has worked alongside Ecuadorian collaborators with support from the Ecuadorian Ministry of Health. The primary focus of the study is to examine serotype-specific incidence of DENV as well as the burden of clinical disease in the region. Since its induction, over 100 serologically positive dengue patients with clinical DF or DHF have been identified. Additionally, approximately 400 febrile seronegative samples have been collected to date.

While El Oro is one of the most endemic regions of Chagas in Ecuador, prior to this study the incidence of neither dengue nor Chagas had been studied in detail in the Machala region (Black, 2009; Walter Reed, 1998). This study seeks to close this research gap by measuring the incidence of Chagas disease and *T. cruzi*/DENV coinfections in the Machala region of Ecuador. Additionally, this research serves as a preliminary study of the incidence of *T. cruzi* in Machala in order to gauge the possibility of launching a larger research initiative in the area focusing on dengue coinfections. Much remains to be elucidated regarding the pathogenesis of dengue. Studying the effects of coinfections on dengue progression may be key to understanding dengue disease progression and what factors contribute or hinder the development of DHF and DSS.



## **Methods**

### Subject Enrollment

*Population Size* – 245,972 residents of Machala

### *Hospitalized Subjects*

Teofilo Davila Hospital (TDH), the central Ministry of Health hospital for El Oro Province, is the primary study site in Machala. TDH is where the Ministry of Health refers severe dengue patients in the region and is also home to the study diagnostic laboratory. In addition, four of the twenty-one surrounding public clinics and hospitals were chosen to be included in the study. These care centers are geographically representative of Machala and include the Ministry of Health clinics: Brisas del Mar, Rayito de Luz, Mabel Estupiñan and El Paraiso (Appendix 1). All patients over six months of age that are clinically diagnosed with dengue at these care centers may be included as study subjects.

Hospitalized patients with a clinical diagnosis of dengue were referred to TDH, where a nurse or laboratory technician obtained informed consent, collected a blood specimen and recorded demographic information including home address, chief complaint/reason for hospitalization, date of onset of symptoms, symptoms during illness, oral temperature, travel history and assessment of vector contact. Additionally, a convalescent blood sample was taken during a two-week follow up visit.

### *Cluster*

Up to five NS1-confirmed dengue patients identified through the care centers were chosen each week as the center of a cluster investigation. Field teams visited these patients' houses and recruited family members. A blood sample and demographic information was collected from up to four family members. CDC backpack aspirators were used to capture mosquitos in and around the home. In addition, household surveys were conducted to identify dengue risk factors such as demographics, housing condition, dengue awareness and access to public services. This process was then repeated in four neighboring households located within a 200 meter radius of the index household; the typical flight range of the *Aedes aegypti* mosquito. Up to five participants were included in the study from each neighboring household.

### Blood Specimen

A 20 ml blood sample (adjusted for age and weight by NIH criteria) was obtained by venipuncture from each hospitalized subject. For cluster participants, a 3-10 ml blood sample was obtained. Serum, cells and plasma were aliquoted into multiple tubes and stored at -70°C. Samples are currently stored in two sites: One aliquot of each type of sample (serum, cells, plasma) is stored in county at the MOH laboratory in Machala; all other aliquots are at Dr. Endy's laboratory at SUNY Upstate Medical University in Syracuse, New York for additional analyses, including the ones outlined in this study.

### Dengue Diagnoses

#### *Laboratory Assays*

Serum samples were tested for the presence of NS1 protein, IgM and IgG antibodies and viral genome. Initial serum screening involved the detection of NS1 protein via rapid STRIP tests.

PanBio Dengue IgM and IgG Capture ELISAs were used to test for the presence of anti-dengue antibodies in serum samples and to differentiate between primary and secondary infections (See Appendix 2). PanBio Dengue NS1 ELISAs were also performed, as they are more reliable, yet slower than the rapid tests. Serotype-specific RT-PCR assays were run to confirm DENV infection by detecting the presence of viral genome and to examine what DENV serotypes were in the region.

### *Dengue Definitions*

Acute dengue was defined as any sample that was positive by NS1 rapid test, NS1 ELISA and/or RT-PCR. Positive IgM ELISA samples were considered to be recent primary infections and positive IgG ELISA samples were defined as secondary infections.

### Chagas Diagnoses

#### *Laboratory Assay*

360 serum samples collected from March to September 2014 were tested at SUNY Upstate Medical University in Syracuse, New York using the ORTHO *T. cruzi* ELISA test system and the corresponding FDA protocol. Some samples from the larger study had limited serum quantities available and were thus excluded from the Chagas analyses for solely this reason. In the first stage of the test, samples and controls were added to microwells pre-coated with *T. cruzi* antigens and incubated for 1 hr at 37°C. If antibodies against *T. cruzi* were present in the serum samples, they attached to the microwells, forming antibody-antigen complexes. Unbound antibodies were removed by eight consecutive washes, each with 300 µL of Ortho Wash Buffer. Wash was left to soak in the plate for 10-30 seconds before removal. During the second stage,

conjugate (murine monoclonal antibodies conjugated with Horseradish Peroxidase) was added to the microwells and incubated for 30 min at 37°C. The conjugate specifically attached to anti-*T. cruzi* antibodies that had bound to the microwells during the first stage. Subsequent washes removed unbound conjugate. The final stage involved an enzyme detection mechanism in which added *o*-phenylenediamine (OPD) was oxidized by bound conjugate peroxidase, causing a color change (See Appendix 3). After a 30 min incubation in the dark, 4N sulfuric acid was used to stop the reaction. The intensity of the resulting color is directly proportional to the number of anti-*T. cruzi* antibodies bound to each microwell. A BioTek  $\mu$ Quant microplate spectrophotometer was used to measure the intensity of the colored substrate in each microwell at 492 nm with a 630 nm reference.

#### *Chagas Validity Tests*

The validity of each ELISA plate was measured as set forth by the Ortho FDA protocol. Each ELISA plate was run with one blank well, two negative controls and three positive calibrators. After adding controls, calibrators and samples, a photometric Sample Omission Monitoring (SOM) test was performed at 610 nm. SOM values were calculated by dividing the optical density (OD) of each well by the optical density of the blank well. In order to continue, each control, calibrator and sample had to have a SOM value  $\geq 1.400$ .

An additional photometric test, Conjugate Omission Monitoring (COM), was performed at 492 nm. In order to continue testing, each well had to have a not blank-adjusted absorption of  $\geq 0.700$  OD.

Each ELISA plate had to meet certain quality control measures in order for the results to be considered valid. First, the absorbance values of blank wells had to be between -0.010 and 0.50 OD and the positive calibrator values had to be between 0.300 and 1.800 OD. Additionally, each positive calibrator had to be within 15% of the positive calibrator mean value. Negative controls had to have a signal to cutoff ratio (S/C) between -0.012 and 0.300 in order for the plate to be considered valid.

#### *Chagas Interpretation of Results*

The cutoff value for each plate was set as 42.5% of the positive calibrator mean. As the ORTHO *T. cruzi* ELISA test is traditionally used to screen donor blood, this cutoff value is set conservatively. A signal to cutoff (S/C) ratio was determined for each sample by dividing the blank-adjusted OD value by the cutoff value determined for that particular plate. Any sample with a S/C of  $\geq 0.700$  was considered positive (Figure 1).

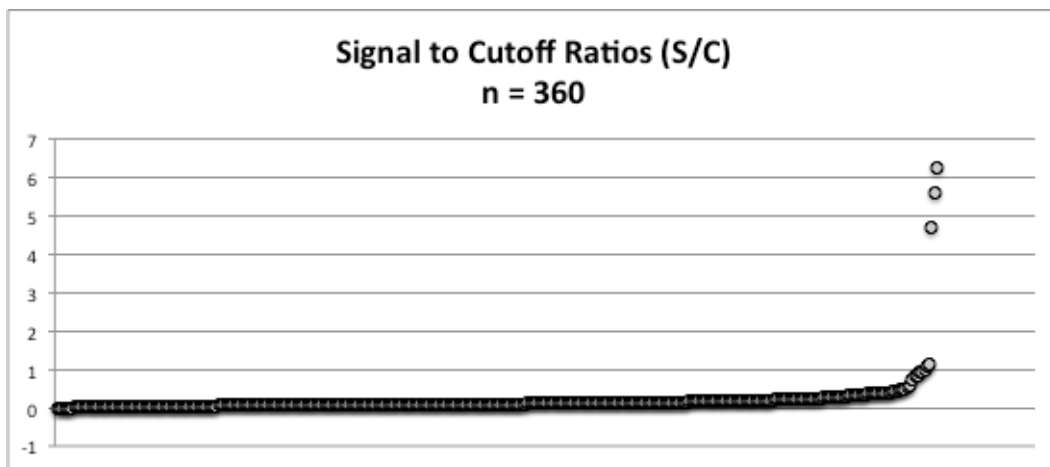
#### Institutional Review Board

This study was reviewed and approval by IRB at SUNY Upstate Medical University as well as by the Luis Vernaza Hospital IRB in Guayaquil, Ecuador, with regional approval by the Ecuador Ministry of Health. Luis Vernaza Hospital is the IRB of record for this protocol. The Chagas analyses were added to the initial IRB via an amendment that received approval from both SUNY Upstate and the Ecuador Ministry of Health.

## Results

### Chagas Positive Samples

Of the 360 samples screened for *T. cruzi* infection, five had signal to cutoff ratios (S/C) above one, indicating that they were positive. Additionally, six samples had OD values just below the cutoff, but substantially higher than the rest of the samples on the same plate (Figure 1). These six samples were included as positives. In future analyses, the 11 samples assumed to be positive will be retested in duplicate to confirm that they are true positives.



**Figure 1: S/C Ratios** – Signal to cutoff ratios were determined for each of the 360 samples tested. 11 samples, all with  $S/C \geq 0.700$ , were deemed positive.

### The Incidence of Chagas and *T. cruzi*/DENV Coinfection

The incidence of Chagas disease in the sample set was found to be 3.1%. This is comparable to other studies in the region, which found the incidence of Chagas in Manabí, Guayas and Lojas provinces to be 5.7%, 1.0% and 3.6%, respectively (Black, 2009). 55% of the Chagas positives came from the same cluster, but not necessarily the same household. *T. cruzi*/DENV coinfections were discovered, with an incidence of 0.6%. Of the samples found to be dengue positive (n=88), 2.3% were also Chagas positive.

## Demographic Distribution

<b>Sample Demographics</b>	
<b>Age</b>	<b>Average (Range)</b>
Sample Set	33 (1-86)
Acute Dengue	25 (1-73)
Chagas	50 (23-77)
Coinfection	48 (38-58)
<b>Gender - Female</b>	<b>n (%)</b>
Sample Set	219 (61.3)
Acute Dengue	44 (50.6)
Chagas	9 (81.8)
Coinfection	1 (50)
<b>Location - Cluster</b>	<b>n (%)</b>
Sample Set	234 (64.8)
Acute Dengue	44 (50.6)
Chagas	9 (81.8)
Coinfection	1 (50.0)

**Table 1: Sample Demographics Comparison** – A comparison of the average age, age range, gender breakdown and location distribution among the entire sample studied (n=360), the samples that were positive for acute dengue (n=87), the samples that were positive for Chagas (n=11) and the samples that were positive for both dengue and Chagas (n=2).

### *Age*

The average age of the Chagas positive samples was substantially above the average age for the sample set in its entirety as well as that for the acute dengue samples. This is consistent with previous studies in the area, which found that Chagas incidence increases with age in the provinces of Manabí and Guayas (Black, 2009). This is likely due to the fact that older people have had more time to be exposed to chronic infections such as *T. cruzi*. Dengue infections, however, are more frequent in younger populations. In fact, the majority of dengue cases recorded in the SUNY Upstate study occurred in individuals between ages 10 and 14 (AM Stewart Ibarra et al, in prep).

### *Gender*

The large majority of Chagas positive samples were female (81.8%). This gender skew was surprising and warrants future investigations into whether females in Machala are at an increased risk of contracting Chagas and, if so, what factors lead to this increased risk. The gender distribution of *T. cruzi*/DENV coinfections was evenly split, but due to the extremely low sample size (n=2), a definitive statement about the gender breakdown cannot be made with confidence. Past studies have found a relationship between gender and risk of dengue coinfection. In French Guiana, males were found to be more likely to have malaria/DENV coinfections (Epelboin, 2012). It is thus possible that gender may be a significant risk factor for other dengue coinfections as well.

### *Location*

The distribution of sample collection location was very similar to that of gender distribution, with the vast majority of Chagas positive samples being collected from cluster investigations rather than patients admitted to the study through sentinel clinics. This is not overly surprising as Chagas is often asymptomatic and those infected with the disease may not seek medical attention.

### Housing

<b>House Condition</b>		
	<b>Sample n (%)</b>	<b>Chagas Positive n (%)</b>
Bad (Old/Poorly Maintained)	16 (11.6)	1 (11.1)
Regular	79 (57.2)	4 (44.4)
Good (New/Well Maintained)	43 (31.2)	4 (44.4)



**Table 2: House Condition Comparison** – A comparison of the housing conditions between Chagas positive households and the entire sample set. House conditions were ranked as ‘Bad (Old/Poorly Maintained),’ ‘Regular’ or ‘Good (New/Well Maintained)’ by field team members conducting household surveys during cluster investigations. The data for 138 of the 241 households included in the sample set and 9 out of 10 of the Chagas positive households were included in Table 2. (Some households were not part of cluster investigations and thus household surveys were not conducted for them.)

The distributions of housing conditions between the entire sample set and the Chagas positives are very similar, suggesting that housing condition is not a notable risk factor for Chagas disease. This contradicts the widely accepted idea that poorly kept homes are more likely to house triatomine bugs and thus have a higher risk of Chagas transmission. It is important to recognize, however, that this analysis included a limited number of housing information (n=9) and that the classification of a house as bad, regular or good was subjective and may have varied amongst surveyors. Interestingly, five of the ten Chagas positive households (50%) were from the same cluster investigation indicating that the houses were within 200 meters of one another.

## Discussion

The incidence of Chagas disease in our subset of the population of Machala, Ecuador was found to be comparable to national averages and previous studies in the surrounding regions. Additionally, this study documented that *T. cruzi*/DENV coinfections occur in Machala, albeit at a low incidence of 0.6%. While this sample set only contained 360 volunteers, the fact that Chagas and *T. cruzi*/DENV coinfections were identified in such a relatively small sample is promising for future studies. These findings also confirm that it is worthwhile to continue testing the incoming samples from the larger SUNY Upstate dengue study for Chagas and *T. cruzi*/DENV coinfections.

As expected, the population that was positive for Chagas was on average older than the general sample set. The average age for the population that was classified as having acute dengue, however, was younger than expected. This age distinction may limit the number of *T. cruzi*/DENV coinfections as older individuals are more likely to have Chagas and younger ones are more likely to have dengue.

The majority of the Chagas positive cases (55%) came from a single cluster investigation, suggesting that Chagas disease in Machala may be concentrated in certain areas or neighborhoods. This could have important policy implications for Chagas prevention and educational campaigns in the area. As it is common for families to immigrate to Machala and live in neighboring houses, it is also possible that the cluster consists of one family that was infected with Chagas in a higher-risk region, such as the highlands, before moving to the coast.

The next step in this investigation is to launch a larger Chagas and dengue initiative in Machala. While the preliminary results discussed here are promising and show some interesting trends, including the potential correlation between gender and Chagas risk, the sample size of

this study was low. In order to make stronger conclusions, the number of samples included in the analysis must be increased. Once the sample size is expanded, the clinical symptoms of *T. cruzi*/dengue coinfections will be compared to independent dengue infections in order to examine whether coinfection affects the severity of dengue illness. The results of this comparison may help elucidate the underlying mechanisms of dengue pathogenesis by exploring how parasitic coinfections modulate this response.

Future samples will also be compared to social determinants of health in order to determine significant risk factors for the independent Chagas and dengue illnesses as well as coinfection. Additionally, future studies should map the incidence of confirmed dengue and Chagas in the area surrounding Machala, Ecuador. This data could be used to develop targeted Chagas and dengue prevention strategies and may also reveal Chagas and dengue hot spots in the city and surrounding area. While this study was a promising preliminary screen for Chagas, a larger study must now be undertaken in order to better assess the *T. cruzi*/dengue burden in Machala and to further the knowledge base of the immune response to dengue via analysis of the effects of coinfections on disease progression.

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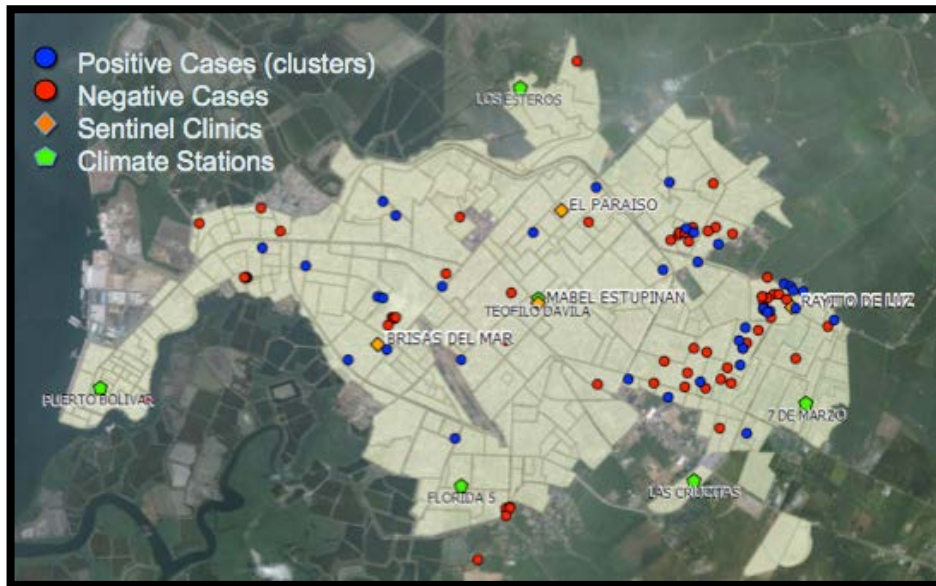
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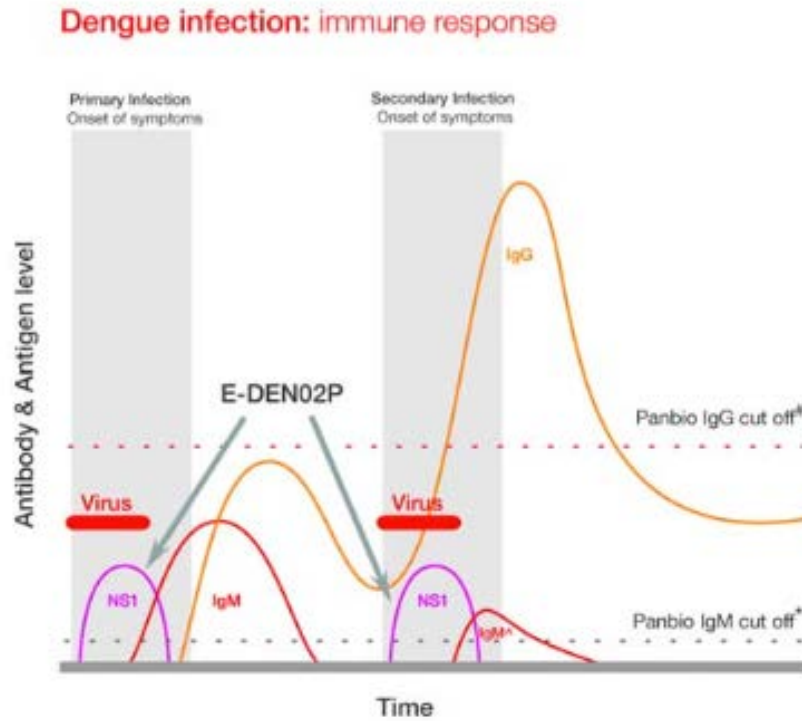
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## Appendix 1 – Study sites in Machala, Ecuador



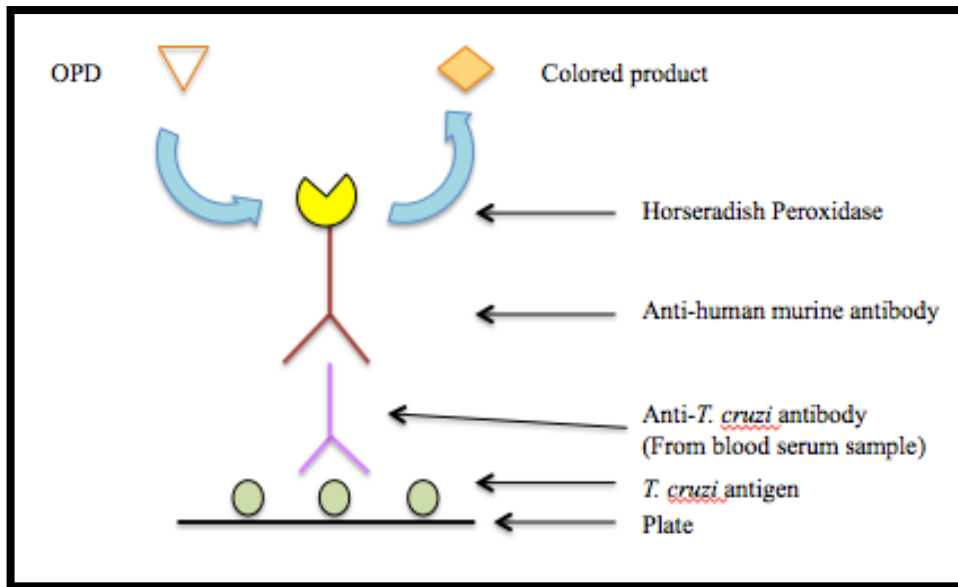
A map of Machala showing the geographic distribution of the five sentinel clinics included in the study as well as the distribution of dengue cases from 2014 (AM Stewart Ibarra et al, in prep)

## Appendix 2 – The immune response to dengue



A timeline outlining the detectable presence of dengue virus, NS1 protein as well as IgM and IgG antibodies in the serum of infected patients. The graph distinguishes between primary and secondary infections and also includes the cutoff levels for PanBio IgM and IgG ELISA kits (PanBio Dengue Early ELISA protocol)

### Appendix 3 – Chagas ELISA design



The ORTHO *T. cruzi* ELISA test system consists of three stages. First, samples and controls are added to microwells pre-coated with *T. cruzi* antigens. If antibodies against *T. cruzi* were present in the serum samples, they attached to the microwells, forming antibody-antigen complexes. Unbound antibodies are washed away. During the second stage, conjugate (murine monoclonal antibodies conjugated with Horseradish Peroxidase) are added to the microwells. The conjugate specifically attaches to anti-*T. cruzi* antibodies that have bound to the microwells during the first stage. Subsequent washes removed unbound conjugate. The final stage involved an enzyme detection mechanism in which added *o*-phenylenediamine (OPD) is oxidized by bound conjugate peroxidase, causing a color change.