UNIVERSITY OF CALIFORNIA
RIVERSIDE

Chagas Disease in the United States and the Prevalence of *Trypanosoma cruzi* in Southern California Woodrats

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Masters of Science

in

Genetics, Genomics and Bioinformatics

by

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March 2012

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Acknowledgements

I would like to thank everyone who helped me complete this project. At UCR, I am thankful for the help I received from Dr. Allen, Dr. Rao, Dr. Vidalakis, Dr. Wang, Dr. Weirauch, the lab of Dr. Roper and librarian Bernice Ridgeway. In addition, I would like to acknowledge Stephen Bennett from Orange County Vector Control District who provided the woodrats used in the experiment. Also, I would like the thank Miguel Pinto and Texas Tech University for providing the infected woodrat tissue. Last but not least, I would like to thank the friends who supported me, put up with me during stressful times, and who gave me advice on my work.

Dedication

I dedicate my thesis to Alexandra Koetter; my co-worker and friend at UCR.

ABSTRACT OF THE THESIS

Chagas Disease in the United States and the Prevalence of *Trypanosoma cruzi* in Southern California Woodrats

by

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Master of Science, Graduate Program in Genetics, Genomics and Bioinformatics
University of California, Riverside, March 2012
Dr. Mike Allen, Chairperson

Trypanosoma cruzi is the protozoan parasite that causes Chagas disease. Millions of people are infected with the incurable disease and it is estimated that thousands die from Chagas every year. It is thought that all triatomine, which are almost exclusive to the Americas, are capable of being a disease vector and all mammals can be a host of the parasite. Parasites are transmitted from wildlife to man primarily in Latin America, but due to migration, blood transfusions, organ donations, and vertical transmission, the disease now exists worldwide. In addition to Latin America, autochthonous transmission has occurred in North America. Most of the reports of *T. cruzi* in the United States wildlife are from the southwest where the primary mammalian host is the woodrat. The health of 55 southern California *N. fuscipes* woodrats was evaluated by PCR using the TCZ1/TCZ2 primer set. None of the woodrats tested were found to be infected with the *T. cruzi* parasite.

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Introduction

Chagas disease was discovered in 1909 by Carlos Chagas, a Brazilian doctor who was studying malaria at the time (Chagas 1909). Some of his patients had symptoms that were not associated with malaria or any other known illnesses. He discovered that a bug (now known as Triatominae) found in many patients' homes harbored a flagellated protozoa. After some investigation, he found that this parasite was causing the locals to become sick. His studies on the protozoa resulted in a description of the *Trypanosoma cruzi* (*T. cruzi*) life cycle in vector and man (Chagas 1911).

Currently, Chagas is a neglected tropical disease (WHO 2011) that is endemic in 22 Latin American countries (Coura 2010). In 2010, the World Health Organization (WHO) estimated that 10 million people are infected with *T. cruzi* and more than 25 million people are at risk of contracting Chagas disease (WHO 2010). Additionally, it is thought that more than 10,000 people died from Chagas in 2008 (WHO 2010). However, there is no gold standard for diagnosis (Otani 2009) and there is no cure. For over 40 years, the only drugs available have been benznidazole and nifurtimox (Clayton 2010 [A]). However, they are only effective if administered soon after infection, treatment requires several months, and both drugs often cause side effects (Clayton 2010 [A], Leslie 2010, WHO 2010). Due to the lack of effective medication, preventative measures and public awareness are important to manage the disease. Control strategies focus on eliminating the triatomine vector by insecticide spraying and improving housing conditions (WHO 2010). However, some triatomine have become resistant to the insecticides (Leslie 2010).

Autochthonous transmission has occurred one place outside of Latin America, in the neighboring United States (Bern 2011). 1916 was the first time *T. cruzi* was reported in nature in the U.S. (Kofoid 1916, 1933), and the first reported human case occurred in 1955 (Woody 1955). Since then, there have been six additional documented autochthonous cases and many studies have found *T. cruzi* in wildlife in the southern part of the United States (Bern 2011). Therefore, Chagas disease may need more recognition in the United States than anywhere else outside of Latin America. The purpose of this thesis is to summarize what is known about Chagas disease, the likelihood of autochthonous transmission in North America, and the prevalence of the infection in the primary host in southern California (Bern 2011).

Although autochthonous transmission had not occurred outside of America, Chagas disease is becoming a worldwide issue due to migration (WHO 2010). Other than contracting the illness from infected tropical triatomine, Chagas can be acquired from blood transfusions, organ transplantations, and by vertical transmission (WHO 2010). Current blood screening in North America indicates that a sizeable population may be infected. Blood screening in the United States began in January 2007 and the overall seroprevalence from the 14 million blood donations tested in 16 months was 1:27,500 (Bern 2009). The highest infection rates were found in Florida (1:3800) and in California (1:8300) (Bern 2009). By the end of April 2010, more than 1000 donations were found to be infected (FDA News Release 2010). It has been estimated that there are over 300,000 infected people living in the US (Bern 2009, FDA News Release 2010, Schmunis 2010).

Moreover, it seems important to investigate Chagas disease within United States. The first part of my thesis consists of a literature review that examines current information about Chagas disease and the likelihood of autochthonous transmission in a southwestern region that directly borders Latin America: southern California. The second part of my thesis is a PCR experiment to research the occurrence of *T. cruzi* in the primary host of southern California.

I searched the published literature and the internet for literature containing information about all aspects of Chagas disease which includes; the *T. cruzi* parasite life cycle, the disease vector (triatomine), the sylvatic cycle, disease transmission, and the course of the disease in humans. Current data on blood screening and disease statistics was also found using the internet.

Literature Review

Triatomine: the vectors of *T. cruzi*:

Triatomine are the vectors of the *T. cruzi* parasite. There are more than 150 species of Triatominae and they are primarily found in the Americas (their approximate latitude range is from 46°N to 46°S) (Coura 2010, Schofield 2009). The only known exception is that *Triatoma rubrofasciata* has been found in some port areas including; the western Pacific region, Southest Asia, the Middle East, and Africa (Coura 2010). Although *T. cruzi* has yet to be found in wildlife outside of the Americans and there are triatomine that have never been found infected, all are probably capable of transmitting the disease (Schofield 2009). In addition, over 100 mammalian species have been found

to be a host of the parasite in the Americas and all mammals are thought to be capable of infection (Coura 2010, Bern 2011).

Triatomine become infected after ingesting *T. cruzi* trypomastigotes while blood feeding from an infected host. Within the Triatomine, the parasites will cycle through several forms. The trypomastigotes transform into epimastigotes in the midgut and asexually multiply by binary fission. The epimastigotes move to the hindgut and some transform into metacyclic epimastigotes which are released in the feces when defecation occurs (CDC 2010, De Souza 2002). Several studies have found that *T. cruzi* cause triatomine to feed more often, a behavior that encourages the survival of the parasite and increases disease prevalence. However, if the insect experiences periods of starvation while infected, it may lead to a loss of parasitemia (Botto-Mahan 2009). Typically, triatomine feed every 3 weeks but they have survived over 6 months without a meal (Bice 1966, Shields 1956).

The infected triatomine can then go on to feed from a human. Triatomine stand next to their sleeping victim and feed on exposed areas at night (Shields 1956). Because bites often occur on the face, the insect is also referred to as a kissing bug (Shields 1956). People usually find the bite to be painless but they may feel some tingling (Wood 1942). Feeding time to repletion of California triatomine seems to range between a few minutes to almost an hour depending on the difficulty in finding a blood vessel (Klotz 2009, Wood 1942, 1951, 1960 [A]). Sometime after feeding, the bug will defecate and release the parasite. The time of defecation in relation to blood feeding depends on the triatomine species and is an important indicator of vector efficiency (Klotz 2009).

Although when these insects feed on their host it is referred to as a bite, the mouthparts of triatomine are not capable of biting (Vetter 2001). The triatomine punctures the host's skin with their mandibles, and then introduces their lengthy, flexible maxillae. The dorsal food tube and ventral salivary tube are found between the right and left maxilla. During this probing phase, saliva is released and the maxillae make whip-like movements (which cause hematomas) until they find a blood vessel for feeding (Soares 2006). If the Triatomine cannot obtain a sufficient amount of food, they make try probing again nearby or in another area. Once it finds a suitable place to feed, the engorgement phase occurs and saliva continues to be pumped (Laviopierre 1959, Soares 2006). The saliva contains anticoagulants and anaesthetic compounds which increase feeding efficiency (Dan 1999, Ribeiro 1981). In the US, there is more emphasis on the allergic reactions to the saliva of Triatomine than to the possibility of contracting Chagas disease (Klotz 2010).

Chagas disease:

The metacyclic trypomastigotes from the triatomine feces may enter the human body though the bite wound or mucous membranes including the eyes, mouth and nose (Clayton 2010 [A]). Once inside, the trypomastigotes penetrate cells and transform into amastigotes which rapidly multiply by binary fission. They remain in the cells cytoplasm where they transform back into trypomastigotes until the host cell bursts, and then they enter the blood stream to reach other tissues. This cycle can occur all over the body and is what causes the clinical manifestations of Chagas (DPDx 2011).

The disease has different phases in the host. The acute phase persists for about two months post infection. During this time, many parasites circulate the blood. Therefore, detection is easy and the disease is curable, however, most people are asymptomatic (Leslie 2011, Rassi 2010, WHO 2010). Following the acute phase, some people develop the indeterminate (or latent) form of the disease. In this phase, people are asymptomatic however they are seropositive for *T. cruzi* (Rassi 2010). During the chronic phase, the parasites are sparse in the blood but they are within body tissues. Although some patients remain asymptomatic, as many as 30% of people will suffer from cardiac illnesses and up to 10% will have affected digestive and/or neurological function (WHO 2010). The chronic phase of the disease can lead to heart failure or sudden death caused by the destruction of the heart regardless of whether symptoms were present (Rassi 2010, WHO 2010).

Current treatment for the disease is only effective around the time of onset. The treatment time for the two drugs available are lengthy (up to 90 days for nifurtimox and 60 days for benznidazole) and as many as 40% of people experience side effects, some serious, therefore, many patients do not complete the regimen (Leslie 2011, WHO 2010). Neither medication is approved by the U.S. Food and Drug Administration (FDA) but they can be acquired through the Centers for Disease Control and Prevention (CDC) (Bern 2011).

Chagas disease and the likelihood of autochthonous transmission in the US:

So far, there have been only 7 reports of autochthonous transmission in the US (Bern 2011). However, eleven triatomine species have been found in the United States.

The reports have come from 28 states on the mainland (from coast to coast and as north as Pennsylvania) and in Hawaii (Bern 2011). Ten of these states have found *T. cruzi*; most of the reports are from the Southwest (Bern 2011). Two of the eleven triatomine species, *Paratriatoma hirsuta* and *Triatoma incrassata*, have never been found infected with *T. cruzi* (Bern 2011). So far, 24 animals have been found to be a host of *T. cruzi* in the United States (Bern 2011).

T. cruzi strains are now categorized into six types; TvI-TcVI (Zingales 2009). Only TcI and TcIV (previously TcIIa) genotypes have been found in the U.S. (Bern 2011, Roellig 2010). In studies on laboratory rodents, US isolates rarely cause symptoms or mortality (Roellig 2010). On the other hand, baboons in Texas have showed clinical signs of Chagas disease; four have died and 182 have tested seropositive for *T. cruzi* (Williams 2009). So far, there is no consensus on the relationship between *T. cruzi* strain and disease severity (Rassi 2010).

Chagas disease in southern California:

In the United States, the *T. cruzi* parasite was first found in Californian *T. protracta* in 1916 (Kofoid 1916, 1933). There are three Triatominae species endemic to California; *Triatoma protracta*, *Triatoma rubida*, and *Paratriatoma hirsuta* (Hwang 2010). These three species mainly associate with woodrats of the genus *Neotoma* and they are considered the primary reservoir in the western US (Bern 2011, Ryckman 1962). *T. protracta* and *T. rubida* will also feed from reptiles and amphibians, however, mammals are the only reservoir of the parasite (Ryckman 1954, Wood 1944). *P. hirsuta* has never been found infected in nature but it can be infected experimentally (Bern 2011,

Ryckman 1965 [A]). The cumulative infection prevalence of *T. protracta* and *T. rubida* in the United States is 17.5% (723/4,124) and 7.2% (96/1,340) respectively (Bern 2011). Included in this national calculation was a recent study on infected southern California *T. protracta*. Escondido and Glendora *T. protracta* had infection rates of 19% and 36% respectively (Hwang 2010). These statistics revealed a higher infection in southern California *T. protracta* than the average for the country.

The defecation time of the triatomine plays a major role in determining its efficacy as a vector of *T. cruzi* (Wood 1951, Zeledon 1977). One recent study found that only 55% *T. rubida* and 26% *T. protracta* defecated during the 1 hour observation period following the blood meal. When the triatomine defecated, it usually occurred within 1 minute of feeding, however, they never defecated on the host (Klotz 2009). A study in 1960 found that the time till defecation for infected *T. protracta* ranged from greater than 15 minutes to an hour (Wood 1960 [A]). In 1951, an experiment found a range of 0.1 minute to 133 minutes for the 69% of *T. protracta* that had their first defecating during the observation time. In the same study, *T. rubida* took about 0.1 to 6 minutes for their first defecation (Wood 1951). Based on defecation time, *T. rubida* would be more likely to transmit *T. cruzi*; however, *T. protracta* is more likely to carry the parasite (Klotz 2009, Wood 1951).

Conclusions based on the Literature

Chagas has been studied for over 100 years now so there is a vast amount of information about the sylvatic cycle and the disease in man. Because triatomine are found

in the Americas and autochthonous transmission has occurred in North America, there should be more recognition of the disease in the United States. Although physicians in the US are usually aware of Chagas disease in Latin America, they may not know about the possibility of transmission in the states (Klotz 2010). Therefore, even if their patient displays symptoms the doctor may not even consider Chagas disease. In the case that the doctor considers Chagas disease, they may not know how to test their patient. If the doctor has been informed that their patient has Chagas, they could be unaware there is available medication (Stimpert 2010)

More studies on the disease prevalence in the United States would help the country become more knowledgeable about the existence of the parasite in nature. Examining the prevalence of infected triatomine in the US, especially outside the southwest, would provide this information. This would lead to more of an idea about whether Americans (Florida and California) are infected due to migration from Latin America, or from autochthonous transmission. Testing for Chagas disease may need to be routine in some regions of the US, not only for blood and organ donations.

Because Chagas disease exists outside of Latin America, physicians and the public should be aware of the disease. Uninformed people travelling to Latin America will not recognize the bug and won't take precautions. Americans, who could be exposed to the disease in their homeland, may be unaware of the disease in addition to the possibility of being infected although asymptomatic (Stimpert 2010). With increased awareness, the prevalence of the disease should decrease with time.

In an attempt to gain more insight into the possibility of autochthonous transmission in southern California, information about the sylvatic cycle was gathered and an experiment was performed to determine the prevalence of infection in the primary mammalian host.

The relationship between woodrats, Triatomine, and *T. cruzi*

The three triatomine species found in southern California, *T. protracta*, *T. rubida*, and *P. hirsuta*, are known to associate with woodrats (Bern 2011, Hwang 2010, Ryckman 1962). There are several woodrats found in southern California; *Neotoma lepida*, *Neotoma albigula* and *Neotoma fuscipes* (Salmon 1994). *T. protracta* collected from *N. fusicpes* nest was the first time *T. cruzi* was found in the US (Kofoid 1916).

The triatomine bugs live in the woodrat nests and blood feed from the woodrat (Wood 1967, Ryckman 1967). The number of kissing bugs found per nest in the US varies by season and has ranged from none to 49 (Bice 1966, Wood 1967) with averages ranging from 1.4 to 6.8 (Bice 1966, Wood 1960 [B]). A single woodrat midden usually houses one woodrat but several kissing bug species may be found there depending on the region (Ryckman 1967). There can be many bugs per nest because female triatomine are capable of producing a large numbers of progeny; 220 for *T. protracta* and 396 for *T. rubida* (Ryckman 1962). One experiment found that Triatomine can feed off the woodrat often (~6 times/day) without any detrimental effect on the rodent (Wood 1943). However, once the Triatomine population in the nest surpasses the woodrats tolerance threshold, they will be eaten (Ryckman 1965 [C]). The consumption of triatomine is the

primary way that woodrats are thought to become infected (Kofoid 1933). One week post infection, *N. fuscipes* was transmitting the parasite to kissing bugs. Parasites remained in their blood at the conclusion of the 64 week study and feeding kissing bugs were still becoming infected (Ryckman 1965 [B]).

The course of infection in the woodrat depends on age, weather, and sex. Studies with laboratory rats saw that young rodents were more likely to become ill and die when infected (Kolodney 1939 [B]). However, when adult rodents were infected, they did not show symptoms and parasites were difficult to find in blood (Kolodney 1939 [B], Ryckman 1965 [B]). Tolerance of the parasite as well as the ability to transmit the disease makes these rodents an ideal host. In addition to age, some studies have seen that male laboratory mice; were more susceptible to the disease, exhibited worse disease symptoms, experienced higher parasitemia, and had a greater likelihood of mortality in comparison to female mice (Chapman 1975, Hauschka 1947, Pinto 2010). Also, temperature has a big effect on diseased rats. Lab rats were much more likely to have higher parasitemia, become sick, and die during the colder winter months (Kolodney 1939 [A], 1940). However, triatomine are more likely to be found blood feeding in the woodrat nests during the winter. During the warmer months when temperatures exceed 65° F (18° C) after dusk, triatomine may fly ¼ of a mile or further. They may end up at another woodrat nest, near another animal they can feed from, or at humans house because they are attracted to light at night (Ryckman 1981). People should not keep a porch light on for this reason, especially because triatomine release more *T. cruzi* in their feces during the summer months (Wood 1954, 1960 [B]).

The prevalence of *T. cruzi* in southern California *N. fuscipes*:

Data obtained from the surveillance of vector-borne diseases is very important because it provides insight into what is occurring in the sylvatic cycle. Surveillance includes monitoring infected vectors and hosts, including humans. With the acquired information, it may be necessary for health officials to work on decreasing transmission of the disease in the wild as a preemptive measure.

There have been several studies that examined the prevalence of infected *N. fuscipes* from southern California. A study in 1937 found that one of 76 *Neotoma fuscipes* from San Diego County were infected by using microscopy (Wood 1937). In 1967, 9 of 99 *Neotoma fuscipes* from LA were found to be infected using xenodiagnosis (Wood 1967). Xenodiagnosis is a diagnostic method that involves having an uninfected triatomine feed on a mammal and checking the insect's feces for *T. cruzi*. In 1974, the last time the prevalence of *T. cruzi* in southern California *N. fuscipes* was elucidated, it was found that one of 19 *Neotoma* woodrats from Juniper Hills (LA county) were infected as determined by xenodiagnoses (Wood 1975).

Recently, a study was done to look at the prevalence of infected *N. micropus* from southern Texas. A PCR analysis using forward primer TCZ1 and reverse primer TCZ2 determined that 26.4% of the woodrats were positive for the parasite. The results of this study revealed higher infection prevalence than other studies that used different methods for diagnosis (Pinto 2010). The prevalence of *T. cruzi* in *N. fuscipes* has never been assessed by PCR.

Detection of *T. cruzi*:

The polymerase chain reaction (PCR) was the method chosen to detect Chagas disease in the experiment. In comparison to detecting *T. cruzi* by microscopic methods, PCR has been shown to detect parasite DNA earlier in the acute phase and more frequently during the chronic phase than by microscopy (Kirchoff 1996). When *T. cruzi* DNA is found by PCR it indicates that parasites were present at the time the sample was extracted (Vera-Cruz 2003, Zhang 1999). In addition, the presence of *T. cruzi* is indicative of disease and the parasite load correlates with the severity of Chagas (Zhang 1999). Parasites are found easily during the acute phase. However, detection is much more difficult during the chronic phase (Cummings 2003). Several studies have found that muscle (Guarner 2001, Tarleton 2003, Zhang 1999), specifically skeletal muscle (Andersson 2002, Postan 1983, Vera-Cruz 2003, Zhang 1999), is one of the tissues that contain the most parasites during the chronic stage of Chagas.

The most abundant DNA sequence in *T. cruzi* is a 195bp repeat of unknown function (Martins 2008). The repeats are grouped in clusters of around 30±10 kb (Elias 2003). It is present in varying amounts in all *T. cruzi* strains (Martins 2008, Virreira 2003) and it has been estimated to comprise about 5% of the genome of *T. cruzi* strain CL Brener (Martins 2008). This 195bp repeat is species specific and when PCR is carried out with the primer set TCZ1/TCZ2, the result is a sensitive and specific Chagas assay (Gonzalez 1984, Moser 1989, Virreira 2003). One study compared *T. cruzi* detection by PCR with 7 primer sets and found the TCZ1/TCZ2 pair amplified *T. cruzi* DNA the best

(Virreira 2003). The TCZ1/TCZ2 primer set amplifies 188bp of the 195bp *T. cruzi* repeat (Moser 1989).

The purpose of my experiment was to determine the prevalence of *T. cruzi* infected southern California *N. fuscipes* by PCR using the TCZ1/TCZ2 primer set.

Materials and Methods

Woodrat DNA extraction:

55 *N. fuscipes* woodrats were collected from Orange County (41), San Bernardino County (6), LA County (5), and Riverside County (3) from 1999 to 2005. Once they were obtained from Orange County Vector Control District, they were stored at -80°C in the lab. In addition, skeletal muscle from infected *N. micropus* was obtained from the Pinto 2010 study and used as a positive control. DNA was extracted from leg muscle tissue using the QIAGEN DNeasy Blood & Tissue Kit following the manufacturers recommended protocol. The DNA purity (260/280 and 260/230 ratios) and concentration was measured using a Thermo Fisher Scientific Nanodrop ND-1000 Spectrophotometer. The DNA samples were stored at -20°C.

T. cruzi PCR Amplification:

Because contamination would result in inaccurate data on the prevalence of *T. cruzi* in woodrats, the PCR reaction was set up/amplified in one building, then the product run in another as done in Kirchoff 2006.

The PCR reaction included 12.5µl Promega GoTaq Hot Start Green Master Mix,

10µM of primer TCZ1 (5'-CGAGCTCTTGCCCACACGGGTGCT-3'), 10µM of primer TCZ2 (5'-CCTCCAAGCAGCGGATAGTTCAGG-3') (Moser 1989), 100ng DNA, and water. The control positive was DNA extracted from skeletal muscle from infected *N. micropus*. The initial denaturation step of the PCR was run at 94°C for 5 minutes. 40 amplification cycles were run at 94°C for 30 seconds, 64°C for 30 seconds and 72°C for 15 seconds. The final extension was run at 72°C for 1 minute, and then it was cooled and held at 4°C. Once PCR was complete, 10 µl of PCR product were run on a 2% agarose gel stained with ethidium bromide at 125V for 45 minutes with 1.5µl of a 1kb ladder.

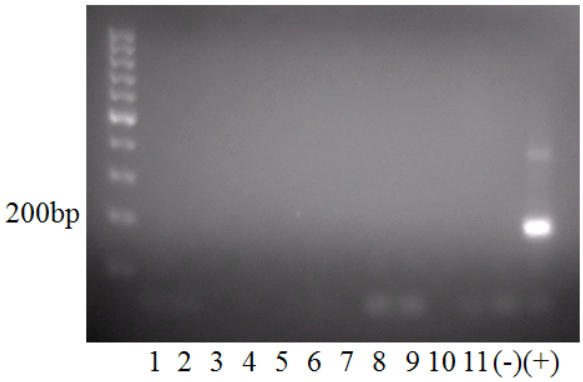
PCR Sensitivity Assay:

The 188bp PCR product amplified with the TCZ1/TCZ2 primer set was purified using the Zymo Clean & Concentrator kit. After Sanger sequencing determined that the band was part of the *T. cruzi* 195bp repeat, it was inserted into the pGEM-T plasmid by TA cloning. The plasmid construct was transformed into Z-competent *E. coli* cells and grown overnight in the presence of amplicillin. Single colonies picked from the plate were cultured overnight. The plasmid DNA was extracted from the cultured cells using the Zyppy Plasmid Miniprep Kit and used in the sensitivity assay. Serial dilutions were made starting with the 7.33ng/µl of plasmid DNA down to 10^{-15} and PCR was performed to test the sensitivity of the assay. The PCR products were visualized on a 2% agarose gel containing ethidium bromide that was run at 125V for 35 minutes with 3µl of a 1kb ladder.

Results

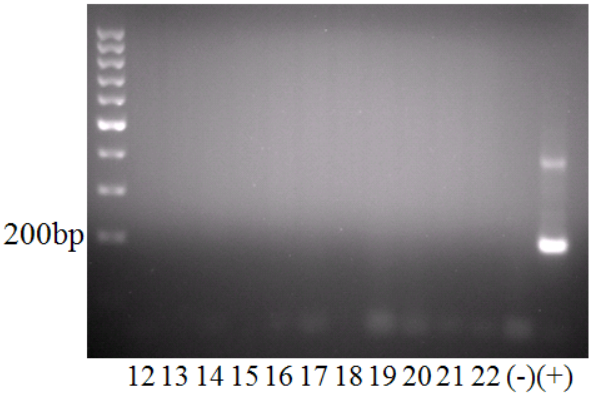
Once the concentration of extracted woodrat DNA was measured with a Nanodrop spectrophotometer, PCR reactions were set up with 100ng of DNA. All 55 woodrats were tested for the presence of a highly repetitive *T. cruzi* sequence with the TCZ1/TCZ2 primer set (Fig. 1a – 1e).

Figure 1a. Detection of *T. cruzi* DNA in woodrat skeletal muscle (woodrats 1-11)



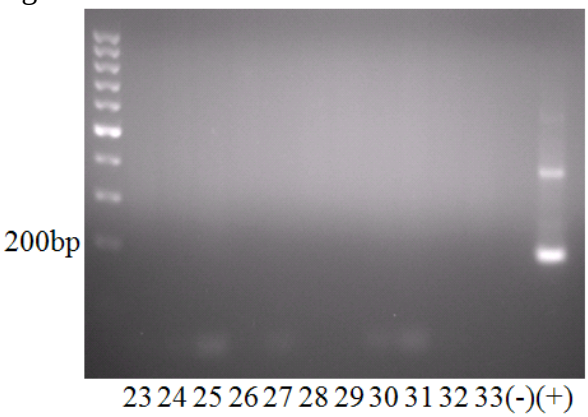
The negative control lane contains 100ng of DNA from a *N. fuscipes* woodrat determined to be healthy in the experiment. The positive control lane contains 100ng of DNA from an infected *N. micropus*.

Figure 1b. Detection of *T. cruzi* DNA in woodrat skeletal muscle (woodrats 12-22)



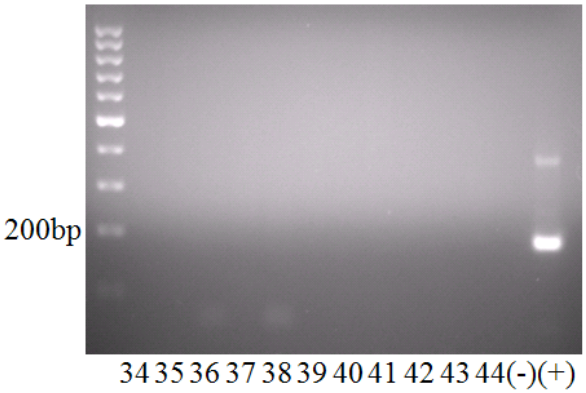
The negative control lane contains 100ng DNA from a healthy woodrat and the positive control lane contains 100ng DNA from infected *N. micropus*.

Figure 1c. Detection of *T. cruzi* DNA in woodrat skeletal muscle (woodrats 23-33)



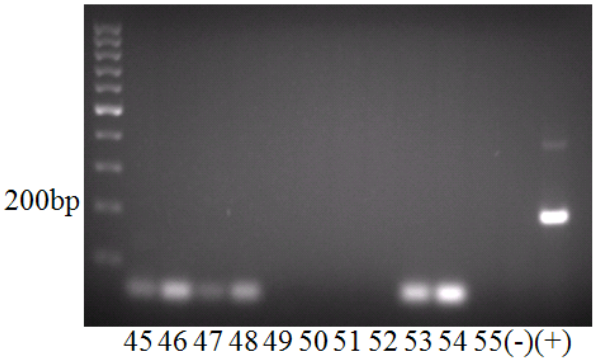
The negative control lane contains 100ng DNA from a healthy woodrat and the positive control lane contains 100ng DNA from infected *N. micropus*.

Figure 1d. Detection of *T. cruzi* DNA in woodrat skeletal muscle (woodrats 34-44)



The negative control lane contains 100ng DNA from a healthy woodrat and the positive control lane contains 100ng DNA from infected *N. micropus*.

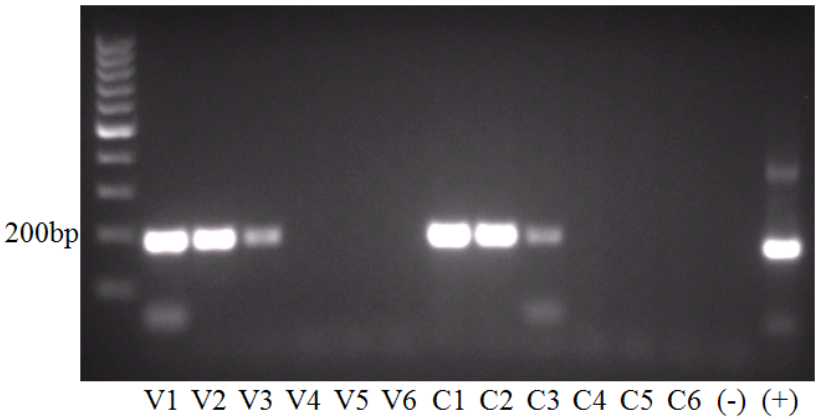
Figure 1e. Detection of *T. cruzi* DNA in woodrat skeletal muscle (woodrats 45-55)



The negative control lane contains 100ng DNA from a healthy woodrat and the positive control lane contains 100ng DNA from infected *N. micropus*.

The gels that were run with PCR product revealed that none of the *N. fuscipes* samples contained *T. cruzi* DNA. The positive control reveals the 188bp band amplified from the 195bp *T. cruzi* repeat. The 188bp product was purified and sequenced to confirm that the band was the *T. cruzi* repeat. Additionally, a band under 400bp is also seen in the positive control lane. There was no non-specific amplification in the lanes of the healthy woodrats. Primer dimers of varying strength were seen in some positive and negative samples. After testing all the woodrats, the sensitivity of the assay was assessed. PCR was run with dilutions of the cloned 188bp PCR product/pGEM-T plasmid DNA alone and with 100ng of DNA from a negative woodrat (Fig. 2).

Figure 2. Sensitivity of the PCR assay for detecting the 188bp *T. cruzi* sequence.



P=plasmid DNA only and C=plasmid DNA combined with 100ng genomic DNA from healthy woodrats. Numbers correspond to copies of the *T. cruzi* repeat; 1=2130, 2=213, 3=21.3, 4=2.13, 5=.213, 6=.0213. The negative control lane contains 100ng DNA from a healthy woodrat and the positive control lane contains 100ng DNA from infected *N. micropus*.

Amplification occurred until there were 21.3 copies of the repeat put into the pcr reactions with and without genomic DNA. PCR artifacts under 100bp were seen in columns V1 and VG3.

Discussion

All 55 woodrats tested in this experiment were found to be negative for Chagas disease. The disease was confirmed in the control positives by sequencing the 188bp product. The additional band seen at ~400bp in the positive control has been seen in other studies that ran pcr with the TCZ1/TCZ2 primer set (Bi 2010, Kirchoff 1996, Virreira 2003). It is thought that the band results from amplification of two copies the 195bp *T. cruzi* repeat which is organized in tandem arrays. The light band in figure 2 under 100bp in two lanes was probably due to non-specific amplification. It was not seen when the gel was run for 45 minutes (versus 35 minutes) and did not seem to have an effect on the results.

Previous studies on infected southern California *N. fuscipes* revealed infection prevalence to be 1.3%, 9%, 5.3% (Wood 1937, 1967, 1975). Therefore, prevalence in the woodrats was expected to be detected with a low percentage. Surprisingly, none of the 55 woodrats were infected. It is also shocking that *N. fuscipes* is easily infected, can maintain infection for a long time (Ryckman 1965 [C]), but in studies that look at the number of infected *N. fuscipes* in nature the percent is often low.

Most of the woodrats (51/55) were collected during the cooler months when parasitemia is the highest in infected rodents. This would make the likelihood of parasite detection greater; however, it could be that the sick woodrats died and the rats collected were healthy. Sex can also influence the likelihood of finding infection as was seen in a Texas study that found more males to be infected (Pinto 2010). The female to male ratio was close (29 females:26 males) but if more specimens were collected, specifically

males, there would be increased the possibility of having an infected woodrat. All of the woodrats were past the juvenile phase (54 adults and 1 subadult) so they had lived past the most vulnerable age. Although their longevity would means the woodrats have had more to time to acquire to disease, none were infected and this was not found to have an influence according to the results in Pinto 2010 either.

In the 1967 study which revealed that 9% of the woodrats were infected, all of the rodents were collected during the winter. In addition, it was found that 24.8% of triatomines also collected from Griffith Park carried the parasite (Wood 1967). The presence of woodrats is a good indicator of triatomine and if positive, the insects are probably positive as well. Because many tratiomine can feed on one woodrat, the rodent has the potential to infect many triatomine. However, if the bugs remain in the nest they may be eaten. If they remain and survive in the nest of an infected woodrat, they will probably become infected. In an experiment where kissing bugs were kept with an infected woodrat, all bugs became infected (Ryckman 1965 [C]). It is surprising that that in nature, not all triatomine collected from an infected woodrat nest are infected (Wood 1967). Therefore, bugs living in the woodrat nest in nature may leave to feed on another animal. Other possibilities could be that the triatomine found haven't fed from the woodrat yet; they could be very young or healthy insects just reached the nest. Bugs are thought to lose infection when they starve (Botto-Mahan 2009), but if they are living near the woodrat it doesn't seem likely that they would go hungry.

The experiment should be undertaken with a larger number of woodrats. Ideally, all of the woodrats would be from a specific area and triatomine from the same area

tested as well. Other animals, possibly raccoons and opossums, which are known to be a host should simultaneously be collected and tested (Bern 2011). The results from this study should find that woodrats have the highest infection prevalence if it truly is the primary reservoir in the southwest.

In addition to the experiment conducted, DNA could have been extracted and tested from heart tissue because it is also often found infected during the chronic phase (Postan 1983, Vera-Cruz 2003, WHO 2010, Zhang 1999). Because one study had difficulty finding *T. cruzi* in the tissues of infected woodrats, extractions from several areas of the heart and skeletal muscle would increase the likelihood obtaining parasite DNA (Ryckman 1965 [B]). PCR could also be done with blood because infection in the acute phase can easily be detected. PCR can only detect current infection but the results of antibody testing indicate whether disease has occurred at some point. The best serologic test for Chagas is the University of Iowa's RIPA assay. If the woodrats were tested using the RIPA assay as well, rodents may be discovered to have been infected in the past (Otani 2009).

The results from the assessment of the PCR assay indicated that it was very sensitive. There only needed to be 21.3 copies of the 188bp sequence put into the reaction to have amplification in the presence or absence of woodrat DNA. It has been estimated that 5.13% of the CL Brener strain genome consists of the 195 bp repeat (Martins 2008). The CL Brener genome is approximately 108.55Mbp (El-Sayed 2005), therefore there are over 28,500 copies of the repeat per parasite. If CL Brener strain DNA was obtained, $3.825x10^{-6}$ parasites could be detected by the assay. Although the assay is sensitive and

all woodrats all tested negative, another possibility is that some have infection but it is below the sensitivity of the PCR assay. Recently, the sensitivity of PCR to detect *T. cruzi* in blood was assessed. *T. cruzi* DNA was extracted from the discrete typing units I, IV (IIa), and VI (IIe) and 48 PCR tests were done. The lowest detection limits were found to be 0.01 fg/µl for I DNA (Silvio X10 strain), 1 fg/µl for IV DNA (CAN III strain), and 0.01 fg/µl for VI DNA (CL-Brener strain) (Schijman 2011). The sensitivity of my assay was reached at 0.0733 fg/µl plasmid construct where 188bp/3188bp, or about 5.9% of the DNA consists of the repeat. 5.57Mbp/108.55Mbp, or 5.13% (as previously mentioned) of the CL-Brener strain consists of the repeat. Therefore, the sensitivity of my PCR assay falls within the range of the lowest detection limits found from testing 48 different PCR methods.

Testing woodrats is not the best indicator of *T. cruzi* in nature. Although the presence of infected woodrats is a good indicator that triatomine are present and are likely to be diseased, triatomine can acquire the disease after blood feeding from any infected mammal (Bern 2011). As long as triatomine exist, there will probably always be infected mammals in nature. Because triatomine are responsible for spreading the disease to mammals, it is more useful to study the prevalence of their infection.

Conclusion

Chagas should no longer be characterized as a tropical disease because autochthonous transmission has occurred in the US and infected people have the potential to infect others wherever they chose to live. Although all woodrats tested in this study

were negative for *T. cruzi*, the presence of positive triatomine in southern California shows that Chagas exists in the sylvatic cycle and woodrat nests should be dismantled. In order to reduce this public health problem, there must be; a standardized *T. cruzi* detection assay, better medication, and continuous wildlife surveillance.

Complete elimination of domestic populations of triatomine has been suggested as a solution to prevent future transmission to people. Due to insecticide spraying, transmission was eliminated from Uruguay in 1997, Chile in 1999, and in sizeable areas of Argentina, Bolivia, Paraguay and some areas in Central America. However, this is only a temporary victory because reinfestation will occur if the spraying is stopped or if the triatomine become resistant to the insecticides. Also, with the success comes a loss of political interest even though the problem still remains (Schofield 2006).

Currently, there are several drugs being tested for efficacy against *T. cruzi*, the one that seems most promising is called K777 (Clayton 2010 [B]). It is a protease inhibitor that selectively targets *T. cruzi* protein, cruzain (McKerrow 2009). Cruzain plays a role in immune evasion (Doyle 2011) and when inhibited, it leads to parasite death (Engel 1998). Because cruzain is expressed in all stages of *T. cruzi*, K777 has effectively cured acute and non-acute infection in mice (McKerrow 2009). The drug has also improved cardiac damage in diseased dogs (Barr 2005). It is predicted human treatment with K777 will consist of an oral dose of about 10 mg/kg for 14-30 days. No adverse side effects were seen with a dose up to approximately 50 mg/kg/day (McKerrow 2009). Therefore, K777 would have shorter treatment time, be effective during all disease

phases, and have fewer side effects than current medication. Recently, K777 has been approved by the FDA for phase I safety trials (Leslie 2011).

Even if a cure is found, a highly sensitive detection assay would be important so that infected people know they need treatment. Because a cure does not prevent people from contracting the disease, wildlife surveillance would remain important in order to be prepared for outbreaks among humans. Hopefully one day soon, diagnostic testing and effective medication will be widely accessible and affordable to the millions of people suffering from Chagas disease.

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