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Trypanosoma cruzi Prevalence in the Domestic Canine Population in Central and Eastern Kentucky

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TRYPANOSOMA CRUZI PREVALENCE IN THE DOMESTIC CANINE
POPULATION IN CENTRAL AND EASTERN KENTUCKY

A Capstone Experience/Thesis Project

Presented in Partial Fulfillment of the Requirements for

the Degree Bachelor of Science with

Honors College Graduate Distinction at Western Kentucky University

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2015

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ABSTRACT

Domestic canines are regarded as natural sentinels for the transmission of vector-borne pathogens since infection in an owner's dog suggests the presence of the vector in or around the household. In collaboration with the University of Kentucky's Veterinary Diagnostic Lab in Lexington, Kentucky, we have investigated the prevalence of *Trypanosoma cruzi* (the causative agent of Chagas disease) in canines from central and eastern Kentucky via a serological test, polymerase chain reaction (PCR), and DNA sequencing. In addition, Canine SNAP 4Dx plus tests (Idexx Laboratories, Inc.) were used to determine the prevalence of four other vector-borne pathogens: *Ehrlichia canis/ewingii*, *Borrelia burgdorferi*, *Anaplasma phagocytophilum/platys*, or *Dirofilaria immitis* in the dogs. Results to date reveal a surprisingly high sero-prevalence of 10.23% for *T. cruzi*. Two positive samples (2.27%) were confirmed using the polymerase chain reaction and one resulted in a known *T. cruzi* sequence. In addition, results of the SNAP 4DX plus tests showed a prevalence of 5.68% for *Ehrlichia canis/ewingii* (the causative agent of ehrlichiosis) and 6.82% for *B. burgdorferi* (the Lyme disease spirochete). We believe that further studies are urgently needed to fully evaluate the role that canines might be playing as reservoir hosts for these as well as other vector borne diseases in Kentucky.

Keywords: *Trypanosoma cruzi*, Chagas disease, *Canis lupus familiaris*

Dedicated to Matthew, Mom, Dad, and Travis and to everyone who I could not list by name here, but from whom blessings have flowed ceaselessly, and for putting up with me through the entire process.

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CHAPTER 1

INTRODUCTION

History:

Carlos Chagas, a Brazilian physician, was the first to discover *Trypanosoma cruzi* (Chagas, 1909) while studying parasitic diseases in South America. Chagas observed symptoms in patients that did not match any known disease. In his life, Chagas was able to determine the clinical symptoms of infection, the epidemiology and life cycle of the parasite within the vector and human host as well as other definitive hosts (Clayton, 2010). In honor of the extensive work Chagas put into the disease, it is now commonly known as Chagas disease. It is also known as American trypanosomiasis due to the fact that the etiological agent is a trypanosome that is endemic in North America, Central America and South America.

Chagas disease in the United States:

Trypanosoma cruzi, the etiological agent of Chagas disease, is endemic to areas of Mexico, Central America, and South America. However, recent studies have shown that the parasite life cycle is also well established within the United States. As of 2009, the estimated number of people with *Trypanosoma cruzi* infection within the United States was 300,167 individuals (Bern et al., 2009). The majority of these cases occur in individuals who have emigrated from Chagas endemic areas.

It is well known that canines can serve as crucial reservoir hosts in Latin America (Gurtler et al., 1991). However, the role that dogs may be playing in the epidemiology of Chagas disease in the United States is not well known (Beard et al., 2003). *Trypanosoma cruzi* infection has been documented in the canine populations of Virginia, Oklahoma, Tennessee, Louisiana, and Texas (Bradley et al., 2000; Burkholder et al., 1980; Kjos et al., 2008; Rowland et al., 2010; Rosypal et al., 2010; Shadomy et al., 1980; Snider et al., 1980; Williams et al., 1977; Meurs et al., 1998). *Trypanosoma cruzi* infection in the domestic canine population implies that the vector is also likely present in the vicinity of the human household.

Within the southeastern United States, there is evidence to support the presence of a sylvatic cycle that involves the transmission of the parasite between Reduviid bugs and wildlife reservoir hosts (Rosypal et al, 2007). There have been studies that show that wild canids are also contracting Chagas disease. This is evident in the Rosypal et al., (2007) study where 2 out of 26 gray foxes (8%) were *T. cruzi* positive out of South Carolina. This shows that the cycle is perpetuated in the environment where canines are potentially exposed.

It is assumed that canines are obtaining the infection by chewing the Reduviid bug that is infected with *T. cruzi*. When infection is acquired, there is the possibility for a dog to develop both acute and chronic Chagas disease. Mortality in the acute phase is more common in very young dogs due to their inability to fight off the parasite. This acute disease includes possible cardiomegaly and/or cardiac arrhythmias depending on the strain of *T. cruzi* (Guedes et al., 2007, Kjos et al., 2008). Chronic disease commonly manifests as heart disease in older canines which will ultimately lead to the death of the

dog (Barr et al., 1991, Kjos et al., 2008). These symptoms are very similar to those that occur within human populations. Unfortunately, at this time there are no approved medications that veterinarians can use to combat this disease in canines.

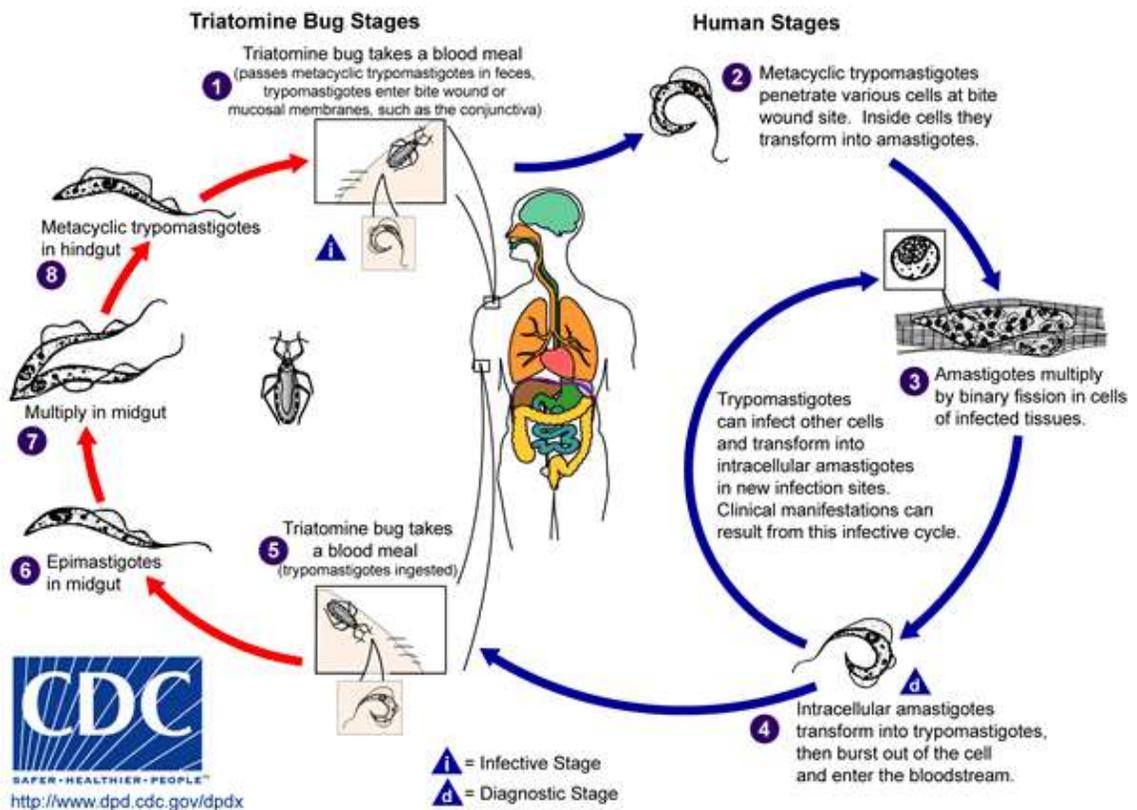
Life Cycle of *Trypanosoma cruzi*:

Triatomine bugs in the insect family Reduviidae serve as intermediate hosts in the life cycle of *T. cruzi*. Within the southeast this is predominately *Triatoma sanguisuga* (Figure 1.1). The triatomine bug feeds on a potential host, typically a mammal, and defecates on the skin as it takes its blood meal. The trypomastigote stages present in the bug's feces are introduced into the bite wound or enter via the mucous membrane of the eyes, nose or mouth. Trypomastigotes enter host cells at the site of entry, and then rapidly transform into amastigote stages. The amastigote stages then replicate through several rounds of binary fission and then transform once again into trypomastigotes. Trypomastigote stages erupt from the cells and disseminate to other cells and tissues. Trypomastigotes circulating in the blood are also infective for the intermediate host, the triatomine bug. The triatomine ingests the trypomastigotes along with the blood meal. Within the mid-gut of the triatomine, the parasites transition into the epimastigote stage. During this stage the epimastigotes proliferate in the gut before once again transforming back to the trypomastigote stage in the hind gut. The trypomastigote stage is then released when the triatomine bug defecates while consuming a blood meal. (CDC, Roberts et al., 2012) (See Figure 1.2)

Figure 1.1: *Triatoma Sanguisuga* (retrieved from CDC.gov)



Figure 1.2: CDC Life cycle of *Trypanosoma cruzi*



Other Vector Transmitted Diseases

There have been surprisingly few published studies that have investigated the prevalence of tick-borne and other vector-borne pathogens within the canine population within the state of Kentucky. These vector-borne pathogens include *Borrelia burgdorferi*, *Ehrlichia canis/ewingii*, *Anaplasma phagocytophilum/platys*, and *Dirofilaria immitis*. *Borrelia burgdorferi*, the causative agent of Lyme disease (Burgdorfer et al., 1982) and *Ehrlichia canis/ewingii*, the causative agents of ehrlichiosis, are present in both human and canine populations. Similar to *T. cruzi*, canines also serve as a reservoir host for these diseases that are also communicable to human (Shaw et al., 2001).

The prevalence of *Borrelia burgdorferi* in Kentucky, according to a 2011 study of 287 ticks, is low, with only one tick (0.4%) confirmed to be positive for *Borrelia lonestari*. This bacterial species causes a variant of Lyme disease known as southern tick associated rash illness (STARI) (Fritzen et al., 2011). None of the ticks were carrying *Borrelia burgdorferi* in that study. The prevalence of *Ehrlichia ewingii*, which was also reported in this study, was 4 out of the 287 ticks (1.4%). In another study published in 2012, the prevalence of *Ehrlichia ewingii* in Kentucky was determined to be 3 out of 16 dogs and 1 out of 16 dogs for *Ehrlichia canis* (Beall et al., 2012).

Anaplasma phagocytophilum/platys, the causative agent of anaplasmosis in both canines and humans, leads to the infection of the host's neutrophils and neutrophil precursors (Sarkar et al., 2007). In an unpublished study from our lab, the prevalence of *Anaplasma phagocytophilum/platys* was 2 out of 361 domestic canines (0.53%). This study was conducted on dogs from the western half of the state.

Dirofilaria immitis, the causative agent of canine heartworm, is transmitted through a mosquito vector. In the same unpublished study from our laboratory, the prevalence of *Dirofilaria immitis* in the western region of the state was determined to be 13 out of 361 dogs (3.7%).

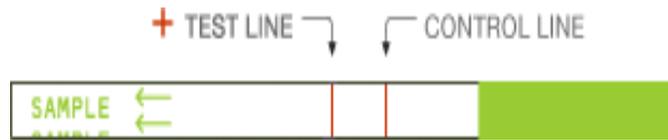
CHAPTER 2

MATERIALS AND METHODS

Canine sera: Serum or blood samples from domestic canines were obtained from the University of Kentucky Veterinary Diagnostic lab and were transported back to Western Kentucky University under refrigerated conditions. Upon arrival, a 250 micro-liter aliquot was removed from each sample, placed into a sterile microfuge tube, and stored at a temperature of -20°C. The collection vials containing the remaining sera or blood samples were stored at 4°C until use. Along with the serum samples, we recorded the county of the reporting veterinarian, as well as the sex, age and breed of the canine.

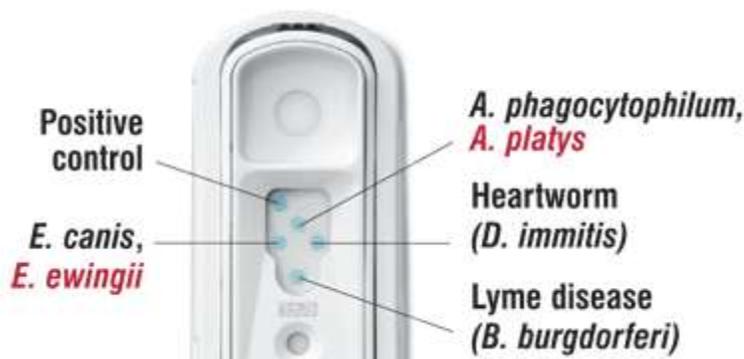
Chagas dipstick chromatography test: Twenty micro-liters of sample canine serum were applied to the dipstick's sample pad and the end of the dipstick was placed into one well of a 96-well, micro-liter plate. Three drops of chase buffer were then added to the well. Additional buffer was added after one minute if the buffer had not yet reached the test line. This additional buffer was typically one drop. After 10 minutes, the results were read. The presence of a positive control line indicated that the test was functioning properly, and the presence of a second test line was indicative of a positive result; whereas no test line indicated a negative result. (See Figure 2.1)

Figure 2.1: INBIOS dipstick example



SNAP 4Dx Plus Test (IDEXX): After being allowed to equilibrate to room temperature for 30 minutes, 3 drops of canine serum were added to a clean microfuge tube. Four drops of the conjugate buffer were then added and the tube was inverted several times to thoroughly mix the contents. The contents of the tube were then applied to the sample well. Once the solvent front reached the activation window, the activator pad was snapped down. Results were read after 8 minutes. The presence of a positive control dot indicated that the test was functioning properly, and the presence of other dots in specific locations indicated positive results for *Ehrlichia canis/ewingii*, *B. burgdorferi*, *Anaplasma phagocytophilum/platys*, or *Dirofilaria immitis* respectively. (See Figure 2.2) Some of the names are in red because they are the additional strains of the disease added to the Snap test that distinguished between the Snap 4DX and the Snap 4DX Plus.

Figure 2.2: IDEXX Snap 4DX Plus Test Results



DNA Isolation: Genomic DNA was isolated from 200 micro-liters of serum or whole blood from each canine that gave a positive result on the Canine Chagas Dipstick test. QIAamp DNA blood mini kits (Qiagen) were used for DNA isolation. DNA concentrations were determined using a Nanodrop 2000 Spectrophotometer (Thermo Scientific), and the DNA samples were stored at -20°C until use.

Polymerase Chain Reaction (PCR): PCR amplification was performed on selected positive and negative DNA samples, using the TCZ1 and TCZ2 primer pair. The TCZ1 and TCZ2 primers were designed to amplify 188 base pairs (bp) of a 195-bp repetitive nuclear sequence of *T. cruzi* (Moser et al., 1989). The TCZ1 and TCZ2 primers represent the following genomic sequences: TCZ1 (5'-CGA GCT CTT GCC CAC ACG GGT GCT 3') and TCZ2 (5'-CCT CCA AGC AGC GGA TAG TTC AGG 3'). Each reaction mixture consisted of 20 micro-liters of the Master Mix, 1 micro-liter of the TCZ1 primer, 1 micro-liter of the TCZ2 primer, 3 micro-liters of nanopure water, and 25 micro-liters of the canine template DNA. A negative control containing sterile nanopure water in place of template DNA and a positive control sample of *T. cruzi* genomic DNA were included with each analysis. Reaction mixtures were placed into an automated DNA thermal cycler to undergo amplification as outlined in Figure 2.3.

Figure 2.3. Thermocycler Protocol used for TCZ1 and TCZ2 primers.

Thermocycler program for PCR amplification using the TCZ1 and TCZ2 primers.			
	Temperature (°C)	Time	# of cycles
Initial Denature	94	2 min	1
Denature	94	1 min	30
Annealing	64.5	30 sec	
Extension	72	15 sec	
Final extension	72	5 min	1
Hold	4	Indefinite	1

Gel Electrophoresis: PCR products, as well as two 1-kb DNA ladders, were loaded onto 2% agarose gels (containing GelRed) and subjected to electrophoresis at 100V for 1 hour. Gels were then taken to the Western Kentucky University Biotechnology Center where images were captured using a Fluor Chem HD2 (Alpha Innotech). These images were then saved permanently for later access.

DNA Sequence Analysis: A Big Dye Terminator v3.1 (Applied Biosystems) sequencing reaction was performed on a subset of DNA samples determined to be positive by PCR. Unincorporated dye terminators were removed from PCR amplification products using a DyeX 2.0 Spin Kit (Qiagen), and samples were subjected to DNA sequence analysis using an ABI 3130 Genetic Analyzer. The sequences were edited before searching the BLAST database of the National Center for Biotechnology

Information (NCBI) Genbank for sequence matches. These edits consisted of removing the first 6-10 bases as well as the last 6-10 bases.

CHAPTER 3

RESULTS

Trypanosoma cruzi:

A total of 88 serum samples, from 30 counties within the state of Kentucky, were tested serologically for antibodies specific for the protozoan parasite, *Trypanosoma cruzi*. The geographic distribution of positive samples by county is depicted in Figure 3.1 and 3.2. When analyzing the data that came with the *T. cruzi* positive serum samples, there were some similarities. Four male and five female canines were sero-positive with an age span ranging from 2.5 years to 8 years. Four of the positive samples were obtained from four-year-old canines and two positive samples were collected from eight-year-old canines. When analyzing the breeds of the canines that tested positive, the only similarity found was that two of the positive canines were coonhounds.

The results of a recent collaboration with the Breathitt Veterinary Diagnostic Lab in Hopkinsville Kentucky are shown in Figure 3.2 (unpublished results). Canine samples from the Breathitt laboratory collaboration were obtained primarily from counties in central and western Kentucky whereas canine samples analyzed in the present study were obtained primarily from counties in central and eastern Kentucky.

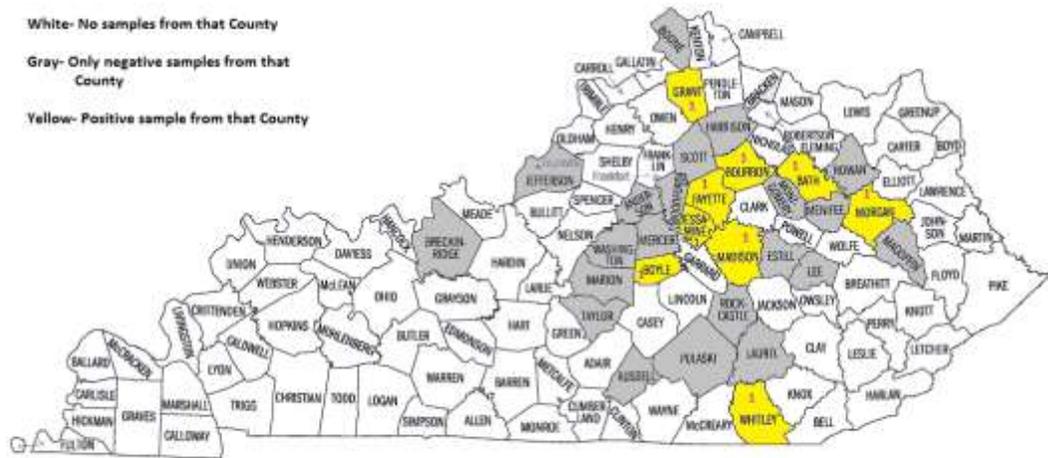


Figure 3.1: Geographic distribution by county of *T. cruzi* positive canine samples (UK Diagnostic Lab Collaboration)

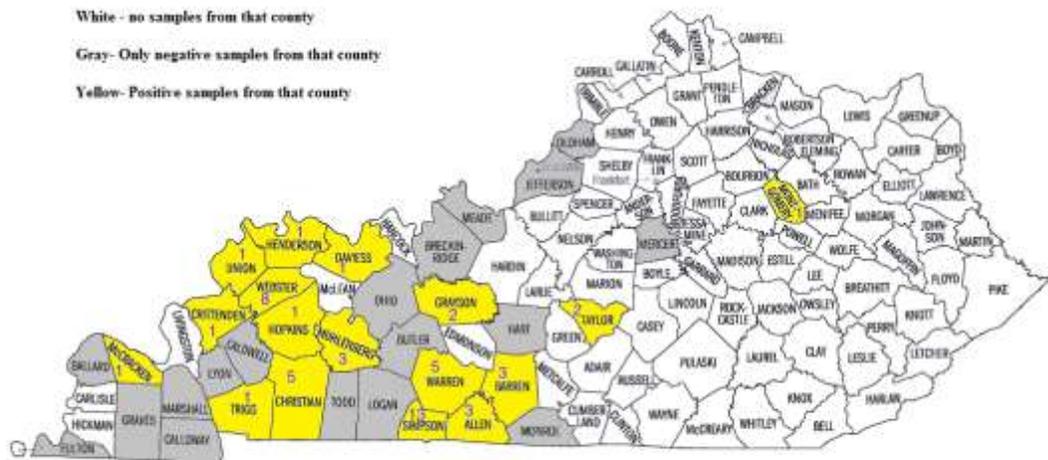


Figure 3.2: Geographic distribution by county of *T. cruzi* positive canine samples (Breathitt Diagnostic Lab Collaboration)

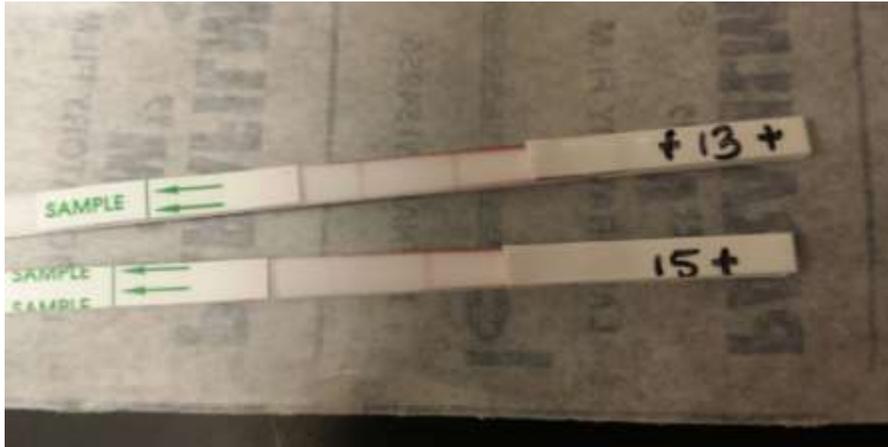


Figure 3.3: Positive *T. cruzi* Serological Dipsticks

DNA isolation

DNA was isolated from positive samples using a QIAamp DNA blood mini kit (Qiagen) according to the manufacturer's instructions. DNA concentrations (See Figure 3.4) were determined using a Nanodrop 2000 and samples were then frozen at -20C until further use. Concentrations ranged from 0.7 ng/microliter to 6.3 ng/microliter.

Sample number	DNA concentration (nanograms /microliter)
13	1.2
15	1.1
25	0.7
32	1.0
38	6.3
72	2.3
77	3.5
82	3.6
83	3.9

Figure 3.4: DNA concentrations for *T. cruzi* positive canine serum samples

PCR and Agarose gel electrophoresis

DNA samples from sero-positive dogs were subjected to PCR using *T. cruzi*-specific primers TCZ1 and TCZ2. Resulting products were then subjected to electrophoresis. Two of the nine canine DNA samples were found to be PCR positive. These canine samples were from Boyle and Grant County.

DNA Sequencing

The PCR products from two positive canine DNA samples were further subjected to DNA sequencing analysis. One of the two samples resulted in a DNA sequence that gave a positive match when blasted using the BLAST database of the National Center for

Biotechnology Information (NCBI) Genbank. The sequence showed 94% identity to the known *T. cruzi* Can III satellite sequence (See Figure 3.6). The sequence was:

“CCGGTTTATGGGGTTGGAGGAAAAAGTTAACTATTACTTGCCGTTATCCAG
 ATTTTGTGCTGCGAATTGTGATGGTGGGAGTCAGAGGCACACTCTGTCACTACG
 TGTCTGCGCGGTCACACACTGGTCACCAAACAACCCTGAACTATCCGCTGCTT
 GGAGGAGG”

Description	Max score	Total score	Query cover	E value	Accession
T. cruzi strain Can III satellite sequence	178	178	70%	2e-41	EU178923.1
T. cruzi strain Can III satellite sequence	160	160	70%	2e-37	GU128864.1
T. cruzi strain R147 clone F31 satellite sequence	145	145	70%	2e-31	FJ584863.1
T. cruzi strain CL Strain 3 (epithelial promastigote) T381.104719320057.1 satellite sequence	146	178	70%	2e-31	HM_000133.1
T. cruzi strain CL Strain 3 (epithelial promastigote) T381.104719320057.1 satellite sequence	145	310	70%	2e-31	HM_751348.1
T. cruzi strain CD600 satellite sequence	140	140	70%	2e-31	AY200661.1
T. cruzi strain CD600 satellite sequence	140	140	70%	2e-31	AY200661.1
T. cruzi strain DC18c11 satellite sequence	140	140	70%	2e-31	AY200661.1
T. cruzi strain MH01 clone 910 satellite sequence	139	139	70%	1e-29	FJ584871.1
T. cruzi strain T318 satellite sequence	138	138	70%	1e-29	EU178924.1
T. cruzi strain CL Strain 3 (epithelial promastigote) T381.104719320168.4 satellite sequence	139	198	70%	1e-29	HM_000133.1
T. cruzi strain T522 satellite sequence	139	139	70%	1e-29	AY200661.1
T. cruzi strain J515C satellite sequence	138	138	70%	1e-29	AY200661.1
T. cruzi strain CD601 satellite sequence	139	139	70%	1e-29	AY200661.1
T. cruzi strain CD601 satellite sequence	139	139	70%	1e-29	AY200661.1
T. cruzi strain 10001 (epithelial promastigote) T381.104719320172.1 satellite sequence	139	388	70%	1e-29	HM_000133.1
T. cruzi strain F301 satellite sequence	137	137	70%	4e-29	AY200661.1
T. cruzi strain CD601 satellite sequence	137	137	70%	4e-29	AY200661.1
T. cruzi strain R147 clone C10 satellite sequence	135	135	67%	1e-28	FJ584863.1
T. cruzi strain HS01 clone 127 satellite sequence	135	135	67%	1e-28	FJ584871.1
T. cruzi strain CL Strain 3 (epithelial promastigote) T381.104719320172.1 satellite sequence	135	1941	70%	1e-29	HM_751348.1

Figure 3.5: NCBI Blast results

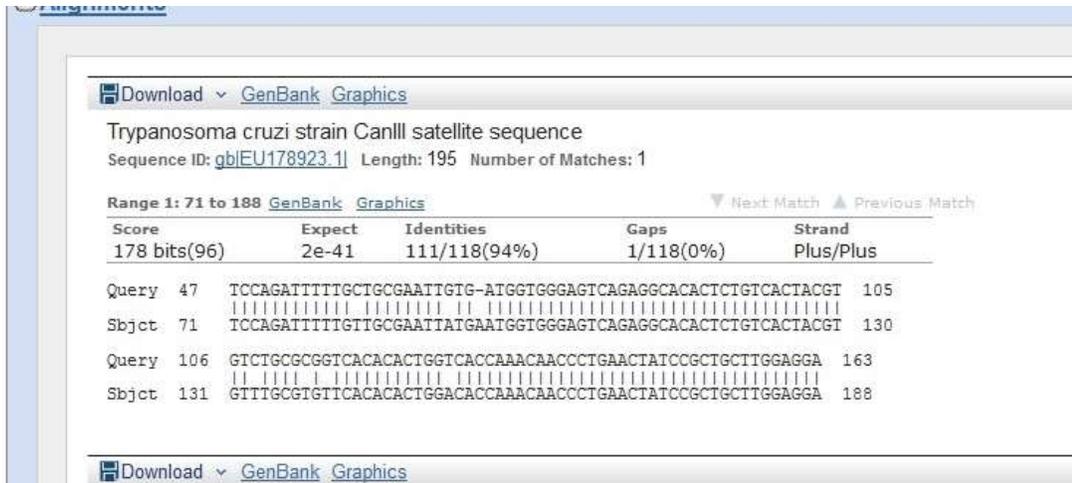


Figure 3.6: Comparing Sample 15 to CAN III satellite sequence

The canine that yielded this confirmed DNA sequence was from Boyle County. It was a three-year-old, female, English bulldog. Although we did not perform strain identification of the positive sequence, its match to the CAN III satellite sequence indicates that it may be a *T. cruzi* Type 4 strain (Figure 3.6).

Other Vector borne diseases:

Eighty-eight canine serum samples from 30 counties were tested for *Ehrlichia canis/ewingii*, *Borrelia burgdorferi*, *Anaplasma phagocytophilum/platys*, and *Dirofilaria immitis* using Snap 4DX Plus tests.

Of the 88 samples, 6 tested positive for *Borrelia burgdorferi*, the causative agent of Lyme disease (Figure 3.7). The gender of the Lyme positive canines was split evenly with 50% being male and 50% being female. Three of the positive samples were from Fayette County which includes the city of Lexington, Kentucky's second largest city. One other sample was from Jessamine, a county neighboring Fayette.

Five of the 88 canine serum samples tested positive for *Ehrlichia canis/ewingii*, the causative agent of ehrlichiosis. (See Figure 3.8) None of the canines in this study tested positive for canine heartworm (*Dirofilaria immitis*) or anaplasmosis (*Anaplasma phagocytophilum/platys*).

Sample Number	County	Breed	Gender	Age
2	Laurel	mountain kurr	F	3
13	Whitley	fiest	F	3
41	Fayette	German shepherd	F	n/a
42	Fayette	doberman pincher	M	7
65	N/a	n/a	M	2.5
83	Jessamine	mixed breed	M	8

Figure 3.7: Data for *Borrelia burgdorferi* positive canine samples

Sample Number	County	Breed	Gender	Age
2	Laurel	mountain kurr	F	3
9	Marion	coonhound	M	5
14	Breckinridge	N/A	F	6
79	Washington	lab	F	3
86	Russell	Australian shepherd	M	4

Figure 3.8: Data for *Ehrlichia canis/ewingii* positive canine samples

CHAPTER 4

DISCUSSION

The results of this study confirm the presence of *Trypanosoma cruzi* infection in the domestic canine population of central and eastern Kentucky. Serological analysis of the serum samples, showed a sero-prevalence of 10.23% (9/88). Two of the nine canine samples were also found to be positive for *T. cruzi* by a highly sensitive PCR analysis. One positive sample was confirmed to be *T. cruzi* based upon a 94% DNA sequence identity to the satellite DNA sequence of the CAN III strain. The CAN III strain of *T. cruzi* is known to be Type IV strain based upon serotype analysis. In an earlier unpublished study conducted in collaboration with the Breathitt Veterinary Diagnostic Research Laboratory, a sero-prevalence of 12.5% was observed from the central and western half of the state. The overall sero-prevalence of *Trypanosoma cruzi* across the state of Kentucky may be as high as 12.9% (58/449).

There have been many studies performed across the southeastern United States that have documented the presence of *T. cruzi* infection in canine populations since 1972. The prevalence of infection reported in these studies ranged- from a low of 1% in a Virginia study, as reported by Rosypal et al. (2010), to as high as 22% in a Louisiana study (Nieto et al., 2009). It should be noted that the Nieto et al. study in 2009 consisted of only 50 dogs. The study performed by Rosypal et al. in 2010 had a sample size of 90 canines. The most recent study reporting sero-prevalence of canine Chagas was

conducted with shelter populations in Texas (Tenney et al., 2014). The sero-prevalence reported in this study was 8.8% of 205 canines.

When comparing the eastern (Figure 3.1) and western (Figure 3.2) halves of Kentucky to look for contradicting results, there were two counties that yielded different results. Montgomery and Taylor counties both had sero-positive canine samples in the Breathitt collaboration, but yielded sero-negative samples in the UK collaboration. During the comparison; Jefferson, Mercer and Breckinridge County yielded exclusively sero-negative samples in both studies. Within this study, no statistical comparisons were performed between the two collaborations. The purpose of the current study was strictly to investigate the prevalence of *T. cruzi*.

In the present study, PCR was used to confirm any serological positives because the InBios assay is a serological test that strictly looks for antibodies to *T. cruzi*. The PCR analysis is more sensitive and actually confirms the presence of *T. cruzi* DNA in canine serum samples. It should be noted that none of the *T. cruzi* positive samples tested positive for any of the other vector-borne pathogens looked at in this study. The TCZ1 and TCZ2 primers were used for the PCR analysis because these primers are specific for 185 base pairs of the highly repetitive 195 base pair sequence also known as the CAN III satellite sequence (Moser et al. 1989). Due to the repetitiveness of CAN III Satellite sequence, it allows for a much larger amplification of the DNA than if the primers were only targeting a small portion of the *T. cruzi* genome. These primers are highly specific and only target this DNA sequence while not replicating other DNA sequences that may be present.

Within our study, there was a difference between the number of serological positives and those that were confirmed by PCR and then by DNA sequencing. This is probably because samples consisted primarily of serum and not whole blood. Consequently, the DNA yield was much smaller, which may have made it difficult to get sufficient quantities of parasite DNA to amplify. Another possible explanation is that the long-term storage of serum samples may have resulted in DNA degradation. By the time the serum samples arrived in our lab, it may have been six weeks or more since the samples were originally submitted to the diagnostic lab by veterinarians. One final possibility is that some of the dogs may have been exposed to *T. cruzi* but did not develop symptomatic Chagas disease.

It should be noted that all canines in this study were cared for by veterinarians in central and eastern Kentucky. Most of the dogs in this study were likely to be on preventatives for heartworm, if they went to a veterinarian on a regular basis, which explains the lack of positive samples for the canine heartworm (*Dirofilaria immitis*). The prevalence of Lyme disease on the Snap 4DX Plus tests may also not be a completely accurate estimate of Lyme disease prevalence since the SNAP test is also an antibody test, and does not confirm the presence of *Borrelia burgdorferi*.

Canine Chagas disease is currently not diagnosed by veterinarians in the state of Kentucky. This diagnosis may not be occurring because either veterinarians aren't aware that the disease has reached Kentucky or because the majority of the canines are asymptomatic. If there are no apparent symptoms, then there is no reason for a veterinarian to submit blood work to the CDC or another diagnostic lab. The difficulty of diagnosis is further compounded by the lack of a clinical serological test for Chagas

disease to be used within veterinary clinics across the state. The InBios Canine Chagas Dipstick was only approved for use in research, and it is now no longer available for purchase due to the low demand. Finally, another reason for a lack of diagnosis is that necropsies are rarely performed on canines. A necropsy is the only way to absolutely confirm that a canine was infected by *T. cruzi* upon its death. Necropsies in the United States are very expensive and are not usually requested by an owner when their pet dies suddenly.

In conclusion, the results of this study demonstrate that dogs are being exposed to *Trypanosoma cruzi* in Kentucky. Determining whether they are acquiring the actual disease or just being exposed will require further studies. Veterinarians within the region need to be notified that this parasitic disease is present in the state and that their patients are at possible risk of infection. Future studies should be performed on canines found within humane societies or shelters to survey canines that may not be actively cared for by veterinarians their entire lives. Another future study that should be pursued based upon the results of this study is to further investigate the higher prevalence of Lyme disease in canines from eastern Kentucky.

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