DOG-VECTOR-PARASITE INTERACTIONS IN THE CHAGAS DISEASE SYSTEM: CARDIAC CLINIC STATUS OF NATURALLY-INFECTED DOGS ACROSS THE US

A Dissertation

by

ALYSSA CATHERINE MEYERS

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Chair of Committee,	Sarah A. Hamer
Committee Members,	Gabriel Hamer
	Ashley Saunders
	Kevin Cummings
Head of Department,	C. Jane Welsh

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ABSTRACT

In the southern U.S., triatomine vectors maintain *Trypanosoma cruzi*- the protozoan parasite that causes Chagas disease in humans and animals- in sylvatic cycles. Infection with *T. cruzi* may be asymptomatic or lead to heart disease and death. Using dogs as a model host system, our objectives were to improve the ecological and clinical understanding of Chagas disease in the U.S. We collected 461 triatomines from Big Bend National Park from 2015-2017 and found an overall infection prevalence of 23.1%. Blood meal analysis on 42 triatomines showed DNA evidence of humans, domestic animals, wild birds and mammalian wildlife. In 2015-17, we sampled 1,660 working dogs from 43 states using three independent serology assays to detect a seroprevalence of 7.3% (CI: 6.1-8.6%). To better characterize the cardiac outcomes in *T. cruzi* infected dogs we applied a 24-hour Holter monitor to 17 *T. cruzi*-infected, 18 uninfected dogs and 4 dogs with discordant serology results. The presence of ECG abnormalities varied by *T. cruzi* infection status ($p<0.0001$) and positive dogs had higher serum concentrations of cardiac troponin I (cTnI), a biomarker for cardiac injury, than both negative dogs ($p=0.044$) and discordant dogs ($p=0.06$). Finally, we performed a retrospective study looking at 375 dogs presented to a teaching hospital in Texas. *T. cruzi*-infected dogs (N=63, 16.8%) were significantly younger than negative dogs (N=312) (mean 5.9 vs. 7.4 yr old respectively; $p=0.0069$) with no difference in infections by sex or breed. Infected dogs were more likely to have ventricular arrhythmias (28.6%), combinations of ECG abnormalities, and cTnI greater than 0.129 ng/mL (ADVIA assay). Combining ecological and clinical approaches to enhance our understanding of Chagas disease should provide insight for vector control and measures to protect veterinary and public health.

DEDICATION

To my parents for their endless support, encouragement and teaching me to never give up.

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NOMENCLATURE

AKC	American Kennel Club
AVB	atrioventricular block
AV	atrioventricular
CBP	Customs and Border Protection
CHF	congestive heart failure
CT	cycle threshold
cTnI	cardiac troponin I
DHS	Department of Homeland Security
DTU	discrete typing unit (strain type)
ECG	electrocardiogram
ELISA	enzyme-linked immunosorbent assay
IFA	indirect fluorescent antibody
OR	odds ratio
SL-IR	spliced leader intergenic region
TSA	Transportation Security Administration
TVMDL	Texas A&M Veterinary Medical Diagnostic Laboratory
VPC	ventricular premature contraction
VMTH	Veterinary Medical Teaching Hospital

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1. INTRODUCTION

1.1 Introduction

Infection with the protozoan parasite *Trypanosoma cruzi* causes Chagas disease, a potentially deadly heart disease of humans and animals. Chagas disease is the leading cause of infectious heart disease across the Americas, where over 6 million people are estimated to be infected. *T. cruzi* is vectored by triatomine insects (‘kissing bugs’) and transmitted among wildlife and domestic animals with occasional spillover to humans. (Bern et al., 2011) In South America dogs are an important reservoir of *T. cruzi* and have been used as a model to better understand the pathogenesis of *T. cruzi* infection, in particular the mechanisms behind the development of chronic Chagas disease. In both dogs and humans, clinical presentation varies widely, ranging from sudden death to chronic heart disease, while an unknown number of infected animals may remain asymptomatic. In humans, approximately 20-30% of infected individuals develop chronic disease, (Bocchi et al., 2017) but the likelihood of developing chronic heart disease in canines is unknown and the variation in disease progression is not well understood. Despite the discovery of Chagas disease in dogs in the U.S. nearly 50 years ago, (Williams et al., 1977) the pathogenesis of Chagas cardiomyopathy is not well understood in dogs.

Chagas disease has three phases: acute, indeterminate, and chronic. (Barr et al., 1991) Acute phase can often be subclinical, where parasitemia can initially be detected, then decreases in experimentally infected dogs. (Barr et al., 1991; de Lana et al., 1992) However more severe symptoms have been documented in experimental models of canine Chagas disease in the acute stage and include lethargy, generalized lymphadenopathy, pale mucous

membrane, slow capillary refill time, ascites, weak pulse, and enlarged liver and/or spleen and sudden death. (Barr et al., 1991) During the indeterminate stage, infected dogs are seropositive but typically asymptomatic and parasitemia is infrequent. (Veloso et al., 2008) Dogs can remain in the subclinical indeterminate stage for life, but an unknown proportion will progress to the chronic stage, developing cardiac arrhythmias and conduction abnormalities including right sided or bilateral heart failure. (Barr et al., 1991) The inability to predict the outcome of infection in dogs limits a veterinarian's ability to effectively treat an infected dog. The first step to achieving better treatment for Chagas disease is to develop a better understanding of the clinical outcomes of infection.

One source of clinical variability from infection with *T. cruzi* could be due to the genetic diversity of the parasite. (Macedo and Pena, 1998) *T. cruzi* is genetically diverse, clustering into seven monophyletic clades called discrete typing units (DTUs; TcI-TcVI and TcBat), each broadly associated with different ecologies, reservoir hosts, geographical distributions and pathologies. (Miles et al., 2009; Ramírez et al., 2010; Zingales et al., 2012) In dogs, limited experimental infections have shown severity of infection can vary based *T. cruzi* strain. (Barr et al., 1991) Similarly, by typing *T. cruzi* from archived tissues in lethal cases of canine Chagas disease, there is some evidence that dogs with chronic heart disease are disproportionately infected with TcI (Hodo and Hamer, unpublished data). These observations suggest that parasite genetics may play an important role in tissue tropism and the pathology of the disease. (Macedo and Pena, 1998)

The majority of documented canine cases in the U.S. are from Texas, but *T. cruzi* infected dogs have been document from at least 7 other southern states including Louisiana, Oklahoma, Tennessee, Virginia, California, Georgia and South Carolina. (Barr et al., 1995;

Bradley et al., 2000; Fox et al., 1986; Kjos et al., 2008; Navin et al., 1985; Rowland et al., 2010; Snider et al., 1980; Sánchez-Camargo et al., 2014; Tomlinson et al., 1981) *T. cruzi* is transmitted via introduction of infected kissing bug's feces into a wound or mucous membrane or through ingestion of the infected bug or its feces. Dogs are more likely to become infected than humans, (Gürtler et al., 1986, 2005) which could be from dog's affinity to consume bugs. (Bradley et al., 2000; Reithinger et al., 2005) Previous studies have examined signalment as risk factors for exposure in dogs in the U.S. and found that infection is widespread across breed, sex, age and location. (Curtis-Robles et al., 2017a, 2017b; Meyers et al., 2017; Tenney et al., 2014) Previous studies have found risk factors for increased exposure including multi-dogs kennels, (Curtis-Robles et al., 2017a) housing dogs outside, (Nieto et al., 2009; Pineda et al., 2011; Rowland et al., 2010; Tenney et al., 2014) or dogs living in older houses, Hispanic neighborhoods, and fragment habitat. (Raghavan et al., 2015)

There are currently no vaccinations or approved anti-parasitic treatments for *T. cruzi* infections in dogs in the U.S. and diagnosis is more commonly made during the chronic state where experimental treatments are least affective. Benznidazole and nifurtimox are used as treatment in human medicine but benznidazole has only been experimentally used for treatment in dogs and has been shown to not be effective in preventing myocardial lesions or dysfunction. (Santos et al., 2016, 2012) Consequently, disease prevention is focused on limiting dog-vector contact. This includes insecticide spraying, housing dogs indoors, avoiding unnecessary outdoor lights that attract bugs, and limiting vegetation growth near the house to reduce wildlife reservoirs and triatomine bug habitat. However, a better

understanding of vector behavior, host preferences, and preferred habitat hinders effective control.

To combat Chagas disease in dogs it is critical to have a comprehensive understanding of the vector, disease distribution, risk factors for infection and clinical outcomes. Importantly, since dogs are able to infect triatomines during parasitemia, (Canale et al., 1996; Gürtler et al., 1992) dogs could potentially serve as a reservoir in the U.S. disease system similar to their role in South America. A better understanding of *T. cruzi* infection in canines could have important public health implications. This dissertation aims to describe the ecological factors affecting the vector and transmission of *T. cruzi*, vector-host interactions, geographic distribution, risk factors and clinical presentation in dogs. The research presented here will provide a baseline for Chagas disease surveillance in dogs, help define an index of suspicion for testing, and better define clinical outcomes.

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2. PARASITIC INTERACTIONS AMONG *TRYPANOSOMA CRUZI*, TRIATOMINE VECTORS, DOMESTIC ANIMALS, AND WILDLIFE IN BIG BEND NATIONAL PARK ALONG THE TEXAS-MEXICO BORDER*

2.1 Introduction

In recent years, the US National Park Service has recorded over 75 million visitors per year to the 59 designated National Parks located throughout 27 states (National Park Service 2018). Many of these parks have unique ecosystems that necessitate individualized conservation and management strategies. One element requiring careful consideration is the threat of disease-causing pathogens. A diversity of zoonotic diseases—such as plague, leptospirosis, rabies, brucellosis, and tick-borne fevers—have been documented in wildlife in national parks (Aguirre *et al.* 1993). Such diseases pose a risk to employees, researchers, visitors, and their pets; the risk may increase for individuals residing within a park for long periods of time, such as long-term employees or extended-stay visitors. Outbreaks of hantavirus and relapsing fever have been recorded in park visitors (Boyer *et al.* 1977; Núñez *et al.* 2014), and incident infections of leptospirosis, bartonellosis, tick-borne fevers, and other diseases have been detected in park employees (Adjemian *et al.* 2012), including a fatal case of plague (Wong *et al.* 2009). While the National Park Service has worked to address disease control (Bosch *et al.* 2013), a lack of site-specific information limits targeted efforts.

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* Reprinted with permission from: Curtis-Robles, R.†, Meyers, A.C. †, Auckland, L.D., Zecca, I.B., Skiles, R., Hamer, S.A. 2018. Parasitic interactions among *Trypanosoma cruzi*, triatomine vectors, domestic animals, and wildlife in Big Bend National Park along the Texas-Mexico border. *Acta Tropica*. 188:225-233. †Co-first authors. Minor grammatical and syntactical changes have been made.

Efforts have focused on documenting vectors (primarily ticks), reservoirs, and zoonotic vector-borne disease risk in National Parks (McLean *et al.* 1981; Ford *et al.* 2015; Johnson *et al.* 2017; Burns *et al.* 2018); however, the risk of vector-borne pathogens in these highly-visited areas remains largely unknown (Eisen *et al.* 2013). An understanding of peak periods of vector activity and quantifying infection prevalence in vectors are key to disease prevention.

Triatomine insects, commonly referred to as 'kissing bugs' or 'cone-nose bugs' in the US, vector the parasite *Trypanosoma cruzi*, which causes Chagas disease—a potentially deadly disease of humans and animals. Triatomines occur throughout the Americas, including the southern US (Bern *et al.* 2011). These primarily nocturnal vectors feed exclusively on blood throughout their life cycle; thus, determining blood meal sources can indicate important local reservoirs of *T. cruzi* as well as the degree of triatomine contact with humans and pets. Although studies have revealed a diversity of blood meal hosts in various focal areas in the US (Stevens *et al.* 2012; Kjos *et al.* 2013; Waleckx *et al.* 2014; Klotz *et al.* 2014), feeding behavior appears opportunistic and site-specific. In the southern US, domestic dogs have been commonly implicated as blood meal hosts for triatomines (Kjos *et al.* 2013; Waleckx *et al.* 2014; Curtis-Robles *et al.* 2017a; Meyers *et al.* 2017), and kissing bug infestations are commonly reported in canine kennels (Williams *et al.* 1977; Kjos *et al.* 2013; Meyers *et al.* 2017; Curtis-Robles *et al.* 2018b). Further, canine infection with *T. cruzi* appears to be widespread across breed, sex, age and location (Bradley *et al.* 2000; Kjos *et al.* 2008; Rowland *et al.* 2010; Tenney *et al.* 2014; Meyers *et al.* 2017). Accordingly, domestic dogs appear to play a key role in supporting triatomines and *T. cruzi* in the southern US.

T. cruzi is classified into six discrete typing units (DTUs), TcI-TcVI, and an additional TcBat genotype (Marcili *et al.* 2009), which have varying ecological and epidemiological associations (Zingales *et al.* 2012). In the US, two DTUs—TcI and TcIV—have been well documented in triatomines, wildlife, and domestic dogs (Bern *et al.* 2011). TcI has been the only definitively documented DTU in autochonthonous cases of human Chagas disease in the US (Roellig *et al.* 2008; Garcia *et al.* 2017). Identifying *T. cruzi* DTUs present in the US is essential, since parasite strains can have different pathologies and ecological niches (Barr *et al.* 1991; Ramírez *et al.* 2010; Zingales *et al.* 2012) which could impact assessments of disease risk and control efforts.

Understanding the transmission of pathogens in highly visited national parks is critical to disease prevention. In and around a highly visited national park in Texas - a state with high triatomine diversity and established sylvatic *T. cruzi* transmission (Kjos *et al.* 2009; Wozniak *et al.* 2015; Curtis-Robles *et al.* 2018a) - we aimed to characterize: 1) *T. cruzi* infection in triatomines; 2) *T. cruzi* infection in dogs; and 3) triatomine blood meal sources.

2.2 Methods

2.2.1. Site description

Big Bend National Park (Fig. 1A) is situated along the Rio Grande River in Brewster County, Texas, bordered on the south and west by Mexico. There is documentation of *T. cruzi*-infected triatomines from the Big Bend area as early as 1941 (Wood 1941) and as recently as 1984 (Ikenga & Richerson 1984). The Park encompasses a total area of 3,242 ha located within the Trans-Pecos region of the Chihuahuan Desert ecoregion. Elevation varies from 550 m to 2,440 m, and is comprised of a variety of habitats, including canyons, desert,

rivers, and forested mountains. Vegetation ranges from creosote (*Larrea tridentate*) desert-scrub grasslands to montane forests of pines (*Pinus cembroides*), junipers (*Juniperus* spp.), and oaks (*Quercus* spp.) (Griffith *et al.* 2004; Texas Parks & Wildlife 2018). The wildlife community includes many nocturnal and crepuscular species, such as coyotes (*Canis latrans*), bobcats (*Lynx rufus*), Black-tailed jackrabbits (*Lepus californicus*), wood rats (*Neotoma* spp.), snakes (including diamondback rattlesnakes, *Crotalus atrox*), and lizards, as well as many species of bats (Wauer & Fleming 2001).

The Park's headquarters, visitor center, and main residential area are located in a central part of the Park known as 'Panther Junction' (Figure 2.1). The average temperature high of 35 °C occurs during June-August, and the average temperature low of 3 °C occurs during December-January; average annual cumulative precipitation is 31.6 cm ("Weather in Big Bend National Park" 2017). In the triatomine activity periods of April-August during the study period (2015-2017), minimum temperature, maximum temperature, and total precipitation were, respectively: 11 °C, 37 °C, 142 mm (2015); 13 °C, 37 °C, 228 mm (2016); 13 °C, 37 °C, 173 mm.

The population density of Brewster County is approximately 0.60 people per square kilometer, and the closest population center to the Park is Terlingua, Texas, which had a population of 58 at the time of the 2010 census (U.S. Census Burea-Data Access and Dissemination System 2010). Approximately 200-250 people live in the park year-round, concentrated mainly in Panther Junction (Figure 2.1), along with approximately 25 pet dogs owned by year-round staff/residents. Panther Junction residential units include slab-on-grade cement block structures build in the 1960's, and wood-frame, pier-and-beam structures (with subfloor) constructed during two later phases, one in the 1980's and another during the

2000's. There are also short-term researchers at the Park throughout the year, generally staying in the 'K-Bar House' (Figure 2.1) to the east of Panther Junction. The K-Bar House is a historic ranch house with multiple potential entry points for triatomines (gaps around doors/windows, pipe chases, skeleton key holes, etc.). From April 2015-August 2017 (the time period of this study), a total of 930,894 recreational visitors were recorded, including 528,633 overnight visitors (National Park Service 2018).

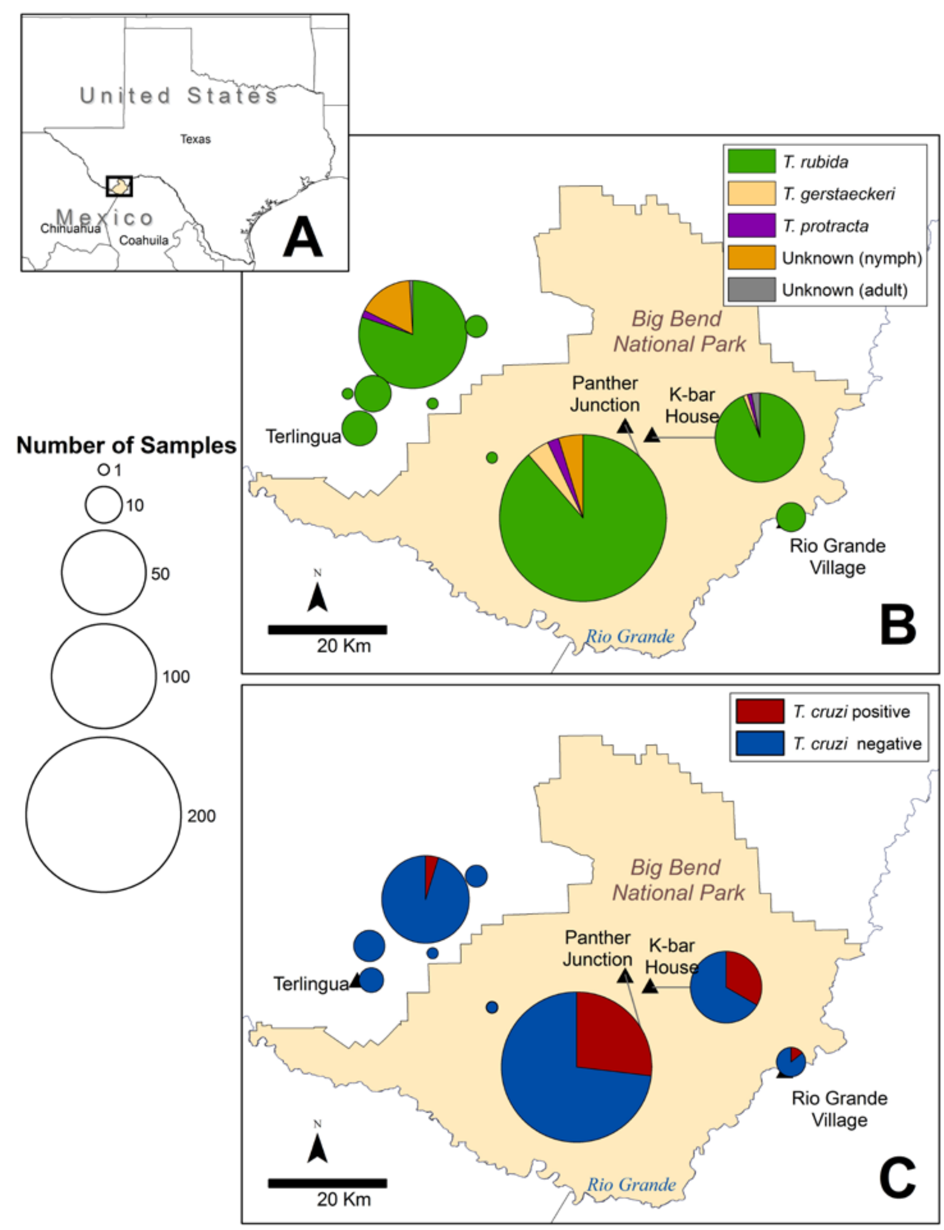


Figure 2.1. Map of Big Bend National Park and surrounding area of Brewster County, Texas. A) The study site was located in southern Brewster County, TX, with the southern border with Mexico defined by the Rio Grande (Río Bravo). B) Triatomine species collected from locations within and outside the Park. C) *Trypanosoma cruzi* infection prevalence in triatomines tested in this study was 21.8% (n = 344). An additional 35 specimens did not have exact collection site noted and were not included in these maps. (Curtis-Robles et al. 2018).

2.2.2 T. cruzi *and blood meals in triatomine samples from Brewster County*

2.2.2.1 Triatomine collection

From 2015-2017, triatomine insect samples were solicited from members of the public through our citizen science program (Curtis-Robles *et al.* 2015), recruitment/outreach by the Park's Wildlife Biologist (RS), and advertisements in local news sources (*The Big Bend Gazette* and *The Terlingua Moon*). Local residents opportunistically collected triatomine insects encountered in and around their homes; samples were collected between April 2015 and August 2017. Triatomine samples were kept at -18 °C until transfer to the laboratory, where they were stored at 4 °C until processing. Samples were collected from other communities in Brewster County as well as within the Park boundaries. Data requested for each triatomine submission included: date and time of capture, location of capture, whether the specimen was alive, and any notes or comments about the behavior of the triatomine at time of capture. In April 2016, triatomines were also collected one night from 7pm to 11pm by four members of our research team within the Park using active searching and black lights placed around the K-Bar House, where triatomines had been collected in 2015 by park staff and residents.

2.2.2.2. Detection and characterization of T. cruzi *in triatomines*

Triatomines were identified to sex and species using morphologic features (Lent & Wygodzinsky 1979). All specimens from 2015, and a subset of specimens from subsequent years, were subjected to *T. cruzi* testing and blood meal analyses. Specimens were washed in 10% bleach solution and rinsed in distilled water, and sterile instruments were used to dissect and isolate hindgut material. Evidence of a recent blood meal was scored based on visual

examination of gut material (1 = no blood, desiccated guts; 2 = no blood, guts visible; 3 = traces of blood in gut; 4 = blood present, but either not much or not fresh [dried]; 5 = large amount of fresh blood) (see Curtis-Robles *et al.* 2018b).

DNA was extracted from hindgut samples using commercial kits (E.Z.N.A. Tissue DNA kit, Omega Bio-Tek, Norcross, GA; KingFisher Cell and Tissue DNA kit, Thermo Fisher Scientific, Waltham, MA) and subjected to a quantitative polymerase chain reaction (qPCR) amplifying a 166-bp repetitive nuclear satellite target using the Cruzi 1/Cruzi 2 primer set with Cruzi 3 probe (Duffy *et al.* 2013). Reactions were run on a Stratagene MxPro3000 (Agilent Technologies, Santa Clara, CA) using previously described conditions (Curtis-Robles *et al.* 2018a). Samples producing amplification curves with cycle threshold (Ct) values of <35 were considered suspect positive for *T. cruzi* DNA. To confirm presence of *T. cruzi* DNA and characterize *T. cruzi* DTU in samples, we used a multiplex qPCR assay to amplify the spliced leader intergenic region (SL-IR) DNA, according to a published protocol (Cura *et al.* 2015) but with some modifications as previously detailed (Curtis-Robles *et al.* 2017). This method uses several probes with unique fluorescent tags specific to the DTUs, and affords the ability to detect mixed-DTU infections. Samples were considered untypeable after three amplification attempts (Curtis-Robles *et al.* 2018a). Samples which produced a Ct value of <35 on the Cruzi 1/2/3 qPCR and were typeable on the SL-IR qPCR were considered positive. Positive controls for the Cruzi 1/2/3 qPCR were DNA extracted from *T. cruzi* Sylvio X10 CL4 (ATCC 50800, American Type Culture Collection [ATCC], Manassas, VA; DTU TcI) and a *T. cruzi*-positive (DTU TcIV) *T. sanguisuga* collected from Lee County, Texas; positive controls for the SL-IR qPCR also included DNA extracted from *T. cruzi* Y strain (ATCC 50832, ATCC; DTU TcII). No-template controls were included in

each set of DNA extractions, and molecular grade water was included as a negative control in all PCR batches.

2.2.2.3. Detection of recent blood meal sources

All samples from 2015 were processed in order to determine the sources of recent triatomine blood meals. We used an iterative process in which hindgut DNA was subjected to PCR amplification of host cytochrome B sequences using previously published 'herp' and 'BM' primers. Reactions using the 'herp' primer set (Cupp *et al.* 2004; Hamer *et al.* 2009) included 1.5 μL template DNA, primers at final concentrations of 0.66 μM each, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a final reaction volume of 15 μL using previously described cycling conditions (Hamer *et al.* 2009). PCR using the 'BM' primer set (Boakye *et al.* 1999; Hamer *et al.* 2009; Kjos *et al.* 2013) included 1.5 μL template DNA, primers at final concentrations of 0.66 μM each, and FailSafe PCR Enzyme Mix with PreMix E in a final reaction volume of 15 μL using previously described cycling conditions (Kjos *et al.* 2013). In cases where no amplicons were generated, DNA was diluted 1:10 and reactions were attempted again. All PCR batches included negative controls (water) and positive controls of DNA extracted from white-tailed deer (*Odocoileus viginianus*) or cynomolgus macaque (*Macaca fascicularis*). Amplicons were visualized on 1.5% agarose gel with ethidium bromide and sequenced using Sanger sequencing (Eton Bioscience Inc., San Diego, CA, USA). Resulting sequences were compared to existing sequences in GenBank (www.ncbi.nlm.nih.gov/genbank/) (Clark *et al.* 2016) using the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information, US National Library of Medicine). A blood meal identification attempt was considered

successful when there was a match with ≥98% BLAST identity in the GenBank database. Various protocols were in place to mitigate risk of exogenous human DNA contamination (bleaching specimens prior to dissection, all work conducted in Class II biosafety cabinets, and dedicated areas of the laboratory for pre-PCR and post-PCR processing). In recognition of potential contamination with human DNA during collection and/or processing, samples that revealed a potential human blood meal were re-run on a second PCR with additional sequencing. Samples were considered positive for a human blood meal only when yielding two independently acquired human DNA sequences.

2.2.3. T. cruzi *infection in dogs in Big Bend National Park*

2.2.3.1. Sample collection

We used a cross sectional study design to collect blood samples from domestic dogs from residences of Panther Junction in Big Bend National Park in April 2016. Selection criteria included dogs over 6 months old and living in the Park for a minimum of 5 months. Information gathered from owners also included whether the dog slept indoors or outdoors at night (when triatomines may be most active (Sjogren & Ryckman 1966; Pippin 1970; Ekkens 1981). A total of 2-4 mL of blood was split between a no-additive tube and an EDTA tube. Samples were centrifuged for 5 minutes and separated into components for further processing. Dog sampling was in adherence with animal use protocols approved by Texas A&M University Institutional Animal Care and Use Committee.

2.2.3.2. Serological methods

Dog serum was screened for anti-*T. cruzi* antibodies using three different serological assays. Two rapid immunochromatographic tests were used: Chagas Stat-Pak (ChemBio Diagnostic Systems, Inc., Medford, NY), which has been validated in dogs (Nieto *et al.* 2009), and Chagas Detect Plus Rapid Test (InBios International, Inc., Seattle, WA). Serum samples were tested according to manufacturers' protocols; any development of a colored band was considered a positive result on a test. Samples were also tested using indirect fluorescent antibody (IFA) detection of anti *T. cruzi*-IgG antibodies at the Texas Veterinary Medical Diagnostic Laboratory (TVMDL; College Station, Texas). All samples were screened at doubling dilutions from 1:20 to 1:1280; according to TVMDL protocol, sample reaction at any dilution (≥1:20) was considered positive. A dog was considered seropositive when at least two of the three tests were positive.

2.2.3.3. Detection of T. cruzi *DNA in dog blood samples*

DNA was extracted from 250 µL of buffy coat using a commercial kit (E.Z.N.A. Tissue DNA kit, Omega Bio-Tek, Norcross, GA). *T. cruzi* nuclear DNA was amplified using the qPCR protocols described above (section 2.2.2).

2.2.3.4. Statistical analyses

Bivariable analysis using Chi square or Fisher's exact tests was used to assess associations between triatomine *T. cruzi* infection status and the following variables: life stage, sex (adults only), species (adults only), location collected, season collected, and year collected.

To evaluate whether presence/absence of a blood meal influenced the likelihood of successful detection of host DNA in a triatomine hindgut, we first categorized blood meal scores into two groups: those without evidence of recent blood meal (scores of 1-2) and those with evidence of recent blood meal (scores 3-5). We assessed the relationship between blood meal presence/absence and successful host determination through bivariable analysis using Fisher's exact test. Logistic regression was used to evaluate success/failure of host determination across different blood meal scores, with odds ratio (OR) and 95% confidence interval (CI) calculated. Additionally, we used bivariable analysis to explore relationships between blood meal score and type of host previously fed upon (wildlife vs. domestic animals and humans).

An independent-samples t-test was conducted to compare the mean ages of seronegative and seropositive dogs; Fisher's exact test was used to assess associations between dog serostatus and sex. All variables with p-values of <0.05 were considered significant. Data were analyzed using R 3.4.2. (R Development Core Team 2008).

2.3 Results

2.3.1. Triatomine characteristics

A total of 461 triatomine insects were collected from Brewster County from April 2015 to August 2017. Specimens (n = 450) collected by residents of Big Bend National Park and Brewster County were collected throughout all seasons, but were mainly (355 specimens) collected from April-June in all years (Table 2.1; Figure 2.2). Of the 450 resident-collected triatomines, 310 (68.9%) were from within the Park and 135 (30.0%) were from outside of Park boundaries (Figure 2.1B), 5 specimens (1.1%) did not have exact

capture location noted. In addition to the resident-collected specimens, in April 2016 our active bug trapping resulted in the capture of 11 triatomines from near the K-Bar House within the Park. Of the 241 triatomines found inside residences, 97.5% were adults. Of the 204 triatomines with a known status at collection, 186 (91.2%) were found alive and 18 (8.8%) were found dead. Collection varied over the three-year study period, with highest number collected in 2016 (332 specimens; 72.0%) and lowest in 2017 (37 specimens; 37%; Table 2.1).

Table 2.1. Bivariable analyses (using chi square or Fisher's exact tests) of predictor variables of *Trypanosoma cruzi* infection status in triatomines found in/around Big Bend National Park, Texas. (Curtis-Robles et al. 2018)

Variable	Total Collected	No. Tested (%)	No. *T. cruzi* Positive (%)	p-value
Life stage				0.04
Adult	429 (93.1)	320(74.6)	74 (23.1)	
Nymph	32 (6.9)	24 (75.0)	1 (4.2)	
Sex of Adults				0.09
Male	213 (49.7)	164 (76.9)	45 (27.4)	
Female	214 (49.9)	155 (72.4)	29 (18.7)	
Unknown[1]	2 (0.4)	1 (50.0)	0	
Species of Adults				0.21
T. gerstaeckeri	*11 (2.6)*	11 (100.0)	1 (9.1)	
T. protracta	10 (2.3)	9 (90.0)	4 (44.4)	
T. rubida	*405 (94.4)*	299 (73.8)	69 (23.1)	
Unknown (adult)[1]	3 (0.7)	1 (33.3)	0	
Collection Site				0.49
Inside Non-Residential Building	19 (4.1)	10 (52.6)	3 (30.0)	
Inside Residence	241 (52.3)	202 (83.8)	44 (21.8)	
Outside Residence	76 (16.5)	47 (61.8)	13 (27.7)	
Unknown	125 (27.1)	85 (68.0)	15 (17.6)	
Season				0.08
January-March	3 (0.65)	2 (66.7)	0	
April-June	355 (70.0)	274 (77.2)	67 (24.5)	
July-September	55 (11.9)	47 (85.5)	4 (8.5)	
October-December	5 (1.1)	5 (100.0)	0	
Unknown	43 (9.3)	16 (37.2)	4 (25.0)	
Year				0.56
2015	90 (19.5)	89 (98.9)	19 (21.3)	
2016	332 (72.0)	219 (66.0)	45 (20.5)	
2017	37 (8.0)	21 (56.8)	7 (33.3)	
Unknown	2 (0.4)	1 (50.0)	0	

[1] Unknowns removed from bivariable analysis.

Of the 336 samples for which exact collection site was noted (72.9%, Table 2.1), 260 (77.4%) were found indoors. The location with the most triatomines captured (229; 49.7% of triatomines collected) was Panther Junction. A private residence northwest of the Park collected 96 triatomines, including 16 nymphs. The K-Bar House had the third highest number of triatomines collected (66; 14.3%).

Three species of triatomines were identified (Table 2.1): *T. rubida* (405; 87.9%), *T. gerstaeckeri* (11; 2.4%), and *T. protracta* (10; 2.2%). Three adult specimens could not be identified to species due to poor condition of the specimens, including two for which sex could not be determined. For those 437 specimens for which sex was determined (Table 2.1), samples were equally male (49.9%) and female (50.1%). A total of 32 nymphs were collected (6.9% of triatomines collected; Table 2.1); species and sex of nymphs were not determined. Adult triatomines with known collection month (n = 372) were most frequently collected in June (48.2%) while nymphs with known collection months (n = 32) were found most often in August (46.2%).

Peak visitor activity to Big Bend National Park is typically in the months of March, April and November ("Recreation Visitors by Month, Big Bend NP" 2017). During the months that the majority of triatomines were captured during our study (April-August of 2015 and 2016, Figure 2.2), an estimated total of 261,000 visitors had visited the Park ("Recreation Visitors by Month, Big Bend NP" 2017).

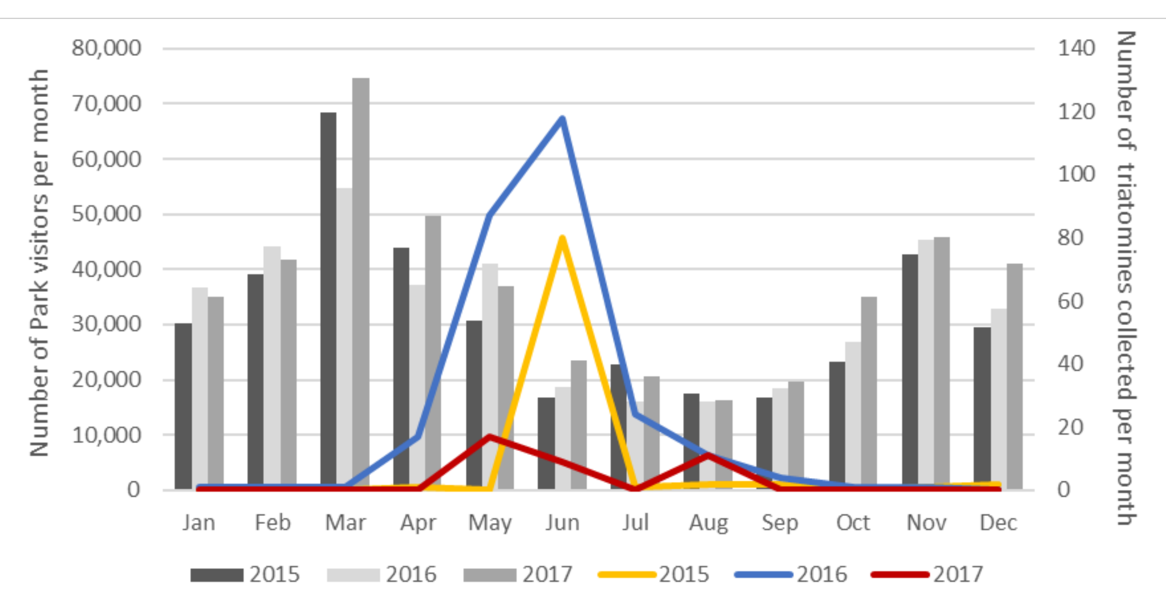


Figure 2.2. Recreational visitors by month in Big Bend National Park from 2015-2017 are represented by the bars (National Park Service 2018) and number of triatomines collected per month during the study period (2015-2017) are represented by the lines. (Curtis-Robles et al. 2018).

2.3.2. Triatomine T. cruzi infection and DTU typing

We tested 344 triatomines for infection with *T. cruzi*, of which 75 (21.8%) were infected (Figure 2.1C). Infection was higher in adults (23.1%, n = 320) compared to nymphs (4.2%, n = 24); (p = 0.04, Table 2.1). Males had a marginally higher infection prevalence (27.4%, n = 164) than females (18.7%, n = 155; p = 0.09). Infection prevalence did not differ across species of adults (p = 0.21) or collection site (p = 0.49). Triatomines collected in April-June showed the highest infection prevalence (24.5%, n = 274) relative to other seasons (p = 0.08, Table 2.1). Infection prevalence ranged from 20.5%-33.3% across the three years of the study (p = 0.56).

Infected triatomines were found at several locations (Figure 2.1). The *T. cruzi* infection prevalences in triatomines collected from the two main locations within the Park—Panther Junction and the K-Bar House—were 26.9% (n = 186) and 33.3% (n = 42),

respectively. In contrast, the *T. cruzi* infection prevalence in triatomines collected at a private residence northwest of the Park was only 4.8% (n = 63). The *T. cruzi* DTU TcI was the only DTU detected in the infected triatomines.

2.3.3. Triatomine blood meal results

A total of 337 specimens were dissected and scored for blood meal; 62.0% did not have evidence of blood in the gut (blood meal scores of 1-2) and 38.0% had evidence of blood in the gut (scores of 3-5). Of the dissected/scored samples, 89 specimens collected in 2015 were subjected to blood meal analysis, of which 42 (47.2%) yielded successful results. Triatomines with scores of 1 (no blood in gut) were associated with only 20% success determining host identity whereas triatomines with scores of 5 (fully engorged) were associated with 100% success determining host identity (Figure 2.3). The odds of successfully determining blood meal host identity were nearly 11 times greater (OR 10.8, 95% CI 4.2-30.6, $p < 0.001$) in specimens with evidence of a recent blood meal (scores 3-5) than specimens with no evidence of a recent blood meal (scores 1-2).

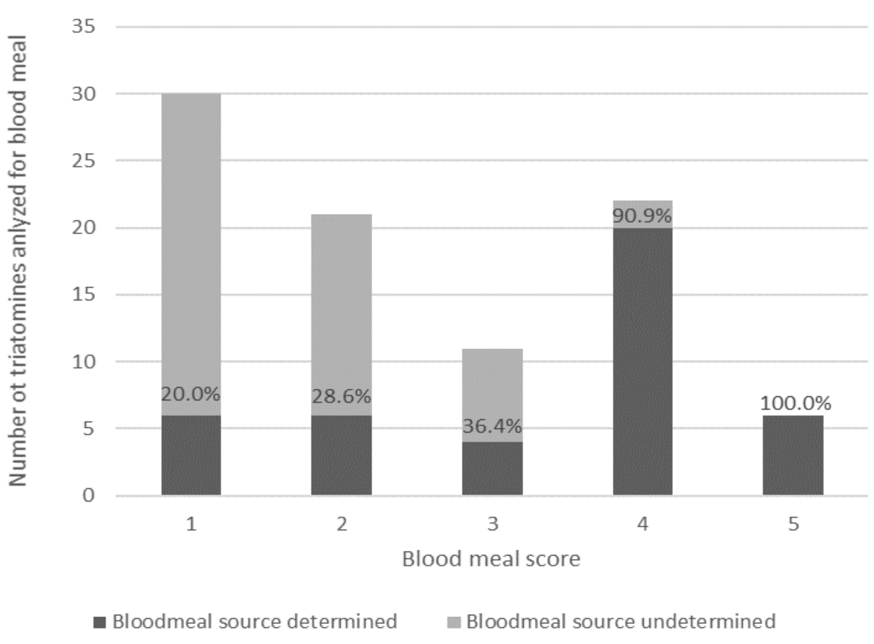


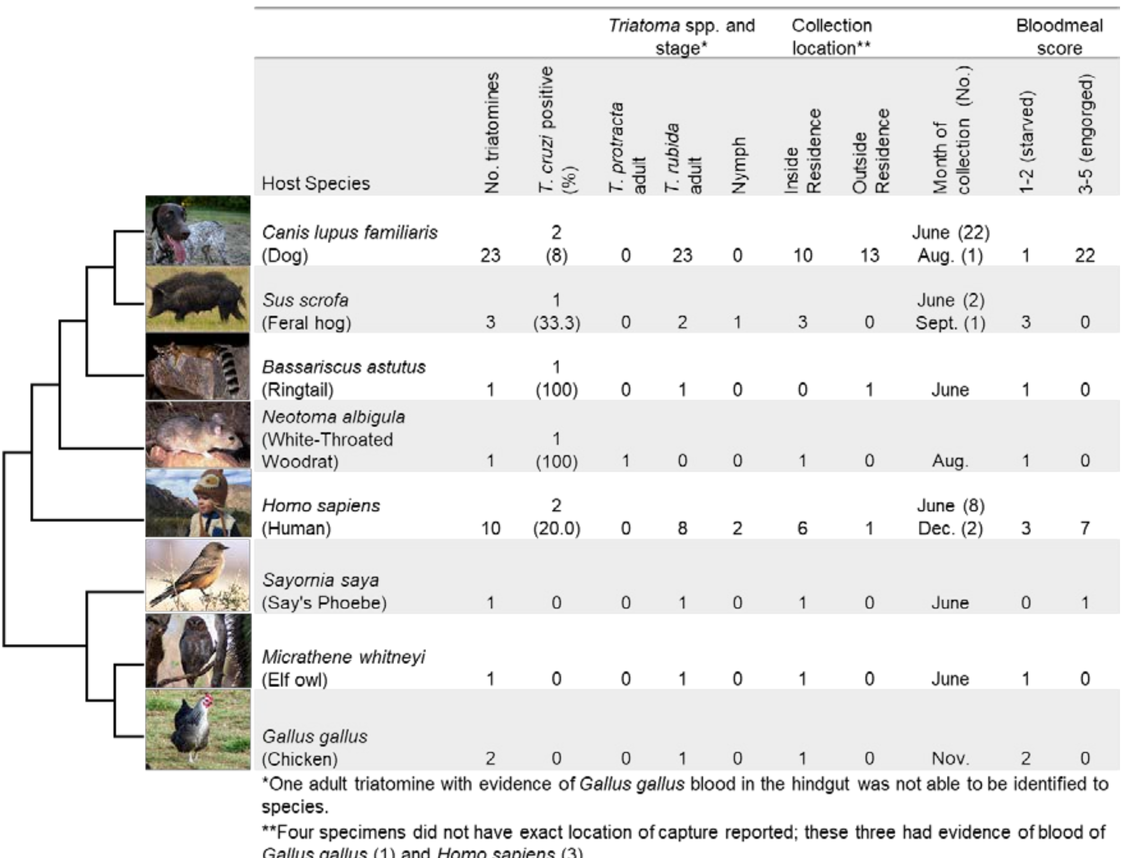
Figure 2.3. Details of blood meal analysis success in triatomine specimens collected in/around Big Bend National Park, 2015. Forty-two (47.2%) of the 89 triatomines subjected to blood meal analyses had blood meal sources successfully determined. The odds of successfully determining blood meal host identity were nearly 11 times greater (OR 10.8, 95% CI 4.2-30.6, $p < 0.001$) in specimens with evidence of a recent blood meal (scores 3-5) than specimens with no evidence of a recent blood meal (scores 1-2). (Curtis-Robles et al. 2018).

Of the 42 samples yielding successful results, the majority (54.8%; Figure 2.4) of blood meals detected were from dogs (*Canis lupus familiaris*). Other animals detected as blood meal sources included 3 feral hogs (*Sus scrofa*), 2 chickens (*Gallus gallus*), 1 ringtail (*Bassariscus astutus*), 1 white-throated woodrat (*Neotoma albigula*), 1 elf owl (*Micrathene whitneyi*), and 1 Say's phoebe (*Sayornia saya*).

There were 10 samples with two lines of evidence (results from multiple independently-run PCRs) of having fed on human (*Homo sapiens*) blood (Figure 2.4). Two of these samples were nymphs from a private residence near Terlingua, both found outside the home. Two adults were from the K-Bar House, one found in the bedroom and one on the

porch. Six adults came from residences in Panther Junction: 1 in a bedroom, 3 in a living room, 1 near a dog bed, and 1 without collection site reported.

Triatomines in which wildlife blood meals were detected were significantly more likely to have low blood meal scores of 1-2 (relatively starved; 85.7%) compared to triatomines in which humans or domestic animals (dogs and chickens) blood meals were detected (17.1%; p = 0.001).



Host Species	No. triatomines	*T. cruzi* positive (%)	*Triatoma* spp. and stage* *T. protracta* adult	*T. rubida* adult	Nymph	Collection location** Inside Residence	Outside Residence	Month of collection (No.)	Bloodmeal score 1-2 (starved)	3-5 (engorged)
Canis lupus familiaris (Dog)	23	2 (8)	0	23	0	10	13	June (22) Aug. (1)	1	22
Sus scrofa (Feral hog)	3	1 (33.3)	0	2	1	3	0	June (2) Sept. (1)	3	0
Bassariscus astutus (Ringtail)	1	1 (100)	0	1	0	0	1	June	1	0
Neotoma albigula (White-Throated Woodrat)	1	1 (100)	1	0	0	1	0	Aug.	1	0
Homo sapiens (Human)	10	2 (20.0)	0	8	2	6	1	June (8) Dec. (2)	3	7
Sayornia saya (Say's Phoebe)	1	0	0	1	0	1	0	June	0	1
Micrathene whitneyi (Elf owl)	1	0	0	1	0	1	0	June	1	0
Gallus gallus (Chicken)	2	0	0	1	0	1	0	Nov.	2	0

*One adult triatomine with evidence of *Gallus gallus* blood in the hindgut was not able to be identified to species.

**Four specimens did not have exact location of capture reported; these three had evidence of blood of *Gallus gallus* (1) and *Homo sapiens* (3)

Figure 2.4. Details of blood meal sources detected in triatomine samples collected in/around Big Bend National Park, 2015. A total of 42 triatomine samples had a blood meal sources successfully identified using PCR-based blood meal analysis. Relationships among vertebrate hosts were determined using analysis of cytochrome B sequences. Phylogenetic tree was created in Mega 7.0.26 using host sequences of cytochrome B. (Curtis-Robles et al. 2018).

2.3.4. T. cruzi *in resident dogs*

A total of 14 dogs were sampled in April 2016 (Table S1), including eleven with indoor sleeping sites and three that slept indoors/outdoors at night. Thirteen breeds were represented, and ages ranged from 9 months to 13 years. Sampled dogs had lived in the Park from 5 months to 8 years. Using the criterion of positivity on at least two of the three serological tests, 28.6% of dogs were seropositive (Table S1). None of the 14 dogs had parasite DNA in the blood at the time of sampling. The mean age of seronegative dogs (5.73 years) was not significantly different ($p = 0.99$) than the mean age of seropositive dogs (5.75 years). Similarly, the mean time a seronegative dog spent in the Park (2.43 years) was not significantly different ($p = 0.48$) than the mean time of seropositive dogs (4.17 years). Female dogs had a marginally higher seroprevalence (60.0%) compared to males (11.1%, $p = 0.09$).

2.4 Discussion

National Parks in the US receive over 75 million visitors per year (National Park Service 2018), and provide environments and recreation activities that allow for exposure to a diversity of vector-borne pathogens. Furthermore, the majority of visitors and outdoor recreational activities take place during the summer months when vectors are most active (Curtis-Robles *et al.* 2018b). We studied the disease ecology of *T. cruzi* at Big Bend National Park, a highly visited national park in a region endemic for triatomine vectors, and found an active transmission of *T. cruzi* among vectors, wildlife, and domestic animals with the potential for spillover to humans.

Triatomines are found across 29 US states (Swanson 2011; Bern *et al.* 2011), but their occurrence in national parks and the risk that they pose to park residents and visitors is largely unknown, with the exception of one previous study including triatomines in Big Bend National Park (Ikenga & Richerson 1984) and an unpublished record of *Paratriatoma hirsuta* in Death Valley National Park in eastern California (R. Ryckman, personal communication). Determining *T. cruzi* presence and characterizing ecoepidemiology details are critical to mitigating transmission in national park settings.

Although triatomines are known to occur across Texas (Kjos *et al.* 2009; Bern *et al.* 2011; Curtis-Robles *et al.* 2018b), few studies of triatomines have been conducted in Brewster County or Big Bend National Park. A 1984 study had found *T. recurva* and *T. gerstaeckeri* from the K-Bar House in the Park (Ikenga & Richerson 1984); that study constitutes the only report of *T. recurva* from Texas. The lack of detection of *T. recurva* in the current study, despite its reported presence in the same area over three decades earlier, could reflect a low population density that our sampling effort was not sufficient to detect, local extirpation of the species in the area, misidentification of the specimens in the prior report, or other factors. Another recent study at the Indio Mountains Research Station, approximately 240 km northwest of the Park, found 61.5% of 39 *T. rubida* infected with *T. cruzi* (Buhaya *et al.* 2015). *T. rubida* was also the most frequently collected species in our study, but the infection prevalence was comparatively lower (23.1%). Compared to our finding of 44.4% infection in *T. protracta*, Wood documented 4.3% infection in *T. protracta woodi* collected from rat nests near the Chisos Mountains in Big Bend National Park (Wood 1941). In our study, only one (9.0%) of 11 *T. gerstaeckeri* was infected, which contrasts our finding of high (63.3%) infection prevalence in this species in other parts of the state (Curtis-

Robles *et al.* 2018a). The one other report of *T. gerstaeckeri* in Brewster County reported an uninfected individual (Sullivan *et al.* 1949). Variation in triatomine infection prevalence across geographic regions (also shown in Curtis-Robles *et al*. 2018a) may reflect different locally-available reservoir populations, reservoir infectiousness, and vector-host contact dynamics (Gürtler & Cardinal 2015; Hodo & Hamer 2017).

We found that male triatomines had a marginally higher infection prevalence compared to females, which is similar to previous findings in Texas, where adult male triatomines were 1.41 times more likely to be infected than females (Curtis-Robles *et al.* 2018a). Similarly, Pippin found higher infection prevalence in males (Pippin 1970). We found no statistically significant variation in the annual *T. cruzi* infection prevalence in triatomines over the 3-year sampling period, suggesting that the level of infection is stable within *T. rubida* in the area. We found a higher *T. cruzi* infection prevalence in triatomines collected from within the Park (26.9-33.3%) compared to a private residence northwest of the Park (4.8%); this striking contrast could possibly be due to different host assemblages at each area. In particular, dogs were a common host of triatomines collected in the Park and are known to be important parasite reservoirs capable of infecting triatomines (Gürtler and Cardinal 2015), whereas dogs were not represented in blood meals of bugs collected outside the Park.

The greatest number of triatomines were captured during May-June of each year, in agreement with other studies finding June to be a peak period of triatomine activity in areas of Texas and Mexico (Martínez-Ibarra *et al.* 1992; Curtis-Robles *et al.* 2018b). Decreasing numbers of captures and reports of triatomines throughout the rest of the summer months could be due to a combination of less triatomine activity and increased awareness after

encounters with triatomines, and renewed fortification of homes to reduce entry points for the insects. Of the 336 triatomines with capture site specified, 241 (71.7%) were found inside residences, and an additional 19 (5.7%) were found indoors in non-residential buildings, where they can pose risk of *T. cruzi* transmission to humans and pets. Adult triatomines accounted for 97.5% of the triatomines collected in residences. A study of triatomines collected across Texas found the majority of bugs collected from a peridomestic setting were adults and concluded that colonization of houses in the US was infrequent (Kjos *et al.* 2009). Similarly, a study in Nuevo Leon, Mexico, found only adult triatomines in houses and a low likelihood of colonization (defined as multiple life stages in a residence) by *T. gerstaeckeri* and *T. lecticularia* (Martínez-Ibarra *et al.* 1992). A study of an introduced population of *T. lecticularia* in a non-endemic area of Mexico found adults and eggs, but no nymphs in houses (Grant-Guillén *et al*. 2018). Results of a study in the Yucatan peninsula of Mexico suggested that seasonal invasions of flying adult triatomines may play a more important role in *T. cruzi* transmission than colonized ('domestic') populations of triatomines (Dumonteil *et al.* 2002). Similarly, our findings of primarily adult triatomines in homes suggest that they are invading or dispersing rather than representative of colonized populations. Of the triatomines tested that were found in residence homes, 21.8% were infected.

Although human bite data was not systematically collected in our study, there are many anecdotal reports of human bites at and around the Park. For example, the individuals that submitted bugs in our study stated that there were at least 12 human bites associated with the bugs that were submitted to our study. All bites were associated with adult *T. rubida*, except in two cases, by nymphs of unknown species. Three triatomines in which we detected human

blood meals were noted as having bitten a person, and all had evidence of a recent blood meal in the triatomines gut (blood meal scores of 3, 3, and 4).

With respect to parasite transmission risk to visitors, visitors to Big Bend National Park generally peak in March and decline until an annual low during July (Figure 2.2), with an average of approximately 36,200 visitors in May of each year, and an average of approximately 19,600 visitors in June of each year (averages based on 2015-2017 data) (National Park Service 2018). The highest number of triatomines collected from the Park peaked in June in both 2015 and 2016 (Figure 2.2). Most importantly, the subset of visitors reported as 'overnight stays' (which are at highest risk of contact with nocturnal triatomines; including individuals in tents, recreational vehicle (RV) campers, and backpacking campers) in May-June of 2015-2017 totaled almost 81,300 person-nights (a person-night is one night within the Park by one visitor; (National Park Service 2018). Triatomine activity peaking in May/June is comparable to findings from other areas of Texas (Pippin 1970; Curtis-Robles *et al.* 2018b), although those collections included very few *T. rubida*, the main species collected in the current study. A study of mainly *T. rubida* in Arizona found peak activity from late May through mid-July, and concluded that high temperatures, low relative humidity, and low wind speeds were the most important environmental factors contributing to *T. rubida* flight dispersal (Ekkens 1981).

Our finding of exclusively DTU TcI in *T. cruzi* infected triatomines is consistent with a recent study that documented only TcI in *T. rubida* from another area of western Texas (Buhaya *et al.* 2015), but is in contrast with our previous findings of near equal proportions of TcI and TcIV in triatomine vectors (namely, *T. gerstaeckeri* and *T. sanguisuga*) across the south (Curtis-Robles *et al.* 2018a). TcI is known to circulate in US populations of

triatomines, wildlife and domestic dogs (Roellig *et al.* 2008; Curtis-Robles *et al.* 2017a, b, 2018a). TcI has been implicated in autochthonous cases of human Chagas disease in the US (Roellig *et al.* 2008). Interestingly, evidence of possible Chagas disease in mummified human remains excavated from approximately 150-200 km northeast of Big Bend National Park is suggestive of *T. cruzi* transmission roughly 1,200 years ago (1,150 BP) (Reinhard *et al.* 2003; Araújo *et al.* 2009).

We found that over one quarter (28.6%) of dogs in our study tested positive on at least two serologic tests and were therefore considered seropositive (Table S1). *T. cruzi* diagnostics for humans and animals is typically based on the detection of anti-*T. cruzi* antibodies. Numerous serologic platforms are available, but results are often discordant (Guzmán-Gómez *et al.* 2015; Meyers *et al.* 2017) and the findings in our study are not an exception. In the current study, 4 dogs were positive on at least 2 testing platforms but only a single dog was positive on the IFA test (titer value 1:40), emphasizing the potential importance of the immunochromatographic tests for detecting infection. We previously examined government working dogs across Texas and found a seroprevalence of 18.8% in an area including Big Bend National Park when using the same diagnostic approach as in the current study (Meyers *et al.* 2017). A previous study using indirect hemagglutination assay (IHA) found 15.6% seroprevalence in 32 dogs tested from Brewster County, though the authors cautioned that the percentage may not be precise because of potential complementary reactions with other trypanosomes (Ikenga & Richerson 1984). Studies of dogs along the southern Texas-Mexico border have revealed seroprevalences of 7.5-19.6% (Beard *et al.* 2003; Curtis-Robles *et al.* 2017b); another study detected 6.7-13.8% seroprevalence across the state of Texas (Tenney *et al.* 2014). None of the dogs tested in this study had evidence of

T. cruzi DNA in their blood, suggesting that these dogs were not parasitemic during testing. This may be related to timing of sampling, since studies indicate that parasitemia peaks between 1 and 5 weeks after initial infection (Barr *et al.* 1991; Lana *et al.* 1992), and our dog sampling occurred in April, prior to peak triatomine season.

Domestic dogs appear to play a key role in transmission in the peridomestic setting in Big Bend National Park, evident by dogs being the most common blood meal source we identified in triatomines. Previous studies looking at blood meal results from triatomines in Mexico and across Texas have also found dogs to be the among the most common blood meal sources (Zárate *et al.* 1980; Villalobos *et al.* 2011; Ibáñez-Cervantes *et al.* 2013; Kjos *et al.* 2013). Additionally, we found that *T. rubida* and *T. protracta* had fed on a diversity of wildlife hosts. We detected DNA evidence of white-throated wood rat (*N. albigula)* blood meal in a *T. protracta*; *T. protracta* and *T. rubida* have previously been documented in the nests of wood rats (Wehrle 1939; Vorhies & Taylor 1940; Wood 1941; Paredes-Gonzalez *et al*. 2015). We found DNA evidence of an elf owl (*Micrathene whitneyi)* blood meal in a *T. rubida*; elf owls are the tiniest owls in the world and are endemic to the deserts and wooded canyons of the southwestern US-Mexico border, breeding in the region and migrating further south in Mexico in the winter (McKinney 2006). Similarly, owl blood meals have been found in *Rhodnius pallescens* in Panama (Christensen & de Vasquez 1981). Two triatomines had evidence of chicken (*Gallus gallus*) blood meals, one triatomine collected from inside a house in November from outside the Park with a bloodmeal score of 1, while the other was from the Panther Junction area within the Park with a bloodmeal score of 2. The bloodmeal scores indicate that the triatomines were starved and possibly dispersed in search of a new host. Since chickens are not allowed on the Park property, this observation may suggest

dispersal of a chicken-fed triatomine into the park. Alternatively, it could be evidence of illegal presence of a chicken within the Park or laboratory contamination/error. Others have noted triatomines in chicken nests/roosts in the US (Sullivan *et al.* 1949; Curtis-Robles *et al.* 2018b), including chicken blood meals in analysis of triatomines from the US and Mexico (Sullivan *et al.* 1949; Zárate *et al.* 1980; Bosseno *et al.* 2006; Villalobos *et al.* 2011; Stevens *et al.* 2012); one study found a significant association between presence of a chicken coop on a property and likelihood of finding *T. sanguisuga* in the house (Moudy *et al.* 2014). A single *T. rubida* that was found inside a house near a doorway was found to have fed on a Say's Phoebe (*Sayornia saya*), a small flycatcher species that is known to occur in the park year-round and often nests in or on buildings (Tweit 2005). Three blood meal results sequenced to *Sus scrofa,* which we interpret to be feral swine given no domestic pigs are kept in the region. While no feral hogs are known to occur in the immediate vicinity of where these bugs were collected, a group of feral hogs is known to reside about 30 kilometers from the location (RS, personal observation). As boars are often solitary and are known to travel long distances (Gaston et al. 2008), feral hogs may have moved into the region. Alternately, given all three triatomines with feral hog DNA were considered starved (bloodmeal scores of 1-2), these insects could have fed on pigs prior to their dispersal into the region of collection. Little is known about maximum flight dispersal capabilities of Triatomines, though some studies suggest flight potential of multiple kilometers (Ekkens 1981; Schweigmann *et al.* 1988; Castro *et al.* 2014). Feral hogs have been found to harbor *T. cruzi* in Texas (Comeaux *et al.* 2016) and previous studies have found evidence of pig blood meals in triatomines (Zárate *et al.* 1980; Kjos *et al.* 2013). These findings suggest that a complex transmission

cycles exists, involving a diversity of wildlife and domestic animals, as well as humans in the Big Bend National Park ecosystem.

Our research illustrates the potential risk of *T. cruzi* exposure for park visitors and staff in a national park setting. Furthermore, this study demonstrates the importance of promoting awareness among park staff and visitors of the potential for *T. cruzi* exposure in this environment, particularly for visitors from non-endemic areas who might be unaware of the vector and disease. This research is a first step towards understanding *T. cruzi* risk to public health in national parks, and can be used to improved education and awareness of park visitors and employees. National parks may consider providing educational materials instructing visitors on precautionary personal protection (long pants/shirt at dusk, repellent, knowing how to identify vectors of concern and their seasonal activity periods; using a bed net when sleeping outdoors/in cabins (bed nets are currently recommended by park staff for residents of the K-Bar House); and signs/symptoms of *T. cruzi* infection. Additional investigation of the transmission dynamics of *T. cruzi* in national park settings will allow for understanding and reducing the risk of *T. cruzi* exposure to park staff, visitors, and their pets.

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3. WIDESPREAD *TRYPANOSOMA CRUZI* INFECTION IN GOVERNMENT WORKING DOGS ALONG THE TEXAS-MEXICO BORDER: DISCORDANT SEROLOGY, PARASITE GENOTYPING AND ASSOCIATED VECTORS [*]

3.1 Introduction

Chagas disease, a potentially deadly cardiac disease of humans and dogs, is caused by the flagellated protozoan parasite *Trypanosoma cruzi*. The parasite is transmitted by infected *hematophagous* triatomine insects, commonly known as 'kissing bugs'. Chagas disease is estimated to infect nearly 6 million people throughout Latin America, and occurs across the southern US in enzootic cycles [1,2], where raccoons and other wildlife serve as reservoirs [2,3]. In many areas of Latin America, such as in the Gran Chaco ecosystem, domestic dogs are an important reservoir of *T. cruzi* and domestic vectors that fed on dogs showed higher infection prevalence than vectors that fed on other domestic hosts [4,5]. The importance of canines in the *T. cruzi* transmission cycle in the US is not yet understood.

The occurrence of *T. cruzi* infected canines in the USA is especially high in the state of Texas [1,6,7], where 439 cases were reported across 58 counties between 2013-2015 when there was mandatory reporting of *T. cruzi* infected dogs [8]. Texas harbors at least seven established species of triatomine vectors capable of transmitting *T. cruzi* [3] and infected wildlife are widespread [1]. The high frequency of canines infected with *T. cruzi* likely reflects robust enzootic transmission in the state. Outside of Texas, dogs infected with *T.*

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[*]Reprinted with permission from: Meyers, A.C., Meinders, M., Hamer, S.A. 2017. Widespread *Trypanosoma cruzi* infection in government working dogs along the Texas-Mexico Border: discordant serology, parasite genotyping and associated vectors. *PLoS Neg Trop Dis.* 11(8): e0005819. Minor grammatical and syntactical changes have been made.

cruzi have been reported in Louisiana [9,10], Oklahoma [11,12], Tennessee [13], and Virginia [14]. Across the studied populations, apparent seroprevalence ranged from 3.6-57.6% and predispositions of infection status with certain breeds or types of dogs do not appear to be strong, with hunting dogs, working dogs, household pets, shelter and stray dogs all impacted [6,7,9,12,14,15].

T. cruzi infection can occur by vector-mediated transmission through the introduction of infected bug feces into the bite site or mucous membrane or through the ingestion of infected bugs or their feces [5]. Additionally, congenital transmission may occur [3]. Dogs are more likely to become infected than humans [16,17], which could be from dog's affinity to consume bugs [12,18–21]. *T. cruzi*-infected dogs may be asymptomatic or may develop debilitating acute or chronic cardiac disease, characterized by myocarditis, hepatomegaly, ascites, cardiac dilatation, or sudden death [22]. There are currently no vaccinations or approved anti-parasitic treatments for *T. cruzi* infections in dogs in the US, and infected dogs are treated symptomatically.

The Department of Homeland Security (DHS) of the US government manages over 3,000 working dogs in various capacities including the Transportation Security Authority, Coast Guard, Secret Service, Federal Protective Services, Customs and Border Protection, and Federal Operations. These dogs are highly trained in working duties performed in the indoor and outdoor environment including search and rescue functions as well as detection of concealed persons, narcotics, or explosives. DHS working dogs may be at increased risk for contact with vector species from working and sleeping outdoors. Some of the working dogs are kept in group kennels, which have previously been shown to be a risk factor for *T. cruzi* infection [7]. Their working environment could further be an attractant to the vector, where

there is high vehicle traffic emitting CO_2- a known attractant [23], bright lights at night, and concentrations of animals and people in otherwise rural areas. In order to provide a baseline for conducting clinical assessments and developing disease management strategies, we conducted a seroepidemiological investigation to quantify the prevalence of *T. cruzi* infection in populations of working dogs along the Texas-Mexico border. Additionally, we aimed to determine the infection status and feeding patterns of triatomine vectors in the environments where these dogs work and are kenneled.

3.2 Materials and Methods

3.2.1 Ethics statement

All canine samples were collected in adherence with animal use protocols approved by Texas A&M University's Institutional Animal Care and Use Committee on 08/17/2015 under the number 2015-0289. Written consent was received for each canine sampled from the handler.

3.2.2 Study population- DHS working dogs

Sampled DHS working dogs were predominantly the breeds Belgian Malinois and German Shepherds. Most dogs were bred in Europe, and less commonly dogs came from vendors within Texas or other parts of the US. Dogs receive over 6 months of training at either a training facility in El Paso, Texas, or Front Royal, Virginia, and specialize in various jobs such as track and trail, detection of humans, narcotics, currency, or agricultural products, and search and rescue. After training, dogs are typically assigned to a specific management area and have limited travel. The dogs in our study perform working duties either

immediately adjacent to the geopolitical border (ports of entry) or north of the border (checkpoints). Off-duty canines are either kenneled individually at their handler's residence or in a group kennel. Residential kennels are indoor-outdoor metal kennels raised 2 feet from the ground, giving the dog the option of sleeping inside or outside. Group kennels are indoor-outdoor, concrete kennels, and dogs are confined inside during the night.

3.2.3 Sample collection

We used a cross sectional study design to collect blood samples from DHS working dogs during November 2015 and April 2016. Working dogs were sampled from all 5 management areas, with a goal of sampling at least 60% of the dogs that occurred within each management area. Additionally, we sampled DHS canines that were in training or waiting for deployment at a training facility in management area #1 (Figure 3.1). Sample criteria included dogs over 6 months in age and on active duty or in training. Demographic information was collected on all dogs sampled including age, sex, breed, canine job, sleeping location and station of duty. A minimum of 1 ml of blood was collected by venipuncture and aliquoted into serum and EDTA tubes.

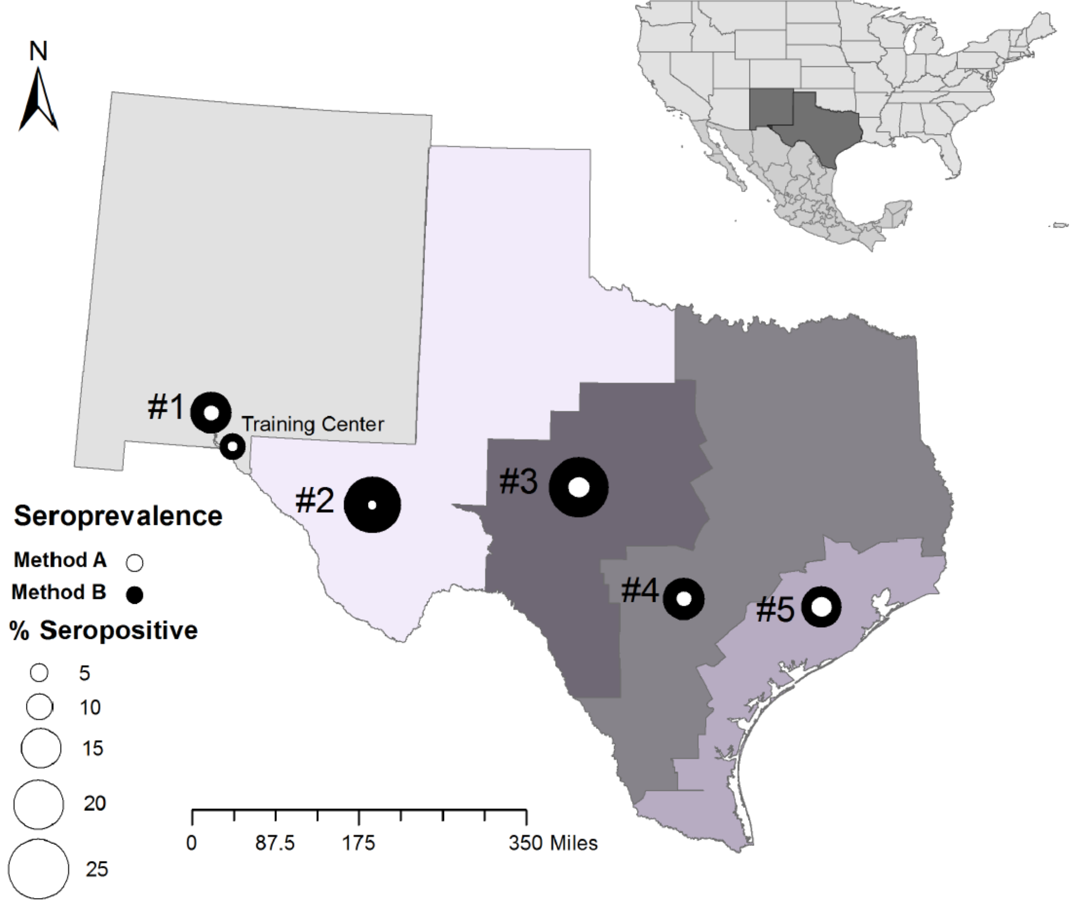

Figure 3.1. Seroprevalence of government working dogs along the Texas-Mexico border by two methods. Government working dogs were sampled from five management areas (#1-5) and a training center along the Texas-Mexico border in 2015-2016; management area #1 spans New Mexico and Texas. Seroprevalence was calculated using Method A- a conservative analysis in which very faint serological bands were interpreted as negative, and Method B- an inclusive analysis in which very faint serological bands were interpreted as positive. Map was created using ArcGIS and with Texas, Mexico, and New Mexico base layers downloaded from www.arcgis.com and the U.S. Department of Commerce, U.S. Census Bureau, Geography Division, Cartographic Products and Services Branch. (Meyers et al. 2017)

3.2.4 Serological methods

Serum and plasma samples were screened for anti-*T. cruzi* antibodies by Chagas Stat-Pak® rapid immunochromatographic test (ChemBio, NY) which was designed for use in humans and has been validated in dogs [9]. Stat-Pak® assay uses three *T. cruzi* recombinant antigens (B13, 1F8 and H49/JL7) that are bound to the assay membrane solid phase. Serum

or plasma samples were tested according to manufacturer's protocol and read for result determination after 15 minutes. Tests were considered negative when no color developed and positive when a clear line developed. Additionally, very faint bands that were not perceptible enough to be consider a clear positive, yet with some low level of color development to differentiate them from negative, were tracked as 'inconclusive' and subjected to additional testing.

All positive or inconclusive samples as determined by Stat-Pak® plus 10% of the negatives were tested by both indirect fluorescent antibody (IFA) test and Trypanosoma Detect™ (InBios, International, Inc., Seattle, WA). IFA detects anti-*T. cruzi* IgG antibodies and was performed by the Texas Veterinary Medical Diagnostic Laboratory (TVMDL, College Station, TX) on serum or plasma samples. Titer values of 20 or higher were considered positive as per TVMDL standard protocol; this titer value cutoff has also been used in human medicine [24]. IFA readers were blinded to previous serologic results. Trypanosoma Detect™ is a rapid immunochromatographic dipstick assay that employs a multi-epitope recombinant antigen for the detection of anti-*T. cruzi* antibodies. The Trypanosoma Detect™ test was designed for use humans but has been found to have high sensitivity and specificity for use in dogs [25]. Serum or plasma were tested according to manufacturer's protocol and read for result determination after 20 minutes. Test results were scored as positive, inconclusive, or negative using the same criteria as described above for the Chagas Stat-Pak®. Serological positivity was assigned to samples that tested positive on at least two independent tests.

3.2.5 Molecular methods

Amplification of parasite DNA from blood samples by real time PCR was performed on all sampled dogs. DNA was isolated from 250 uL of buffy coat by using E.Z.N.A. Tissue DNA kit (Omega Bio-Tek, Norcross, GA). Negative controls (no template or water template) were included in the DNA extractions and the PCR. To determine if analysis of clot rather than buffy coat may result in a greater ability to detect parasite DNA, we conducted additional work with a subset of samples as follows. From 12 dog samples, we extracted DNA from 1 mL of clot for PCR analysis. These 12 dogs comprised 10 that were seropositive and PCR negative based on buffy coat; 1 that was seropositive and PCR positive based on buffy coat; and 1 that was seronegative and PCR positive based on buffy coat analysis.

Samples were first screened for presence of *T. cruzi* satellite DNA using the Cruzi 1/2 primer set and Cruzi 3 probe in a real-time assay to amplify a 166-bp segment of a repetitive nuclear DNA [26, 27]. Reactions consisted of five microliters of extracted DNA, primers I and II each at a concentration of 0.75 µM, 0.25 µM of probe, and iTaq University Probes Supermix (BioRad Laboratories, Hercules, CA), in a 20 µL reaction volume. Previously published thermocycling parameters were followed except with a 3-minute initial denaturation using a Stratagene MxPro3000 (Agilent Technologies, Santa Clara, CA). *T. cruzi* DNA extracted from isolate Sylvio X10 CL4 (ATCC 50800, American Type Culture Collection [ATCC]) was used for a positive control. Machine-calculated thresholds and reaction curves were visually checked to assure successful amplification. Samples with Ct values less than 34 were considered suspect positive and subjected to further testing.

Suspect positive samples by qPCR were run on a second, independent PCR using *T. cruzi* 121/122 primers to amplify a 330-bp region of kinetoplast DNA [28,29]. Reactions included 1µL template DNA, primers at final concentrations of 0.75 µM each, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a final reaction volume of 15 µL. Amplicons were visualized on 1.5% agarose gels stained with GreenGlo™ safe DNA dye (Denville Scientific Inc., Metuchen, NJ). Samples that yielded a band of the appropriate size were interpreted as positive in our analyses. Parasite positive dogs were defined as those that tested positive on both the rt-PCR screening and PCR assays.

3.2.6 Determination of T. cruzi *strain types*

We used a multiplex quantitative, real time PCR to determine *T. cruzi* DTUs of samples that were positive or suspect positive on the Cruzi 1/2/3 assay based on amplification of the nuclear spliced leader intergenic region (SL-IR) [30]. Using a QIAGEN Multiplex PCR Kit (QIAGEN, USA) reactions were performed using 2µL template DNA in a final volume of 20 µl and run on a BioRad CFX96 (Hercules, CA, USA). The only deviation from the previously described protocol was the extension of cycles from 40 to 45 and substitution of dyes as previously described [7]. Positive controls consisted of DNA from triatomines collected across Texas that were previously characterized as infected with TcI or TcIV based on amplification and sequencing of the TcSC5D gene [31]. Samples with Ct values less than 34 were considered positive, and fluorescence signal determined the strain type.

3.2.7 Vector collection and testing for T. cruzi

Triatomine bugs were opportunistically collected by dog handlers in summer 2016 from group kennels, outside handler's residence around canine housing, and at stations where dogs worked. To encourage collections, outreach materials with photos of triatomines and look-alike species were disseminated by email and in printed format to dog handlers prior to the summer peak of adult triatomine activity. Bugs were identified to species using morphologic features [32] and sexed. After bugs were washed in 10% bleach solution and rinsed in distilled water, sterile instruments were used to dissect the bugs, isolate hindgut material and evidence of a recent bloodmeal were noted. DNA was extracted from hindguts and tested for *T. cruzi* DNA and determination of *T. cruzi* DTU using the same methods as the above testing of dog samples. In order to determine the source of recent bloodmeals, hindgut DNA was subjected to PCR amplification of vertebrate cytochrome B sequences using previously published primers and cycling conditions [33,34]. Reactions included 3 µL template DNA, primers at final concentrations of 0.66 µM each, and FailSafe PCR Enzyme Mix with PreMix E (Epicentre, Madison, WI) in a final reaction volume of 50 µL. Amplicons were visualized on 1.5% agarose gel, prepared for sequencing using ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA), and Sanger sequencing was performed (Eton Bioscience Inc., San Diego, CA, USA). Resulting sequences were compared to existing sequences using Basic Local Alignment Search Tool (National Center for Biotechnology Information, US National Library of Medicine). In recognition of the potential for contamination from the environment, samples that aligned to human were re-run on another PCR assay to provide a secondary line of evidence.

3.2.8 Statistical methods

Due to the uncertainty of sample serostatus associated with the inconclusive band development, antibody-positive dogs were defined using two methods; a) in the conservative method, inconclusive band development was interpreted as negative, and b) in the inclusive method, inconclusive band development was interpreted as positive. In the absence of gold standard serological methodology, these two different criteria of positivity (method A and B) were analyzed separately to provide a range of results.

To evaluate the relationship between potential risk factors and the serostatus of canines, data were imported into RStudio 1.0.136 software [35] for analysis. Assessed variables were dog age (young=6 months to <3 years, middle age=$\geq$ 3 years to <6 years, senior=$\geq$ 6 years), sex, breed, sleeping location (individual kennel at handler's residence or group kennel) and management area (locations 1-5 or training center). Due to the small sample size of dogs in some jobs, canine job was dichotomized based on type of detection. Bivariable analysis using the chi-squared or Fisher's exact was used to identify putative risk factors. Factors with a $p \leq 0.25$ from the initial screening were used in a logistic regression model, while controlling for management area as a random effect. Generalized linear mixed models were calculated and factors with values of $p < 0.05$ were considered significant. Odds ratios and 95% confidence intervals were calculated. To determine variation in seroprevalence across management areas, a logistic regression model was used in which the training center served as the referent to which all five management areas were compared. Kappa index was used to test the agreement between each pairwise combination of the results of the three serological assays for the samples that were tested on all three assays.

3.3 Results

A total of 528 dogs from along the Texas-Mexico border were evaluated using a variety of serologic and molecular techniques to detect *T. cruzi* exposure and infection. Distribution of samples among the five management areas ranged from 47 (8.9%) to 135 (25.6%), and 86 (16.3%) dogs were sampled from the training center. The most common breeds were Belgian Malinois and German Shepherd, which together comprised 86% of the sampled dogs, with Dutch Shepherds, Sable Shepherds, Groenendael and Labrador Retriever comprising the remainder. Age ranged from approximately 6 months to 13 years with a median of 4.47 and a mean of 4.79. There were 351 males (66.5%) and 177 females (33.5%). Of the dogs sampled, 55.9% spend their off-duty time in individual residential kennels whereas 44.1% were group kenneled. The sample sizes of dogs within each canine job category are not disclosed because it is law enforcement sensitive information.

3.3.1 Serological results interpreted using Method A: Conservative method

In considering inconclusive bands on immunochromatographic tests as negative, 39 of 528 (7.4%) of dogs were seropositive for antibodies to *T. cruzi* on at least 2 assays. Across management areas and the training center, seroprevalence ranged from 4.3% to 10% (Figure 3.1). In the bivariable analysis, *T. cruzi* seroprevalence was significantly different across dog breed (p=0.03), with seroprevalence of German Shepherds being lowest (3.7%) and ‘other’ breeds being highest (14.3%; Table 3.1). Dogs that spent off-duty time in residential kennels had a significantly higher seroprevalence (29/295, 9.8%, p=0.02) than those that were group-kenneled (10/233, 4.3%). Seroprevalence was significantly different between age groups (p=0.04), where senior dogs had a seroprevalence of 10.4%, middle age

dogs a seroprevalence of 7.9% and young dogs 3.2%. Seroprevalence did not vary significantly by sex or canine job.

Table 3.1. Results of bivariable analysis of potential risk factors for seropositive government working dogs (defined by method A and B) in five management areas and training center along the Texas-Mexico border, 2015-2016. (Meyers et al. 2017)

		Method A: Conservative Method			Method B: Inclusive Method		
Variable	Sample size No. (%)	Seropositive No. (%)	Seronegative No. (%)	P-value	Seropositive No. (%)	Seronegative No. (%)	P-value
Sex				0.39			0.6
Female	177 (33.5)	16 (9.0)	161 (91.0)		37 (20.9)	140 (79.1)	
Male	351 (66.5)	23 (6.5)	328 (93.4)		64 (18.2)	287 (81.8)	
Breed				0.03			0.86
Belgian Malinois	267 (50.6)	23 (8.6)	244 (91.4)		54 (20.2)	213 (79.8)	
German Shepherd	188 (35.6)	7 (3.7)	181 (96.3)		32 (17.0)	156 (83.0)	
Dutch Shepherd	31 (5.9)	3 (9.7)	28 (90.3)		6 (19.4)	25 (80.6)	
Other	42 (8.0)	6 (14.3)	36 (85.7)		9 (21.4)	33 (78.6)	
Canine Job[a]				0.19			0.18
Detection A		(8.3)	(91.7)		(20.5)	(79.5)	
Detection B		(4.2)	(95.8)		(13.7)	(86.3)	
Sleeping location				0.02			0.09
Residential	295 (55.9)	29 (9.8)	266 (90.2)		65 (22.0)	230 (78.0)	
Group Kennel	233 (44.1)	10 (4.3)	223 (95.7)		36 (15.4)	197 (84.6)	
Age				0.04			0.18
Young	156 (29.5)	5 (3.2)	151 (96.8)		22 (14.1)	134 (85.9)	
Middle Age	190 (36.0)	15 (7.9)	175 (92.1)		39 (20.5)	151 (79.5)	
Senior	182 (34.5)	19 (10.4)	163 (89.6)		39 (21.4)	143 (78.6)	

[a] Sample sizes of dogs in each canine job as well as the specific detection abilities of dogs are not disclosed because this information is law enforcement sensitive.

Multivariable logistic regression analysis showed a significant association (odds ratio [OR] 0.41, 95% CI 0.17-0.99, p=0.047, Table 3.2) between breed and seropositive dogs, after controlling for management areas as a random effect (Table 3.2), in which German Shepherds were associated with a significantly lower seroprevalence (3.7%) than Belgian Malinois (8.6%). No significant association was found between age, job, or sleeping location and seroprevalence.

Table 3.2. Association between seropositive dogs (defined by method A and B) and age, breed, job and sleeping location. (Meyers et al. 2017)

	Method A: Conservative Method			Method B: Inclusive Method		
Variable	Odds Ratio	95% CI	P-value	Odds Ratio	95% CI	P-value
Age						
Young	referent			referent		
Middle Age	2.03	0.69–5.96	0.19	1.4	0.77–2.56	0.27
Senior	2.34	0.78–7.02	0.13	1.37	0.72–2.58	0.33
Sleeping location						
Residential	referent			referent		
Group kennel	0.54	0.24–1.24	0.15	0.76	0.46–1.25	0.28
Canine Job						
Detection A	referent			referent		
Detection B	0.77	0.27–2.19	0.62	0.85	0.45–1.60	0.61
Breed						
Belgian Malinois	referent			*Not included in model*		
German Shepherd	0.41	0.17–0.99	**0.04**			
Dutch Shepherd	1.19	0.33–4.32	0.78			
Other Breed	1.71	0.62–4.69	0.29			

3.3.2 Serological results interpreted using Method B: Inclusive method

In considering inconclusive bands on serologic tests as positive, 100 of 528 (18.9%) of dogs were seropositive for antibodies to *T. cruzi* on at least 2 assays. Seroprevalence ranged from 11.6% to 26.7% across management areas and the training center (Figure 3.1). When running bivariable analysis, dogs that spent off-duty time in residential kennels (65/295, 22%) were marginally (p=0.09, Table 3.2) more likely to be seropositive than dogs sleeping at a group kennel (36/233, 15.4%). Seroprevalence did not vary significantly by age, breed, sex or canine job.

Multivariable logistic regression analysis showed that there was no association between age, job, or sleeping location and seroprevalence. Backwards elimination was performed and when only age was included in the model there was a marginal association in which old dogs had a higher seroprevalence (39/182, 21.4%) than young dogs (22/156, 14.1%; p=0.09), after controlling for management areas as a random effect.

3.3.3 Seroprevalence across management areas

While seroprevalence did not significantly differ across management areas and the training center when positivity was defined according to Method A, dogs from management area #2 (OR 2.6, 95% CI 1.0-6.7, p=0.04) and #3 (OR 2.8, 95% CI 1.2-6.8, p=0.02) had significantly higher seroprevalence compared to the training center when seropositivity was determined according to Method B (Table 3.3).This indicates that area #2 and #3 were both associated with many samples that produced very faint (inconclusive) bands on the immunochromatographic tests.

Table 3.3. Association between management areas or training center and seropositive dogs with seropositivity defined using method A (including very faint serological bands as negative) and method B (including very faint serological bands as positive). (Meyers et al. 2017)

	Method A: Conservative Method				Method B: Inclusive Method			
Management Area	Seropositive No. (%)	Odds Ratio	95% CI	P-value	Seropositive No. (%)	Odds Ratio	95% CI	P-value
# 1	10 (7.4)	1.6	0.5–6.1	0.42	25 (18.5)	1.7	0.8–4.0	0.17
# 2	2 (4.3)	0.9	0.1–4.9	0.92	12 (25.5)	2.6	1.0–6.7	**0.04**
# 3	6 (10.0)	2.3	0.6–9.3	0.22	16 (26.7)	2.8	1.2–6.8	**0.02**
# 4	7 (7.3)	1.6	0.5–6.3	0.46	18 (18.8)	1.8	0.8–4.2	0.18
# 5	10 (9.6)	2.2	0.7–8.2	0.20	19 (18.3)	1.7	0.8–4.0	0.20
Training Center	4 (4.7)	referent			10 (11.6)	referent		

3.3.4 Discordant serology

In comparing the results across all three serological testing platforms (Table 3.4), all IFA positive samples are positive on Trypanosoma Detect™, and all but two samples are Stat-Pak® positive-both of these samples having a titer of 20. When comparing the IFA negative samples 71.3% are positive or inconclusive on Stat-Pak® and 48.4% are positive or inconclusive on Trypanosoma Detect™. The majority of the IFA-positive dogs were strongly positive (band score of 3 or 4) on Stat- Pak® (68%) and Trypanosoma Detect™ (78.9%). From the 528 dog samples in the study, 215 samples were tested on all three

serology assays. Overall test agreement ranged from slight to moderate agreement based on the Kappa Indices (Table 3.5), with agreement between tests being better when interpreting immunochromatographic test results using the conservative method A (kappa range 0.37-0.48) compared to inclusive method B (kappa range 0.05-027). The best agreement was using method A between Stat- Pak® and Trypanosoma Detect™, with a Kappa index of 0.48 (moderate agreement).

Table 3.4. **Comparison of results from three serological assays for the detection of anti-*T. cruzi* antibodies: Chagas Stat-Pak® rapid immunochromatographic test (ChemBio, NY), indirect fluorescent antibody (IFA) test performed by the Texas Veterinary Medical Diagnostic Laboratory (TVMDL, College Station, TX) and Trypanosoma Detect™ (InBios, International, Inc., Seattle, WA).** Number of samples with positive, inconclusive, or negative results on each test are given over the total number of samples with specified IFA endpoint titers. (Meyers et al. 2017)

IFA Titer[a]	Stat-Pak			Trypanosoma Detect		
	Pos.	Inconclusive	Neg.	Pos.	Inconclusive	Neg.
0	34/196	106/196	56/196	46/196	49/196	101/196
20	1/3	1/3	1/3	3/3	0	0
160	3/3	0	0	3/3	0	0
320	9/9	0	0	9/9	0	0
640	4/4	0	0	4/4	0	0

[a]no samples had a titer of 40 or 80

Table 3.5. **Kappa Index comparing three serological assays; Chagas Stat-Pak® rapid immunochromatographic test (ChemBio, NY), indirect fluorescent antibody (IFA) test and Trypanosoma Detect™ (InBios, International, Inc., Seattle, WA).** Panels A-C represent Kappa Index when interpreting immunochromatographic test results using the conservative method A (including very faint serological bands as negative); Panels D-F represent Kappa Index when interpreting immunochromatographic test results using the inclusive method B (including very faint serological bands as positive). (Meyers et al. 2017).

A.	IFA +	IFA -	Total
Stat-Pak +	17	34	51
Stat-Pak -	2	162	164
Total	19	196	215
Moderate agreement:	**0.41**		
B.	IFA +	IFA -	Total
Trypanosoma Detect +	19	46	65
Trypanosoma Detect -	0	150	150
Total	19	196	215
Fair agreement:	**0.37**		
C.	Stat-Pak +	Stat-Pak -	Total
Trypanosoma Detect +	36	29	65
Trypanosoma Detect -	15	135	150
Total	51	164	215
Moderate agreement:	**0.48**		
D.	IFA +	IFA -	Total
Stat-Pak +	18	140	158
Stat-Pak -	1	56	57
Total	19	196	215
Slight agreement:	**0.05**		
E.	IFA +	IFA -	Total
Trypanosoma Detect +	19	95	114
Trypanosoma Detect -	0	101	101
Total	19	196	215
Slight agreement:	**0.16**		
F.	Stat-Pak +	Stat-Pak -	Total
Trypanosoma Detect +	98	16	114
Trypanosoma Detect -	60	41	101
Total	158	57	215
Fair agreement:	**0.27**		

Of the 57 randomly-selected Stat-Pak® negative samples that were subjected to additional serologic testing, one was positive on both IFA (titer 20) and Trypanosoma Detect™; this sample was counted as positive in the seroprevalence estimates. Nine (15.8%)

samples that were both Stat-Pak® and IFA negative were positive on Trypanosoma Detect™; these dogs were counted as negative in the seroprevalence estimates, but could be false negatives. When applying this prevalence of potential false negatives to the total number of dogs that were negative by Stat-Pak®, an additional 49 dogs are extrapolated to be potential false negatives; including these samples as positive would increase seroprevalence to 15.9% (84 dogs) by conservative method A, and 25.4% (149 dogs) by inclusive method B.

Inconclusive bands were reported from 108 (20.5%) samples screened on Chagas Stat-Pak®. When tested on IFA only 1 (0.9%) inconclusive tested positive with a titer of 20. When inconclusive samples were run on Trypanosoma Detect™, 37 (29.6%) had inconclusive bands on Trypanosoma Detect™, 20 (18.5%) had bands scoring between 1-4, and 51 (47.2%) were negative.

3.3.5 Molecular detection of parasite DNA and T. cruzi strain types

T. cruzi DNA was detected in the buffy coat fraction of the blood in three of 528 (0.6%) dog samples according to our diagnostic method which included amplification in both a screening and confirmatory assay. The first PCR-positive dog was sampled from area # 5 in November and was positive for antibodies by all three serology assays with a relatively high titer (640) on IFA. Using the multiplex real time PCR to determine *T. cruzi* DTUs, we found that this dog harbored DTU TcIV. The second PCR-positive dog was from the canine training center, sampled in April, positive on all serology assays with a titer of 320 and harbored a mix TcI/TcIV. The third dog was from area # 2, sampled in April, was negative by all serological assays, and strain type could not be determined. When this PCR positive yet serologically-negative dog was included in binomial analysis of risk factors and the

logistical regression model, no difference was found in significant associations. The subset of 12 samples that were subjected to an additional DNA extraction from 1mL of clot produced PCR results that were identical to the results obtained from the 250 uL buffy coat extractions with the exception of the sample from the seronegative, buffy coat-positive sample. This sample was negative based on clot analysis.

3.3.6 Infection of triatomine vectors with T. cruzi

In the summer of 2016, a total of 20 adult triatomine bugs of two species (18 *Triatoma gerstaeckeri* and 2 *T. rubida)* were opportunistically collected by canine handlers from three management areas (Table 3.6). Kissing bugs were collected from stations where dogs and handlers work (n=6), handler's residence near canine housing (n=7), group kennels (n=4) and 1 bug was removed from a dog while working. Nine (45%) triatomines were positive for *T. cruzi* including half of the *T. gerstaeckeri* specimens but neither of the two *T. rubida* specimens. Of the 9 positive bugs, parasite strain typing revealed DTU TcI in 6, TcIV in 1, and a mixed TcI/TcIV coinfection in 2. From dissection, 13 of the 20 bugs had evidence of a recent blood meal in their hind gut, and 11 of these yielded results after the blood meal analysis protocols, revealing human, canine, coastal-plain toad (*Bufo nebulifer)* and rat (*Rattus rattus)* DNA (Table 3.6).

Table 3.6. *Triatoma* spp. collected from locations where canines sleep or work were tested for *Trypanosoma cruzi* presence, strain type of *T. cruzi* and bloodmeal source were determined. (Meyers et al. 2017).

Triatomine species	Management area	Location	Sex	Bloodmeal source	Strain Type
T. gerstaeckeri	#3	Group kennel	M	n/a	Negative
T. gerstaeckeri	#3	Station	F	Dog (*Canis lupus familiaris*)	Negative
T. gerstaeckeri	#3	Station	F	Dog (*Canis lupus familiaris*)	TcI
T. gerstaeckeri	#3	Station	M	Human (*Homo sapiens*)	TcI
T. rubida	#2	Station	F	n/a	Negative
T. gerstaeckeri	#3	Station	M	n/a	TcI
T. gerstaeckeri	#3	Group kennel	F	n/a	Negative
T. gerstaeckeri	#3	Group kennel	M	n/a	TcI
T. gerstaeckeri	#3	On canine	F	n/a	TcIV
T. gerstaeckeri	#5	Residential kennel	F	Dog (*Canis lupus familiaris*)	Negative
T. gerstaeckeri	#5	Residential kennel	M	Dog (*Canis lupus familiaris*)	TcI
T. gerstaeckeri	#5	Field	F	n/a	Negative
T. gerstaeckeri	#5	Residential kennel	M	Coastal-Plain toads (*Bufo nebulifer*)	TcI
T. gerstaeckeri	#5	Field	M	Rat (*Rattus rattus*)	Negative
T. gerstaeckeri	#5	Residential kennel	F	n/a	TcI/TcIV
T. gerstaeckeri	#5	Residential kennel	M	Coastal-Plain toads (*Bufo nebulifer*)	Negative
T. gerstaeckeri	#5	Residential kennel	F	n/a	Negative
T. rubida	#2	Residential kennel	F	Dog (*Canis lupus familiaris*)	Negative
T. gerstaeckeri	#3	Station	F	Dog (*Canis lupus familiaris*)	TcI/TcIV
T. gerstaeckeri	#3	Group kennel	M	Human (*Homo sapiens*)	Negative

3.4 Discussion

We found widespread *T. cruzi* infection in government working dogs along the Texas-Mexico border. DHS working dogs play an important role in detection and security functions in the Unites States and the clinical manifestation of infection may be associated with significant future economic and security consequences. We are aware of only two prior epidemiological investigations of *T. cruzi* infection in working dogs in the US. In 2007, a serological survey was conducted on military working dogs (MWD) in San Antonio, TX, after veterinarians noted an increase in Chagas disease diagnoses, revealing 8% of the kenneled dogs was positive by IFA [36]. Such findings are of utmost importance in these dogs; in 2009, MWDs deployed in Iraq were evacuated due to cardiac symptoms and diagnosed with *T. cruzi* infection leaving troops vulnerable without explosive detection dogs [36]. Recently, populations of working hound dogs in south central Texas that are used for scent detection and track/trail were characterized with an extremely high seroprevalence of

57.6% (n=85) in which positive dogs were reactive on both Stat-Pak® and IFA [7]. The study population also included many dogs with parasite DNA in the blood and other organs, and infected triatomines collected from the dog kennels were determined to have fed on dogs, allowing the authors to conclude that multi-dog kennels can be high risk environments of *T. cruzi* transmission [7].

Exposed dogs were present in all five management areas and the canine training school, with an overall apparent seroprevalence of 7.4-18.9%. This seroprevalence is similar to that reported from dogs in Chagas-endemic areas in Latin America including populations in Peru (12.3%) [37], Argentina (45.6%) [38], Panama (11.1%) [39], Costa Rica (27.7%) [19], Yucatan State, Mexico (9.8%-14.4%) [40] and Mexico State, Mexico (10%-15.8%) [41]. Previous epidemiological investigations of *T. cruzi* in canines in the US are limited, and most have focused on stray dogs or those sampled from animal shelters, which may be considered as high risk populations due to outdoor activity. A serosurvey of high risk kenneled dogs in southern Louisiana found that 22.1% [9] of dogs tested positive for *T. cruzi* antibodies using the same three serology assays performed in this study. A study in Oklahoma sampling shelter dogs and pet dogs concluded that 3.6% dogs were seropositive when testing by radioimmunoprecipitation assay (RIPA) [12]. Earlier studies in southern Texas stray dogs 375 dogs were tested and 7.5% were positive by indirect immunofluorescence [15]. Similarly, across Texas shelter dogs had a seroprevalence of 8.8% when testing dogs on Chagas Stat-Pak® [6]. These studies and ours suggest that despite the regular veterinary care, quality food and shelter, highly-valued working dogs can have similar or greater *T. cruzi* infection than stray and shelter dogs in the US and free roaming or pet dogs in endemic countries.

Both population-level and individual-level *T. cruzi* studies of naturally-infected hosts suffer from a lack of gold standard tests or diagnostic recommendations. Discordance among tests results is prevalent in human and veterinary Chagas diagnostics. For example, a study looking at seroprevalence in people from Veracruz, Mexico used 5 assays and found that test agreements ranged from 0.038-0.798 on the Kappa index [42]. Similarly, using the Kappa index we found a high discordance among serology assays used, with agreement ranging from slight to moderate depending on the interpretation method. Assay discordance could be affected by the single freeze-thaw cycle, or the age of the sample. These diagnostic challenges make it difficult to directly compare seroprevalence across populations and diagnostic methods, and presents a challenge in clinical settings for diagnosis. Two of the three serological tests we used are available for research use only for dogs in the US, and a limited number of commercial laboratories offer canine *T. cruzi* diagnostic test services. As in most diagnostic tests, there is some subjectivity in the interpretation of results, and the development of very faint 'equivocal' serological bands on both Chagas Stat-Pak® and Trypanosoma Detect™ posed particular complexities in our analysis. The Stat-Pak® and Trypanosoma Detect™ instructions state that band intensity will vary, but faint bands should be interpreted as positive [43,44] and that variation is dependent on the concentration of antibodies present [44]. However, some previous canine studies have counted faint bands as negative [6,9] while others have interpreted them as positive for analysis [45]. Our presentation of a seroprevalence calculated both conservatively (very faint bands interpreted as negative) and inclusively (very faint bands interpreted as positive) is an effort to account for imperfect diagnostics. Until refined *T. cruzi* diagnostic tools are available, we encourage

transparency in presenting results on single vs. multiple tests across all strengths of test response.

The discordance between test results and the within assay variation could be caused by parasite heterogeneity [45]. *T. cruzi* is notably heterogeneous with seven major genotypes or discrete typing units (DTUs) described as TcI-TcVI and TcBat which vary be region [46,47]. Additionally, a notable intra-DTU variability has been found [47,48]. Previous research has found that assay reactivity varies by geographic origin of the patient [49]. O'Connor and others found that strain TcI clusters geographic between North and South America [50]. The Chagas Stat-Pak® was validated with human sera from Central America to detect strains circulation in that region [51] and may not be as sensitive to *T. cruzi* clones from Texas. When very faint bands were interpreted as positive (method B), seroprevalence was significantly higher in two western management areas (OR 2.6-2.8, 95% CI: 1.0-6.8 p =0.02-0.04) compared to the training center (Table 3.3), whereas this difference was not evident when very faint bands were interpreted as negative (method A). The disproportionate abundance of very faint bands in this geographic area may be driven differences in the locally-circulating *T. cruzi* clones. Diosque et al. performed a genetic survey of *T. cruzi* isolates within a restricted geographical area (~300 km^2) and found five different clones circulating [52]; such findings are clinically and diagnostically relevant because parasite heterogeneity has been shown to cause varying infectivity and immune response [52–54]. In addition, host biological factors (exposure history, coinfection, genetic makeup) could also cause reaction variability within and across serology assays.

Sleeping location (group housed indoors vs. individually housed outdoors) appeared to be independently associated with *T. cruzi* status with a higher seroprevalence in dogs

sleeping outdoors than indoors by method A (p=0.02), and marginally significant by method B (p=0.09) in bivariable analysis. Previous studies have indicated dogs housed outdoors where vector contact is more likely to be at a higher risk for exposure [6,9,39]. Dogs in Tennessee spending 100% of their time outdoors were significantly more likely to be seropositive for *T. cruzi* than dogs spend $\leq$50% of their time outdoors [13]. Seroprevalence did increase with age in both method A and B, but was only significant in bivariable analysis in method A, where senior (>6 years) and middle age ($\geq$ 3 years to <6 years), were more likely to be seropositive than young dogs (<3 years old) (Table 3.1). This is anticipated in infectious disease since exposure increases with age and has been found in previous studies [7,13,17,55]. We found that German Shepherds were associated with a significantly lower seroprevalence (3.7%) than Belgian Malinois (8.6%) in our study; although the driving factors for this difference are currently unknown, it may relate to host behavior, differences in host immune response, or a physical characteristic.

We found three dogs (0.6%) harbored parasite PCR in their blood, suggesting that these dogs are parasitemic. While two of the three PCR-positive dogs also harbored detectable anti *T-cruzi* antibodies, one did not, suggesting this dog may have been in the acute stage of infection [56]. The two dogs with successfully typed infections harbored DTUs TcIV and a TcI/TcIV mix, consistent with previous studies on dogs in the US [57,58]. Both strain types infect a variety of hosts and vectors in the southern US [3]. DTU TcI is an ancient strain found throughout South and Central America and the predominant strain infecting humans in the US [3], where it is also associated with wildlife reservoirs including opossums (*Didelphis virginiana*) [57]. TcIV is also associated with wildlife, especially raccoons (*Procyon lotor*) [3] and to our knowledge has not been implicated in the small

number of typed human infections in the US. This study found a lower prevalence of dogs PCR positive then previous studies, which likely reflects the time of sampling (November and April) when the vector is less active and dogs in Texas are less likely to come in contact with the kissing bug [58]. In recognition of other datasets that have shown that analysis of clot, rather than buffy coat, may afford a greater chance of detecting parasite DNA [59], we subjected 12 clot samples to PCR and compared results to previously results from analysis of buffy coat. We found that buffy coat and clot results were identical across this subset with the exception of a sample from a single seronegative dog which was positive from buffy coat and negative from clot. Based on this small comparison trial, we suggest that the low frequency of encountering PCR-positive dogs in our study was not due to the blood fraction used in the analysis.

We found an infection prevalence of 45% in the kissing bugs collected from areas where the working dogs frequent, including kennels, stations and handler's residence, including DTUs TcI, TcIV, and TcI/IV mix. This infection is slightly lower than previous estimates across the state of Texas of 63% and 51% [59,60]. Bloodmeal analysis revealed canine, human, and wildlife DNA within the hindguts of these insects, underscoring the generalist feeding strategies of triatomines that often use the most locally abundant hosts. Strict protocols were used to reduce the risk of contamination of samples by exogenous DNA (i.e., human DNA), including surface sterilization of vectors and dissection of the hindguts, and it is biologically plausible that the insects associated with suspected human blood feeding encountered humans at their residence or station or work. A study in California and Arizona that collected bugs by light traps found that 5 of 13 bugs (38%) bugs were positive for a human blood meal, 4 fed on canine and 1 each for rat, pig, chicken and mouse [61].

Another study in Texas found 65% (n=65) of bugs positive for human blood meal and 32% for canid bloodmeal; in contrast, another Texas study found only 1% of vectors (n=96) collected from residential settings had fed on a human, and 20% on dogs [62]. Larger sample sizes of engorged vectors from the working dog environments will assist in learning the local vector-host interactions that sculpt disease risk.

Using dogs as sentinels has been suggested for targeted vector control programs endemic areas such as Peru [37] and to monitor transmission in Argentina [55]. However, the relative importance of dogs as reservoirs, and whether or not they can be a sentinel species for human disease risk in the US, is unknown. Further, because the triatomines in the US tend not to be colonized within homes, dogs are less likely to be useful sentinels at the household level. Nonetheless, given these infected working dogs signal the presence of infected vectors in the environment, there are public health implications of these findings especially with respect to the human handlers who are exposed to the same environments.

Because not all *T. cruzi*-infected dogs will develop disease [21], the prognosis and clinical implications of the widespread presence of *T. cruzi*-infected government working dogs along the US-Mexico border is unknown. Nonetheless, the potential loss of duty days resulting in an inadequate canine workforce must be considered. Additionally, given that the canine training school in west Texas (Fig. 3.1) occurs in an area where triatomines are endemic, vector and canine surveillance must be conducted to determine if young dogs may be exposed to the parasite while in training, which would not only have implications for the health of the dog but also potentially afford dispersal of the parasite to the new areas across the US where these dogs are stationed. Understanding the epidemiology of *T. cruzi* infection

is the first step toward implementing control measures to protect the health of these high-value working dogs.

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4. NATIONWIDE EXPOSURE OF U.S. WORKING DOGS TO THE CHAGAS DISEASE PARASITE, *TRYPANOSOMA CRUZI*

4.1 Introduction

Trypanosoma cruzi is a protozoal parasite and the etiologic agent of Chagas disease. Chagas disease is a zoonotic disease that affects over eight million people throughout the Americas and a diversity of domestic and wild animals.[1] *T. cruzi* infections in humans and animals may be asymptomatic or may be associated with debilitating acute or chronic cardiac disease, characterized by myocarditis, hepatomegaly, ascites, cardiac dilatation, or sudden death.[2,3] There are currently no vaccinations, and anti-parasitic treatments are limited in humans and not approved for dogs. The southern half of the U.S. harbors an established enzootic cycle of *T. cruzi*, where the parasite is vectored by several triatomine species and mammalian hosts including raccoons, opossums, and domestic dogs.[1,4,5] Contact between humans and triatomine vectors usually occurs when sylvatic vectors are attracted to lights, compromised housing structures, or deconstruction of host or triatomine habitat, which can attract vectors to invade human dwellings. [1,6,7] This epidemiological setting contrasts with what is commonly found in Central and South America, where triatomines more commonly colonize homes and dogs are recognized to play an important role as *T. cruzi* reservoirs. [8–11] The role of domestic dogs in the *T. cruzi* transmission cycle in the U.S. is not completely understood, although an increasing number of studies demonstrate exposure of diverse dog populations in the south, especially Texas, with reported seroprevalence ranging from 3.6-57.6%. [12–19]

Infection with *T. cruzi* is more likely in dogs than humans,[20,21] presumably owing to several ecological and behavioral factors including that dogs may readily consume insects including triatomines, allowing for oral *T. cruzi* transmission [15,22–25] and that dogs more commonly sleep outside, increasing their contact with nocturnal peridomestic vectors.[13,16,26,27] In South America dogs have been used as sentinels of human disease risk,[10,28] yet the degree to which infected dogs may signal human disease risk in the U.S. is not well understood. However, understanding spatial risk factors associated with *T. cruzi* infected dogs could allow new insight to inform vector control initiatives with benefits for both veterinary and public health.

We conducted an epidemiological investigation of dogs infected with *T. cruzi* through studying a population of government-owned working dogs from across the U.S. Our objectives were to (*i*) determine the seroprevalence of dogs across the U.S. with attention to exposures in dogs that work in southern states with established triatomines versus northern states without established triatomines; and (*ii*) use a comparative diagnostic approach and multiple independent testing platforms to compare prevalence of antibody positive dogs versus dogs with circulating parasite DNA. We hypothesize that the prevalence of *T. cruzi* exposure is higher within the range of triatomine vectors, and that any seropositive dogs outside the range would be attributed to canine travel history to an endemic region. As it pertains to the particular study population of working dogs, symptomatic *T. cruzi* infections may limit a dog's ability to work with follow-on security consequences. With a better understanding of the distribution of *T. cruzi* infection in dogs in working dogs, we can determine risk factors for exposure and provide targeted interventions to populations most at risk.

4.2 Materials and Methods

4.2.1 Ethics statement

All canine samples were collected in adherence with animal use protocols approved by Texas A&M University's Institutional Animal Care and Use Committee on 03/22/2017 under the number 2015-0289. Written consent was received for each canine sampled from the handler.

4.2.2 Study population- DHS working dogs

The United States Department of Homeland Security (DHS) owns over 3,000 working dogs across the US assigned to the following task forces: Federal Protection Services, U.S. Coast Guard, Secret Service, Transportation Security Administration (TSA), or two task forces within the Customs and Border Protection (CBP): Border Patrol or Port of Entry. Many of the dogs were bred in Europe, but some were purchased from vendors across the U.S. Dogs receive approximately 3-6 months of training at one of four training facilities in Texas, Virginia or Georgia, and specialize in various jobs such as explosives detection, track and trail, detection of humans, narcotics, currency, agricultural products, and search and rescue. After training, dogs are typically assigned to a specific task force and management area and have limited travel (with the exception of Secret Service dogs which travel both within and outside of the country). When dogs are off-duty they are either kenneled individually at their handler's residence or in a group kennel.

4.2.3 Sample collection

A cross sectional study design was used to collect blood samples from DHS working dogs across the U.S. from March 2017 to May 2018. In addition, test results from CBP dogs

we previously sampled in Texas and New Mexico in 2015-2016[18] were included in the analysis unless they were resampled in 2017-2018 in which case the more recent test results were used. Samples were collected in two ways: from field sampling in California, Arizona, and Texas and from submissions by the dog's veterinarians across 41 states, Washington D.C., and the U.S. Virgin Islands with a goal of sampling at least 50% of all DHS working dogs. Detailed instructions were provided to veterinarians during a dog's routine veterinary visit. For both the field sampling and the sampling at veterinary clinics, a minimum of 5 ml of blood was collected by venipuncture and aliquoted into serum and EDTA tubes. Sample criteria included dogs over 6 months in age and on active duty or in training. Demographic information was collected on all dogs sampled including age, sex, breed, canine job, sleeping location (home or kennel, indoors/outdoors), station of duty and address.

4.2.4 Serologic and molecular testing

After an aliquot of anticoagulated whole blood was taken, the blood tubes were spun and separated into serum, clot, plasma, and buffy coat and frozen at -20°F until analysis. Serum samples were screened for anti-*T. cruzi* antibodies by Chagas Stat-Pak® rapid immunochromatographic test (ChemBio, NY) using previously described methods.[18] All positive or inconclusive (faint line on lateral flow assay) samples as determined by Stat-Pak® plus 10% of the negatives were tested by both indirect fluorescent antibody (IFA) test performed by the Texas Veterinary Medical Diagnostic Laboratory (TVMDL, College Station, TX) and Trypanosoma Detect™ (InBios, International, Inc., Seattle, WA). On the IFA, titer values of 20 or higher were considered positive as per TVMDL standard protocol. If two or more of the three tests were positive, an individual was classified as seropositive.

When faint bands (inconclusive results) were present they were counted as negative in the calculation of prevalence.

DNA was extracted from 250uL of buffy coat or clot samples using E.N.Z.A. Tissue DNA kit (Omega Bio-Tek, Norcorss, GA). Samples were screened for *T. cruzi* DNA using Cruzi 1/2 primer set and Cruzi 3 probe for amplification of a 166-bp segment of repetitive nuclear DNA by real time PCR as previously described.[29,30] Samples with Ct values less than 34 were run on a confirmatory PCR amplifying a 330-bp region of kinetoplast DNA using *T. cruzi* 121/122 primers.[31,32] Amplicons were visualized on 1.5% agarose gels and samples that yielded a band of the appropriate size were interpreted as positive in our analyses. In order to determine the DTUs of the positive samples a multiplex quantitative, real time PCR was used based on amplification of the nuclear spliced leader intergenic region (SL-IR)[33] as previously described.[14,18]

4.2.5 Statistical analysis

To evaluate the relationship between potential risk factors and the serostatus of canines, we performed bivariable analysis and logistic regression using Program R.[34] Variables included, task force (Federal Protection Services, U.S. Coast Guard, Secret Service, Transportation Security Administration (TSA), or two task forces within the Customs and Border Protection (CBP): Border Patrol or Port of Entry, or dogs in training), job/type of detection (agriculture, currency/firearms, human/narcotics, track and trail, search and rescue/cadaver, or explosives), sleeping location (indoors or outdoors), sex, age, breed, and if the location the dog worked was within triatomine range or outside it. [35] Triatomine range was assigned on a state-level based on the CDC distribution map.

Due to the small sample sizes of breeds other than Belgian Malinois, remaining breeds were combined into breed groups as follows: Shepherd (German Shepherd, Dutch Shepherd, Belgian Shepherd, Belgian Turvuren, Bohemian Shepherd, Czech Shepherd, Dutch Shepherd, Groenendael, and Sable Shepherd) Retriever (Labrador Retriever and Flat Coated Retriever), Pointer (German Shorthair Pointer, German Wire Hair Pointer, Vizsla), and other (Beagle, Springer Spaniel, Weimaraner). Bivariable analysis using the chi-squared or Fisher's exact was used to identify putative risk factors and age was analyzed using a T-test. Factors with a $p \leq 0.25$ from the initial screening were used in a logistic regression model, while controlling for task force as a random effect. Generalized linear mixed models were calculated including odds ratios and 95% confidence intervals and factors with values of $p < 0.05$ were considered significant. To determine variation in seroprevalence across task force, a logistic regression model was used in which dogs in training (sampled at a training school) served as the referent to which all five management areas were compared.

4.3 Results

A total of 1,610 DHS working dogs were sampled from across the U.S. from six tasks forces and two training locations, comprising approximately half of the canine work force. Of these, 498 dogs were sampled in 2015-2016[18] and 1,112 were sampled in 2017-2018. Dogs came from 41 states plus Washington D.C. and the U.S. Virgin Islands; one third (33.2%) of the dogs came from Texas (Figure 4.1). Overall 1 to 534 dogs were sampled from each state/location, with a median of 8 dogs. The greatest number of dogs sampled were from Border Patrol (32.9%), followed by TSA (30.2%), then Port of Entry dogs (21.2%), followed by Secret Service (2.0%), Federal Protective Services (1.4%) and U.S. Coast Guard

(0.87%), finally 11.4% of dogs were sampled while in training (training facilities in El Paso, TX or Front Royal, VA). Most dogs sampled (58.9%) were human/narcotic detection dogs, or explosive detection dogs (34.4%). Of the 1,111 dogs for which sleeping location was known, 71.5% slept indoors and 28.5% slept outdoors. There were 1,110 males (68.9%) and 500 females (31.1%). Age ranged from approximately 6 months to 13 years and 8 months, with a median of 4.4 and a mean of 4.8. The most common breed was Belgian Malinois (n=583) followed by German Shepherds (n=489), Labrador Retriever (n=254), German Shorthair Pointer (n=147) and Dutch Shepherds (n=74). Of the dogs sampled, 92.4% were inside the triatomine range.

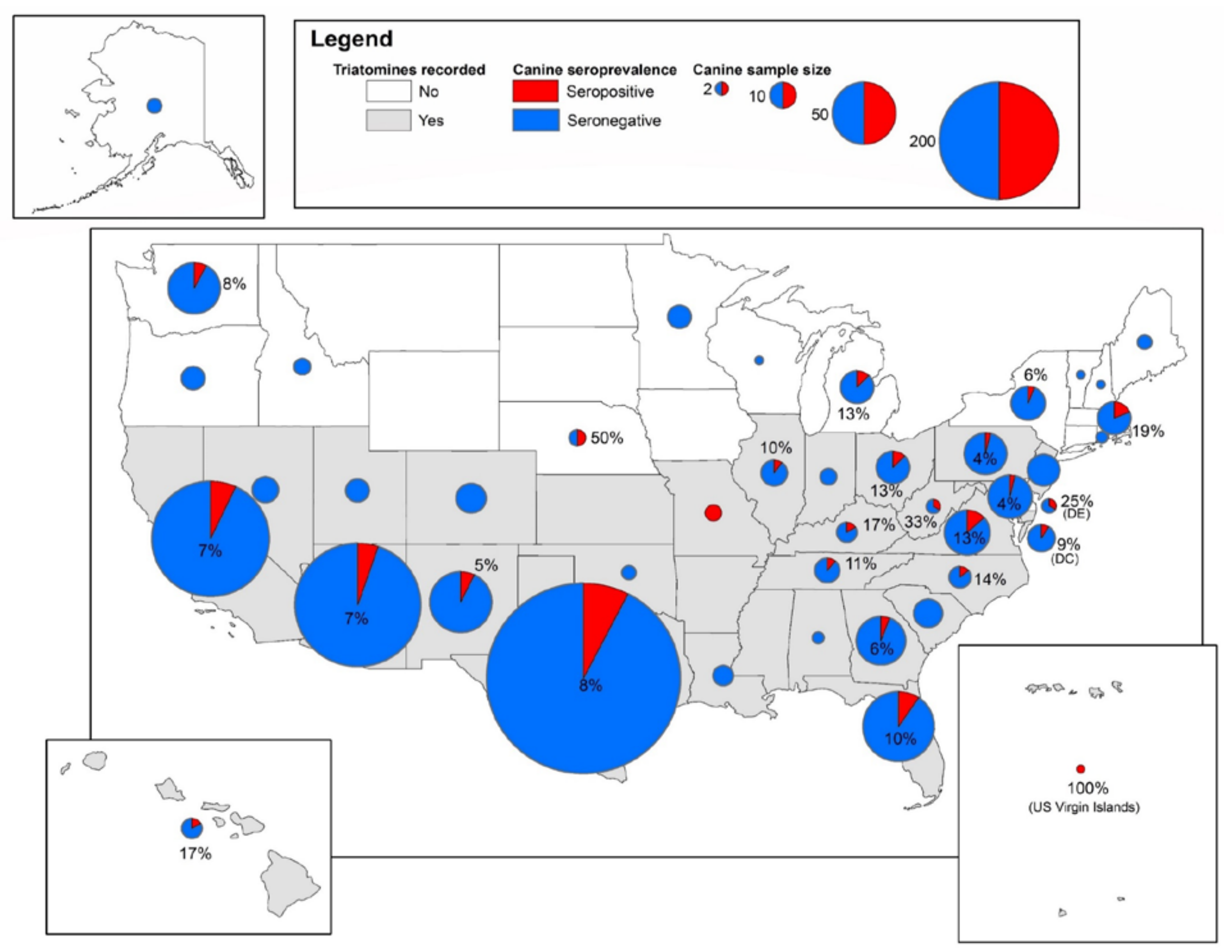

Figure 4.1. Seroprevalence of antibodies to *T. cruzi* in Deparment of Homeland Security dogs across the U.S. Circles are proportional to the sample size and red represents the percent of seropositive dogs. Canines were sampled from six different task forces, but all train in the southern U.S. Grey states represent the geographic range of the kissing bugs as reported by the CDC https://www.cdc.gov/parasites/chagas/. Map was created using ArcGIS with a U.S. base layer of U.S. states and the Virgina Islands downloaded from www.census.gov.

4.3.1 Seroprevalence

Of the total dogs sampled 7.5% (120/1610) were seropositive on the basis of responding on at least two independent tests, discounting samples that only produced a very faint band in the immunochromatographic tests. In the bivariable analysis, *T. cruzi* seroprevalence was significantly different across task force (p=0.013; Table 4.1). Logistic regression demonstrated that dog task force was also marginally associated with serostatus

(OR 3.19, 95% CI 0.82-10.28, p=0.065), in which seroprevalence was higher in dogs in the Federal Protective Services (18.2%) than dogs at the training center (6.5%). Logistic regression showed a significant association (odds ratio [OR] 2.21, 95% CI 1.12-4.10, p=0.01) between breed and seropositive dogs after controlling for Task Force as a random effect, in which Retrievers were associated with a significantly higher seroprevalence (12.0%) than Belgian Malinois (7.2%). Seroprevalence was marginally associated with breed (p=0.054), in which the Retriever breed group had the highest seroprevalence of 12.0%. Seroprevalence did not vary significantly by sex, age, detection type/job, sleeping inside/outside or location within or outside triatomine range.

Table 4.1. Results of bivariable analysis of potential risk factors for seropositive Department of Homeland Security dogs with antibodies to *T. cruzi* across the U.S.

	Variable	Sample size No. (%)	Seronegative No. (%)	Seropositive No. (%)	P-Value
Task Force	Border Patrol	530(32.9)	480(90.6)	50(9.4)	0.013[a]
	Coast Guard	14(0.87)	12(85.7)	2(14.3)	
	Federal Protective Services	22(1.4)	18(81.8)	4(18.2)	
	Port of Entry	342(21.2)	329(96.2)	13(3.8)	
	Secret Service	32(2.0)	29(90.6)	3(9.4)	
	TSA	486(30.2)	450(92.6)	36(7.4)	
	Training	184(11.4)	172(93.5)	12(6.5)	
Breed Group[c]	Shepherd	576(35.8)	539(93.6)	37(6.4)	0.054[a]
	Belgian Malinois	583(36.2)	541(92.8)	42(7.2)	
	Pointer	174(10.8)	165(94.8)	9(5.2)	
	Retriever	258(16.1)	227(88.0)	31(12.0)	
	Other	19(1.2)	18(94.7)	1(5.3)	
Detection	Agriculture	25(1.6)	23(92.0)	2(8.0)	0.89[a]
	Currency/Firearms	22(1.4)	20(90.9)	2(9.1)	
	Human/Narcotics	949(58.9)	883(93.0)	66(8.6)	
	Track & Trail	35(2.2)	32(91.4)	3(8.6)	
	Search & Rescue/Cadaver	25(1.6)	23(92.0)	2(8.0)	
	Explosives	554(34.4)	509(91.9)	45(8.1)	
Sex	Male	1110 (68.9)	1029(92.7)	81(7.3)	0.80
	Female	500 (31.1)	461(92.2)	39(7.8)	
Range	Inside triatomine range	1488(92.4)	1379(92.7)	109(7.3)	0.61
	Outside triatomine range	120(7.5)	109(90.8)	11(9.2)	
Sleeps	Indoors	794(49.3)	739(93.1)	55(6.9)	0.55
	Outdoors	317(19.7)	289(91.2)	28(8.8)	
	Unknown	499(31.0)	462(92.6)	37(7.4)	
	Average Age[b]		4.8	5.0	0.39[b]

[a] expected cell count in the contingency table <5, Fisher's exact test reported instead of chi square
[b] t-test performed instead of chi square

Twenty-eight of the dogs sampled in 2015-2016 that were in training at that time were resampled in 2017-2018 after being deployed to a task force; all were working in Border Patrol or at Ports of Entry dogs in 2017-2018. Of these 28 dogs, 2 were positive during training in 2015-2016 on two or more independent serological assays. One of these dogs remained positive during the 2017-2018 sampling, whereas the other was positive on 2 assays (Stat-Pak® and Trypanosoma Detect™) in April 2016, to have two inconclusive (faint bands) test results in June of 2017 and therefore considered negative in the current analysis.

4.3.2 Molecular detection of parasite DNA and T. cruzi *strain types*

Trypanosoma cruzi DNA was detected in the buffy coat fraction of the blood in 4 of 1,610 (0.25%) dog samples using both a screening and confirmatory assay. Additionally there were three dogs with samples that amplified in the screening PCR with a CT value of 31, yet these samples were negative on the subsequent assay and therefore considered negative Three of the PCR-positive dogs were sampled only in 2015-2016 and were from Texas, one dog had a Ct value of 33.5 and DTU TcIV, another had a Ct of 30.3 and was coinfected with TcI/TcIV, and one had a Ct value of 33.1 and was untypable, as previously reported.[18] The remaining PCR-positive dog was a 3-year-old female Labrador Retriever that worked in Border Patrol to perform narcotics/human detection functions at the airport; this dog was working in Washington D.C. at the time of sampled. She is reported to sleep indoors, and was sampled in June 2017 at which time she tested positive for antibodies by all three serology assays with a high titer (1280) on IFA. This dog had a Ct of 26.1 and using the

multiplex real time PCR to determine *T. cruzi* DTUs, we found that this dog harbored DTU TcIV.

4.4 Discussion

T. cruzi transmission in the U.S. was first reported in dogs in 1972[36] and locally-acquired human infections were first recognized in 1955,[37]although triatomines have been recognized from human dwellings since the 1930s.[38,39] Infection in dogs has been reported from at least 8 southern states including Texas, Louisiana, Oklahoma, Tennessee, Virginia, California, Georgia and South Carolina.[12,15,27,40–46] Herein, we tested 1,610 dogs for *T. cruzi* exposure from 41 states as well as Washington D.C. and the U.S. Virgin Islands. To our knowledge, this is the largest domestic dog serosurvey on *T. cruzi* infected dogs performed in the U.S., and the first to include dogs from 41 states.

We found that working dogs had widespread exposure to *T. cruzi* across the U.S. Overall, we found that seroprevalence in DHS working dogs across the U.S. was 7.5%. Many dogs had inconclusive results (faint bands) on Stat-Pak® or Chagas Detect™ and were considered negative for our analysis. However, if our criteria for cateregorizing a dog as positive in this study were more inclusive- still requiring positive reactions on two independent serologic testing platforms, yet allowing for the inclusion of such faint bands as a positive result- the seroprevalence could be as high as 23.1%. Surprisingly we found no significant difference between seroprevalence of dogs within and outside the triatomine range (p=0.61), with 9.2% (n=120) and 7.3% (n=1488) of dogs seropositive, respectively. The 11 seropositive dogs outside the kissing bug range likely demonstrate movement of *T. cruzi* exposed dogs from locations where transmission naturally occurs. Given that DHS

working dogs train at one of four centers in the southern U.S.- all in states with established triatomine populations- training may represent an at-risk time for exposure. We followed up on the procurement and training histories of the 11 of seropositive dogs found outside of the kissing bug range and found that all had spent at least some time training or living in the south where local transmission could have occurred. Individual dogs can move for various reasons including owner relocation, travel, and adoption programs; such movements allow for the translocation of infections that might not be acquired or transmitted in the new environment as has been described for other vector-borne diseases such as heartworm, Lyme disease, ehrlichiosis, and anaplasmosis.[47,48] These data demonstrate the need for heightened veterinary awareness for infection with vector-borne diseases in dogs outside endemic areas. Further, in dogs with heart disease, knowledge of a travel history to a southern state with endemic triatomines may raise the index of suspicion for Chagas disease.

Antibodies to *T. cruzi* have been found in 48 different dog breeds in the U.S.[42] We found that the Retriever breed group was associated with a significantly higher seroprevalence (12.0%) compared to Belgian Malinois (7.2%, p=0.01) after controlling for Task Force as a random effect. Similarly to our findings in Retrievers, a retrospective study of serologically and/or histopathologically *T. cruzi* positive dogs in Texas found that sporting breeds-primarily made up of Labrador Retrievers and English Pointers- made up 51.6% of the cases, compared to 8.1% of their cases being herding dogs (which includes Belgian Malinois and German Shepherds).[42] The high seroprevalence seen in Retrievers could be due to life history prior to arrival and training at DHS facilities and could be a result of difference in type of housing (indoor/outdoor kennel), geographic location of kennel or an individual propensity for consuming bugs, rather than breed predilection. The Retrievers are also bred

in the U.S., whereas most of the Belgian Malinois are bred in Europe. Dog task force was also marginally associated with serostatus (p=0.065), where seroprevalence was higher in dogs in the Federal Protective Services (18.2%). The dogs serving in the Federal Protective Services are the only dogs that train at a facility in Georgia- their higher exposure could be due to exposure at that facility or differences in procurement prior to training as Federal Protective Service dog.

Although previous studies of *T. cruzi* infection in dogs found that exposure increased with age,[10,14,18,21,27], owing to older dogs having a longer time for exposure to *T. cruzi*, we observed no statistical difference in ages of exposed versus unexposed dogs (p=0.39). Additionally, previous studies in Texas, Tennessee and Louisiana have concluded that dogs sleeping outdoors have greater exposure to the parasite,[13,16,18,27] yet the working dogs we study showed no difference in exposure based on sleeping location (p=0.55). The unique life histories of these working dogs, which includes months or years of training outside early in life, may account for different transmission environments than other naturally-exposed dogs.

Twenty-eight dogs were tested while in training during our prior study[18], then again after deployment to their task force one to two years later in the current study. Twenty-six dogs were negative at both time points, and one dog was consistently positive at both time points. One dog, however, was associated with different test results between the years. This dog was in training in in El Paso, TX, before being deployed to San Ysidro, CA, and its status changed from being positive on both immunochromatographic assays in 2016 to having inconclusive reactions (faint bands) on these assays in 2017 that were interpreted as negative; during both years of testing the dog was IFA negative. Discordant test results are common in *T. cruzi* diagnostics and testing is limited by a lack of a gold standard; [16,18] the

use of multiple serology assays is widely employed to assign positivity.[13,14,18,23,26,28,49] Although *T. cruzi* infections are commonly thought to be life-long in the absence of antiparasitic treatments, spontaneous seroreversion has been documented in mice, humans and dogs.[8,10,50–54] and this dog may have seroreverted. Alternatively, the two assays could be inaccurate due to test cross reactions or other reasons for a false positive result. A need for improved diagnostics for both veterinary epidemiological research and individual diagnoses is critical to allow for improved estimation of infection prevalence and allow for earlier detection which could improve prognosis.

Only four dogs were positive for *T. cruzi* DNA circulating in their blood by test criteria which required positve PCR results on two independent assays, although an additional 3 dogs were positive only on the screening PCR. All dogs were residing within the kissing bug range- three were in Texas (DTUs TcI and TcI/TcIV mix) and one was from Washington D.C. (DTU TcIV). TcI and TcIV have been commonly found in hosts and vectors in Texas.[4,14,18] Although there are no prior reports of canine Chagas disease in Washington D.C., infection with *T. cruzi* has been reported in wildlife and dogs in the neighboring state of Virginia[55–57], including a finding of TcIV in a Cocker Spaniel from Virginia.[58] Additionally, the Kissing Bug Citizen Science program has received multiple specimens of triatomines from Virginia and surrounding areas, including individuals infected with TcIV.[59] An infection prevalence of 33% (n=464) was found in raccoons (*Procyon lotor*) in urban/suburban areas outside of D.C.[55]-which have previously been shown to be primarily infected with TcIV.[1,4,60] It is possible that this dog was infected locally since TcIV has been found to be circulating in the local wildlife. Understanding of *T. cruzi* strain types circulating

in dogs is important since different strain types are potentially associated with different clinical outcomes.

Determining the prevalence of *T. cruzi* in dogs has practical implications for disease risk management and could assist with improved control measures. These findings should raise awareness among medical practitioners regarding *T. cruzi* infection throughout the U.S. Furthermore, understanding the distribution and risk factors for zoonotic parasite infection in natural populations of dogs could potentially be informative for human health.

4.5 References

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5. SELECTED CARDIAC ABNORMALITIES IN *TRYPANOSOMA CRUZI* SEROLOGICALLY POSITIVE, DISCORDANT, AND NEGATIVE WORKING DOGS ALONG THE TEXAS-MEXICO BORDER

5.1 Introduction

Despite a century of research since the discovery of *Trypanosoma cruzi* as the etiologic agent of Chagas disease, the associations between parasite infection and disease outcome are incompletely understood, complicating the diagnosis and prognosis of Chagas disease. In humans, a subset of infected individuals develop Chagas cardiomyopathy, which can cause sudden death, arrhythmias, heart failure or ventricular aneurysms. [1,2] A similar range of clinical outcomes is reported from dogs with no current morbidity or mortality estimates. Four mechanisms are attributed to the development of Chagas cardiomyopathy in humans: cardiac dysautonomia, microvascular disturbances, parasite induced myocardial damage, and immune-mediated myocardial injury, [1] with these mechanisms relevant to dogs and other hosts as well.

In South and Central America, dogs are important sentinels and a reservoir for *T. cruzi* transmission [3–7] and are good models for understanding the pathogenesis of *T. cruzi* infection in humans. Currently, dogs and non-human primates are the only other known species that develop acute, indeterminate and chronic stages of infection similar to humans. [8–11] Chagas disease can take years to manifest after initial infection, making the use of animal models challenging and in some cases, prohibitively expensive. However, naturally-infected dogs are widespread in the southern US and Texas in particular [12–17] , and the study of these dogs can be informative for understanding disease progression and prognostic

indicators. Furthermore, dogs have served as experimental models for benznidazole treatment during the acute and chronic stage of infection, [18,19] however, a better understanding of disease progression is necessary for interpretation of drug and vaccine effectiveness.

T. cruzi is transmitted through the introduction of infected triatomine feces through the bite site or mucous membrane, or orally through ingestion of the triatomines or their feces. After infection the trypomastigote invades the mammalian host cells, [20] where *T. cruzi* has a tissue tropism for cardiomyocytes and skeletal muscle. [21] Invasion of cardiomyocytes causes cellular injury, causing release of cytokines which can have proarrhythmic effects. Persistent parasite infection of the heart causes continued stimulation of the immune response, which can cause cell death leading to replacement fibrosis and secondary hypertrophy. [1] In dogs as in humans, electrocardiogram (ECG) abnormalities detected during *T. cruzi* infection can vary widely, and reported abnormalities in experimentally and naturally infected dogs include changes to the ECG complex morphology (i.e. T wave abnormalities, small complexes), axis shifts, conduction disturbances (i.e. atrioventricular block, bundle branch block) and arrhythmias (supraventricular and ventricular). [22,23] During the subacute stage clinical signs may not be evident, and ECG recordings can be normal or have minimal abnormalities. [1] Cardiac troponin I (cTnI), a biomarker of cardiac injury, can assist in the diagnosis of acute myocardial injury, and elevated cTnI has been associated with Chagas myocarditis in dogs. [24] There are currently no vaccinations or approved anti-parasitic treatments for *T. cruzi* infections in dogs in the U.S., consequently, disease prevention is focused on limiting canine contact with vectors.

The goal of this study is to identify and describe selected cardiac abnormalities associated with dogs naturally infected with *T. cruzi* as a model population for further defining parasite-host interactions. Since the cardiac abnormalities associated with Chagas disease in humans and animals are generally not specific and may also arise from other conditions, the specific comparison of selected cardiac measures between an infected and uninfected population is a useful approach for identifying abnormalities that are associated with infection status. Understanding the cardiac manifestations of natural *T. cruzi* infections in dogs is critical for prognostication and to establish a baseline for interpreting data from drug and vaccine studies.

5.2 Materials and Methods

5.2.1 Ethics statement

All canine samples were collected in adherence with animal use protocols approved by Texas A&M University's Institutional Animal Care and Use Committee (protocol number 2015-0289) and Clinical Research Review Committee, with informed consent obtained by canine handlers prior to sampling.

5.2.2 Study population- working dogs along the Texas-Mexico border

The Department of Homeland Security (DHS) of the U.S. government utilizes highly trained dogs for border security and detection purposes. Previous research has shown that these dogs have widespread exposure to *T. cruzi* along the Texas-Mexico border with 7.4-18.9% seroprevalence depending on test interpretation (n=528). [14] Additionally, 45% of triatomines found in the working dog's environments were infected with *T. cruzi,* and the

majority had recently fed on canines. [14] Despite this high level of exposure and vector contact, many infected dogs continue to work regularly with no perceptible adverse health outcomes noted by handlers, and so the degree to which their hearts are impacted is unknown. DHS working dogs are predominantly bred in Europe but the population also includes some animals that were bred domestically. The breeds of sampled DHS working dogs consisted of Belgian Malinois, German Shepherds, Sable Shepherds, Dutch Shepherds, Groenendael and Labrador Retrievers. DHS working dogs receive 6 months training at a working dog training facility either in El Paso, Texas, or Front Royal, Virginia, in order to specialize in various jobs such as track and trail; detection of humans, narcotics, currency or agricultural products; or search and rescue. In the post-training period, dogs are assigned to work at a specific location across the U.S. and have limited travel. Dogs included in this study worked either immediately adjacent to the geopolitical Texas-Mexico border (ports of entry) or north of the border (checkpoints). All dogs receive vaccines and deworming annually, are on tick and flea preventative and receive bi-annual health exams.

Dogs were selected from a population of DHS working dogs that were initially tested for *T. cruzi* DNA and antibodies by our research team in November of 2015 [14] , and we performed follow-up testing in 2017. In addition to PCR, we use up to three independent antibody detection tests on each dog per year, and require a positive reaction with at least two tests to code a dog as positive in a given year with the exception of two dogs which had a history of being positive on IFA prior to the start of our study and then had positive reactions on at least one test in both 2015 and 2017 (methods below). We classified dogs into three categories for analysis: ‘positive’ (positive test result on two or more assays in 2015 or indirect fluorescent antibody (IFA) testing prior to 2015 and at least one test in both 2015 and

2017); ‘discordant’ (either (i) positive on only a single test each year (ii) or had a history of being IFA-positive prior to 2015 testing but negative on all tests in 2015 and 2017); and ‘negative’ (negative on all tests in 2015 and 2017 and no history of positivity prior to our study). After the identification of positive and discordant dogs, ‘negative’ dogs were then selected to match the positive and discordant dogs based on age (+/-1 year), sex, breed and location of sector (Del Rio, Laredo and Rio Grande Valley). In two cases, positive or discordant German Shepherds could not be matched by breed, and were instead matched to a negative Sable Shepherd and Dutch Shepherd. In addition to negative serology, all negative dogs were required to be PCR negative as a step to reduce the chance that acutely infected dogs (i.e., prior to development of detectable antibodies) were classified as negative.

5.2.3 ECG monitoring

To characterize cardiac arrhythmias and conduction abnormalities, we applied a 5-lead continuous read ambulatory ECG ‘Holter’ monitor (LabCorp, Burlington, NC). This model has previously been used in both canine and human medicine, and records heart rate, pauses, R-R variability (total duration of ventricular depolarization) and rhythm and conduction abnormalities. On each dog, an area was shaved and cleaned with alcohol for electrode placement. Electrodes were placed according to manufacturer instructions on the lateral chest walls of the dog. The electrodes and battery were secured using 3-inch Ultra-Light™ elastic adhesive tape (Covidien, Dublin, Republic of Ireland), over which a working vest was placed. Dogs conducted their normal working routine to provide an accurate representation of daily cardiac stress. Monitors were removed after 24-48 hours. Tracings obtained from the ECG were recorded at a speed of 25mm/s using a lead configuration of

V1, V2 and V5, then transferred to LabCorp for automatic analysis. During ECG application, each canine handler was asked if he or she had observed a change in performance in their dog, or fatigue, or if the dog had any other major health concerns over the past two years. All responses were recorded.

Full disclosure tracings were reviewed by a board-certified veterinary cardiologist (ABS) who was blinded to the dog's infection status to characterize the occurrence and severity of arrhythmias and conduction abnormalities. Recordings made unreadable by excessive background noise or artifact, or with read times less than 19 hours were excluded from further analysis. The following were recorded: analyzed duration, heart rate (max, min, mean), number of pauses > 3000ms, longest RR interval, and sinus arrest > 4 seconds. The presence of ventricular premature contractions (VPCs) was recorded and the number was normalized to 24 hours. Ventricular arrhythmias were graded based on a modified Lown score using the following criteria: 0- no VPCs; 1-single uniform VPCs; 2-bigeminy, trigeminy, or multiform VPCs; 3- accelerated idioventricular rhythm; 4- VPCs in couplets or triplets; 5- "R on T" phenomenon or ventricular tachycardia. [25,26] Also recorded were presence of supraventricular arrhythmias and presence and degree of atrioventricular block. Supraventricular and ventricular tachycardia were defined as >3 abnormal complexes in a row at a heart rate of >100 bpm if greater than 4 beats. The anatomic level of the ECG abnormality was recorded as being present or absent at the atria, atrioventricular (AV) node, or ventricle. The following ECG abnormalities were assigned as atrial level (supraventricular premature beats, supraventricular tachycardia including atrial fibrillation, sinus arrest > 4 seconds), AV node (atrioventricular block – any degree), and/or ventricle (ventricular premature beats, ventricular tachycardia, bundle branch block).

5.2.4 Serologic and molecular testing

At the time the Holter monitor was applied, a minimum of 6 ml of blood was collected and aliquoted into serum and EDTA tubes, spun, separated, and stored at -20ºC until analysis. All dog serum was tested for anti-*T. cruzi* antibodies by two rapid immunochromatographic assays, Chagas Stat-Pak® (ChemBio, NY), and Chagas Detect™ Plus Rapid Test (InBios, International, Inc., Seattle, WA) using previously described methods. [14] All dogs that gave a positive result on one or both rapid tests were also tested on indirect fluorescent antibody (IFA) test at the Texas Veterinary Medical Diagnostic Laboratory, College Station, TX. Titer values of 20 or higher were considered positive as per TVMDL standard protocol.

All dogs were tested for amplification of parasite DNA from buffy coat of blood by real time PCR as previously described; [27,28] in the case of a positive qPCR reaction, additional qPCR was carried out to assign the parasite discrete typing unit. [29] All deviations from protocols are previously described. [14,30]

To rule out cardiac abnormalities caused by other vector-borne diseases, we used a commercially available rapid format ELISA, the SNAP 4Dx Plus (IDEXX, Westbrook, ME), for detection of *Dirofilaria immitis* (heartworm) antigen and antibodies to *Ehrlichia canis*, *E. ewingii*, *Borrelia burgdorferi*, *Anaplasma. phagocytophilum*, and *A. platys*.

5.2.5 Cardiac biomarker

Cardiac troponin I (cTnI) is a regulatory protein used in cardiac muscle contraction and is a sensitive and specific marker of myocardial injury. cTnI concentrations are considered a quantitative measurement for the degree of injury sustained by the heart. [31]

Frozen serum samples were slowly thawed at room temperature immediately prior to analysis. cTnI analysis was performed using an ADVIA Centaur TnI-Ultra immunoassay at the Gastrointestinal Laboratory at Texas A&M University on 250 uL of serum. The reported range for cTnI detection by the manufacturer is 0.006 to 50.0 ng/mL.

5.2.6 Statistical methods

To evaluate the relationship between dog *T. cruzi* infection status (positive, discordant, or negative) and clinical outcomes, data were imported into RStudio 3.4.2 (2017-09-28) software for analysis. Bivariable analysis using Fisher's exact was used to determine if *T. cruzi* infection was associated with the presence of a cardiac abnormality. Since there is no pre-defined threshold number of VPCs/24 hours that is accepted as normal in healthy dogs, we used the seronegative dog population to establish a within-study threshold value of 4.2 VPCs/24 hours; this threshold value was calculated as three standard deviations above the mean from negative dogs (μ=0.56, σ=1.2). One negative dog (#258) had 762 VPCs/24 hours and was considered an outlier and was removed from the calculation for a threshold. VPCs were included as a cardiac abnormality in analysis when there was >4.2 VPCs/24 hours and/or the modified Lown score was 2 or more. Quantitative variables from the ECG were analyzed using one-way ANOVA followed by a Tukey's test. For variables that were not normally distributed, statistical significance was determined by the non-parametric Kruskal Wallace ranked sum followed by Dunn's test with a Holm adjustment of p-values. This includes the evaluation of infection and the number of cardiac abnormalities, modified Lown score, number of VPCs/24 hours and cTnI concentration. Significance was defined as $P \leq 0.05$.

5.3 Results

5.3.1 Study population

In 2017, 48 dogs had Holter monitors placed. Seven monitors were non-diagnostic due to dogs either chewing or removing the electrodes. Of the 41 successful recordings, 17 were from positives dogs, 6 were from discordant dogs and 18 were from negative dogs. Two discordant dogs had read times less than 19 hours and were excluded from the ECG analysis. The matching criteria resulted in 14 of the negative dogs individually matched to 14 positive and discordant dogs based on age, breed, sex, and location criteria with an additional 6 positive dogs, 2 discordant dogs, and 3 negatives dogs included in analysis that were not specifically matched to each other. Detailed antibody testing history for each individual dog is reported in the Supplemental Table (S2).

The majority of 41 dogs were male (n=26). Age ranged from 3.3-11.2 years with the average age of 6.6 years and median of 7.0. Breeds sampled included Belgian Malinois (n=22), German shepherd (n=11), Dutch shepherd (n=3), Labrador retriever (n=2), Sable Shepherd (n=2) and Groenendael (n=1). Dogs worked in 3 management locations in Texas: Del Rio (n=14), Laredo (n=9) and the Rio Grande Valley (n=23). When not performing working duties, most of the dogs (n=32) were housed at home with their handlers, but 9 were group kenneled.

5.3.2 Overall health

Of all 41 handlers questioned about their dog's health, 5 (12.2%) reported that they had seen a performance decline in their dog over the past two years. The 5 dogs reported to have a performance decline included 4 that were *T. cruzi* positive and 1 that was negative for

which the handler attributed the performance decline to the dog's age (10 years old). Positive dogs experiencing performance decline ranged from 4.78-7.39 years. Handlers of 5 positive dogs reported fatigue (3 of these handlers had also reported performance decline); the age of dogs with fatigue ranged from 4.78-8.30 year. No handlers of discordant or negative dogs reported fatigue. Other major health concerns reported by owners included: one dog was receiving allergy shots, one dog had had a splenic torsion, one dog had chronic ear infections, one had had coccidiosis. Blood samples from all dogs were tested on the SNAP 4Dx Plus assay (Table S2). Three dogs (7.3%) were positive for antigen of *Dirofilaria immitis,* including one each of positive, discordant, and negative for *T. cruzi* and none of which had cardiac abnormalities, as assessed in this study. One *T. cruzi*-positive dog tested positive for antibodies to *Ehrlichia* sp., and this dog had first degree AV Block and 5 VPCs per 24 hours (#53).

5.3.3 ECG findings

The thirty-nine Holter analyzed recording times ranged from 19:04-48:00 hours, with a median read time of 47:26. Twenty-six of the recordings were of good quality and the remaining 13 were a good quality with some baseline artifact but were still able to be interpreted. Overall, 19/39 (48.7%) dogs were characterized as having at least one ECG abnormality. When dogs were dichotomized as having 1 or more ECG abnormality vs. no abnormality, there was a significant difference between presence of abnormality and *T. cruzi* infection ($p<0.0001$), with abnormalities detected in 76.5% (13/17) of positive dogs, 100.0% (4/4) of discordant dogs, and 11.1% (2/18) of negative dogs. Ventricular arrythmias were the most common ECG abnormality (when defined as $\geq$4.2 VPCs/24 hours and a Lown score of $\geq$2), and were present in 47.1% of positive dogs, 50.0% of discordant dogs and 5.6% of

negative dogs (Table 5.1). Additional ECG abnormalities observed included first and second degree AV block, and supraventricular premature beats/tachycardia. Neither atrial fibrillation or bundle branch block were identified in any dog.

Table 5.1. Anatomic level (atria, AV node, or ventricle) and presence of various types of arrhythmias and conduction abnormalities found in government working dogs with a $\geq$2 year known serological history of being antibody positive for *T. cruzi* (n=17), discordant (n=4), or negative (n=18).

		Atria			**AV node**		**Ventricle**		
Infection Status	**Dog #**	**Supraventricular premature contractions**	**Supraventricular Tachycardia**	**Sinus Arrest (pauses$\geq$4secs)**	**First degree AV Block**	**Second degree AV Block**	**Ventricular Tachycardia**	**VPCs/24 hours**	**Modified Lown Score for ventricular arrhythmias**
Positive	*238*	X						0	0
	369				X			0	0
	200			X				0	0
	62			X				0	0
	159	X						3	1
	53				X			5	2
	203							11	4
	105	X	X					12	1
	60							38	1
	153	X			X			79	2
	65						X	691	5
	92					X	X	1163	5
	64							6594	2
Discordant	*234*	X		X				0	0
	2					X		0	0
	198							25	1
	227							286	2
Negative	*54*			X				0	0
	94							1	1
	530							1	1
	16							3	1
	193							4	1
	258				X	X		762	5

*16 dogs are not displayed on the table because they had 0 VPCs and no apparent abnormalities in the 24 hour monitoring. This includes 4 positive dogs and 12 negative dogs.

There was a significant difference between mean number of different ECG abnormalities among *T. cruzi* infection status ($\chi^2(2) = 15.8$, $p<0.001$), in which the mean number of abnormalities was higher in both positive compared to negative dogs ($p<0.001$), and discordant compared to negative dogs ($p=0.014$) and there was no difference between positive and discordant dogs ($p=0.63$, Table 5.2).

Table 5.2. Total dogs by serostatus with the number of different types of ECG abnormalities found using a 24-hour ECG Holter monitor. Types of ECG abnormalities were defined as: sinus arrest with pauses >4 seconds, presence of any number of supra ventricular premature complexes or supraventricular tachycardia, ventricular tachycardia, the presence of AV block (first or second degree) and VPCs greater than 4.2 per 24 hours with Lown score of 2 or more. Dogs had a $\geq$2 year known serological history of being seropositive for *T. cruzi*, seronegative, or having discordant test results.

	Number of different types of ECG abnormalities				
	0	**1**	**2**	**3**	**Total**
Negative	16	1	0	1	18
Discordant	0	3	1	0	4
Positive	4	8	2	3	17
Total	20	12	3	4	39

The number of VPCs present in 24 hours was not significantly different across *T. cruzi* status ($\chi^2(2)=4.58$, $p=0.10$). Number of VPCs ranged from 0-4 in negative dogs, with one outlier that had 762 VPCs/24 hours. The discordant dogs ranged from 0-286, and the positive dogs ranged from 0-6594 VPCs/24 hours (Table 5.1, Figure 5.1).

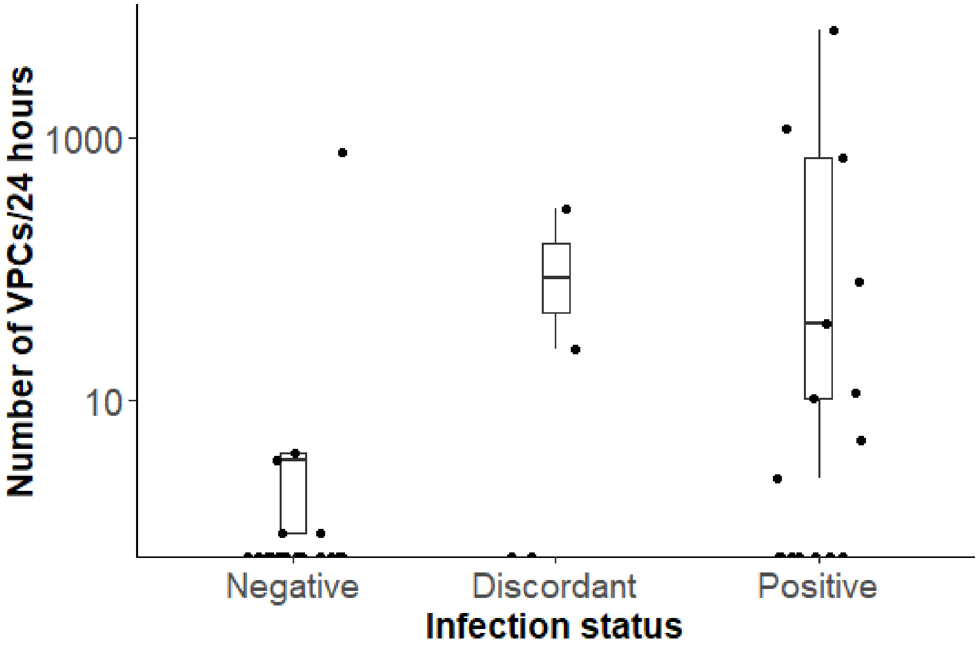

Figure 5.1. Box plots illustrating the normalized number of VPCs per 24 hours in *T. cruzi* positive, discordant and negative dogs. Y-axis scaled to log10.

All dogs were assigned a modified Lown score quantifying the severity of the ventricular arrhythmias present. Overall, 35.3% of *T. cruzi* positive dogs had a Lown score of 2 or more compared to 25.0% of discordant dogs and 5.5% of negative dogs ($\chi^2(2)=3.27$, p = 0.20).

Table 5.3. Modified Lown score for severity of ventricular arrhythmias found on ambulatory ECG (Holter) from government working dogs with a ≥2 year known serological history of being seropositive for *T. cruzi*, seronegative, or having discordant test results. Ventricular arrhythmias were graded based on a modified Lown score using the following criteria: 0- no VPCs; 1-single uniform VPCs; 2-bigeminy, trigeminy, or multiform VPCs; 3- accelerated idioventricular rhythm; 4- VPCs in couplets or triplets; 5- "R on T" phenomenon or ventricular tachycardia.

		Modified Lown Score					
	Total dogs	**0**	**1**	**2**	**3**	**4**	**5**
Negative	18	13	4	0	0	0	1
Discordant	4	2	1	1	0	0	0
Positive	17	8	3	3	0	1	2
Total dogs	39	22	8	4	0	1	3

Quantitative variables from the ECG including heart rate range and pauses are displayed in Table 5.4. Maximum heart rate was not significantly different between positive and negative dogs (p=0.11), positive and discordant dogs (p=0.98) or between discordant and negative dogs (p=0.32). Analysis on all other quantitative variables showed no significant difference among *T. cruzi* infection status (Table 5.4). Overall, there were 11.8% (2/17) positive dogs that had more than 25 pauses over 3 seconds during the time observed, and no negative or discordant dogs with more than 25 pauses over 3 seconds. Sinus arrest >4 seconds was the reason for pauses in 4 dogs (#200, 62, 234 and 54); two of these dogs were positive, one was discordant and one was negative.

Table 5.4. Quantitative variables found on ECG from government working dogs with a ≥2 year known serological history of being seropositive for *T. cruzi*, seronegative, or having discordant test results. Mean and range is reported for each variable.

ECG variable	Positive (N=17)	Negative (N=18)	Discordant (N=4)	ANOVA P-Value
Max HR (bpm)	218.5 (131-285)	245.5 (206-308)	214.8 (173-264)	0.09
Min HR (bpm)	28.3 (18-56)	28.4 (20-45)	24.3 (22-28)	0.58
Mean HR (bpm)	76.4 (54-124)	76.4 (58-103)	69.8 (64-79)	0.65
Pauses > 3s	14.8 (0-164)	2.7 (0-25)	3.8 (1-10)	0.40
Longest RR Interval(s)	3.0 (1.7-4.2)	2.9 (1.7-4.1)	3.4 (3.1-4.1)	0.40

T. cruzi-positive, discordant, and negative dogs showed ECG abnormalities at all three anatomic levels (atria, AV node or ventricles). Individual dogs with abnormalities present at multiple levels were more common in the positive dogs compared to the discordant and negative dogs (Figure 5.2).

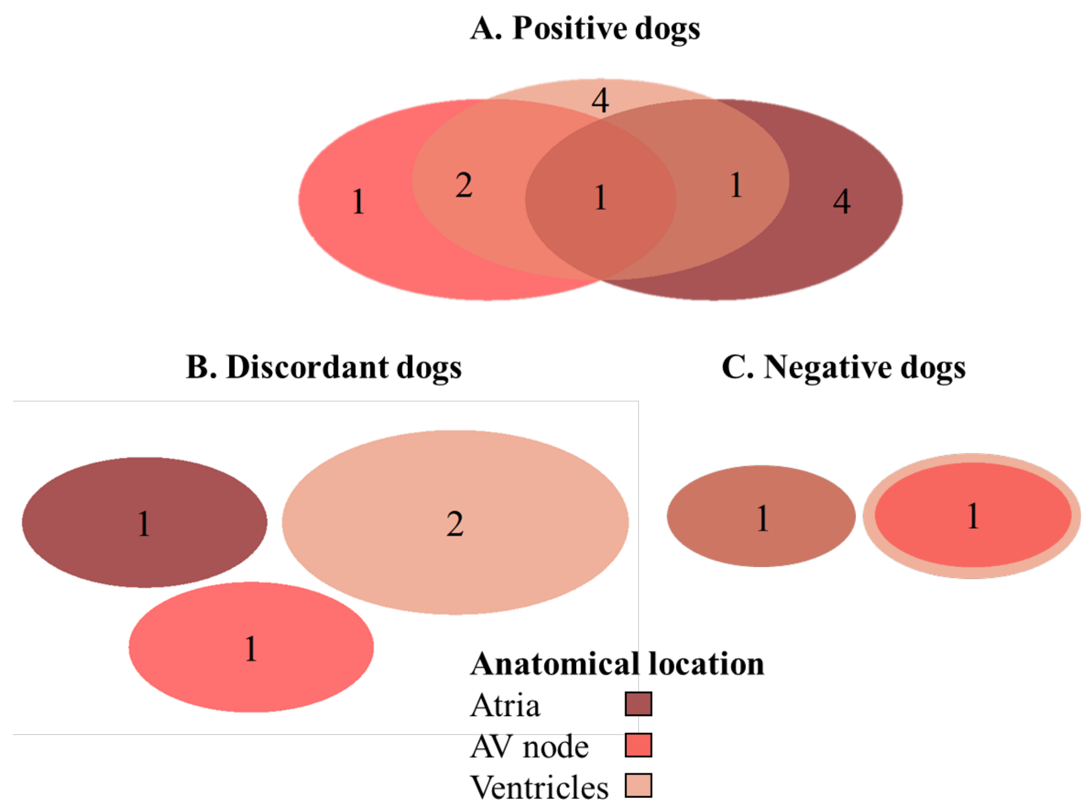

Figure 5.2. Venn diagrams demonstrating the anatomic level (atria, ventricles and/or AV node) of cardiac abnormalities. Of the 39 dogs tested, some dogs demonstrated cardiac disease in one location, two locations or all three. Dogs were determined to be infected with *T. cruzi* (A), have discordant test results (B) or negative for *T. cruzi* (C).

5.3.4 Molecular analysis

All 41 dogs were negative for *T. cruzi* by PCR in 2015. Forty of these dogs remained negative by PCR in 2017. A single dog- a 8-year-old female Belgian Malinois from Rio Grande Valley management area- was PCR-positive in 2017 with a CT value of 31.32 on the Cruzi 1/2/3 real-time assay. The discrete typing unit present was TcI. This dog was serologically positive with an IFA titer of 320 in both 2015 and 2017 and was positive on both Chagas Stat-Pak® and Chagas Detect™ both years tested.

5.3.5 Cardiac biomarker

All positive (n=17) and discordant (n=6) dogs, and a random subset of negative (9/18) dogs had serum submitted to measure cTnI concentrations. The cTnI concentrations ranged from <0.006 ng/mL to 0.57 ng/mL in the positive dogs with a median of 0.085 ng/mL; while the discordant dogs ranged from <0.006 ng/mL to 0.043 ng/mL, with a median of 0.011 and the negative dogs ranged was from <0.006 ng/mL to 0.18 ng/mL, with a median of 0.007 ng/mL (Figure 5.3). The cTnI level was below the limit of detection in 5.9% (1/17) of positive dogs, 33.3% (2/6) of discordant dogs and 22.2% (4/9) of negative dogs. There was a significant difference between *T. cruzi* infection status and the cTnI concentration (χ^2 (2)=8.22, p = 0.016) with higher concentrations in positive than negative dogs (p =0.044) and discordant dogs (p=0.06), and no difference between discordant and negative dogs (p=0.96).

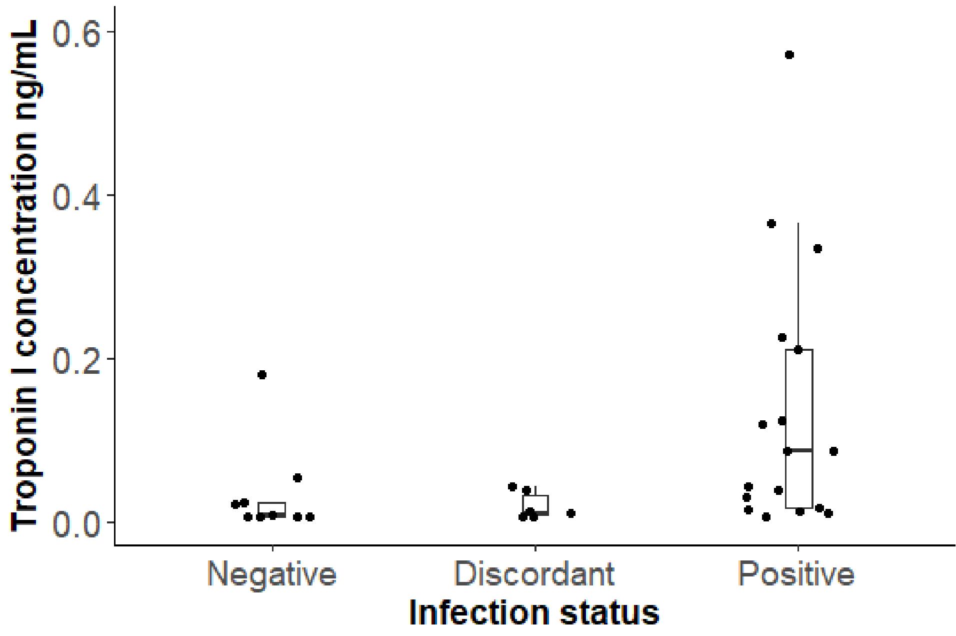

Figure 5.3. Box plots illustrating the range of cardiac troponin I concentrations in *T. cruzi* positive, discordant and negative dogs. Median cardiac troponin I concentration was 0.085 for positive dogs (N=17), 0.007 for negative dogs (N=9) and 0.011 for discordant dogs (N=6).

5.4 Discussion

Holter (24-hour ambulatory ECG) monitors are a useful tool for monitoring the cardiac rate and rhythm abnormalities of *T. cruzi*-infection in hosts. We found that the presence ($p<0.0001$) and number ($p<0.001$) of ECG abnormalities was higher is seropositive dogs than seronegative dogs. The mean number of ECG abnormalities was also higher in positive and discordant dogs than negative dogs ($p<0.001$, $p=0.014$). Supraventricular premature contractions, AV block, ventricular arrhythmias (premature beats and tachycardia) were more commonly found in seropositive dogs than seronegative dogs. Similarly, Barr et al. 1992 found that experimentally infected beagles in the acute stage had AV block and in the chronic stage had multiform ventricular premature contractions and ventricular tachycardia. [22] Other studies in canines have also reported first degree AV block and ventricular conduction disturbances. [8,32,33] Second degree AV block, which we observed in only one *T. cruzi* positive dog, has been less commonly reported; prior studies have found second degree AV block in 1/18 *T. cruzi* infected dogs and third degree AV block in 1/10 *T. cruzi* infected dogs. [22,23] Some arrhythmias previously documented in dogs with Chagas disease, such as atrial fibrillation and third degree AV block, were not found in this study. It is important to determine the prevalence and type of cardiac abnormalities in *T. cruzi* infected dogs to better predict clinical outcomes and so future treatments can be evaluated.

Overall 76.5% (13/17) of seropositive dogs had ECG abnormalities during the study period. This is in-line with previous study findings where 90%-100% of dogs have cardiac abnormalities. [23,34] This percentage, which is based on $\geq$2 year known infection, is higher than what has been reported in for humans in which approximately 20-40% of *T. cruzi* infected humans develop cardiac abnormalities resulting in heart failure, arrhythmias, heart

blocks, thromboembolism, stroke and sudden death. [35–37] This variation in percentage of dogs that development cardiac abnormalities could be due to host immune response, physiology exposure route or dose of parasite upon exposure. Dogs have an affinity to eat bugs, and oral transmission in dogs from the consumption of infected kissing bugs has been suggested as a likely route, [15,38–41] which could expose them to a higher density of parasite. Previous studies have shown that pathogenicity in mice and dogs is relative to the quantity or parasite inoculated and to the route of inoculation. [42–44]

There is no single gold standard diagnostic test for Chagas disease in humans or animals and so samples are commonly subjected to multiple independent tests and conflicting test results are commonly reported. [12–14,45–47] We found that 'discordant' dogs – dogs that were positive on a single but not multiple independent tests- were somewhat intermediary with respect to their clinical status. Notably, we found that all four discordant dogs in our study showed ECG abnormalities, with abnormalities occurring in all anatomic levels of the heart. This is evidence that the result of a single test (in some cases a single immunochromatographic test used in an off-label manner) may be useful in signaling the potential for clinical disease and should not immediately be dismissed. The significance of a single positive serologic test result in studies of human Chagas disease- where discordant serology is a long-standing problem [45,48–50] remains to be determined. Further research on a larger population of dogs, or over a longer time period, is warranted to better understand clinical outcomes in a population of discordant dogs.

We found that ventricular arrythmias were the most common cardiac abnormality seen in the working dogs. The number of VPCs/24 hours and complexity of VPCs is frequently used in veterinary medicine to evaluate clinically significant ventricular

arrhythmias, [25,51–53] yet determining a threshold value of an acceptable number of VPCs above which would signal an abnormality proved challenging. In previous studies, a low number of VPCs has been suggested to be normal in dogs as it is in humans, [54–56] however, repetitive, numerous or polymorphic VPCs could be suggestive of cardiac disease or increased risk of sudden death. Although prior cardiac research documents populations of healthy dogs with <50 VPCs/24 hour, this value likely varies by breed and with co-morbidities. Based on characterizing the data from seronegative dogs, we established a within-study threshold and categorized as abnormal dogs with ≥4.2 VPCs/24 hours and a Lown score of ≥2; using these criteria, nearly half (47.1%) of the seropositive dogs had abnormal ventricular arrhythmias. Similarly, in humans with Chagas disease VPCs are one of the most common ECG abnormalities. [57] Barr et al. found that 100% of dogs inoculated with *T. cruzi* developed ventricular arrhythmias, which were polymorphic and developed into ventricular tachycardia. [22] Previous research has shown >50 VPC/24 hour, polymorphic VPCs, and the presence of ventricular tachycardia in dogs is significantly associated with shorter survival times. [53] In seronegative dogs, 27.8% (5/18) of dogs had VPCs, but only 5.6% (1/18) were above the threshold of what was established as normal for this population. Our findings of VPCs in a healthy population of dogs is in-line with previous findings of the presence of VPCs hours in 21.5% (n=228) of clinically normal beagles, 12.5% (n=16) and in a healthy population of pet dogs, and 32.0% in 50 healthy dogs. [54,55,58] Furthermore, a study evaluating large-breed healthy dogs by ambulatory ECG for ≥20 hours found that only 8% (n=50) had a Lown score of 2 or more and concluded this could be suggestive of cardiac or systemic disease. [58]

Heart rate is related to the intensity of cardiovascular workload and can be useful for monitoring animals. [59,60] We found no significant associations between the quantitative variables from the ECG and infection status in dogs, including number of pauses longer than 3 seconds, longest RR interval, or minimum, mean, or maximum heart rate. Camacho et al. found heart rate (beats per minute) in *T. cruzi* experimentally infected dogs to be significantly higher than control dogs during acute infection, but no difference was found during the indeterminate phase. [32] In contrast, a study in humans found that patients seropositive for *T. cruzi* in the indeterminate phase have significantly lower heart rates than seronegative patients. [61] Furthermore, it is documented that chronic Chagas cardiomyopathy in humans can cause a decreased response to exercise and stress due to failure of cardiac compensation. [61,62] The DHS working dogs are highly active and participate in weekly training throughout their working lives. Benefits of exercise training in chronic heart failure patients have been well studied, but new research suggests that exercise could assist with cardiac function in chronic Chagas disease in humans. [63,64] A randomized trial by Lima et al. compared humans with chronic Chagas disease who underwent a 12 week exercise training program compared to those who did not, and found that those who completed exercise training had improvements in oxygen uptake, exercise time, walking distance and heart rate peak. [63] Since the DHS working dogs are regularly exercising, similar to what is demonstrated in humans, it is possible that regular exercise could benefit cardiac function, but more research is needed to explore this further.

When we examined anatomic level of ECG abnormality (atria, AV node or ventricles) we found that in all populations of dogs (positive, discordant, negative) abnormalities were present at each level, however, individual positive dogs were more likely

to have abnormalities detected at multiple levels of the conduction system within the heart. The slow progressing myocarditis that results from infection can cause decreased contractile function and dilatation of all four chambers. [65] Histological evidence in humans and dogs shows that there is destruction of myocardial cells throughout the heart and scarring of the conduction system. [24,33,66] In *T. cruzi* infection focal inflammation causes destruction of cardiac fibers and subsequent fibrosis in nonregenerating cells can occur which can result in myocardial dysfunction, heart failure and arrhythmias. [67,68] This damage to cardiac myocytes explains why infection with *T. cruzi* can cause combinations of arrhythmias, conduction disturbances, myocardial dysfunction and heart failure.

Cardiac troponin I is an indicator of myocardial damage, and previous studies have shown that dogs and humans with Chagas cardiomyopathy can have elevated cTnI concentrations. [24,69] We found that positive dogs had higher cTnI concentrations than discordant dogs ($p=0.06$) and negative dogs ($p=0.044$). cTnI can be elevated for multiple reasons associated with cardiac disease, so alone it is not a dependable test for Chagas disease screening. However, used in conjunction with serology and ECG, it provides insight into the presence of cardiac damage and could assist in diagnosis of Chagas disease. [24]

This study is limited in that ECGs were only taken from one time point. To better understand the clinical outcomes of *T. cruzi* infection it would be important to follow these dogs with additional ECGs to assess progression and variability at future time points. These dogs are working at checkpoints along the U.S. border and are likely exposed to other agents or environments that could impact cardiac health that could affect cTnI or arrhythmia results. To account for this potential variation we performed individual-level matching of positive and negative dogs in our study design, with the matching protocol accounting for breed, age,

sex, and geographic location. All DHS dogs were reported to receive monthly flea, tick and heartworm preventative and dogs had been tested annually or bi-annually for common parasites including *Dirofilaria immitis, Borrelia burgdorferi, Ehrlichia* spp., and *Anaplasma* spp. Additionally, to rule out cardiac abnormalities caused by these pathogens, we tested dogs using the IDEXX 4DX. The three dogs that were positive for *D. immitis* did not have ECG abnormalities and based on prior negative testing were thought to have been infected within the past year. One *T. cruzi* positive dog had antibodies to *Ehrlichia,* undetectable cTnI concentration (<0.006 ng/mL), and ECG abnormalities that included first degree AV block and VPCs (Lown score 2). A study of dogs naturally infected with *E. canis* found that 3.33% (n=150) of infected dogs had first degree AV block, and 2.7% had VPCs. [70] This study also found that dogs infected with *E. canis* had higher concentrations of cTnI than uninfected dogs. [70] While we cannot rule out that this dog's cardiac abnormalities were due to *E. canis*, the dog had a history of being infected with *T. cruzi* since 2013, whereas the infection with *E. canis* was likely in the past year since dogs are checked annually for *E. canis*. Furthermore, VPCs and AV block are frequently found in *T. cruzi* positive dogs with VPCs in up to 100% of infected dogs. [8,22,32] This study is further limited by the ECG tracings being read by automatic analysis. Although all tracings were reviewed and abnormalities confirmed by a board-certified veterinary cardiologist, it is possible abnormalities were missed in the automatic analysis. Finally, supraventricular premature beats were reported as being present or absent and the number/24 hours was not reported. A scoring system for supraventricular arrhythmias is not available and use of the modified Lown score was not readily applicable.

Understanding the cardiac manifestations of natural *T. cruzi* infections in dogs is critical for prognostication and also as a baseline for interpreting data from intervention studies. The DHS working dogs play a critical role in border security, cardiac manifestations of Chagas disease in these highly valued working dogs is likely associated with a yet-unquantified impact on their ability to work, economic and security consequences. As a model of naturally and locally-infected hosts across the landscape, understanding the epidemiology and clinical outcomes of *T. cruzi* infection in these dogs can advance not only veterinary but also human medicine.

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6. RISK FACTORS AND SELECT CARDIAC CHARACTERISTICS IN DOGS NATURALLY INFECTED WITH *TRYPANOSOMA CRUZI* PRESENTING TO A TEACHING HOSPITAL IN TEXAS[†]

6.1 Introduction

Infection with the protozoan parasite *Trypanosoma cruzi* causes Chagas disease in humans and animals across the Americas, and is the leading cause of infectious myocarditis in humans worldwide. [1] *T. cruzi* infection can occur by vector-mediated transmission by triatomine insects after the introduction of infected bug feces into the bite site or mucous membrane, through the ingestion of infected bugs or their feces, or through congenital transmission. [2,3] In both dogs and humans, clinical outcome is variable, ranging from sudden death to chronic heart disease, but also including asymptomatic infections. In some ecological settings dogs can be sentinels of human disease risk. [3,4] Chagas disease is increasingly recognized in the southern U.S., where the parasite is maintained among diverse wildlife in sylvatic transmission cycles. [2,5] The seroprevalence of *T. cruzi* in dogs is particularly high in the state of Texas, with seroprevalence estimates of 6-13% in shelter dogs across the state, 6-18% in government working dogs along the Texas-Mexico border, and seroprevalence exceeding 50% in some multi-dog kennel environments with triatomine infestations. [6–8] Seropositive dogs occur in Louisiana, Oklahoma, Tennessee, Georgia and Virginia. [9–15]

[†] Reprinted with permission from: Meyers A.C., Hamer S.A., Matthews D., Gordon S.G., Saunders A.B. 2019. Risk factors and select cardiac characteristics in dogs naturally infected with *Trypanosoma cruzi* presenting to a teaching hospital in Texas. *J Vet Intern Med*. 2019;1-12 Minor grammatical and syntactical changes have been made.

Clinical presentation of Chagas heart disease varies widely depending on the extent of myocardial damage that results in a combination of arrhythmias, conduction abnormalities, heart enlargement and heart failure. [16,17] There are four recognized pathogenic mechanisms for the development of Chagas heart disease in humans: 1) parasite induced damage to the cardiac tissue; 2) cardiac dysautonomia, microvascular circulation disturbances; 3) immune mediated myocardial injury; and 4) neurogenic disorders. [18,19] In the limited experimental models of Chagas disease in dogs, acute clinical signs were detected between 14-21 days post exposure. [20] Acute signs are non-specific and include lethargy, generalized lymphadenopathy, pale mucous membrane, slow capillary refill time, ascites, weak pulse, enlarged liver, enlarged spleen and sudden death. [20] During this stage arrhythmia and conduction abnormalities can be detected in infected dogs. [18,21] Both human and dogs can then enter an indeterminate period where clinical signs are not evident. [18,21] Chronic Chagas disease in dogs is associated with ventricular arrhythmias, predominantly premature contractions, which can be multiform and progress to ventricular tachycardia. [21] As chronic Chagas disease progresses the dogs could develop myocardial dysfunction and heart failure. [18,21–23] Experimental treatments, such as benznidazole in dogs can cause a temporary reduction in parasite load, but the treatment does not prevent myocardial lesions or dysfunction. [24,25] In the U.S. there are currently no vaccinations or approved anti-parasitic treatments for *T. cruzi* infections in dogs, and infected dogs are typically treated symptomatically.

Limited studies have been conducted in dogs naturally infected with *T. cruzi*, and a better understanding of Chagas disease in dogs is important for veterinarian medicine. *T. cruzi* infection is considered to be lifelong, with self-cure highly unlikely; [2,3] therefore dogs

with indirect fluorescent antibody (IFA) test IgG antibody titers are considered infected. [7,8] In this study dogs were counted as infected if their IFA IgG antibody titer was $\geq$ 20 or they had a histopathologic diagnosis of Chagas disease. The objective of this study was to report epidemiologic, cardiac troponin I (cTnI) and electrocardiographic characteristics, including presence of arrhythmia or conduction abnormality and approximate anatomic origin of the abnormality within the heart, associated with *T. cruzi* infection status in dogs presenting to a teaching hospital in Texas.

6.2 Materials and Methods

A retrospective review of electronic medical records was performed to identify all dogs evaluated at Texas A&M University's Small Animal Veterinary Medical Teaching Hospital (VMTH) in College Station, Texas that were tested for anti-*T. cruzi* antibodies or for which *T. cruzi* was detected using histology of cardiac tissue. The approximately 7-year period of analysis was January 21, 2010, through December 20, 2016. Data collected from the medical records on each dog included signalment (age, sex, breed), geographic location of residence, year referred, reason tested for *T. cruzi*, IFA test result, histopathology findings of *T. cruzi*, and as available, cTnl concentration and ECG abnormalities (listed below).

In the case of multiple *T. cruzi* serological results from different dates for the same dog, results were compared to assess concordance or discordance. For dogs with concordant results, clinical data taken at the time of the initial appointment were used in the analysis to determine risk factors and clinical outcomes. For dogs with discordant results (i.e., at least one positive and one negative test result from two different blood samples), data from the appointment with a positive titer was used for analysis.

The method of serological testing for *T. cruzi* in these dogs was an IFA performed by the Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL, College Station, TX), and samples with IgG titers of ≥ 20 were considered positive per TVMDL standard protocol; this cutoff has been used in dog and human studies.[7,26] In three dogs, no serologic testing was performed, but dogs were included in the analysis due to necropsy records indicating histopathological confirmation of intramyositic *T. cruzi* amastigotes. A positive serology result or histopathology diagnosis of Chagas disease qualified dogs as naturally infected for purposes of the analysis.

Cardiac troponin I concentrations were measured using one of two commercially available assays. For dogs admitted from 2010 to 2013, cTnI analysis was performed using a two-site chemiluminescent immunometric assay (Immulite 2000, Siemens Healthcare Diagnostics, Los Angeles, CA) at the Gastrointestinal Laboratory at Texas A&M University. The Immulite has a lower detection limit of 0.2 ng/mL, and has been validated in dogs.[27,28] Since 2013, the Gastrointestinal Laboratory measures serum cTnI concentrations with the ADVIA Centaur CP® immunoassay (Ultra-TnI, Siemens Medical Solutions USA, Inc., Malvern, PA). The reported range for cTnI by the manufacturer is 0.006 to 50.0 ng/mL, and has been validated in dogs.[29]

Electrocardiogram reports and traces were reviewed, abnormalities recorded and categorized as follows: presence of an abnormality (none detected, arrhythmia, conduction abnormality, or arrhythmia and conduction abnormality); and approximate anatomic location of the abnormality when present (atria including sinus node, atrioventricular (AV) node, ventricles, or combination). Conduction abnormalities included AV block, bundle branch block, and sinus node dysfunction.

To evaluate potential risk factors of *T. cruzi* infection, data were imported into RStudio 1.0.136 software. [30] Putative risk factors with respect to signalment were age at time of serology or histopathology testing, sex, American Kennel Club (AKC) breed group, year of evaluation and geographic location of residence (dogs from Texas were categorized into 10 ecoregions [31]; dogs from other states were grouped as "outside Texas" including Connecticut, Washington, Arkansas, Montana, Michigan, Oklahoma, California and Louisiana). For analysis, three ecoregions had less than 4 dogs each, so these dogs were grouped with the nearest ecoregion (Figure 6.1). Reason tested included arrhythmias-atrioventricular block (AVB); arrhythmias-ventricular; arrhythmias-other (including supraventricular premature contractions, atrial fibrillation, atrial standstill, sinus arrest, sinus bradycardia, sick sinus syndrome and right bundle branch block); congestive heart failure (CHF) (including left sided, right sided or both); littermate/housemate tested or affected; ventricular enlargement and systolic dysfunction; or other. Other included blood donor, esophageal disease, systemic illness or infectious disease. For cTnI concentration, two assays (Immulite 2000 and ADVIA Centaur CP® immunoassay) were analyzed independently, and divided into three categories: within the reference range of healthy dogs; [32,33] 1-2 times the upper reference limit; and $\geq$2 times the upper reference limit. For Immulite 2000 these values were $\leq$0.5 ng/mL, 0.51-1.0 ng/mL and $\geq$1.01 ng/mL, respectively. [28] In two studies in dogs without cardiac disease, cTnI concentration was not detectable in the majority (90-100%) of dogs at baseline evaluation using the Immulite assay. [32,33] Dogs with a concentration of cTnI above 1.01 ng/mL have more severe cardiac disease including arrhythmogenic right ventricular cardiomyopathy, ventricular tachycardia, AVB or neoplasia/myocardial infiltration and a significantly shorter median survival time compared

to dogs with cTnI ≤ 0.15 ng/mL using the Immulite Troponin I assay.[34] For the ADVIA Centaur CP® immunoassay these values were ≤0.128 ng/mL, 0.129-0.255 ng/mL and ≥0.256 ng/mL, respectively.[29] A cohort of healthy dogs had an average cTnI concentration of 0.017 ng/mL with a range of 0.006-0.128 ng/mL using the ADVIA Centaur CP® immunoassay.[29] Bivariable analysis using the chi-squared or Fisher's exact and T-test were used to identify factors with a $p \leq 0.25$; these factors were used in a logistic regression model. Additionally, linear regression was run on the number of dogs tested that were referred to VMTH (dependent) per year (explanatory) to determine if the number of dogs tested varied by year. Logistic and linear regression models were calculated and factors with values of $p < 0.05$ were considered significant. Odds ratios and 95% confidence intervals were calculated for logistic regression models.

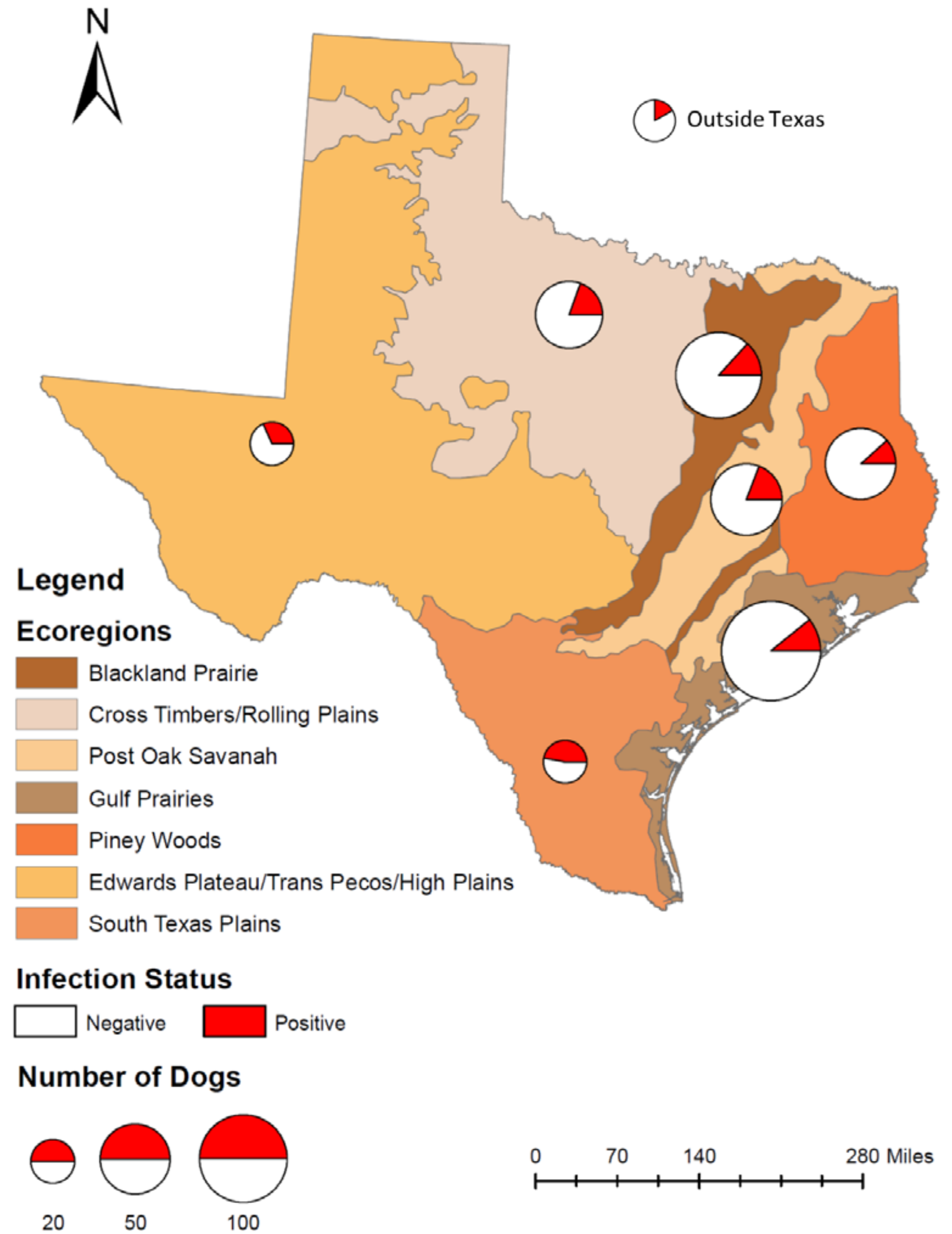

Figure 6.1. Distribution of *T. cruzi* infection in 375 dogs referred to Texas A&M University's Small Animal Veterinary Medical Teaching Hospital between 2010-2016 that received *T. cruzi* serology testing or had *T. cruzi* histological findings across Texas ecoregions. Twelve dogs resided outside Texas and percent positive are depicted by a pie chart. Circles are proportional to the number of dogs sampled per ecoregion. Map was created using ArcGIS and the base layer is from Gould Ecological Regions created by Texas Parks and Wildlife Department GIS lab, downloaded from http://aampo-mpo.opendata.arcgis.com/.

6.3 Results

6.3.1 Study population

A total of 375 dogs had serological testing for anti-*T. cruzi* antibodies or *T. cruzi*-positive histology findings during the approximately 7-year study period. Dogs were considered to be infected if they had an IgG titer ≥ 20 or histologic evidence of *T. cruzi*. Of the 375 dogs, 63 (16.8%) were considered to be infected, including 59 which had a positive serology test result only, and four with a histological diagnosis of Chagas disease. Of the four dogs with a histologic diagnosis, one had a negative IgG titer which is most likely a consequence of an acute infection and insufficient time for seroconversion. [35] Thirty dogs had multiple visits with repeat serological testing, including 20 with negative serology at both evaluations, five with positive titers at the first appointment and negative results at the second appointment (i.e., seroreversion), and five with negative titers at the first appointment and positive titers at the second appointment (i.e., seroconversion).

In the total population tested, age ranged from 0.2 to 16 years (median of 7.6 years, mean of 7.1 years), and 91 dog breeds were represented. The most common breeds tested were Labrador retrievers (14.4%) and boxers (10.6%). Negative dogs had a higher mean age (7.4 years) than infected dogs (5.9 years; p=0.006), in which the odds of infection are 0.91 times lower for each year increase in age (odds ratio [OR] 0.91, 95% CI 0.85-0.97, p=0.0069). In this referral population, the breeds with the highest percent of infected dogs were Bulldogs (English and French) (7/14), German Shepherd (3/10), Chihuahua and Australian Heeler (both 2/7), and Doberman Pinscher (2/8) (Table 6.1). The most common AKC breed group tested was sporting dogs, which comprised 26% of the sampled dogs (Table 6.2). The non-sporting breed group had the highest percentage of dogs that were

infected (29%) followed by the toy breed group (24%), although there were no statistically significant associations between breed group and *T. cruzi* infection status. The most frequent reason why dogs in the toy and non-sporting breed group were tested for *T. cruzi* were presence of AV block (toy; 60%, non-sporting; 49%) and ventricular arrhythmias (toy; 33%, non-sporting; 31%). There were 207 males (55.2%) and 168 females (44.8%) tested with no difference in the proportion of infected dogs by sex (p=0.69; Table 6.2).

Table 6.1. Breed characteristics for 375 dogs that received *T. cruzi* serology testing or had *T. cruzi* histological findings between 2010-2016. Breeds with less than 5 dogs total are grouped into "other".

Breed	Breed Group	Sample size No. (%)	Positive *T. cruzi* infection No. (%)	Positive dogs with ECG abnormalities at the level of ventricles (%)
Bulldog-English/French	Non-Sporting	14 (3.7)	7(53.9)	5 (83.3)*
German Shepherd	Herding	10 (2.7)	3 (30.0)	3 (100.0)
Chihuahua	Toy	7 (1.9)	2 (28.6)	1 (50.0)
Australian Heeler	Herding	7 (1.9)	2 (28.6)	1 (50.0)
Doberman Pinscher	Working	8 (2.1)	2 (25.0)	1 (50.0)
Border Collie	Herding	9 (2.4)	2 (22.2)	1 (100.0)*
German Short Haired Pointer	Sporting	10 (2.7)	2 (20.0)	- *
Dachshund	Hound	5 (1.3)	1 (20.0)	1 (100.0)
Spaniel - Cavalier	Toy	10 (2.7)	2 (20.0)	1 (100.0)*
American Pit Bull Terrier	Terrier	12 (3.2)	2 (16.7)	1 (100.0)*
Australian Shepherd	Herding	12 (3.2)	2 (16.7)	2 (100.0)
Labrador Retriever	Sporting	54 (14.4)	9 (16.7)	4 (50.0)*
Yorkshire Terrier	Toy	7 (1.9)	1 (14.3)	- *
Mixed	N/A	26 (6.9)	3 (11.4)	3 (100.0)
Golden Retriever	Sporting	9 (2.4)	1 (11.1)	0 (0.0)
Boxer	Working	41 (10.9)	3 (7.3)	1 (50.0)*
Rhodesian Ridgeback	Hound	6 (1.6)	0 (0.0)	-
Boston Terrier	Non-Sporting	6 (1.6)	0 (0.0)	-
Other	N/A	122 (32.5)	19 (15.6)	9 (64.3)

* not all positive dogs had an ECG performed

Twelve dogs presented for evaluation from outside of Texas, while the remaining 363 dogs resided in 70 Texas counties spanning all ecoregions.[31] Infected dogs were from 33

counties, and 8 ecoregions. Bivariable analysis demonstrated a significant difference in infection status across ecoregion (p=0.014), in which the highest proportion (47.4%) of infected dogs came from the South Texas Plains ecoregion followed by Edwards Plateau (31.6%; Table 6.2).

Table 6.2. Results of bivariable and logistic regression analysis of signalment, year of evaluation and geographic location of residence for 375 dogs that received *T. cruzi* serology testing or had *T. cruzi* histological findings between 2010-2016.

Variable		Sample size No. (%)	Positive *T. cruzi* infection No. (%)	Bivariable P-Value	Logistic Regression		
					Odds Ratio	95% confidence interval	P-Value
Sex				0.69			
	Male	207(55.2)	38(18.4)		-	-	-
	Female	168 (44.8)	25(14.9)				
AKC Breed Group				0.29*			
	Stock Service	2(0.5)	0(0.0)		-	-	-
	Herding	48(12.8)	10(20.8)		-	-	-
	Hound	20(5.3)	3(15.0)		-	-	-
	Mixed	26(6.9)	3(11.5)		-	-	-
	Non-Sporting	35(9.3)	10(28.6)		-	-	-
	Sporting	96(25.6)	16(16.7)		-	-	-
	Terrier	30(8.0)	2(6.7)		-	-	-
	Toy	42(11.2)	10(23.8)		-	-	-
	Working	76(20.3)	9(11.8)		-	-	-
Ecoregion				0.013*			
	Outside Texas	12(3.2)	2(16.7)		*referent*		
	Blackland Prairie	75(20.0)	10(13.3)		0.79	0.17-5.73	0.79
	Cross Timbers	46(12.3)	9(19.6)		1.34	0.28-9.92	0.73
	Edwards Plateau	19(5.1)	6(31.6)		2.33	0.41-18.72	0.36
	Gulf Prairie	101(26.9)	11(10.9)		0.67	0.14-4.79	0.63
	Piney Woods	51(13.6)	6(11.8)		0.74	0.14-5.63	0.74
	Post Oak Savanah	52(13.9)	10(19.2)		1.41	0.30-10.31	0.69
	South Texas Plains	19(5.1)	9(47.4)		4.70	0.88-37.28	0.092
Year				0.043			
	2010	60(16.1)	14(23.3)		*referent*		
	2011	59(15.7)	10(16.9)		0.67	0.26-1.70	0.41
	2012	42(11.2)	7(16.7)		0.64	0.22-1.79	0.41
	2013	48(12.8)	13(27.1)		1.17	0.47-2.93	0.73
	2014	57(15.2)	11(19.3)		0.68	0.26-1.71	0.41
	2015	56(14.9)	3(5.4)		0.20	0.04-0.68	0.017
	2016	53(14.1)	5(9.4)		0.30	0.09-0.90	0.039

*expected cell count in the contingency table <5, Fisher's exact test reported instead of chi square

Between 2010-2016, 42 to 60 dogs per year had serological tests or histological findings of *T. cruzi* and the proportion of infected dogs varied across years from 5-27% (p=0.043; Table 6.2). The odds of infection in dogs in the last two years of the study decreased and were 3.3 (2016) to 5.0 (2015) times lower than the initial year of study in 2010 (OR 0.20-0.30, 95% CI 0.04-0.9, p=0.017-0.039, Table 6.2). In a linear regression analysis, the overall number of dogs tested did not vary significantly across year (p=0.76).

6.3.2 Reason for testing

Overall, ventricular arrhythmias were the primary reason for testing for *T. cruzi* infection in the total population (175/375, 46.7% of dogs, Table 6.3). Among dogs that tested positive, the predominant reasons for testing was the presence of an infected littermate or housemate (8/12, 67%), CHF (left sided, right sided, both) (10/25, 40%), and ventricular enlargement with systolic dysfunction (11/40, 28%). Overall, eight dogs had both CHF and ventricular enlargement with systolic dysfunction, and 5 of them were considered infected. *T. cruzi* infection was significantly higher in dogs with CHF as the reason for testing (10/25, 40%, p=0.0037) than those that did not report CHF (53/350, 15.1%) in which the odds of a being infected with *T. cruzi* were over 4 times greater in dogs with CHF (OR 4.34 95% CI 1.79-10.17, p<0.001, Table 6.3). *T. cruzi* infection was significantly different between dogs with a known infected house or littermate (8/12, 67%, p<0.001) and dogs that did not report infected house or littermates as the reason for testing (55/363, 15.7%). The odds of infection were 13 times higher in dogs that had a known infected house or littermate than among dogs that did not report an infected house or littermate (OR 13.0, 95% CI 3.94-50.45, p<0.001, Table 6.3). Overall, there was no significant difference between infected and negative dogs

when reason for testing including the presence of arrhythmias (AV block, ventricular, and 'other' arrhythmias) or 'other' reason for testing.

Table 6.3. Results of bivariable and logistic regression analysis of reason tested for *T. cruzi* infection in 375 dogs that had *T. cruzi* serology performed or *T. cruzi* histological findings between 2010-2016.

				Logistic Regression		
Variable	**Sample size No. (%)**	**Positive *T. cruzi* infection No. %**	**Bivariable P-Value**	**Odds Ratio**	**95% confidence interval**	**P-Value**
Arrhythmias-AVB			0.28	-	-	-
Present	120(32.0)	16(13.3)				
Absent	255(68.0)	47(18.4)				
Arrhythmias-Ventricular			0.39	-	-	-
Present	175(46.7)	33(18.9)				
Absent	200(53.3)	30(15.0)				
Arrhythmias-Other			0.24	-	-	-
Present	57(15.2)	6(10.5)				
Absent	318(84.8)	57(17.9)				
Congested Heart Failure			0.0037*			
Present	25(6.6)	10(40.0)		4.34	1.79-10.17	<0.001
Absent	350(93.3)	53(15.1)		*referent*		
Littermate/Housemate Tested			<0.001*			
Present	12(3.2)	8(66.7)		13.00	3.94-50.45	<0.001
Absent	363(96.8)	55(15.2)		*referent*		
Heart Enlargement/Systolic dysfunction			0.091	-	-	-
Present	40(10.7)	11(27.5)				
Absent	335(89.3)	52(15.5)				
Other			0.52	-	-	-
Present	48(12.8)	6(12.5)				
Absent	327(87.2)	57(17.4)				

*expected cell count in the contingency table <5, Fisher's exact test reported instead of chi square

6.3.3 Electrocardiographic and cardiac troponin I findings

Data assessed included ECG abnormalities and cTnI concentration. ECG abnormalities were approximate anatomic location in the heart in three parts defined as 1) atria which includes sinus node, 2) AV node, or 3) ventricles and type of abnormality. In

bivariable analysis the *T. cruzi* infection status was not significantly associated (p=0.73) with the presence or absence of an ECG abnormality (Table 6.4). Dogs with ECG abnormalities (N=289) were analyzed using bivariable analysis as either having an abnormality at a specified anatomic location or not. For those dogs with abnormalities, infection with *T. cruzi* was significantly higher in dogs with an abnormality that originated from the ventricles (21.8%, p=0.027, Table 6.4) than from the AV node or atria (11.3%). The odds of *T. cruzi* infection were approximately 2 times greater (OR 2.19, 95% CI 1.15-4.33, p=0.019) among dogs that had ventricular arrhythmias than among dogs that had arrhythmias at other anatomic locations. *T. cruzi* infected dogs had significantly more anatomic locations (2 or 3 out of 3) in which an ECG abnormality was present (p=0.0062, Table 6.4). The odds of being infected were approximately 3 times greater (OR 2.91, 95% CI 1.37-5.99, p=0.0042) among dogs that had abnormalities at 2 or more anatomic locations than among dogs that had abnormalities at only one location. Only one *T. cruzi* negative dog had ECG abnormalities at all 3 anatomic locations. In bivariable analysis significant association (p=0.013) between *T. cruzi* infection status and the type of ECG abnormality (none, conduction, arrhythmia, or both) was found, with infection being highest in dogs with both a conduction abnormality and arrhythmia (32%) and infection lowest in dogs having only a conduction abnormality (8.8%; Table 6.4). Overall, the percent of infected dogs is significantly lower in the population of dogs that have only conduction abnormalities without arrhythmias (8.8%) than in the population of dogs without any type of abnormality (20%, OR 0.39, 95% CI 0.15-0.98, p=0.046).

Table 6.4. Results of bivariable and logistic regression analysis of ECG findings and cTnI concentrations for dogs that had *T. cruzi* serology performed or *T. cruzi* histological findings between 2010-2016. The lowest range of cTnI was used as the referent in logistic regression.

Variable	Sample size No. (%)	Positive *T. cruzi* infection No. %	Bivariable P-Value	Odds Ratio	95% confidence interval	P-Value
Troponin I concentration						
Immulite N=107			0.19*			
≤ 0.5 ng/mL	58(15.5)	14(24.1)		*referent*		
0.51-1.0 ng/mL	16(4.3)	3(18.8)		0.73	0.15-2.66	0.65
>1.01 ng/mL	33(8.8)	3(9.1)		0.31	0.068-1.06	0.088
Advia N=160			0.0012*			
≤ 0.128 ng/mL	46(12.3)	1(2.2)		*referent*		
0.129-0.255 ng/mL	26(6.9)	5(19.2)		10.71	1.60-212.21	0.035
>0.256 ng/mL	88(23.5)	22(25.0)		14.99	2.98-273.40	0.0093
Not tested or either assay	*108(28.8)*	*15(13.9)*				
Electrocardiographic results						
Abnormality N=375			0.73	-	-	-
Present	289(77.1)	49(17.0)				
Absent	66(17.6)	13(19.7)				
Not tested	*20(5.3)*	*1(5.0)*				
Location of abnormality N=289 †						
Atria	53(18.3)	12(22.6)	0.31	-	-	-
Other location	236(81.6)	37(15.7)				
AV node	124(42.9)	17(13.7)	0.26	-	-	-
Other location	165(57.1)	32(19.4)				
Ventricles	156(54.0)	34(21.8)	0.027	2.19	1.15-4.33	0.019
Other location	133(46.0)	15(11.3)		*referent*		
No. of locations abnormalities present N=289						
1	246 (85.1)	35 (14.2)	0.0062	*referent*		
≥2	43 (14.9)	14 (32.5)		2.91	1.37-5.99	0.0042
Type of Abnormality N=355			0.013			
None	66(18.6)	13(19.7)		*referent*		
Conduction	102(28.7)	9(8.8)		0.39	0.15-0.98	0.046
Arrhythmia	159(44.8)	31(19.5)		0.98	0.49-2.09	0.97
Both present	28(7.9)	9(32.1)		1.93	0.70-5.24	0.20

†When dogs had abnormalities present at more than one location, it was counted in both locations

*expected cell count in the contingency table <5, Fisher's exact test reported instead of chi square

Of the 375 dogs, 267 dogs had cTnI testing performed, with 107 tested on the Immulite 2000, and 160 tested on the ADVIA Centaur CP® immunoassay. For the dog samples run on the Immulite, the cTnI concentration ranged from 0.19 ng/mL to 104.0 ng/mL with an overall median of 0.45 ng/mL, mean of 3.95 ng/mL and IQR 0.19-1.79 ng/mL. Twenty-eight of these dogs had cTnI concentrations below the limit of detection. Using bivariable analysis, no significant difference was found between *T. cruzi* infected dogs and the three pre-defined ranges of cTnI concentration (≤0.5 ng/mL, 0.51-1.0 ng/mL and ≥1.01 ng/mL, p=0.19). No significant difference was found between *T. cruzi* infected and negative dogs with cTnI concentrations of 0.51-1.0 ng/mL and ≤0.5 ng/mL. On the dog samples analyzed on the ADVIA Centaur CP® immunoassay, cTnI concentration ranged from <0.006 ng/mL to 51.0 ng/mL with an overall median of 0.31 ng/mL, mean of 2.51 ng/mL and IQR of 0.11-1.48 ng/mL. Three dogs had concentrations below the limit of detection. Bivariable analysis demonstrated a significant difference between the three ranges of cTnI concentration ≤0.128 ng/mL, 0.129-0.255 ng/mL and ≥0.256 ng/mL) and *T. cruzi* infection (p=0.0012). Furthermore, the odds of being infected were approximately 11 and 15 times greater in dogs with cTnI concentrations of 0.129-0.255 ng/mL and ≥0.256 ng/mL, respectively (OR 10.71, 95% CI 1.60-212.21, p=0.035; OR 14.99, 95% CI 2.98-273.40, p=0.0093) than among dogs that had cTnI concentrations of ≤0.128 ng/mL.

6.4 Discussion

This study demonstrated that dogs infected with *T. cruzi* are found in all ecoregions in Texas and in many breed groups including breeds affected by well described heart diseases that mimic Chagas disease. Further, a clinical index of suspicion is not well defined and understood in dogs with Chagas disease and an understanding of risk factors for infection and

the pathogenesis of chronic Chagas cardiomyopathy will be useful for diagnosis and development of treatment.

T. cruzi infection has been found in over 48 different breeds in the U.S. with a high prevalence in the AKC sporting and working breed groups. [14,36,37] In contrast, we found the highest prevalence in non-sporting (29%), toy (24%) and herding (21%) breed groups. This difference is likely due to these dogs being a part of a referral population, where multiple breeds with a predisposition for acquired heart diseases that result in a similar phenotype as Chagas heart disease are included in this population. For example, German Shepherds (30% positive in this population) that can develop breed related inherited ventricular arrhythmias and sudden death at a very young age, Doberman Pinschers (25% positive) predisposed to idiopathic dilated cardiomyopathy characterized by cardiac enlargement, ventricular myocardial dysfunction, ventricular arrhythmias, CHF and sudden death, and Bulldogs (54% positive) and Boxers (7% positive) that can develop arrhythmogenic right ventricular cardiomyopathy. [38–41] This is an important point to consider when making a diagnosis of a heart disease that can mimic Chagas disease in a region known to have Chagas disease, as discrimination between Chagas disease and other etiologies of arrhythmias and ventricular systolic dysfunction could be difficult. Since *T. cruzi* infection is widespread and all breeds are at risk, it is important for veterinarians to consider *T. cruzi* testing in any dog when there is a clinical index of suspicion, including dogs with known risks for breed related heart diseases.

T. cruzi positive dogs were reported from all 10 ecoregions present in Texas, demonstrating that *T. cruzi* is widespread across Texas. Occurrence of *T. cruzi* infection in dogs in Texas is well documented in the U.S., and Texas could be a hotspot for infection with

seven triatomines capable of transmission, the highest nationwide.[2,7,8,36,37,42–46] The high prevalence of dogs infected with *T. cruzi* likely demonstrates an established enzootic transmission cycle, as Texas harbors the highest species diversity of triatomine vectors that can transmit *T. cruzi* and a diversity of wildlife reservoirs.[2,5,47] The South Texas Plains ecoregion had the highest percent of infected dogs (47%), while the next highest region was Edwards Plateau (32%). There is a greater than expected density of *Triatoma gerstaeckeri* in south central Texas, which is where we found the highest percent of infected dogs.[48] There is a significant difference between infection in triatomines collected from different ecoregions ($p<0.001$), where triatomines collected from Edwards Plateau had the highest infection prevalence of 65.0% (n=562).[47] The high density of infected vectors in these areas could increase exposure and infection in dogs. Furthermore, there is a high *T. cruzi* infection rate in dogs from south central Texas which includes the border with Mexico (this is the south Texas Plains ecoregion).[7,8,36,43] In a population of dogs with *T. cruzi* testing from 1993-2007 over 60% of the cases came from central and southern Texas.[36] Similarly, working hound dogs in south central Texas found a seroprevalence of 57.6% (n=85).[7] Finally, dogs in the Lower Rio Grande Valley havea seroprevalence of 19.6% (n=209).[43] This information suggests all regions of Texas and surrounding states should be involved in future efforts for increasing veterinary and public awareness for Chagas disease. It is important to note that specific ecoregions can be even broader than reported here since dogs were not confirmed to have lived in only one site for the entirety of their lives and dogs are known to move and travel.

The overall number of dogs tested stayed consistent over the years ($p=0.76$). This temporal difference could reflect differences in the referral population over time, or could

reflect the biology of the disease system. For example, in a multi-year (2013-2016) citizen science initiative to collect triatomines across Texas, the infection prevalence in vectors was highest in 2013 and decreased thereafter, authors suggested the decline in infection was due to climate variability. [47] Such fluctuations in the population of infected vectors in nature could account for temporal variation in infection in dogs.

The principal reason reported for testing in *T. cruzi*-infected dogs was the presence of an infected littermate or housemate (67%), and the odds of being infected were 13 times greater among dogs that had an infected litter or housemate. *T. cruzi* can be transmitted congenitally from dam to pup and has been shown to infect multiple littermates. [49,50] While direct dog-to-dog transmission through blood contact is unlikely, an infected housemate typically indicates that infected vectors are in the dog's environment, in the absence of travel history. In Argentina the presence of at least one infected dog in a household is significantly associated with more infected dogs in the same household. [51] Reasons for testing for *T. cruzi* infection including CHF (p=0.0037), ventricular enlargement and systolic dysfunction and a variety of ECG abnormalities including AVB and sinus node dysfunction are based on clinical index of suspicion and include consideration of other factors such as age of the dog or unusual breed in context in an individual case.

Overall, *T. cruzi* infection status was not significantly associated (p=0.73) with the presence or absence of an ECG abnormality, demonstrating that *T. cruzi* infection is one of many reasons for ECG abnormalities in tested dogs. While infected dogs were more likely to have ventricular arrhythmias and had significantly more combinations of ECG abnormalities, there were 13 infected dogs (20%) without an ECG abnormality detected. When abnormalities were analyzed based on the location (atria, AV node or ventricles), the odds of

a *T. cruzi*-infected dog having ventricular arrhythmias were two times greater (p=0.019) than other anatomic locations. In an experimental infection of dogs with *T. cruzi*, the chronic stage of Chagas disease was characterized by the development of ventricular premature contractions and ventricular tachycardia.[21] Similarly in humans with Chagas disease ventricular premature contractions are one of the most common ECG abnormalities.[52] Mechanisms of myocardial damage in *T. cruzi* infected dog and human patients include focal inflammation, diffuse fibrosis, edema, and scarring of the myocardium and conduction system.[35,53,54] This destruction can lead to a combinations of arrhythmias, conduction disturbances, myocardial dysfunction and heart failure in Chagas patients.

T. cruzi tissue tropism is primarily in the myocardium and can cause an inflammatory response that results in myocyte damage. cTnI is exclusively in cardiac myocytes and is released immediately after cardiac injury making it a sensitive and specific marker of myocardial injury. Cardiac troponin I is an indicator of myocardial damage, and Chagas cardiomyopathy can have elevated cTnI concentration in dogs and humans.[35,55] Serum cTnI levels slowly increase in infected dogs and spike at 10-30 mg/mL at approximately 21 days after infection.[56] Furthermore, cTnI serves as a rigorous biomarker since healthy individuals have negligible levels and cTnI circulates for days to weeks after cardiac injury,[57] and is a useful biomarker for Chagas disease development since it is minimally invasive. Paired with ECG results and serology, cTnI can help quantify infection and provide a more thorough prognosis. For dogs that were evaluated between 2010-2013 and cTnI concentrations were tested on the Immulite 2000, 24% of *T. cruzi* positive dogs fell into the lowest concentration of cTnI ($\leq$0.5 ng/mL), while only 9.1% of the dogs in the highest group ($\geq$1.01 ng/mL) were *T. cruzi* positive. The odds of having cTnI concentration of $\geq$1.01 ng/mL were 0.31 times

lower in the *T. cruzi* infected dogs than in the uninfected dogs. Because this population was referred to the cardiology service many of the dogs have cardiac abnormalities which can also cause elevated cTnI. Starting in 2013, cTnI concentration was tested on a more sensitive assay with a lower limit of detection of <0.006 ng/mL. With this assay, the majority of dogs had detectable cTnI, and *T. cruzi* infected dogs were significantly more likely to have cTnI concentrations in the elevated ranges of 0.129-0.255 ng/mL (19%) and ≥ 0.256 ng/mL (25%). Approximately 18% of dogs on both the Immunite 2000 and Advia assay fell in to the middle concentration of cTnI and were *T. cruzi* positive. Chagas disease can effect cardiac health acutely or over a long period. [18,20,21] This finding of infected dogs with cTnI concentrations in the middle range could indicate low levels of cardiac damage over time and even damage as histopathology demonstrated in an infected litter of Boxer puppies. [35] Chagas disease is one potential cause of elevated cTnI concentrations when considered in conjunction with other signs and should be taken into consideration while evaluating the overall health of the dog.

Regarding signalment as a risk factor for infection, *T. cruzi* in this population was widespread and not disproportionately associated with any breed group or sex. Older dogs have a higher prevalence of *T. cruzi* infection then younger dogs, likely because older dogs have had more opportunities for exposure. [5,8,14,49] In our study population, infected dogs were significantly younger (mean=5.9 years, $p=0.006$) than negative dogs (mean=7.4 years). Dogs presenting to a teaching hospital could be more likely to have clinical disease. During an experimental infection of dogs, clinical signs were more severe in younger infected dogs then older dogs, and dogs inoculated at an earlier age had higher parasitemia than older dogs. [20]

Five dogs had positive titers ranging from 1:20 to 1:160 at the first appointment and had negative results at the second appointment. Seroreversion in the absence of treatment

occurs in mice, humans and dogs. [3,49,58–62] Few studies have reported on seroreversion in dogs. One study reported 6% (2/36) of dogs demonstrated seroreversion. [3] Another study found 5% (1/21) dogs seroreverted; however, this dog was concurrently positive by xenodiagnoses, and then became seropositive again two years later. [49] In two dogs with an IFA titer of 1:20 in the present study, it is possible that the change in result is due to inter-observer variability or test cross reaction. [63] Host biological factors such as exposure history, coinfection and genetic makeup could have an effect on reaction variability of the IFA. Further characterization of IFA test variability in dogs is needed, as IFA is the only available test for diagnosis in dogs and test variability is not established.

This study is limited in that it is retrospective and the quality of the data could vary, and in some cases, data was missing. Despite missing data for some variables, the sample sizes for each variable were relatively large and allowed for reliable statistical analysis (smallest sample size n=267). Furthermore, this study is limited in that the majority of dogs were referred to the hospital causing a selection bias. This limits the ability to apply the findings to the general dog population, however this study still provides important insight into risk factors associated with *T. cruzi* infection. This study is further limited by being centered on dogs from Texas (n=363), however dogs from eight other states were included, and Texas has a high prevalence of Chagas disease in dogs making it ideal for a retrospective study. [2] Other limitations include changes in the cTnI assay, from the Immulite 2000 with an analytical sensitivity of 0.2 ng/mL to the more sensitive ADVIA Centaur CP® Ultra-TnI immunoassay which detects cTnI to 0.006 ng/mL. Due to the change in assay during the seven-year study, we analyzed the concentrations of cTnI separately, and the results cannot be directly compared between assays. One limitation to *T. cruzi* testing in dogs that there is

no gold standard for diagnosis and discordant tests results are common.[8,10] The use of multiple serology assays to determine positivity can be useful[7,8,37,64–67] and is in accordance with the World Health Organization guidelines for human medicine.[68] Unfortunately, using multiple serology assays is not possible for veterinary medicine since the IFA is the only test approved for use in dogs. Therefore the true infection rate in dogs is not known. While other tests have been validated in dogs, they are not clinically available for diagnosis.[10] In contrast, human medicine relies on a suite of serology assays to determine if a patient is positive. The ECG abnormalities reported here did not include 24-hour ambulatory ECG analysis which could improve detection of abnormalities. Finally, detailed classification of echocardiographic abnormalities was not specifically assessed in this study because of the wide variety of diseases present in this population.

In summary, dogs infected with *T. cruzi* based on a titer $\geq$ 20 or histologic evidence, were identified in all ecoregions in Texas and in a variety of breed groups. Dogs from breeds affected by well described heart diseases that mimic Chagas heart disease can test positive for Chagas disease. The most common reasons for testing were an affected litter or house mate and the presence of cardiac abnormalities (CHF, ventricular enlargement with systolic dysfunction). Infected dogs were more likely to have ventricular arrhythmias and combinations of ECG abnormalities were commonly diagnosed. These findings suggest a need for an increased awareness of Chagas disease in dogs including knowledge of when to consider testing for it.

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7. SUMMARY

The Chagas disease system is characterized by complex vector-host-parasite interactions and the clinical outcomes of Chagas disease are not well understood in humans or dogs. In humans, Chagas disease is a major cause or morbidity and mortality in Latin America, and is the leading cause of infectious heart disease across the Americas. Domestic dogs play an important role in the ecology and epidemiology of Chagas disease throughout the Americas. Dogs are considered an important domestic reservoir of *T. cruzi* and a sentinel for human infection in South and Central America, but the significance of dogs as a sentinel and reservoir in the U.S. warrants further investigation. Further, dogs are considered good models hosts to study the pathogenesis of Chagas disease- particularly the mechanisms involved with chronic Chagas disease. Our ecological aims included characterizing the triatomine vector communities in environments where dogs live and quantify the *T. cruzi* infection prevalence and genetic parasite strains, and determine the host feeding patterns of the vector. Our clinical aims included to elucidate the relationships between markers of exposure, circulating parasite infection, strain type, and cardiac abnormalities in populations of naturally-infected dogs using both cross sectional and retrospective study designs.

Chagas disease is increasingly recognized in the southern U.S., where triatomine vectors transmit the parasite among wildlife and domestic dogs with occasional spillover to humans. Understanding vector-host interactions is critical for optimizing vector control efforts. Ecological tracking of the host-vector-parasite interactions provides insight on the sylvatic maintenance and spillover risk of *T. cruzi*. For the ecological objectives, we studied *T. cruzi* transmission in Big Bend National Park in west Texas where triatomines are

endemic and park visitors may be at risk for exposure. We received 461 triatomines of three species (*Triatoma rubida*, *T. gerstaeckeri*, and *T. protracta)* through a citizen science collection from Big Bend National Park. Triatomine encounters peaked in June of each year (44.3% of collections) when recreational activities are frequent, potentially increase human exposure. We found an infection prevalence of 23.1% in adult triatomines (n = 320) and TcI was the only *T. cruzi* strain detected. Blood meal analyses revealed a complex transmission cycle, involving a diversity of wildlife and domestic animals, as well as humans in the Big Bend National Park. Domestic dogs appear to play a key role in transmission in the peridomestic setting in Big Bend National Park, evident by dog being the most common blood meal source we identified in triatomines and a 28.6% seroprevalence in 14 dogs. These findings reveal interactions between infected triatomines, humans, dogs, and wildlife in and around Big Bend National Park, with potential risk of human disease.

To further quantify *T. cruzi* infection prevalence, strain types, and risk factors in dogs we sampled 528 government working dogs in 2015-206 and characterized associated triatomine vectors along the Texas-Mexico border. These dogs showed significant variation in seroprevalence across different management areas in Texas 18.3-26.7% (P=0.02-0.04) compared to 11.6% seroprevalence in young dogs at a training center based three independent serological tests. We found three dogs (0.6%) had parasite DNA in the blood, including TcI and TcI/TcIV mix. Nine of 20 (45%) *T. gerstaeckeri* and *T. rubida* from the canine environments were infected with TcI and TcIV; insects analyzed for bloodmeals (n=11) fed primarily on canine (54.5%) but also human and wildlife. The seroprevalence in these working dogs was in line with previous findings of vulnerable dog populations, such as shelter and stray dogs, that live outdoors. This high seroprevalence could be due to the

government dogs working and sleeping outdoors in areas endemic for *T. cruzi* increasing their exposure.

Many of the government working dogs training in Texas, so to determine if they were infected nationwide, we expanded our sampling to include a total of 1,660 working dogs from 43 states using three independent serological tests to detect an overall seroprevalence of 7.3% (CI: 6.1-8.6%). This finding suggests that mandatory canine training in the south prior to deployment, where dogs may encounter vectors, is a risk factor for infection. Determining the prevalence of *T. cruzi* in these working dogs and looking at spatially associated risk factors has practical implications for disease risk management and could assist with improved control measures to protect both animal and human health.

Working in a hotspot of local transmission in the southern U.S. in the state of Texas, we tracked a cohort of 41 working dogs along the Texas-Mexico border comprised of naturally-infected dogs and age/sex/breed/location-matched negative dogs. Our objective was to determine the extent to which electrocardiogram (ECG) abnormalities and elevated cardiac troponin I (cTnI; a biomarker for cardiac injury) are associated with *T. cruzi* infection. We found that the presence and number of ECG abnormalities was higher in infected than uninfected dogs ($p<0.0001$). Furthermore, cTnI concentrations were higher in seropositive than seronegative dogs ($p=0.044$), indicating more severe cardiac injury in positive dogs. Furthermore, we examined a population of 'discordant' dogs – dogs that were positive on a single but not multiple independent tests- and found that they were somewhat intermediary with respect to their clinical status, but further investigation is needed on a larger sample size. Using naturally-infected dogs as model host, which are exposed to the locally relevant parasite strains in the southern US, these data expand the understanding of the variation in

clinical outcomes of *T. cruzi* infections with relevance for both veterinary and human medicine.

Finally, we performed a retrospective study to report epidemiologic and select cardiac characteristics associated with *T. cruzi* infection in 375 dogs presenting to a teaching hospital in Texas. Data analyzed from medical records revealed dogs infected with *T. cruzi* were identified in all ecoregions in Texas in a diversity of breed groups- including breeds affected by well described heart diseases that mimic Chagas disease. The most common reasons for testing were an affected litter or house mate and the presence of cardiac abnormalities (CHF, ventricular enlargement with systolic dysfunction). Infected dogs were more likely to have ventricular arrhythmias and combinations of ECG abnormalities were commonly diagnosed. These results help define a clinical index of suspicion for *T. cruzi* testing. This suggests a need for increased awareness of Chagas disease in dogs presenting with heart disease, including knowledge of when to consider testing.

This research advances our knowledge of the Chagas disease system through a comprehensive examination of host, vector, parasite, and environment. This study aimed to characterize the epidemiology and cardiac abnormalities of infected dogs and develop prognostic indicators of disease progression. The distribution of cases, prevalence and clinical outcomes described here are a reference for future studies determining prognostic indicators for Chagas disease and establishing a baseline for drug and vaccine studies for both human and canine medicine. These findings will help build awareness of *T. cruzi* infection, triatomine vectors and risk factors among veterinarians, physicians and public health practitioners.

APPENDIX

Table S1. *Trypanosoma cruzi* infection status of domestic dogs in Big Bend National Park, 2016. Using the criterion of positivity on at least two of the three serological tests, 28.6% of dogs were seropositive. None of the 14 dogs had *T. cruzi* DNA in the blood at the time of sampling. (Robles-Curtis et al. 2018).

Dog	Breed	Sex	Age (yrs)	Sleeping site (night)	Duration in Park (yrs)	Chagas Stat-Pak	Trypanosoma Detect	IFA Titer	Overall Serology Result	qPCR Result
1	Cocker Spaniel/Golden retriever mix	F	13	Indoor	1.5	-	-	-	-	-
2	Pomeranian	M	5	Indoor	1.5	+	+	-	+	-
3	Blue Heeler	F	8	Indoor/ outdoor	8	+	+	-	+	-
4	Pitbull	M	1.5	Indoor	1.5	-	+	-	-	-
5	Mixed	M	10	Indoor	2.5	-	+	-	-	-
6	Labrador retriever/Shar pei mix	M	5	Indoor/ outdoor	5	-	+	-	-	-
7	Blue Heeler/Border Collie mix	M	6	Indoor	0.9	-	+	-	-	-
8	Chihuahua	M	2	Indoor	2	-	+	-	-	-
9	Golden Retriever	F	5	Indoor	3	+	+	-	+	-
10	German Short Hair Pointer	M	8	Indoor	7	-	+	-	-	-
11	Akita	F	5	Indoor	Unk.	+	-	40	+	-
12	Beagle/Terrier mix	M	2	Indoor	0.5	-	+	-	-	-
13	German Shepherd	F	0.75	Indoor/ outdoor	0.4	-	-	-	-	-
14	Golden Retriever	M	9	Indoor	3	-	+	-	-	-

Table S2. Demographics and test results for government working dogs. Serology included testing for anti-*T. cruzi* antibodies by two rapid immunochromatographic assays, Chagas Stat-Pak® (ChemBio, NY), and Chagas Detect™ Plus Rapid Test (InBios, International, Inc., Seattle, WA), indirect fluorescent antibody (IFA) testing at Texas Veterinary Medical Diagnostic Laboratory (College Station, TX) and a commercially available ELISA, the SNAP 4Dx Plus, which allows for simultaneous detection of canine antibodies to *E. canis*, *E. ewingii*, *B. burgdorferi*, *A. phagocytophilum*, and *A. platys*, and to *D. immitis* antigen. Dogs were also tested for amplification of *T, cruzi* DNA by real time PCR.

Demographics							2015 testing				2017 testing					4Dx testing			
Infection Status	Dog #	Sex	Breed	Age	Sector	Sleeping Location	StatPak	IFA	InBios	rt-PCR	StatPak	IFA	InBios	rt-PCR	Pre-study history of IFA	*Dirofilaria immitis*	*Borrelia burgdorferi*	*Ehrlichia* spp.	*Anaplasma* spp.
Discordant	211	M	Belgian Malinois	6.2	Del Rio	Kennel	-	**<20**	**+**	-	-	**<20**	**+**	-		-	-	-	-
	234	F	Belgian Malinois	10.5	Del Rio	Home	-	**<20**	**+**	-	-	**<20**	**+**	-		-	-	-	-
	100	M	Belgian Malinois	5.7	RGV	Home	-	**<20**	-	-	-	**<20**	-	-	**2012-1:20**	**+**	-	-	-
	2	M	German Shepherd	6.6	RGV	Home	-	**<20**	-	-	-	**<20**	-	-	**2012-1:80**	-	-	-	-
	198	F	Belgian Malinois	8.9	Laredo	Home	-	**<20**	**+**	-	-	**<20**	**+**	-		-	-	-	-
	227	M	German Shepherd	8.9	Del Rio	Home	-	**<20**	-	-	-	**<20**	-	-	**2013-1:20**	-	-	-	-
Negative	147	M	Dutch Shepherd	8.1	Laredo	Kennel	-	**<20**	-	-	-		-	-		-	-	-	-
	164	M	Lab	6.0	Laredo	Kennel	-	**<20**	-	-	-		-	-		-	-	-	-
	168	F	Belgian Malinois	8.2	Laredo	Home	-			-	-		-	-		-	-	-	-
	210	M	Belgian Malinois	7.1	Del Rio	Kennel	-	**<20**	-	-	-		-	-		-	-	-	-
	257	F	Belgian Malinois	10.0	Del Rio	Home	-			-	-		-	-		**+**	-	-	-
	236	F	Belgian Malinois	8.2	Del Rio	Home	-			-	-		-	-		-	-	-	-

Table S2 continued

Infection Status	Dog #	Sex	Breed	Age	Sector	Sleeping Location	2015 testing: StatPak	2015 testing: IFA	2015 testing: InBios	2015 testing: rt-PCR	2017 testing: StatPak	2017 testing: IFA	2017 testing: InBios	2017 testing: rt-PCR	Pre-study history of IFA	4Dx testing: *Dirofilaria immitis*	4Dx testing: *Borrelia burgdorferi*	4Dx testing: *Ehrlichia* spp.	4Dx testing: *Anaplasma* spp.
Negative	245	M	German Shepherd	5.1	Del Rio	Kennel	-	**<20**	-	-	-		-	-		-	-	-	-
	48	M	Belgian Malinois	4.4	RGV	Kennel	-			-	-		+	-		-	-	-	-
	58	F	Belgian Malinois	7.5	RGV	Home	-			-	-		-	-		-	-	-	-
	180	F	Groenendael	4.9	Laredo	Home	-			-	-		-	-		-	-	-	-
	11	M	Dutch Shepherd	3.3	RGV	Home	+	**<20**	-	-	-	**<20**	+	-		-	-	-	-
	94	F	Belgian Malinois	7.5	RGV	Home	-			-	-		-	-		-	-	-	-
	59	F	Belgian Malinois	7.0	RGV	Home	-	**<20**	-	-	-		-	-		-	-	-	-
	530	M	Belgian Malinois	5.2	Del Rio	Home	**N/A**	**N/A**	**N/A**	**N/A**	-	**<20**	+	-		-	-	-	-
	16	M	German Shepherd	7.6	RGV	Home	-	**<20**	-	-	-		-	-		-	-	-	-
	193	F	German Shepherd	5.2	Laredo	Home	-			-	-		+	-		-	-	-	-
	54	M	Belgian Malinois	3.9	RGV	Kennel	-			-	-		+	-		-	-	-	-
	258	M	German Shepherd	4.0	Del Rio	Home	-	**<20**	-	-	-		-	-		-	-	-	-
Positive	238	F	Belgian Malinois	7.9	Del Rio	Home	+	**<20**	+	-	-	**<20**	+	-		-	-	-	-
	369	F	Belgian Malinois	7.2	RGV	Home	+	**<20**	+	-	+	**<20**	+	-		-	-	-	-

Table S2 continued

	Demographics						2015 testing				2017 testing					4Dx testing			
Infection	Dog #	Sex	Breed	Age	Sector	Sleeping Location	StatPak	IFA	InBios	rt-PCR	StatPak	IFA	InBios	rt-PCR	Pre-study history of IFA	*Dirofilaria*	*Borrelia*	Ehrlichia spp.	Anaplasma
Positive	61	M	Belgian Malinois	3.5	RGV	Home	+	<20	+	-	+	<20	+	-		-	-	-	-
	10	M	Belgian Malinois	3.9	RGV	Home	+	<20	+	-	+	<20	+	-		-	-	-	-
	252	M	Dutch Shepherd	5.3	Del Rio	Home	+	160	+	-	+	320	+	-		-	-	-	-
	200	M	Sable Shepherd	8.3	Del Rio	Home	+	20	+	-	-	<20	-	-		-	-	-	-
	66	M	Belgian Malinois	7.3	RGV	Home	-	<20	+	-	-	<20	+	-	2012-1:20	+	-	-	-
	159	F	German Shepherd	4.8	Laredo	Kennel	+	640	+	-	+	320	+	-		-	-	-	-
	62	M	German Shepherd	6.1	RGV	Home	n/a	n/a	n/a	n/a	+	640	+	-	2013-1:20	-	-	-	-
	53	M	Belgian Malinois	5.8	RGV	Home	-	<20	+	-	+	<20	-	-	2013-1:20	-	-	+	-
	203	M	Sable Shepherd	5.4	Del Rio	Home	+	320	+	-	+	320	+	-		-	-	-	-
	105	M	German Shepherd	7.4	Laredo	Home	+	320	+	-	+	320	+	-		-	-	-	-
	60	M	German Shepherd	7.1	Del Rio	Home	n/a	n/a	n/a	n/a	+	320	+	-	2016-1:8192	-	-	-	-
	153	M	Lab	5.1	Laredo	Kennel	+	<20	+	-	-	<20	+	-		-	-	-	-
	65	M	German Shepherd	8.0	RGV	Home	+	320	+	-	+	320	+	-		-	-	-	-
	92	F	Belgian Malinois	8.4	RGV	Home	+	320	+	-	+	320	+	31.32 (TcI)		-	-	-	-
	64	F	Belgian Malinois	7.8	RGV	Home	+	<20	+	-	+	<20	+	-		-	-	-	-