ECOLOGY OF *TRYPANOSOMA CRUZI* AND A SURVEY OF OTHER PARASITES IN THE SOUTHERN PLAINS WOODRAT (*NEOTOMA MICROPUS*)

by

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(Under the Direction of Michael J. Yabsley)

ABSTRACT

Trypanosoma cruzi, a flagellated, protozoan parasite in the class Kinetoplastida, is the causative agent of Chagas' disease or American Trypanosomiasis in humans and dogs. Human cases of Chagas' disease are rare in the United States (only seven autochthonous cases), but domestic dogs and wildlife are frequently infected. To date, *T. cruzi* has been detected in more than 20 wildlife species including various species of woodrats (*Neotoma* spp.). The goal of this thesis was to determine the ecology of *T. cruzi* in various mammalian species with emphasis on the southern plains woodrat (*Neotoma micropus*) and to better understand the ecto- and endoparasitic fauna of these rodents in Uvalde County, Texas.

INDEX WORDS: *Trypanosoma cruzi*, Chagas' disease, United States, wildlife, southern plains woodrat, *Neotoma* spp., parasites

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CHAPTER 1

INTRODUCTION

Trypanosoma (*T. cruzi*) is a flagellated, protozoan parasite that is endemic to the Americas. It is the causative agent of Chagas' disease or American Trypanosomiasis in man and dogs. The parasite was first discovered in Brazil in 1909 and today ~8 million people are infected with a further 200,000 new cases and ~50,000 deaths occurring annually throughout Latin America (Centers for Disease Control and Prevention, 2010; Moncayo, 1993). *T. cruzi* is transmitted mainly by hematophagous triatomine bugs via contamination of bite sites or intact mucous membranes with infected feces, although infection can also occur through blood transfusions, tissue transplants, lab accidents, ingestion or congenitally (Hoff et al., 1978; Ianni and Mady, 2005; Muños et al., 2007). Only about 60% of infected individuals develop symptoms of Chagas' disease. The three stages of the clinical form of the disease are acute, indeterminate and chronic phase. The acute phase lasts from four to eight weeks and may proceed unnoticed or accompanied by fever, general malaise, lymphadenopathy, edema and a characteristic chagoma at the site of parasite entry. Mortality rates range from 2-8% and is usually due to myocarditis or encephalitis. The indeterminate phase occurs after the acute and the patient is usually free of clinical symptoms. After twenty years or more, an individual may develop the chronic phase which is a major cause of disability and mortality in endemic areas. Chronic Chagas' disease manifests as myocarditis, megacolon or megaesophagus.

To date, only seven autochthonous human cases have been reported in the southern United States (Dorn et al., 2007; Greer, 1956; Herwaldt et al., 2000; Ochs et al., 1996; Schiffler et al., 1984; Woody and Woody, 1955) but *T. cruzi* is present in wildlife populations throughout the southern US. Over 20 wildlife species including Virginia opossums (*Didelphis virginiana),* raccoons (*Procyon lotor*), striped skunks (*Mephitis mephitis*), nine-banded armadillos (*Dasypus novemcinctus*) woodrats (*Neotoma* spp.), and smaller rodents, have been reported to be infected (Brown et al., 2010; Packchanian, 1942). Numerous studies have been conducted on the role of wildlife as hosts for *T. cruzi* and it was found that Virginia opossums and raccoons are the two major hosts in the US (Brown et al., 2010). Studies have been conducted on *T. cruzi* in woodrats (*Neotoma* spp.) previously; however, multiple diagnostic methods were not utilized and *T. cruzi* genotypes infecting the woodrats were not determined. Additionally, the parasitic fauna of southern plains woodrats (*N. micropus*) are understudied. Therefore, this study was conducted to determine the importance of southern plains woodrats as reservoirs of *T. cruzi* and to determine what other parasites may be important to the health of these woodrats (Burkholder et al., 1980; Pinto et al., 2010). Because these woodrats hoard food and other items and cohabitate with other species including small mammals, reptiles, insects and arthropods, including reduviid bugs, we hypothesized that they could be important hosts to numerous parasites and/or pathogens (Braun and Mares, 1989; Ikenga and Richerson, 1984; Raun, 1966). Our study area was in Uvalde County, Texas which is located on the southwestern region of the state. This thesis research had three main aims:

1. To determine if southern plains woodrats are important reservoirs of *T. cruzi* in Uvalde County, Texas using multiple diagnostic assays.

2. To genetically characterize *T. cruzi* strains detected in woodrats and known sympatric reservoirs such as raccoons, skunks, and other rodents.

3. To determine what other common parasites infect the southern plains woodrat and to evaluate if any of them pose significant health threats to woodrats or other animals or people.

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CHAPTER 2

LITERATURE REVIEW

History of *Trypanosoma cruzi*

Trypanosoma cruzi, a flagellated, protozoan parasite in the class Kinetoplastida, is the causative agent of Chagas' disease or American Trypanosomiasis in humans and dogs. It was first discovered in Lassance, Brazil by the physician Carlos Chagas. He initially detected trypomastigotes in the mid-gut of the triatomine insect, *Triatoma infestans,* and subsequently found the parasite in a sick cat and a three-year old girl of the same household. The girl had been febrile for two weeks and exhibited a high parasitemia, splenomegaly, hepatomegaly, swollen lymph nodes and myxedema. Unfortunately, she died three days after diagnosis. Later that year, Dr. Chagas diagnosed another patient who survived until 1989 at which time she was still parasitemic with no evidence of pathology (Bastien, 1998; Chagas, 1909). To determine the ability of *T. cruzi* to cause the disease (fever and heart disease) he had observed, Dr. Chagas experimentally inoculated three callithrix monkeys (*Callithrix penicilliata*) with reduviid bug intestinal contents and all three died within days of inoculation ((Chagas, 1909)). Chagas and his mentor, Oswaldo Cruz, later proved that *T. cruzi* was transmitted by *Triatoma infestans* through its fecal matter to mammals via the bite site (Chagas, 1909). Over 100 years later, Chagas' disease is still a major cause of morbidity and mortality in Latin America.

Life Cycle and Transmission

The life cycle for *T. cruzi* was described by Chagas over a century ago (Chagas, 1909). It includes a vertebrate host (e.g. humans, dogs, wildlife, etc.) and an insect vector (reduviid bug) (Figure 2.1). This parasite enters the mammalian host via stercorarian transmission unlike other pathogenic trypanosomes (e.g. *T. brucei*) which are transmitted in the saliva of a vector. Two stages of *T. cruzi* occur in the mammalian host, intercellular trypomastigotes and intracellular amastigotes. Trypomastigotes can be found in two morphologic forms, broad and slender, which are both infective but non-proliferative (Tyler and Engman, 2001). Amastigotes are the replicative stage which is found within any nucleated cell, primarily myocytes, of a vertebrate host. When the reduviid bug takes a blood meal from an infected mammal, it primarily ingests trypomastigotes but can ingest amastigotes present in some cells such as macrophages (Andrews et al., 1987; Ley et al., 1988). As the trypomastigotes enter the mid-gut of the vector, they differentiate into amastigotes which begin to swell and their flagella become extended at which point they are known as sphaeromastigotes which then transform into epimastigotes. One study found that the transformation from amastigotes to elongate epimastigotes was reversible and glucose dependent (Tyler and Engman, 2000). These epimastigotes replicate by binary fission in the mid-gut of the vector. As they migrate to the hindgut, the parasites attach to the walls hydrophobically and then transform into metacyclic trypomastigotes by a process called metacyclogenesis (Bonaldo et al., 1988; Kleffmann et al., 1998). These metacyclic trypomastigotes detach from the hindgut wall only when fully formed and are then excreted in the feces of the bug. If defecated on a mammalian host, the parasites can enter at the reduviid bite site or through mucous membranes. This route (vector-borne) is the most common route of transmission in endemic areas throughout Latin America. The parasite can also be transmitted

via the oral route by ingestion of the infected vector in food or drink, congenital transmission, blood transfusion, organ transplant or laboratory accidents (Hoff et al., 1978; Ianni and Mady, 2005; Muños et al., 2007). Similar transmission routes can occur with domestic animals and wildlife with ingestion of bugs likely being an important route of transmission (Roellig et al., 2009; Yaeger, 1971).

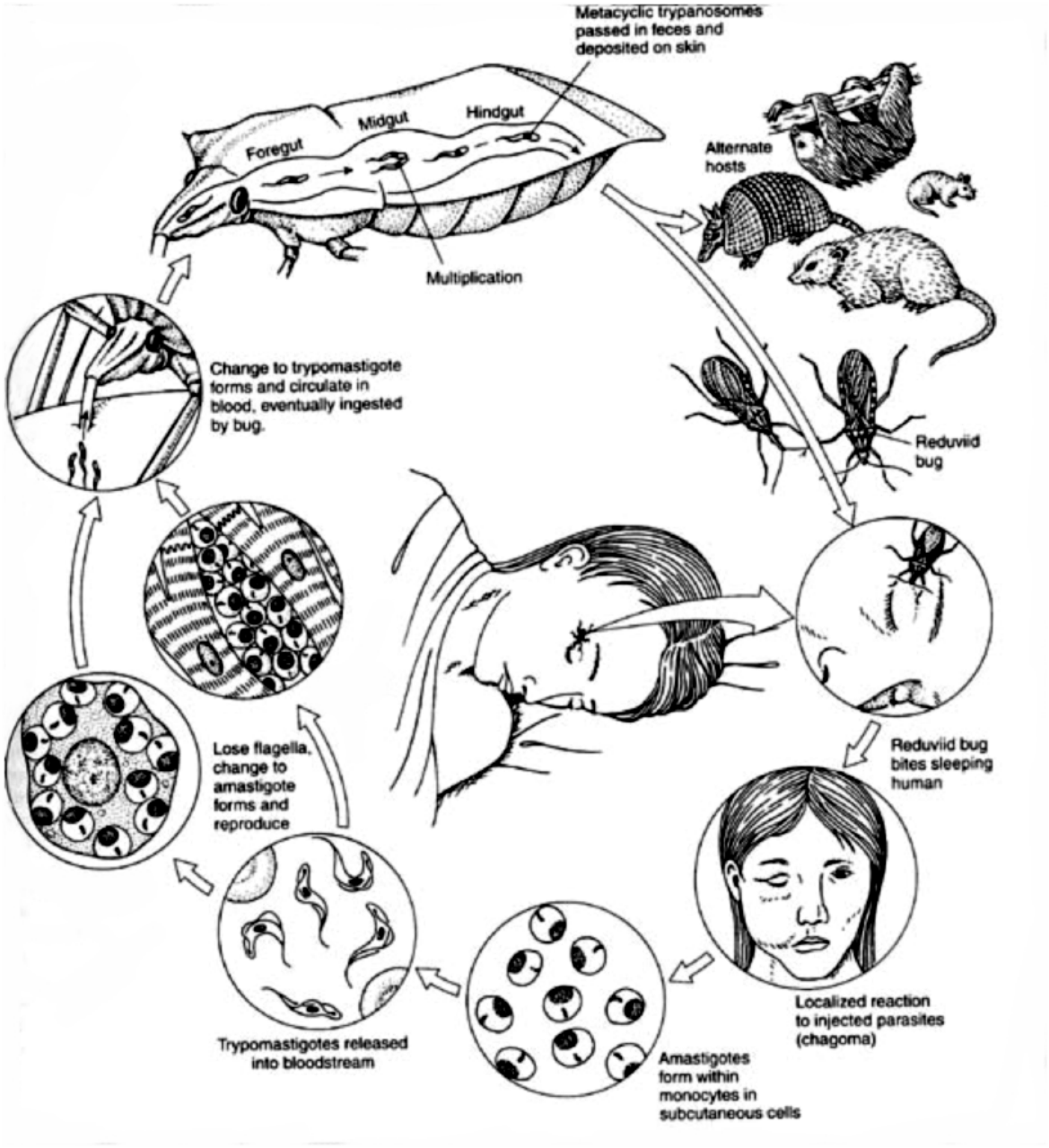


Figure 2.1. Life cycle of *Trypanosoma cruzi*

Disease

Humans

Infection with *T. cruzi* may result in acute and/or chronic disease or no appreciable health issues (indeterminate). Only about 25% of infected individuals show clinical signs during the acute phase of the infection which typically lasts 2-8 weeks. Signs may include fever, fatigue, sweating, swollen lymph nodes, edema, hepatosplenomegaly, anorexia, irritability and sometimes vomiting, diarrhea or a skin rash (Santos-Buch, 1985). A characteristic sore or chagoma may form at the site of parasite entry and if transmission occurs through mucus membranes around the eyes, conjunctivitis and unilateral palpebral edema (Romaña's sign) may develop. Severe myocarditis or encephalitis leading to death usually occurs in about 5-10% of acute infections; children are most likely to die from acute infections (Santos-Buch, 1985). The acute phase is characterized by a high number of circulating trypomastigotes in peripheral blood, making detection of the parasite by blood smear, isolation, and/or xenodiagnosis easiest at this stage. About 70-80% of infected individuals enter a prolonged intermediate phase after the acute infection and even if left untreated, remain asymptomatic for life. This phase is characterized by very few or no circulating parasites in the blood (Centers for Disease Control and Prevention, 2010). However, about one third of individuals eventually develop the chronic phase of Chagas' disease which may not develop for 10-20+ years. This symptomatic stage is characterized by cardiomyopathy, megacolon, megaesophagus or sudden death which is thought to be due to the intracellular form of the parasite (amastigote) causing damage to the tissue in which it replicates. Parasites present in tissues in indeterminate cases can be reactivated and cause chronic Chagas disease if the patient becomes immunosuppressed (.e.g., from AIDS or chemotherapy) (Ferreira et al., 1997).

Animals

Domestic dogs (*Canis lupus familiaris*) show similar clinical signs of Chagas' disease as humans with 3 stages- acute, intermediate, and chronic. The acute stage is characterized by non-specific signs such as lethargy, pale mucous membranes, generalized lymphadenopathy and hepatosplenomegaly (Barr et al., 1991b). Electrocardiographic abnormalities also can be observed. If infected dogs survive this stage, similar to humans, they may enter a prolonged latent stage or may eventually develop chronic disease with progressive right-sided cardiac dysfunction characterized by exercise intolerance, ascites, pleural effusion, distended jugular veins and/or hepatomegaly (Barr et al., 1991b; Meurs et al., 1998). Other animals, mostly exotics or some primates may also develop chronic disease. In contrast, numerous wildlife species are common hosts for *T. cruzi* yet no reports have been made on the development of clinical disease (Packchanian, 1942).

Trypanosoma cruzi in the United States

Human infections

To date, seven autochthonous cases of American trypanosomiasis have been reported in humans in the United States, although serologic testing suggests that undiagnosed infections have occurred (Woody et al., 1961; AABB, 2011). Although *T. cruzi* was first discovered in the vector (Kofoid, 1916) and subsequently in woodrats by Wood in 1934, the first autochthonous human case was not reported until 1955 in a 10-month-old infant in Corpus Christi, Texas (Woody and Woody, 1955). The second case was also found that year, in another infant from Bryan, Texas (Greer, 1956). These cases prompted further studies in southern Texas, particularly in pediatric patients. A serological study on 500 children in southern Texas and surrounding areas found that seven children from five families (all rural) were seropositive and two adults

from these families also tested positive (Woody et al., 1961). All nine individuals reported having been bitten on numerous occasions by *T. gerstaeckeri*, a common vector of *T. cruzi* in that area (Woody et al., 1961). Of interest, six subjects reported having past symptoms consistent with acute trypanosomiasis (systemic illness pattern, heart disease and Romaña's sign) and five had current conditions (hepatosplenomegaly, elevated serum gamma globulin and incomplete right bundle branch block) possibly related to *T. cruzi* infections. No parasites were isolated from blood samples from any of the patients.

The third confirmed case of Chagas' disease occurred in 1983 in a 56-year-old woman from Lake Don Pedro, California (Schiffler et al., 1984). This patient did not exhibit any classic symptoms of *T. cruzi* infection but was confirmed by demonstration of parasites on blood smears, culture, and serology. The fourth case was also found in 1983 in a seven-month-old child from south Texas which died from myocarditis; however, diagnosis was not made until 1996 when histology and polymerase chain reaction (PCR) assay was conducted on archived cardiac tissue (Ochs et al., 1996). The fifth case was diagnosed in 1998 in an 18-month-old boy from rural Tennessee after an engorged and *T. cruzi*-infected adult *T. sanguisuga* was found in his crib (Herwaldt et al., 2000). The infant was later diagnosed by PCR after showing non-specific symptoms (fever, cough, irritability, slight pharyngeal erythema and anorexia) and multiple insect bite wounds. The sixth case was diagnosed in 2006 from a 74-year-old woman from rural southern Louisiana who complained of being bothered by numerous insect bites. She tested positive using indirect fluorescent antibody (IFA) test, a dipstick assay (Trypanosoma Detect; Inbios International Inc., Seattle, WA, USA) and hemoculture (Dorn et al., 2007). The most recent case came from a child near Brownsville, Texas with no previous history of travel outside the US (Kjos et al., 2009).

Several serological surveys on *T. cruzi* in humans have been conducted in the US including Georgia, Arizona, Texas and California. In 1962, the complement fixation test (CFT) was used to test 951 adults from Atlanta, Georgia for antibodies to *T. cruzi* (Farrar et al., 1962). Serum samples from four persons (0.4%) tested positive, while two of 28 (7.1%) patients with diffuse myocardial disease were seropositive. Another study of 122 patients from Georgia found that only 2 (1.6%) were weakly seropositive (Farrar et al., 1972). A study conducted on a population of Native-American (Papago) Indians in Arizona revealed that 20 individuals had elevated *T. cruzi* antibody titers (Miller et al., 1977). In addition, seven of 60 dogs and 20% of *Triatoma rubida* on the reservation were positive for *T. cruzi* (Miller et al., 1977).

During 2006-2007, the American Red Cross conducted a clinical trial to evaluate an investigational assay for *T. cruzi* in donated blood (Centers for Disease Control and Prevention, 2007). Screening was conducted on 148,969 samples at three blood collection centers and revealed that 32 persons were positive for *T. cruzi* antibodies. As a result of these preliminary findings, the FDA approved the use of the Ortho *T. cruzi* ELISA Test System to screen blood donors in the United States. Since 2007, over1,400 blood donations tested by the Red Cross have been positive for *T. cruzi* antibodies (AABB, 2011). This assay is not labeled for general clinical diagnosis of Chagas' disease but the American Red Cross and the Blood Systems Inc. (which are responsible for ~65% of the blood supply in the US) routinely test all donor samples for *T. cruzi*.

Domestic and exotic infections

Cases of acute and chronic Chagas' disease in dogs have been reported in Texas (2.6-8.8%) (Kjos et al., 2008; Meurs et al., 1998; Nabity et al., 2006; Shadomy et al., 2004; Williams et al., 1977), Louisiana (2.3-4.7%) (Barr et al., 1986; Barr et al., 1989; Snider et al., 1980), Oklahoma (3.6%) (Fox et al., 1986), South Carolina (1 case) (Nissen et al., 1977),southern

California (52%) (Navin et al., 1985) and Virginia (1 of 90 cases in one study and one dog in another study) (Patel et al., 2010; Rosypal et al., 2010). Reports of canine infections are represented in Figure 2.2.

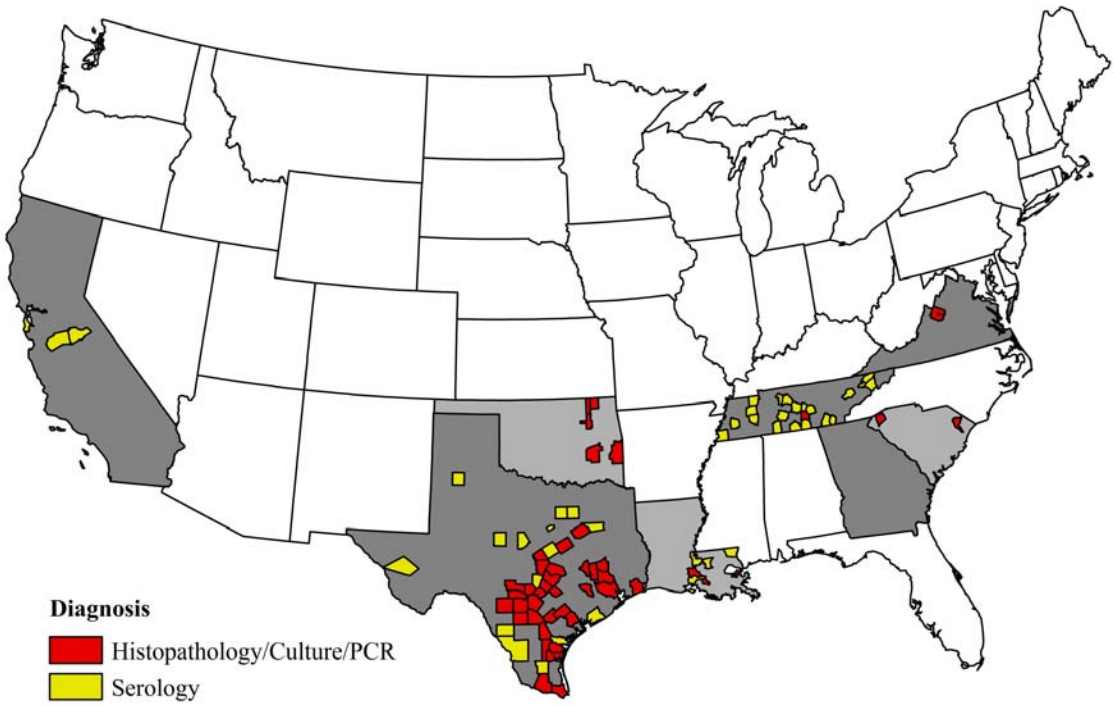


Figure 2.2. Reports of canine *Trypanosoma cruzi* infections. Clinical cases confirmed by parasitologic or histologic methods shown in red. Serologic confirmed cases are shown in yellow. Serologic surveys were conducted in numerous counties with confirmed cases. In some states (California, Georgia, Tennessee, Virginia, shown in dark grey), additional clinical cases were reported but no locality specified.

Infections of non-human primates (exotic to the United States) with *T. cruzi* have also been reported on St. Catherine's Island, Georgia. Investigators discovered that 7 of 11 (64%)

lion-tailed macaques (*Macaca silenus*) and 1 of 19 (5%) ring-tailed lemurs (*Lemur catta*) were infected with *T. cruzi* by PCR (Pung et al., 1998). Another study, almost a decade later on that same island reported a 45% seroprevalence of *T. cruzi* (25 of 56) in the lemur population (Hall et al., 2007).

Wildlife Infections

In the United States, *T. cruzi* has been reported in more than 20 mammalian species including raccoons, opossums, gray foxes, armadillos, striped skunks, coyotes, and various rodent species including woodrats (Tables 2.1-2.4). The first report of *T. cruzi* in a US wildlife species was in dusky-footed woodrats, *Neotoma fuscipes* in 1934 (Wood, 1934). This was followed by detection of natural infection with *T. cruzi* in other wildlife including opossums, nine-banded armadillos, and southern plains woodrats (*Neotoma micropus)* from Texas (Packchanian, 1942). During a Q fever study in the early 1950's, over 2,260 animals (>1,700 rodents) were tested for *T. cruzi* and of the 461 woodrats tested, 161 (35%) were infected with trypanosomes which resembled *T. lewisi*. Even though xenodiagnosis testing for *T. cruzi* with *T. lenticularis* and *T. woodi* were negative, 28% of *Triatoma* collected from the woodrat nests were infected with *T. cruzi* (Eads and Hightower, 1952). A study done by Upton et al., (1989) to determine if *T. cruzi* was present in eastern woodrats (*Neotoma floridana*) from Kansas failed to show the presence of this parasite. However another trypanosome (*T. kansasensis*) similar to *T. neotomae* was discovered.

Table 2.1. Studies of *Trypanosoma cruzi* in procyonids in the United States.

Species	State	Total tested	Number positive (%)	Assay (sample or specific assay)	Reference
Raccoon	AL	35	5 (14)	Culture (heart and blood)	(Olsen et al., 1964)
(*Procyon lotor*)	AZ	5	1 (20)	Serology (IFA[1])	(Brown et al., 2010)
	FL	184	4 (2)	Blood smear	(Telford and Forrester, 1991)
	FL	33	4 (12)	Culture (blood)	(Schaffer et al., 1978)
	FL	70	38 (54)	Serology (IFA)	(Brown et al., 2010)
	FL, GA	608	9 (1.5)	Culture (kidney)	(McKeever et al., 1958)
	GA	54	12 (22)	Culture (blood)	(Pung et al., 1995)
	GA	30	13 (43)	Culture (blood)	(Pietrzak and Pung, 1998)
	GA	510	168 (33)	Serology (IFA)	(Brown et al., 2010)
	GA	10	5 (50)	Culture (blood)	(Schaffer et al., 1978)
	GA, SC	221	104 (47)	Serology (IFA)	(Yabsley and Noblet, 2002)
	KY	44	17 (39)	Culture (blood)	(Groce, 2008)
			19 (43)	Serology (IFA)	
	MD	472	2 (0.4)	Culture (heart)	(Herman and Bruce, 1962)
	MD	NK[2]	5	Culture (blood)	(Walton et al., 1958)
	MO	109	74 (68)	Serology (IFA)	(Brown et al., 2010)
	NC	20	3 (15)	Culture (blood)	(Karsten et al., 1992)
	OK	8	5 (63)	Culture (blood)	(John and Hoppe, 1986)
	TN	3	2 (66)	Culture (blood)	(Herwaldt et al., 2000a)
	TN	706	206 (29)	Serology (IFA)	(Maloney et al., 2010)
	TX	25	6 (24)	Culture (blood)	(Schaffer et al., 1978)
	TX	9	0	Serology (IHA[3])	(Burkholder et al., 1980)
	VA	464	153 (33)	Serology (IFA)	(Hancock et al., 2005)
Ringtail (*Bassariscus astutus*)	AZ	1	1 (100)	Serology (IFA)	(Brown et al., 2010)

[1] IFA, indirect immunofluorescent assay.
[2] NK, not known.
[3] IHA, indirect hemagglutination assay.

Table 2.2. Studies of *Trypanosoma cruzi* in marsupials in the United States.

Species	State	Total tested	Number positive (%)	Assay (sample or specific assay)	Reference
Virginia Opossum	AL	126	17 (14)	Culture (blood and heart)	(Olsen et al., 1964)
(*Didelphis virginiana*)	FL	27	14 (52)	Serology (IFA[1])	(Brown et al., 2010)
	GA	39	6 (15)	Culture (blood)	(Pung et al., 1995)
	GA	421	118 (28)	Serology (IFA)	(Brown et al., 2010)
	GA	29	3 (10)	PCR[2] (liver)	(Parrish and Mead, 2010)
	GA, FL	552	88 (16)	Culture (kidney)	(McKeever et al., 1958)
	KY	48	0 (0) 6 (13)	Culture (blood) Serology (IFA)	(Groce, 2008)
	MD	219	0 (0)	Culture (heart)	(Herman and Bruce, 1962)
	NC	12	1 (8)	Culture (blood)	(Karsten et al., 1992)
	OK	10	0	Culture (blood)	(John and Hoppe, 1986)
	LA	48	16 (33)	Culture (blood)	(Barr et al., 1991a)
	TX	8	5 (63)	Culture (blood)	(Packchanian, 1942)
	TX	391	63 (16)	Blood smear	(Eads et al., 1963)
	VA	6	1 (17)	Serology (IFA)	(Brown et al., 2010)

[1] IFA, indirect immunofluorescent assay.
[2] PCR, polymerase chain reaction.

Table 2.3. Studies of *Trypanosoma cruzi* in various mesomammals in the United States.

Species	State	Total tested	Number positive (%)	Assay (sample or specific assay)	Reference
Nine-banded Armadillo (*Dasypus novemcinctus*)	LA	98	1 (1)	Culture (blood)	(Barr et al., 1991a)
	LA	80	23 (29)	Culture (blood)	(Yaeger, 1988)
			30 (38)	Serology (direct agglutination)	
	TX	15	1 (7)	Culture (blood)	(Packchanian, 1942)
Striped Skunk (*Mephitis mephitis*)	AZ	34	3 (9)	Serology (IFA[1])	(Brown et al., 2010)
	CA	1	1 (100)	Serology and histology	(Ryan et al., 1985)
	GA, FL	306	3 (1)	Culture (kidney)	(McKeever et al., 1958)
	GA	1	1 (100)	Serology (IFA)	(Brown et al., 2010)
Gray Fox (*Urocyon cinereoargenteus*)	GA, FL	118	2 (2)	Culture (kidney)	(McKeever et al., 1958)
	GA	21	0	Serology (IFA)	(Brown et al., 2010)
	SC	26	2 (8)	Serology (IFA)	(Rosypal et al., 2007)
Bobcat (*Felis rufus*)	GA	62	2 (3)	Serology (IFA)	(Brown et al., 2010)
American Badger (*Taxidea taxus*)	TX	8	2 (25)	Serology (IHA)	(Burkholder et al., 1980)
Coyote (*Canis rufus*)	GA	23	1 (4)	Serology (IFA)	(Brown et al., 2010)
	TX	134	19 (14)	Serology (IFA)	(Grogl et al., 1984)
	TX	156	20 (13)	Serology (IHA[2])	(Burkholder et al., 1980)
	VA	26	1 (4)	Serology (IFA)	(Brown et al., 2010)
Feral swine (*Susscrofa*)	GA	110	0	Serology (IFA)	(Brown et al., 2010)

[1] IFA, indirect immunofluorescent assay.
[2] IHA, indirect hemagglutination assay.

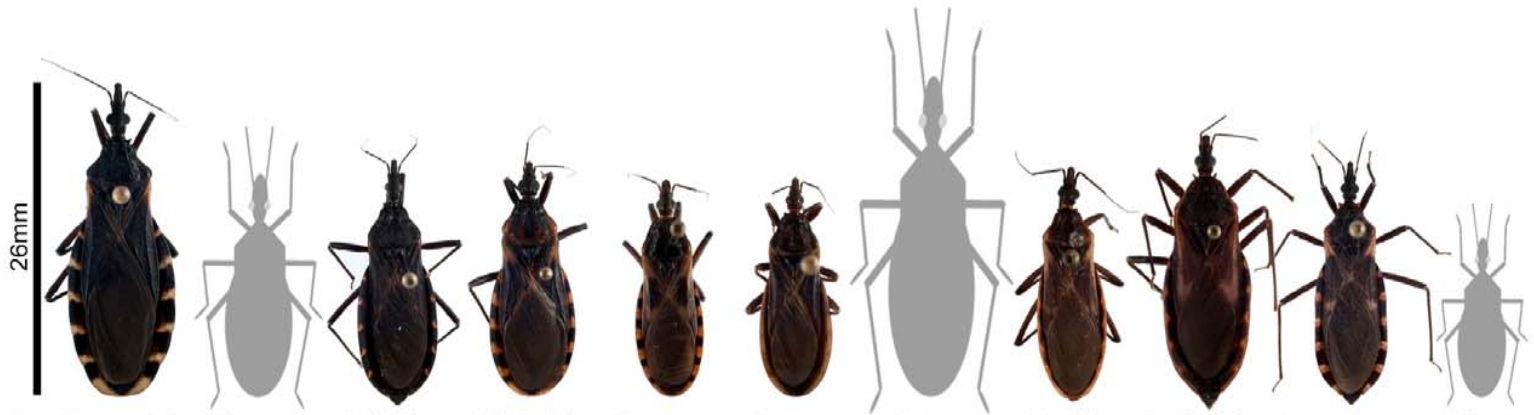
Table 2.4. Studies of *Trypanosoma cruzi* in various rodent species in the United States.

Species	State	Total tested	Number positive (%)	Assay (sample or specific assay)	Reference
Southern plains woodrat (*Neotoma micropus*)	TX	30	5 (17)	Culture (blood)	(Burkholder et al., 1980)
	TX	100	31 (31)	Culture (blood)	(Packchanian, 1942)
	TX	159	42 (26)	PCR[1] (liver)	(Pinto et al., 2010)
	TX	461	161(35)	Blood smear	(Eads and Hightower, 1952) (Pippin, 1970)
	TX	56	12 (21)	Blood smear	(Ikenga and Richerson, 1984)
	TX	13	6 (46)	Serology (IHA)	(Ikenga and Richerson, 1984)
	TX	18	7 (40)	Serology (IHA)	
	NM	NK[2]	1	Xenodiagnosis	(Wood and Wood, 1961)
White-throated woodrat *(Neotoma albigula)*	AZ	NK	2	NK	(Wood, 1952)
	NM	NK	1	Xenodiagnosis	(Wood and Wood, 1961)
Dusky-footed woodrat *(Neotoma macrotis =N. fuscipes* subsp. *macrotis)*	CA	99	9 (9)	Xenodiagnosis and blood smear	(Wood and Wood, 1937; Wood and Wood, 1967; Wood, 1952; Wood, 1975)
Brush mouse (*Peromyscus boylii rowleyi)*	AZ	NK	1	NK	(Wood, 1952)
Gilbert white-footed mouse (*Peromyscus truei gilberti)*	CA	NK	2	NK	(Wood, 1952)
Pinon mouse (*Peromyscus truei montipinoris)*	CA	NK	11	Xenodiagnosis	(Wood, 1975)
Western harvest mouse (*Reithrodontomys megalotis*)	CA	NK	1	Xenodiagnosis	(Wood, 1962)
Hispid pocket mouse (*Perognathus hispidus*)	TX	25	4 (16)	Culture (blood)	(Burkholder et al., 1980)
House mouse (*Mus musculus*)	TX	2	1 (5)	Culture (blood)	(Packchanian, 1942)
Mexican spiny pocket mouse (*Liomys irrorattus*)	TX	11	1 (9)	Culture (blood)	(Burkholder et al., 1980)
Grasshopper mouse (*Onychomys leucogaster*)	TX	9	1 (11)	Culture (blood)	(Burkholder et al., 1980)
CA ground squirrel (*Spermophilus beecheyi*)	CA	19	2 (11)	Culture (blood)	(Navin et al., 1985)
Whitetail antelope squirrel (*Ammospermophilus leucurus*)	NM	NK	3	Xenodiagnosis	(Wood, 1975; Wood and Wood, 1961)

[1] PCR, polymerase chain reaction
[2] NK, not known.

Insect vectors

Similar to the first detection of *T. cruzi* in Brazil, *T. cruzi* was first reported in the United States in a reduviid vector, *Triatoma protracta*. The bugs were collected from the nests of woodrats (*Neotoma* sp.) in San Diego, California (Kofoid, 1916). To date, 11 species of triatomine bugs (and potential vectors of *T. cruzi)* have been reported in the US including: *T. protracta, T. sanguisuga, T. lenticularia, T. neotomae, T. recurva, T. rubida, T. gerstaeckeri, T. indictiva, T. incrassata, T. rubrofasciata* and *Paratriatoma hirsuta* (Figure 2.2). These vectors are distributed across the southern half of the US from the Pacific to Atlantic coasts with the exception of *T. rubrofasciata* which is found in Hawaii (Figure 2.3). Many of these bugs have been found in homes and will feed on humans. Infected bugs have been reported in California, Georgia, Florida, Alabama, Tennessee, Arizona and Louisiana (Beard et al., 1988; Herwaldt et al., 2000; Olsen, 1965; Pung et al., 1995; Yaeger, 1961). The prevalence of *T. cruzi* in US vectors has been highest in Texas (17-50.4%), Arizona (7.1-41.5%) and California (14-40%). (Burkholder et al., 1980; Kjos et al., 2009; Pippin, 1970; Reisenman et al., 2010; Ryckman and Ryckman, 1967; Sullivan et al., 1949; Wood, 1949, 1975; Wood and Wood, 1964). Despite the relatively high prevalence of *T. cruzi* in many populations of US vectors, few human cases have been reported. This discrepancy could be due to under-reporting or misdiagnosis of cases, better infrastructure of homes in the US that better exclude bugs, lack of bug colonization of homes, or delayed time from blood meal ingestion to defecation in vectors. However, it should be noted that *T. sanguisuga* and *T. protracta* were implicated in at least two endemic human cases in the United States (Dorn et al., 2007; Herwaldt et al., 2000).



U.S. records	*T. gerstaeckeri*	*T. incrassata*	*T. indictiva*	*T. lecticularia*	*T. neotomae*	*T. protracta*	*T. recurva*	*T. rubida*	*T. rubrofasciata*	*T. sanguisuga*	*P. hirusta*
T. cruzi prevalence	57.7% (1038/1800)	not reported	33.3% (4/12)	51.1% (144/282)	75.5% (40/53)	17.5% (723/4124)	12.6% (71/565)	7.2% (96/1340)	100% (2/2)	14.6% (151/1031)	0.0% (0/66)
Human bites	Y	N	N	Y	N	Y	Y	Y	Y	Y	Y
Allergic reactions	Y	N	N	N	N	Y	Y	Y	Y	Y	N
Collection sites	woodrat nest house dog kennel livestock pen lights cave bird nest	lights	woodrat nest lights	woodrat nest house dog kennel hollow log lights	woodrat nest dog kennel	woodrat nest house hollow log lights roadbed	woodrat nest house mine shaft lights rocks	woodrat nest house lights	house livestock pen woodpile	woodrat nest house dog kennel livestock pen woodpile lights tree bark/hollows	woodrat nest house lights

Figure 2.3. General information on the vectors of *Trypanosoma cruzi* in the United States. Figures courtesy of Dr. Sonia Kjos.

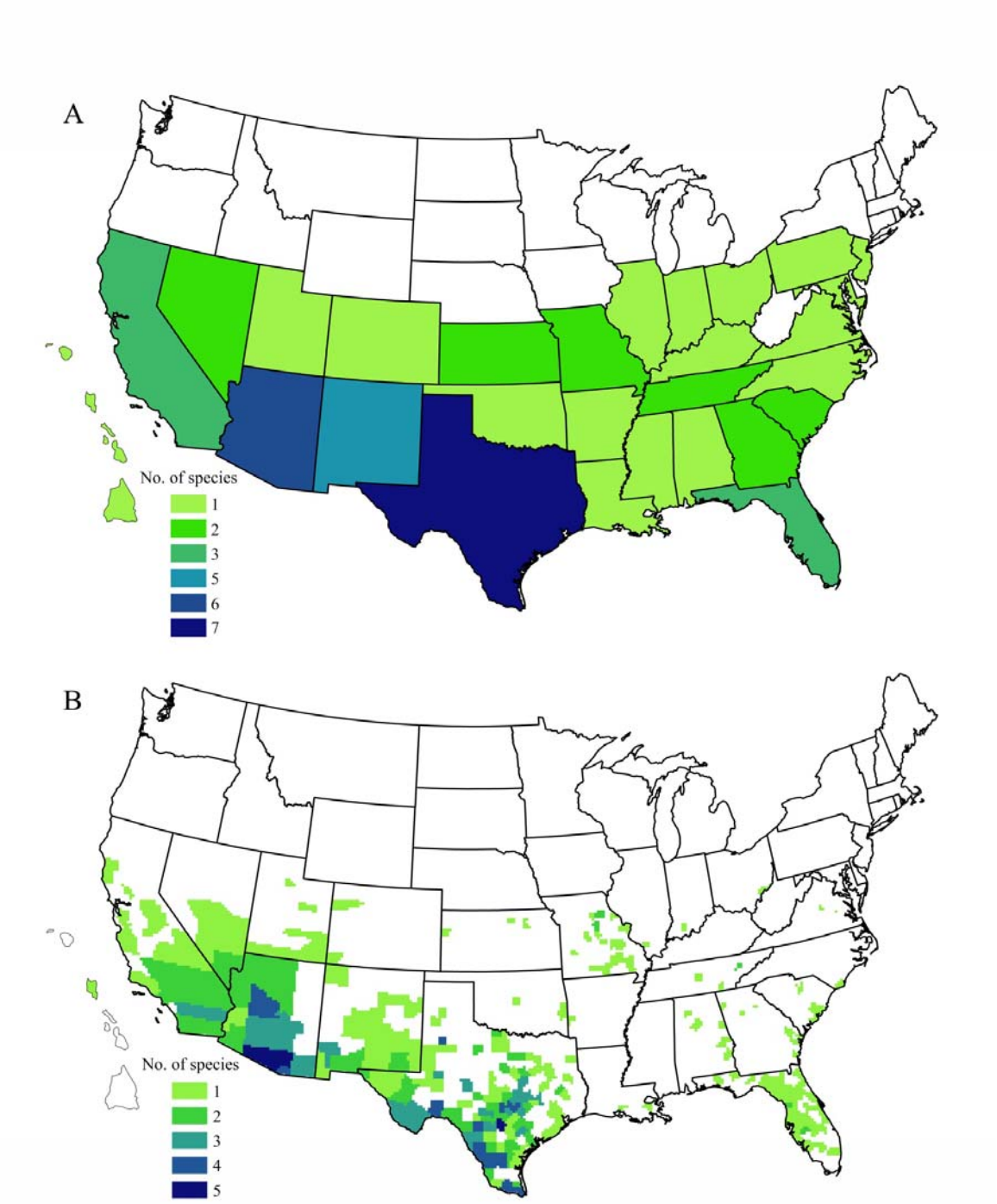


Figure 2.4. Triatomine species diversity in the continental United States and Hawaii, 1939-2010, by (A) state and (B) county. Maps courtesy of Dr. Sonia Kjos.

Diagnostic testing for *Trypanosoma cruzi* infections

A wide range of tests are available for the diagnosis of Chagas' disease in humans including microscopy, culture, xenodiagnosis, molecular techniques (PCR), and serology. Microscopy, hemoculture, PCR, and xenodiagnosis are most useful for diagnosis of acute infections and there is no true gold standard test for chronic *T. cruzi* infection, especially in wildlife (Tarleton et al., 2007). As a result, serologic testing has become increasingly important in the diagnosis of chronic *T. cruzi* infections since detectable antibodies (particularly IgG) persist for years after an initial infection (Dusanic, 1991). Historically, the complement fixation test (CFT) was the main serological test for diagnosis of chronic chagasic infections and although it is very specific and sensitive, the test is time consuming and requires skilled personnel to conduct and interpret (Dusanic, 1991). Currently, numerous serological tests are available including the indirect fluorescent antibody test (IFAT), flocculation test (FT), the direct agglutination test (DAT), the enzyme immunoassay (EIA), the latex agglutination test (LAT) and the enzyme-linked immunosorbent assay (ELISA). The IFAT and ELISA are commonly used in the US but the radio-immune precipitation assay (RIPA) and trypomastigote excreted-secreted antigen immunoblot (TESA-blot) are often used as confirmatory tests (Brashear et al., 1995; Winkler et al., 1995). Recently rapid patient-side assays (e.g., Chagas STAT-pak® and *Trypanosoma* Detect™) have been developed that have high sensitivity (98.5-100%) and specificity (96.0-100%) in people (Cardinal et al., 2006; Luquetti et al., 2003; Ponce et al., 2005). These rapid assays have also been used to test for infection in dogs and some wildlife species including raccoons and degus (*Octodon degus*) (Nieto et al., 2009; Rosypal et al., 2011; Yabsley et al., 2009). Importantly, these rapid assays were unable to detect sero-conversion in two different marsupial species (Virginia opossum and short-tailed opossum (*Monodelphis*

domestica)) which highlights the need to validate new assays with each species of wildlife (Yabsley et al., 2009).

Trypanosoma cruzi genotypes in the United States

As a species, *T. cruzi* is very genetically, biologically and biochemically diverse (Campbell et al., 2004; Macedo and Pena, 1998; Miles et al., 1981; Miles et al., 2003). Initial studies on the diversity of this parasite using iso-enzyme analysis divided the parasite into 2 groups (Miles et al., 1981). Subsequent molecular characterization work divided the parasites into two major lineages TcI and TcII, with TcII further divided into five subgroups, TcIIa-TcIIe (Miles et al., 2009; Souto et al., 1996; Westenberger et al., 2005). Recently, this nomenclature was changed to geno-groups TcI- TcVI as a means to improve communication among researchers in the *T. cruzi* field. TcI remained as TcI, TcIIb became TcII, TcIIc became TcIII, TcIIa became TcIV, TcIId became TcV, and TcIIe became TcVI (Zingales et al., 2009). TcI and TcII lineages appear to be ancestral lines, whereas TcV and TcIV are hybrid lines (Westenberger et al., 2005; Zingales et al., 2009). The origins of TcIII and TcVI are still unknown although some investigators believe that TcIII is a third ancestral line, while others consider it a hybrid of TcI and TcII (Westenberger et al., 2005; Westenberger et al., 2006; Zingales et al., 2009).

In the United States, only two of these six genotypes (TcI and TcIV) have been reported from mammals (including humans) and vectors (Table 2.5). However, a recent report stated that TcII and TcVI isolates were found in the vector, *T. protracta* (Uhler) in California (Hwang et al., 2010). TcI is the only genotype that has been reported in the Virginia opossum, (*D. virginiana*) which is consistent with findings in South America where TcI is the predominate strain reported from *Didelphis* species (Barnabe et al., 2001; Clark and Pung, 1994; Roellig et al., 2008). Both TcI and TcIV have been reported in nine-banded armadillos, raccoons, domestic dogs, and

Rhesus macaques, but TcIV is the predominate strain detected in these placental species (Clark and Pung, 1994; Rosypal et al., 2007). The TcIV lineage has also been reported from a limited number of striped skunks and ring-tailed lemurs (Roellig et al., 2008). Interestingly, the 5 autochthonous human cases isolates from the US that have been typed were TcI which is consistent with data from Mexico and Central America where TcI is the predominate genotype detected in humans (Fernandes et al., 1998). Genotyping of only two woodrat isolates of *T. cruzi* has been conducted; one was TcI and the other was TcIV (Roellig and Yabsley, 2010).

Other parasites of the southern plains woodrat *(Neotoma micropus)*

In addition to being hosts of *T. cruzi* and other trypanosomes sp., southern plains woodrats are hosts to a number of other parasites including helminths, protozoa, and arthropods (Table 2.6). Some of these parasites may cause pathology to woodrats e.g., cestodes while others (especially arthropods) are known vectors of diseases of zoonotic and/or veterinary importance. Such diseases include plague, Q fever, Rocky Mounted spotted fever, tularemia, *Toxoplasma gondii*, relapsing fever, and possibly others (Dubey et al., In press; Johnson, 1966).

Table 2.5. Review of genotypes of *Trypanosoma cruzi* detected in the United States.

Species	N	State(s)	Genotypes (No. of each)	Reference
Human	5	CA, Texas, LA	TcI (5)	(Roellig et al., 2008)
Opossum	15	GA, FL, LA, AL	TcI (15)	(Clark, 1994; Roellig et al., 2008)
Raccoon	79	GA, FL, TN, MD, LA, KY	TcI (2), TcIV (74), mixed (2)	(Brisse et al., 2003; Clark, 1994; Roellig et al., 2008)
Ring-tailed lemur	3	GA	TcIV (3)	(Roellig et al., 2008)
Rhesus macaque	2	GA	TcI (1), mixed (1)	(Roellig et al., 2008)
Nine-banded armadillo	3	LA, GA	TcI (2), TcIV (1)	(Roellig et al., 2008)
Striped Skunk	1	GA	TcIV (1)	(Roellig et al., 2008)
Southern plains woodrat	2	TX	TcI (1), TcIV (1)	(Roellig and Yabsley, 2010)
Domestic dog	7	TN, OK, SC, CA, unknown	TcIV (6), mixed (1)	(Brisse et al., 2000; Brisse et al., 2003; Roellig et al., 2008)
Triatoma spp.	8	GA, FL, TX, CA	TcI (6), TcIV (1) mixed (1), TcII (NK), TcVI (NK)	(Barnabe et al., 2001; Clark, 1994; Hwang et al., 2010; Machado and Ayala, 2001; Roellig et al., 2008)

Table 2.6. Reported parasites of southern plains woodrats (*Neotoma micropus*) in the United States.

Class (order)	Species	Reference
INSECTA	*Neohaematopinus neotomae*	Finley (1958)
Anoplura (suborder)	*Hoplopleura hirsuta*	Johnson (1966)
Brachycera	*Cuterebra* sp.	Johnson (1966)
Hemiptera	*Triatoma neotomae*	Thurman (1944); Ryckman (1962)
	T. gerstaeckeri	Thurman (1944); Johnson (1966)
	T. occulta	Thurman (1944); Ryckman (1962)
	T. protracta	Thurman (1944); Ryckman (1962)
	T. sanguisuga	Johnson (1966)
	T. lenticularis	Johnson (1966)
Siphonaptera	*Foxella ignota*	Eads & Menzies (1949)
	Orchopeas leucopus	Eads & Menzies (1949); Johnson (1966)
	O. sexdentatus	Eads & Menzies (1949)
	Malaraeus sp.	Finley (1958)
	Thrassis fotus	Eads & Menzies (1949)
	T. campestris	Eads & Menzies (1949)
	Anomiopsyllus sp.	Finley (1958)
	Anomiopsyllus hiemalis	Eads & Menzies
	Meringis arachis	Eads & Menzies (1949)
	M. bilsingi	Eads & Menzies (1949)
	M. parkeri	Eads & Menzies (1949)
	Monopsyllus exilis	Eads & Menzies (1949)
	Echidnophaga gallinacean	Miles *et al*. (1952)
	Hoplopsyllus affinis	Eads & Menzies (1949)
	Pulex irritans	Eads & Menzies (1949); Miles *et al*. (1952); Johnson (1966)
	Xenopsylla cheopis	Finley (1958); Johnson (1966)
ARTHROPODA	*Androlaelaps johnstoni*	Eads & Hightower (1951a)
Acarina	*Haemolaelaps glasgowi*	Finley (1958); Eads *et al.*(1952)
	Brevisterna utahenis	Eads *et al.*(1952)
	B. morlani	Strandtmann & Allred (1956)
	Ischyropoda armatus	Eads *et al.*(1952)
	Bdellonyssus bacoti	Eads *et al.*(1952); Johnson (1966)
	Neoichoronyssus neotomae	Eads & Hightower (1951b)
	Uropoda sp.	Eads *et al.*(1952)
	Amblyomma inornatum	Eads *et al.*(1952)
	Dermacentor variabilis	Johnson (1966)
	D. parumapertus	Eads *et al.*(1952)
	Ixodes woodi	Eads *et al.*(1952); Johnson (1966)
	Ornithodoros turicata	Eads *et al.*(1952)
	O. talaje	Eads *et al.* (1956)
CESTODA	*Taenia taeniaeformis*	Johnson (1966)
NEMATODA	*Litomosoides carinii*	Johnson (1966)
	Trichuris muris	Johnson (1966)
KINETOPLASTIDA	*Trypanosoma cruzi*	Packchanian (1942); Burkholder et al., (1980)
	Trypanosoma lewisi	Pinto et al., (2010)
CONOIDASIDA	*Besnoitia neotomofelis*	Dubey and Yabsley, (2010); Charles et al., (2011)

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CHAPTER 3

SOUTHERN PLAINS WOODRATS (*NEOTOMA MICROPUS*) FROM SOUTHERN TEXAS ARE IMPORTANT RESERVOIRS OF TWO GENOTYPES OF *TRYPANOSOMA CRUZI* AND HOST OF A PUTATIVE NOVEL *TRYPANOSOMA* SPECIES[1]

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Abstract

Human cases of Chagas' disease are rare in the United States, but domestic dogs and wildlife are frequently infected. In 2008 and 2010, we investigated the *Trypanosoma cruzi* prevalence in possible vertebrate reservoirs in southern Texas with an emphasis on southern plains woodrats (*Neotoma micropus*). Infection status was determined using a combination of culture isolation, polymerase chain reaction (PCR), and serologic testing. Based on PCR and/or culture, *T. cruzi* was detected in 35 of 104 (34%) woodrats, 3 of 4 (75%) striped skunks (*Mephitis mephitis*), 12 of 20 (60%) raccoons (*Procyon lotor*), and 5 of 28 (18%) other rodents including a hispid cotton rat (*Sigmodon hispidus*), rock squirrel (*Otospermophilus variegatus*), black rat (*Rattus rattus*), and two house mice (*Mus musculus*). Additionally, another *Trypanosoma* sp. was detected in 41 woodrats, of which, 27 were co-infected with *T. cruzi.* Genetic characterization of *T. cruzi* revealed that raccoon, rock squirrel and cotton rat isolates were genotype TcIV while woodrats and skunks were infected with TcI and TcIV. Based on the Chagas Stat-Pak assay, antibodies were detected in 27 woodrats (26%), 13 raccoons (65%), 4 skunks (100%), and 5 other rodents (18%) (two white-ankled mice (*Peromyscus pectoralis laceianus)*, two house mice, and a rock squirrel). Seroprevalence based on indirect immunofluorescent antibody testing was higher for both woodrats (37%) and raccoons (90%) compared with the Chagas Stat-Pak. This is the first report of *T. cruzi* in a hispid cotton rat, black rat, rock squirrel, and white-ankled mouse. These data indicate that, based on culture and PCR testing, the prevalence of *T. cruzi* in woodrats is comparable with other common reservoirs (i.e., raccoons and opossums) in the United States. However, unlike raccoons and opossums which tend to be infected with a particular genotype, southern plains woodrats were infected with TcI and TcIV at near equal frequencies.

Introduction

Trypanosoma cruzi, a flagellated protozoan parasite, is the etiologic agent of American trypanosomiasis or Chagas' disease. The parasite is primarily transmitted to vertebrate hosts in the feces of blood-sucking triatomine bugs but infection of vertebrate hosts can also occur via blood transfusion, organ transplant, ingestion, lab accidents or vertical transmission from mother to offspring (Hoff et al., 1978; Ianni and Mady, 2005; Muños et al., 2007). Chagas' disease is endemic to the Americas and is a major cause of morbidity and mortality throughout Latin America with 8-11 million infected and ~50,000 deaths annually (Centers for Disease Control and Prevention, 2010; Moncayo, 1993). In the United States (US), only seven autochthonous cases have been reported in humans; four were from Texas and one each from Louisiana, California and Tennessee (Dorn et al., 2007; Greer, 1956; Herwaldt et al., 2000; Kjos et al., 2009; Ochs et al., 1996; Schiffler et al., 1984; Woody and Woody, 1955). Chagas' disease is very important from both a public health and veterinary perspective since it can cause fatal myocarditis during the chronic stage in humans, domestic dogs, and some other vertebrates (e.g., baboons (*Papio hamadryas* sp.), macaques (*Macaca silenus*), polar bear (*Ursus maritimus*), and sugar gliders (*Petaurus breviceps*) (Jaime-Andrade et al., 1997; Latas et al., 2004; Pung et al., 1998; Williams et al., 2009).

The first report of *T. cruzi* in the United States was in a reduviid vector, *Triatoma protracta.* The bugs were collected from the nests of woodrats (*Neotoma sp.)* from San Diego, California (Kofoid, 1916). Approximately two decade later, *T. cruzi* was reported in the dusky-footed woodrat, (*Neotoma fuscipes*) (Wood, 1934). This was followed by detection of natural infections in other wildlife species in southern Texas including Virginia opossums (*Didelphis virginiana*), nine-banded armadillos (*Dasypus novemcinctus*), and southern plains woodrats

(*Neotoma micropus*) (Packchanian, 1942). To date, in the US, *T. cruzi* has been detected in more than 20 wildlife species including several species of woodrats (*Neotoma* spp.). In addition to being hosts of *T. cruzi*, woodrats have been reported to be infected with at least two other *Trypanosoma* species, *T. neotomae* and *T. kansasensis* (Upton et al., 1989; Wood, 1936; Wood and Wood, 1937). These *Trypanosoma* spp. use fleas as intermediate hosts whereas *T. cruzi* is transmitted by various species of reduviid bugs.

Historically, hemoculture and examination of blood smears have been used to detect *T. cruzi* in wildlife in the US (John and Hoppe, 1986). Recently, serological tests such as the indirect immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and various commercial rapid tests (e.g. Chagas Stat-Pak® assay) have been utilized due to low sensitivities of hemoculture and blood smear analysis for detection of chronic infections. Rapid tests have been reported to be very sensitive and specific for detecting infections in humans, dogs and some wildlife species, but these assays do not work on all species and must be validated for use in new species (Nieto et al., 2009; Partel and Rossi, 1998; Yabsley et al., 2009).

Several studies have been conducted on the role of woodrats as hosts for *T. cruzi* (Burkholder et al., 1980; Eads and Hightower, 1952; Eads et al., 1963; Pinto et al., 2010; Pippin, 1970). It has been assumed that they are important reservoirs because they share nesting sites with reduviid vectors and prevalence rates have been relatively high ranging from 17-46% (Burkholder et al., 1980; Ikenga and Richerson, 1984; Pinto et al., 2010). The primary aim of the current study was to examine potential reservoirs in Uvalde Co., Texas by determining the prevalence of *T. cruzi* in southern plains woodrat and other known reservoir species using multiple diagnostic methods. Additionally, we genetically classified samples of *T. cruzi* detected in woodrats and other hosts.

Materials and Methods

Trapping and blood collection

During July 2008 and March and May 2010, 104 southern plains woodrats (*Neotoma micropus*), 20 raccoons, four striped skunks, and 28 small rodents were trapped at a total of four sites in Uvalde County, Texas. Small rodents included 14 house mice (*Mus musculus*), eight white ankled-mice (*Peromyscus pectoralis laceianus*), two hispid cotton rats (*Sigmodon hispidus*), a black rat (*Rattus rattus*), a white-footed mouse (*Peromyscus leucopus*), a rock squirrel (*Otospermophilus variegatus*), and a Mexican ground squirrel (*Ictidomys mexicanus*). Live traps (large Sherman traps -H.B. Sherman, Traps, Tallahassee, Florida and small squirrel cage traps (Havahart, Lititz, Pennsylvania) were baited with dried apricots for rodents and cage traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) were baited with sardines for raccoons and skunks. Trappings were done during the afternoon and checked the following morning. Captured animals were weighed, anesthetized and examined for ectoparasites. Rodents were anesthetized by intramuscular injection of 100mg/kg of ketamine (Fort Dodge Laboratories, 43 Inc., Fort Dodge, Iowa). Skunks and raccoons were anesthetized using the same method with a mixture of 20 mg/kg ketamine (Fort Dodge Laboratories, Inc., Fort Dodge, IA) and 4 mg/kg xylazine (Mobay Corporation, Shawnee, KS). Whole blood was collected by cardiocentesis from rodents and jugular venipuncture from skunks and raccoons and placed in potassium ethylenediaminetetraacetic acid (K_2EDTA) BD Vacutainer® tubes (Beckton Dickinson, Franklin Lakes, New Jersey). Rodents were euthanized by cervical dislocation and skunks and raccoons by intracardiac injection with an overdose of sodium pentobarbital (Butler Company, Columbus, OH) followed by exsanguination. All techniques were reviewed and approved by the IACUC committee at the University of Georgia.

Demographic parameters, including age and gender, were recorded for woodrats, skunks, raccoons and other rodents. Raccoons and skunks were classified as adults or juveniles based on weight, tooth wear and development of reproductive organs (Grau et al., 1970). Woodrats and other rodents were aged based on weight and development of reproductive organs.

Histopathology

Animals were necropsied and tissue samples (brain, lung, liver, heart, kidney, spleen, lymph nodes, quadriceps, gonads and sections of the gastrointestinal tract) were preserved in 10% buffered formalin for histopathological examination. Small sections of formalin-fixed tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) for light microscopic examination. Sections were closely examined for the presence of amastigote nests.

Diagnostic methods for *T. cruzi*

Blood smears

Blood smears were made from only woodrats sampled in 2010 (n=56). Smears were made directly with fresh cardiac blood, air-dried, fixed in absolute alcohol for 5 minutes and stained with Geimsa stain.

Hemoculture

One-ten mL aliquots of whole blood from raccoons, skunks, and larger rodents (woodrats, cotton rats, ground squirrel, and rock squirrel) was cultured in DH82 macrophages as described (Hall et al., 2007; Yabsley et al., 2004). Isolation attempts were not made on small rodents such as black rats, house mice and white-ankled mice due to insufficient blood volume. Briefly, to lyse erythrocytes, blood was mixed with 35mL of ammonium chloride potassium (ACK) solution, followed by gentle inversion for 5 minutes and centrifugation at 1620 x g for 10

minutes. The supernatant was discarded and the process repeated. The resulting buffy coat was resuspended in 5 mL minimum essential media (MEM) with 5% fetal bovine serum (FBS), and added to a confluent monolayer of DH82 cells. The culture was maintained at 37°C, fed twice weekly with fresh MEM supplemented with 5% FBS, and examined daily for the presence of trypomastigotes. If no parasites were evident after six weeks the sample was considered negative.

Serology

To detect antibodies to *T. cruzi,* we used two assays, the rapid Chagas Stat-Pak® assay (Chembio Diagnostics Inc., Medford, New York) and an indirect immunofluorescent antibody assay. The Chagas Stat-Pak® assay was conducted according to the manufacturer's instructions using 5μL serum or plasma. The indirect immunofluorescent antibody assay was performed as previously described (Yabsley et al., 2001) with the following modifications. Antigen slides were made by placing mixed cultures of trypomastigotes of several *T. cruzi* strains on serology slides (Erie Scientific, Portsmouth, NH, USA) which were then allowed to air dry and subsequently fixed in acetone for 5 minutes. Serum/plasma samples diluted 1:40 with phosphate buffered saline (PBS) and positive and negative controls were incubated on the slides for 30 minutes at 37°C. This was followed by two 5-minute washes of slides with PBS and a final wash with distilled water. Slides were then air dried. A commercial fluorescein (FITC-labeled) anti-species IgG antibody (1:25 dilution) was added to each well on the slides and incubated for a further 30 minutes at 37°C. Secondary antibodies used for rodents were goat anti-mouse or anti-rat IgG (Kirkegaard and Perry Laboratories (KPL), Gaithersburg, Maryland, USA). FITC-labeled goat anti-ferret and anti-raccoon IgG (KPL) were used for skunk and raccoon samples respectively. Incubation dishes were covered with aluminum foil to prevent photo-bleaching of

the fluorescein dye. Slides were washed twice in PBS for 5 minutes and finally in a 1.65% Eriochrome Black T counterstain (Sigma, St. Louis, Missouri, USA) in distilled water for five minutes. Slides were viewed under a Zeiss microscope equipped with a 50 watt Hg illuminator; green trypomastigotes indicated the sample was positive for *T. cruzi* and red was considered negative.

Molecular detection and characterization

DNA was extracted from 100µL whole blood using the DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA) according to manufacturer's protocol. The extracted DNA was used as a template in a nested PCR which amplified the D7 divergent domain of the 24Sα rDNA gene of *T. cruzi* using D75 5'-GCAGATCTTGGTTGGCGTAG-3' and D76 5'-GGTTCTCTGTTGCCCCTTTT-3' primers (Briones et al., 1999) in the primary reaction followed by D71 5'-AAGGTGCGTCGACAGTGTGG-3' and D72 5'-TTTTCAGAATGGCCGAACAGT-3' primers in a secondary reaction (Souto et al., 1996). Samples were also tested for *T. cruzi* using the primers S35 5'-AAATAATGTACGGGTGGAGATGCATGA and S36 5'-GGGTTCGATTGGGGTTGGTGT which target the kinetoplast minicircle DNA (Vallejo et al., 1999). DNA extractions, primary and secondary amplifications and product analysis were performed in separate dedicated laboratory areas. A negative water control was also included in each set of DNA extractions and PCR reactions as contamination controls. The expected 125- or 110-bp 24Sα and 330-bp minicircle amplicons were visualized by trans-illumination of an ethidium bromide-stained 1.5% agarose gel. *T. cruzi* samples were classified in one of two lineages previously detected in the US based on the 24Sα amplicons which yields a 125-bp for TcI or a 110-bp for TcIV (formally TcIIa) (Souto et al., 1996). Representative amplicons from the D71 and D72 primer pair were

sequenced at the University of Georgia Genomics Institute (Athens, Georgia) to confirm *T. cruzi* genotype results. Additionally, primary reactions that were positive but were subsequently negative on the nested reaction with primers D71 and D72 were presumed to be the other *T.* sp we detected in these woodrats so five random amplicons were sequenced to obtain phylogenetic information.

Statistical Analysis

The prevalence rates of *T. cruzi* and the other *T.* sp. were compared by age, gender, diagnostic method, and host species (for *T. cruzi* only) were compared for statistical significance using the Fisher's exact test (p=0.05). Kappa statistics in R was used to compare the level of agreement between the *T. cruzi* diagnostic tests.

Results

Of the 156 mammals tested from Uvalde Co., TX, 101 (65%) were infected with *T. cruzi* based on direct microscopic examination of blood smears, hemoculture, serology, or molecular testing. Trypomastigotes typical to *T. cruzi* were observed on stained blood films of only three of 56 woodrats (5%) sampled in 2010. These parasites measured between 15-17 μm in length, including the flagellum, and had a characteristic C or S-shaped conformation. The kinetoplast was large and sub-terminal giving the cell membrane a distorted appearance (Hoare, 1972; Kreier and Baker, 1991).

Trypanosoma cruzi was isolated in DH82 cell cultures from 28 (18%) animals (Table 3.1). By species, *T. cruzi* was isolated from 18 woodrats (26%), three skunks (75%), five raccoons (25%), a single rock squirrel (100%) and a hispid cotton rat (50%). Cultures from 35 woodrats were lost due to contamination with fungi and bacteria despite use of aseptic techniques during blood collection and inclusion of antimicrobials and anti-fungals in culture

media. Similar results were obtained with PCR testing; *T. cruzi* was detected in 23 of 104 woodrats (22%), three of four skunks (75%), 11 of 20 raccoons (55%) and five of 28 other rodents (18 %) (a cotton rat, rock squirrel, black rat, and two house mice) (Table 3.1). A total of 25 of 104 (24%) woodrats were infected with the *T. neotomae*-like trypanosome based on PCR and six were co-infected with *T. cruzi* as determined by PCR (Table 3.2). Based on PCR, no significant difference in prevalence was noted between woodrats and other rodents but prevalences in raccoons and skunks were significantly higher than woodrats ($p=0.005$; $p=0.043$ respectively).

Seventy-seven of 156 mammals (49%) had antibodies reactive with *T. cruzi* (Table 3.3). Based on combined results of the IFAT and Chagas Stat-Pak® assay, 50 of the 104 woodrats (48%), all 4 skunks (100%) and 18 of 20 raccoons (90%) were seropositive. All of the small rodents were negative based on IFAT testing, but five (18%) of the small rodents (two white-ankled mice, two house mice, and a rock squirrel), were positive for *T. cruzi* antibodies by the Chagas Stat-Pak assay. Seven of the 50 woodrats (14%) positive for *T. cruzi* by serology only were co-infected with the *T. neotomae*-like trypanosome (Table 3.2). Significantly more raccoons were seropositive compared with woodrats ($p=0.0004$) and other rodents ($p=0.0001$). No significant difference was noted between woodrats and skunks ($p=0.118$); however, woodrats had a significantly higher seroprevalence compared with other small rodents ($p=0.0046$). Overall, there was only a 55% agreement ($\kappa < 0.20$) between the IFAT and Chagas Stat-pak assays.

Another *Trypanosoma* sp. was detected in 41 of 104 (39%) woodrats (Table 3.2). This *Trypanosoma* sp. was observed in the blood smears of three of 56 (5%) woodrats (Table 3.2) but was most often observed in DH82 cultures (31 of 69 (45%) woodrats) (Table 3.2). These

parasites were observed in cell cultures for several days and were slowly removed when cells were fed fresh media. Geimsa-stained cytospins of scraped DH82 cells failed to show any intracellular stages of this *Trypanosoma* sp.; however, the parasites were frequently observed attached to the cell membranes. This *Trypanosoma* sp. failed to survive in liver infusion tryptose (LIT) media. No non-*T. cruzi* trypanosomes were observed in cultures from the skunks, raccoons, rock squirrel, or hispid cotton rat. Measurements of this woodrat *Trypanosoma* sp. were made from trypomastigotes observed in the blood smears of three woodrats. The total body length was 26.7 ± 3.15 (22 to 31 μm), length of body excluding flagellum was 20.3 ± 2 (17-23 μm), free flagellar length was 5.61 ± 0.86 (4-7 μm), distance from end to kinetoplast was 4.9 ± 0.7 (4-6 μm), body width was 1.8 ± 0.43 (1-2.5 μm), nucleus length was 1.7 ± 0.37 (1.5-2.5 μm), kinetoplast length was 0.9 ± 0.18 (0.5-1 μm), and posterior end to nucleus was 13.7 ±1.12 (12-15 μm). One woodrat had a mixed infection with this *Trypanosoma* sp. and *T. cruzi* on blood smear and several other co-infections were noted based on culture and PCR testing (Table 3,2).

Histology

Only mild histopathologic lesions were observed in woodrats and were primarily in skeletal muscle, heart, liver and brain. Some lesions were characteristic of histopathology due to infection with other parasites such as *Hepatozoon* or *Sarcocystis* species. *T. cruzi* amastigote nests were not identified in any of the tissues examined.

Genetic characterization

Only two genotypes (TcI and TcIV) were detected among our isolates. Woodrats (10 TcI and 13 TcIV) and skunks (1 TcI and 3 TcIV) were infected with both genotypes while all of the other positive rodents (hispid cotton rat and ground squirrel) and five raccoons were infected

with TcIV. Genotypes were not determined for all PCR positive animals since they could have been negative with D71/D72 primers but positive with the S35/S36 primers.

Partial sequences (233 bp) of the 24Sα gene of the *T. neotomae*-like sp. from five woodrats were identical. Sequences were very similar to several rodent trypanosomes including *T. kuseli* (99.1% identical, AB175626) from Siberian flying squirrels (*Pteromys volans*), *T. otospermophili* (99.1% identical, AB190225 and AB190228) from Richardson's and Columbian ground squirrels (*Urocitellus richardsonii* and *U. columbianus*, respectively), and *T. grosi* (98.7% identical, AB175624, AB175623, and AB175622) from *Apodemus speciosus* from Japan and *A. peninsualae* and *A. agrarius* from Vladivostok, Russia (Sato et al., 2005).

Population parameters

A total of 88 females (56 woodrats, 11 raccoons, 1 skunk and 20 other rodents) and 68 males (48 woodrats, 9 raccoons, 3 skunks and 8 other rodents) were tested in this study of which 121 were adults (84 woodrats, 14 raccoons, 3 skunks and 20 other rodents) and 28 were juvenile (20 woodrats, 6 raccoons, one skunk and one other rodent). The ages of seven rodents were undetermined. Although a slightly greater number of female woodrats (38 of 56) versus males (32 of 48) were PCR/culture positive and seropositive, no significant difference was noted in prevalence of *T. cruzi* by gender (p=1.000). A similar trend was observed in skunks (one female vs. three males) (p=1.000), raccoons (11 females vs. seven males) (p=0.190) and other rodents (five females vs. four males) (p=0.690). No differences in prevalence were noted according to age for any of the species in this study.

Regarding the *T.* sp., there was no significant difference between male and female woodrats (p=0.842) (21 of 55 females and 20 of 49 males) but significantly more adults compared with juveniles were infected (p=0.0113) (38 of 83 adults and 3 of 21 juveniles).

Discussion

Rodents are common hosts for *T. cruzi* throughout the Americas, but little research has been done on prevalence rates in the United States. To date, only 13 species have shown evidence of *T. cruzi* infection and only two isolates acquired from a single species (southern plains woodrat) have been genetically characterized (Burkholder et al., 1980; Navin et al., 1985; Packchanian, 1942; Roellig and Yabsley, 2010; Wood, 1952, 1962, 1975; Wood and Wood, 1961). Many rodents, including woodrats, are insectivorous, and their burrows may be infested with *Triatoma* spp., which would increase exposure to *T. cruzi* (Eads et al., 1963; Ryckman et al., 1965; Wood and Wood, 1976).

In the current study, using a combination of diagnostic assays, we detected a much higher prevalence of *T. cruzi* infection in southern plains woodrats (66%) compared with previous studies of the same species which detected prevalence rates between 17 and 35%. One previous study found a prevalence rate of 46%; however, few woodrats were examined (n=13) and the indirect hemagglutination (IHA) assay used for diagnosis of infections in that study has low specificity and reproducibility (Ikenga and Richerson, 1984; Remesar et al., 2009). Although we detected a high prevalence of *T. cruzi* in woodrats, raccoons and skunks sampled within the same county had significantly higher prevalence rates.

As expected, the combined prevalence rate of *T. cruzi* in woodrats based on hemoculture, blood smear and PCR was lower (34%) than the serologic prevalence rate since these diagnostic methods are most useful during acute infections when relatively high parasitemias are present. Other surveillance studies conducted on woodrats utilizing blood smear and/or culture reported similar prevalence rates ranging from 17% to 35% (Burkholder et al., 1980; Eads and Hightower, 1952; Packchanian, 1942). Similarly, our prevalence rate based on PCR of blood samples (22%)

was comparable to a recent study (26%) that utilized PCR to survey tissue samples from museum specimens of southern plains woodrats (Pinto et al., 2010). Numerous studies have shown that serologic testing is more sensitive for detecting chronic infections with *T. cruzi* since antibodies to *T. cruzi* can develop as early as 14 days post-infection and antibodies persist in circulation for years (Dusanic, 1991; Hall et al., 2010; Jansen et al., 1985; Yabsley et al., 2001).

A *T. neotomae*-like sp. was also common in the southern plains woodrats. Based on morphological characteristics and measurements, this trypanosome was more similar to *T. neotomae* than *T. kansasensis. T. kansasensis* is easily distinguished from *T. neotomae* based on several morphologic measures including a longer total body length (29.6-34 μm versus 22.5-33.7 μm), longer free flagellum length (6.4-11.2 versus 4.4-5.0) and greater length between posterior end to kinetoplast (4.8-7.6 μm versus 2.7-4.4 μm) (Davis, 1952; Upton et al., 1989). The total length, free flagellar length and distance from posterior end to kinetoplast of the southern plains woodrat *T*. sp. is similar to *T. neotomae* but can be distinguished by a shorter distance from the posterior end to the kinetoplast and shorter free flagellum length. No other measurements were available for *T. neotomae* for comparison. Several morphologic measures can distinguish our *T.* sp. from *T. kansasensis*. Additionally, *T. kansasensis* has a significantly larger kinetoplast compared with our *T*. sp. and *T. neotomae*. Previous studies have detected non-*T. cruzi* trypanosomes in *N. floridana (*eastern woodrat)*,* (*T. kansasensis*- 3 of 23 (13%), *N. micropus* (*T. neotomae,* 3 of 50, (6%)) and *N. fuscipes* (*T. neotomae*, 1 of 78 (1.3%) and 12 of 61 (20%) (Packchanian, 1942; Upton et al., 1989; Wood, 1936). All are believed to be transmitted by fleas which were found in our study (Hoare, 1972; Wood, 1936). In general, prevalence rates with these trypanosomes were low compared to our study; in which, 39% were infected with the *T. neotomae*-like species. Also of interest was the relatively high rate of co-infection of *T. neotoma-*

like sp. infected woodrats (27 of 41 (66%) with *T. cruzi.* Hemoculture proved to be the most efficient method of detecting co-infections of *T. cruzi* and the *T. neotomae*-like trypanosome, but it should be noted that a number of cultures were lost due to contamination which led to a number of diagnoses being based on PCR. We believe that this *Trypanosoma* sp. from the southern plains woodrat may be novel since some morphologic characteristics were different from those of *T. neotomae*; however, further work is needed to characterize this species since our partial 24Sα gene sequence is the only sequence available from *Trypanosoma* spp. from *Neotoma*.

The detection or isolation of *T. cruzi* from a hispid cotton rat, a black rat, two white-ankled mice, and a rock squirrel represent new species reports. We also detected a high prevalence (32%) of *T. cruzi* among non-woodrat rodents when multiple diagnostic methods were used compared to previous studies (5%-16%) that were based on only one diagnostic assay (i.e., blood smear analysis, xenodiagnosis, or culture) (Burkholder et al., 1980; Navin et al., 1985; Packchanian, 1942). We suspect that the lower prevalence of *T. cruzi* among these rodents compared with woodrats is related to biology of the woodrats and triatomine vectors. The close association of woodrats and triatomines is well documented (Burkholder et al., 1980; Kofoid, 1916); however, little is known about triatomine interactions with other rodents. In addition, none of the rodents were positive with the IFAT assay which could be due to lack of cross-reactivity of our rodent-antibody. The only seropositive non-woodrat rodents were detected with the Chagas Stat-Pak® assay which has been used to detect antibodies in experimentally-infected degus (*Octodon degus*), laboratory rats, and laboratory mice; however, this assay did not have very high sensitivity (Yabsley et al., 2009).

Although raccoons are commonly exposed to and infected with *T. cruzi*, the seroprevalence (90%) detected in raccoons from Uvalde County, Texas was higher than all previous studies, even when individual locations are examined (maximum of 70% at one site in northern Florida) (Brown et al., 2010; Maloney et al., 2010). Previous work in Arizona and the states east of the Mississippi River found seroprevalence rates between 20% and 20-68%, respectively (Brown et al., 2010; Burkholder et al., 1980; Hancock et al., 2005; Maloney et al., 2010; Yabsley and Noblet, 2002). Within Texas, two previous studies have investigated *T. cruzi* in raccoons; one isolated *T. cruzi* from blood samples from 6 of 25 (24%) raccoons in Brown County (Schaffer et al., 1978) and another study failed to detect antibodies in 9 raccoons from southern Texas (Cameron and Hidalgo Counties) using an IHA assay (Burkholder et al., 1980). The high seroprevalence in southern Texas is likely related to increased host-vector interactions as there is a high diversity and density of vectors in this region (Kjos et al., 2009; Woody and Woody, 1961).

Results of the current and previous studies suggest that skunks are important hosts for *T. cruzi*. All four striped-skunks we tested were positive for antibodies to *T. cruzi*. Although our sample size was small, previous studies, also with low samples sizes, have found similarly high seroprevalence rates; 3 of 34 (9%) from Arizona, 1 of 1 from California, and 1 of 1 from Georgia. (Brown et al., 2010; Ryan et al., 1985). Striped skunks are omnivorous but consume a large number of insects (Feldhamer et al., 2003). Additional work on the prevalence of *T. cruzi* among skunk populations is warranted.

In the United States, only two (TcI and TcIV) of the six known genotypes have been detected in vectors and vertebrate hosts (Roellig et al., 2008). A host-genotype dichotomy has been observed with Virginia opossums being naturally and experimentally infected with only TcI

while raccoons predominately being infected with TcIV, although they are susceptible to natural and/or experimental TcI infections (Clark and Pung, 1994; Roellig et al., 2008; Roellig et al., 2010). To date, all characterized autochthonous human infections with *T. cruzi* in the US have been TcI (Roellig et al., 2008) which is the predominant cause of Chagas' disease north of the Amazon basin and Mexico (Bosseno et al., 2002). Interestingly, in the current study, no predominate genotype was noted in woodrats, TcI accounted for 43% of isolates and TcIV accounted for 57%. Previously, only two southern plains woodrat *T. cruzi* isolates had been typed and one was a TcI and the other was a TcIV (Roellig and Yabsley, 2010). Previously, only a single isolate of *T. cruzi* from a striped skunk from Georgia had been typed and it was a TcIV (Brown et al., 2010); our data indicate that skunks can be hosts for both TcI and TcIV genotypes. As has been found in previous studies, TcIV was the predominate genotype detected in raccoons (Roellig et al., 2008). The finding of TcIV in the cotton rat and a rock squirrel represents the first genetic characterization of rodent isolates of *T. cruzi* except for two previously characterized southern plains woodrat isolates (Roellig and Yabsley, 2010).

In summary, our data indicate that woodrats, raccoons, skunks and other rodents are reservoirs of *T. cruzi* in Uvalde County, Texas and that multiple diagnostic methods should be employed to acquire the best estimate of *T. cruzi* infection status in hosts. We also report *T. cruzi* infection in a rock squirrel, black rat, two white-ankled mice, and a hispid cotton rat for the first time. In our study area, woodrats are as important or more important as reservoirs compared with raccoons, skunks, and other rodents. Genetic characterization data suggest that woodrats can become infected with both TcI and TcIV genotypes with similar frequency (Roellig and Yabsley, 2010). We also report the first infection of a striped skunk with a TcI isolate. Unfortunately, no Virginia opossums, the most common host of TcI in the United States in

previous studies, were captured during our field work. These alternative placental hosts may be important reservoirs of TcI in southern Texas in areas where opossum densities are not as high as other hosts. Further work on infectivity and virulence studies should be done on the two genotypes found in this study combined with a larger geographic survey of woodrats as reservoirs of *T. cruzi* in the United States. Additionally, isolates of *T. cruzi* should be genetically characterized to determine if other woodrat species or woodrats in other regions of the US or Mexico are common hosts to multiple genotypes. Of interest with this species reservoir is the high rate of co-infections with another species of trypanosome which is not observed in other common *T. cruzi* reservoirs in the United States. Additional studies on the interactions of these two species are warranted to determine if infection dynamics are altered based on previous or concurrent infections.

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Table 3.1. Blood smear, hemoculture, and polymerase chain reaction assay results for *Trypanosoma cruzi* in 156 mammals from Uvalde, Texas.

Species	Diagnostic Assay No. positive/No. tested (%)			Total infected (%)
	Blood smear	Hemoculture	PCR*	
Southern plains woodrat (*Neotoma micropus*) n=104	3/56 (5)	18/69 (26)	23/104 (22)	35/104 (34)
House mouse (*Mus musculus*) n=14	n.d.†	n.d.	2/14 (14)	2/14 (14)
White-ankled mouse (*Peromyscus pectoralis laceianus*) n=8	n.d.	n.d.	0/8 (0)	0/8 (0)
White-footed mouse (*Peromyscus leucopus*) n=1	n.d.	n.d.	0/1 (0)	0/1 (0)
Hispid cotton rat (*Sigmodon hispidus*) n=2	n.d.	1/2 (50)	1/2 (50)	2/2 (100)
Black rat (*Rattus rattus*) n=1	n.d.	n.d.	1/1 (100)	1/1 (100)
Mexican ground squirrel (*Ictidomys mexicanus*) n=1	n.d.	n.d.	0/1 (0)	0/1 (0)
Rock squirrel (*Otospermophilus variegatus*) n=1	n.d.	1/1 (100)	1/1 (100)	1/1 (100)
Raccoon (*Procyon lotor*) n=20	n.d.	5/20 (25)	11/20 (55)	12/20 (60)
Striped skunk (*Mephitis mephitis*) n=4	n.d.	3/4 (75)	3/4 (75)	3/4 (75)

*PCR, polymerase chain reaction.
†n.d., not done.

Table 3.2. Results of diagnostic testing of woodrats for a *Trypanosoma neotomae*-like species and association with *T. cruzi* infection

	n	No. infected (% of total tested)	No. co-infected with *T. cruzi* based on PCR(% of infected) n=23	No. co-infected with *T. cruzi* based on culture (% of infected) n=18	No. co-infected with *T. cruzi* based on serology (% of infected)* n=50
Blood smear	56	3 (5)	0 (0)	0 (0)	1 (2)
Culture	69	31 (45)	5 (22)	10 (56)	5 (10)
PCR	104	25 (24)	6 (26)	14 (78)	1 (2)
Total unique individuals		41 (39)	6 (26)	14 (78)	7 (14)

*these woodrats were only seropositive for *T. cruzi* and were PCR and culture negative

Table 3.3. Serology results for *Trypanosoma cruzi* in 156 mammals from Uvalde, Texas.

Species	Chagas Stat-Pak® Assay	IFAT*	Total infected (%)
	No. positive/No. tested (%)		
Southern plains woodrat (*Neotoma micropus*) n=104	27/104 (26)	38/104 (37)	50/104 (48)
House mouse (*Mus musculus*) n=14	2/14 (14)	0/14 (0)	2/14 (14)
White-ankled mouse (*Peromyscus pectoralis laceianus*) n=8	2/8 (25)	0/8 (0)	2/8 (25)
White-footed mouse (*Peromyscus leucopus*) n=1	0/1 (0)	0/1 (0)	0/1 (0)
Hispid cotton rat (*Sigmodon hispidus*) n=2	0/2 (0)	0/2 (0)	0/2 (0)
Black rat (*Rattus rattus*) n=1	0/1 (0)	0/1 (0)	0/1 (0)
Mexican ground squirrel (*Ictidomys mexicanus*) n=1	0/1 (0)	0/1 (0)	0/1 (0)
Rock squirrel (*Otospermophilus variegatus*) n=1	1/1 (100)	1/1 (100)	1/1 (100)
Raccoon (*Procyon lotor*) n=20	13/20 (65)	18/20 (90)	18/20 (90)
Striped skunk (*Mephitis mephitis*) n=4	4/4 (100)	4/4 (100)	4/4 (100)

*IFAT, indirect immunofluorescent antibody test.

CHAPTER 4

SOUTHERN PLAINS WOODRATS (*NEOTOMA MICROPUS*) FROM UVALDE COUNTY, TEXAS ARE HOSTS TO A HIGH DIVERSITY OF PARASITES, SOME OF WHICH ARE OF VETERINARY OR MEDICAL SIGNIFICANCE[1].

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[1]Charles, Roxanne A., Angela E. Ellis, J.P. Dubey, Barbara C. Shock, and Michael J. Yabsley. To be submitted to *Veterinary Parasitology.*

Abstract

From 2008-2010, southern plains woodrats (*Neotoma micropus*) from Uvalde County, Texas, were examined for parasites and selected pathogens. Intestinal tracts and other major organs were examined from 97 woodrats and we recovered eight helminth species including, *Trichuris neotomae* from 78 [prevalence=80%]), *Ascarops* sp. from 42 (43%), *Nematodirus neotoma* from 31 (32%), *Raillietina* sp. from 9 (9%), *Taenia taeniaeformis* larvae from 8 (8%), and an unidentified female spiurid, a *Scaphiostomum* sp. and a *Zonorchis* sp. each from a single woodrat. *Besnotia neotomofelis* cysts were detected in tissues from 3 (3%) woodrats and microfilaria were detected in blood samples from 7 (7%). PCR testing of blood samples from 104 woodrats detected a novel *Babesia* sp. in 1 (1%) and *Hepatozoon* sp. in 17 (16%) woodrats. Partial 18S rRNA gene sequence of the *Babesia* species was most similar (94% similarity) to *B. conradae*, a parasite of dogs in California. Histologic examination of tissues detected intestinal coccidia in 7 of 104 (7%), *Sarcocystis neotomafelis* in various muscles of 26 (25%), *Hepatozoon* sp. in the liver of 21 (20%), and *Dunnifilaria meningica* in the brain of 4 (4%) woodrats. Three woodrats (5%) were seropositive for *Toxoplasma gondii*. Ectoparasites recovered included two fleas (*Orchopeas sexdentatus* and *O. neotomae*), two ticks (*Ixodes woodi* and *Ornithodoros turicata),* two mites (*Trombicula* sp. and *Ornithonyssus (Bdellonyssus) bacoti*) and one bot fly (*Cuterebra* sp.). There was no difference in prevalence for any parasite related to gender (p=0.460), except for *N. neotoma* (males > females, p=0.029). Prevalence of *T. neotomae* and all intestinal parasites combined was significantly higher in adults compared with juveniles (p=0.0068 and p=0.0004), respectively. Lesions or clinical signs were associated with *Cuterebra*, *T. gondii*, and *B. neotomofelis* infections. Collectively, these data indicate that southern plains woodrats from southern Texas harbor several parasites of veterinary and/or medical importance.

Introduction

The southern plains woodrat (*Neotoma micropus*), commonly called a packrat, is a medium-sized, nocturnal rodent that inhabits semi-arid brush lands, low valleys and plains of the south-central and southwestern United States and northeastern Mexico. In Texas, *N. micropus* inhabits areas dominated by thorny desert shrubs or cacti (Braun and Mares 1989) and their diet consists mainly of vegetation such as succulent leaves and fruit of cacti, seeds and acorns (Raun 1966). Woodrats (*Neotoma* spp.) are common hosts for ticks and fleas which are potential vectors of tularemia (*Francisella tularensis*), plague (*Yersinia pestis*), Q fever (*Coxiella burnetti*), relapsing fever (*Borrelia* spp.) and Rocky Mountain spotted fever (*Rickettsia rickettsi*). Other pathogenic organisms reported from woodrats include *Trypanosoma cruzi* (causative agent of Chagas' disease in humans and domestic animals), *Besnoitia neotomofelis*, and *Leishmania mexicana* (McHugh et al. 1990; Dubey and Yabsley 2010; Pinto et al. 2010).

Although numerous studies have looked at the ectoparasitic fauna of woodrats in Texas, to date, only a few studies have looked at endoparasites of southern plains woodrats. Collectively, in the United States and Mexico, only nine species have been reported including: *Taenia taeniaeformis, Litomosoides carinii, Dunnifilaria meningica, Trichuris muris*, *L. mexicana*, *Trypanosoma cruzi, Trypanosoma neotomae*, *Sarcocystis neotomafelis*, and *B. neotomofelis* (Packchanian 1942; Johnson 1966; Burkholder et al. 1980; Gutierrez-Pena 1989; Galaviz-Silva et al. 1991; Pinto et al. 2010; Charles et al. 2011). Because higher diversities of parasites have been reported in other species of woodrats in the southwestern United States, we conducted this study to better understand the endo- and ectoparasitic fauna of southern plains woodrats from Uvalde Co., Texas.

Materials and Methods

Trapping

A total of 104 southern plains woodrats (56 females and 48 males) were trapped during July 2008 and March and May 2010 at four sites in Uvalde Co., Texas. Animals were live trapped by small squirrel cage traps (Havahart, Litz, Pennsylvania) and large Sherman traps (H.B. Sherman Traps, Tallahassee, Florida) baited with dried apricots. Trap stations were selected based on fresh tracks and rodent droppings at the base of presumed woodrat nests built among cactus (*Opuntia* spp.) plants. Traps were set in the afternoon and checked the following morning.

Anesthesia and blood collection

Captured animals were anesthetized and weighed. Briefly, woodrats were anesthetized with 100mg/kg ketamine (Fort Dodge Laboratories, Fort Dodge, Iowa) followed by blood collection via cardiocentesis into potassium ethylenediaminetetraacetic acid (K_2EDTA) BD Vacutainer® tubes (Beckton Dickinson, Franklin Lakes, New Jersey) using aseptic techniques. In 2010, blood smears were made with fresh blood, air-dried, fixed in absolute alcohol for five minutes, and stained with Geimsa stain. All animals were euthanized by cervical dislocation and adult and juvenile (not pups) were then necropsied and examined for parasites. All techniques were reviewed and approved by the IACUC committee at the University of Georgia.

Parasite collection and identification

Each woodrat was examined for ectoparasites by combing back the fur and collecting specimens with fine forceps. Collected ectoparasites were preserved in 100% ethanol. Bot-fly larvae were removed by gentle traction with forceps and characterized using polymerase chain reaction (PCR) and sequencing of two regions of the cytochrome oxidase subunit I (COI) gene as

described in Table 4.1. Fleas, mites, and ticks were mounted on slides using saline solution and identified to species with a light microscope and published taxonomic keys (Eads 1950; Keirans and Litwak 1989; Lewis 2000; Haas et al. 2004).

During necropsy, the viscera of all woodrats were grossly examined for the presence of parasites such as *Taenia* and *Besnoitia* cysts. The entire length of gastrointestinal tract and some organs (pancreas, liver, and spleen) were removed from the abdominal cavity, dissected under a dissecting scope, and closely examined for helminths. Contents were filtered through a 100μm sieve (W.S. Tyler Incorporated, Mentor, Ohio) for concentration of parasites. All parasites were stored in 100% ethanol and examined under a light or dissecting microscope for identification. Large nematodes were cleared with a 70% ethanol/30% phenol solution.

Histopathology

Tissue samples including brain, lung, liver, heart, kidney, spleen, lymph nodes, quadriceps, gonads and sections of the gastrointestinal tract were preserved in 10% buffered formalin for histopathological examination. Small sections of formalin-fixed tissues were embedded in paraffin, sectioned at 5μm and stained with hematoxylin and eosin (H&E) for light microscopic examination.

Serologic testing for *Toxoplasma gondii*

Sera samples from 66 woodrats were tested for antibodies to *T, gondii* by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

Molecular detection of parasites

Several PCR assays were used to test woodrats for hemoparasites and vector-borne bacteria. DNA was extracted from 100 μL of whole blood using the DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. The extracted DNA was

used as a template to test for species of *Babesia, Hepatozoon*, *Bartonella*, and *Rickettsia* as described in Table 3.1. Briefly, for primary amplification, 5 µl of DNA was added to 20 µl of a master mix containing 11 µL of molecular grade biological water (MGBW), 2.5 µL of $MgCl_2$, 5 µL of GoTaq Flexi Clear Buffer (Promega, Madison, Wisconsin), 0.25 µL of dNTP, 0.5 µL of each primer, 0.25 µL of GoTaq Flexi (Promega). For each secondary reaction (if needed), 1 µL of primary product was used as a template in a 25 µL reaction containing similar PCR components with the exception of an additional 4 µl of water and different primers. Stringent protocols and controls were utilized in all PCR reactions to prevent and detect contamination. Separate dedicated laboratory areas were used for DNA extraction, primary amplification, secondary amplification, and product analysis. A negative water control was included in each set of DNA extractions, and one water control for each set of primary and secondary PCR reactions. Amplicons were visualized by trans-illumination of an ethidium bromide-stained 1.5% agarose gel.

Statistical analyses

Parasite prevalence, intensity and range were calculated as defined by Bush et al. (1997). Fisher's exact test was used to test for differences in parasite prevalence (by species and collectively) among age classes and gender. A two-way ANOVA implemented by SAS, was used to determine if gastro-intestinal nematode intensity varied according to age and/or gender.

Results

A total of nine helminth species were recovered from 90 of 97 woodrats (54 females/43 males and 79 adults/18 juveniles) including four species of nematodes (*Trichuris neotomae, Nematodirus neotoma, Ascarops* sp. and a single unidentified female spiurid), two species of cestodes (*Raillietina* sp. and *Taenia taeniaeformis)* and two species of trematodes

(*Scaphiostomum* sp., a pancreatic fluke, and *Zonorchis* sp., a liver fluke) (Table 4.3). Additionally, microfilaria of an unknown filarial nematode species (likely either *Litosomoides carinii* or *Dunnifilaria meningica*) was found in the blood. The *T. taeniaformis* cysticerci were found encysted in the liver and the adult stage of a *Raillietina* sp. was found in the lumen of the small intestine. The three nematodes were all found in the gastrointestinal tract, *Ascarops* sp. in the stomach, *N. neotomae* in the small intestine, and *T. neotomae* in the large intestine (Table 4.3). The only helminth that was associated with gross lesions was the *Ascarops* sp. which were surrounded by areas of thickened gastric mucosa (~1cm diameter) which housed multiple worms, up to 17 in one case. Seven woodrats (three females/four males and two adults/five juveniles) were negative for all helminths.

No difference in prevalence of whipworms was noted for gender (p=1.000) but adults had a significantly higher prevalence compared with juveniles (p=0.0068). In contrast, prevalence of *Nematodirus neotomae* was significantly higher in males (19 of 43) compared to females (12 of 54) (p=0.0285), but no difference was noted for age (p=0.165). Overall gender was not associated with differences in prevalence of intestinal parasites (p=0.460) but age was, with more adults 77 of 79 (98%) being infested than juveniles (12 of 18 (67%) (p=0.0004). Based on ANOVA, the interaction of age and gender on parasite intensity for all gastro-intestinal nematodes was not significant (p>0.05).

A total of 181 ectoparasites (118 fleas, 9 ticks, 39 mites and 15 bots) were collected from 42 of the 104 (40%) woodrats (Table 4.2.). Bot fly larvae were found predominantly on the chest and neck regions of infested woodrats but one (first instar) larva was found in the nasal cavity. A single woodrat (juvenile female, weight, 118g) that had 4 larvae (1.5-2 cm in length) under the chin and on the chest region was found severely emaciated, lethargic, and non-responsive a few

inches outside one of our traps. We were able to collect the woodrat by hand after which it died while we performed an external examination. Sequence of a fragment of the COI gene (411 bp) from a single bot fly was 87.9% similar to *C. grisea* (AY507182) and 86.8% similar to *C. fontinella* (AY507188). Sequences of another region of the COI gene (544bp) from two bot fly larvae from two different animals were identical and 90.4% similar to *C. fontinella* (AY507197) and 89.9% similar to *C. grisea* (AY507222).

Based on PCR testing, a novel *Babesia* was detected in 1 of 42 (2%) woodrats in 2008; all were negative in 2010 (Table 4.4). Partial 18S rRNA gene sequence of the *Babesia* species was most similar (434 of 460bp, 94.4% similarity) to *B. conradae* (AF158702), a parasite of domestic dogs in California. Other similar *Babesia* species included *B. lengau* (94.1%) from cheetahs (*Acinonyx jubatus*) (GQ411405-GQ411417), *B. vesperuginis* (92.4%) from a *Pipistrellus* sp. bat from England (AJ871610), and *B. duncani* (92%) from humans in California (AY027815). *Hepatozoon* sp. was commonly detected in blood samples of woodrats by PCR (Table 3.4); no gamonts were observed in blood smears. All woodrat blood samples were PCR negative for *Bartonella* and *Rickettsia*. Three of 66 (5%) woodrats were positive for antibodies to *T. gondii* (titers of 1:25, 1:25, and 1:400). Additionally, *T. gondii* was isolated from digested brain, heart and tongue tissue from one seronegative woodrat, but not from any of the three seropositive woodrats (data not shown, Dubey et al., in press).

Several parasites were detected during histologic examination of tissues. A meningeal worm (*Dunnifilaria meningica*), associated with mild lymphocytic and eosinophilic meningitis, was observed in the meningeal and sub-meningeal spaces of four (4%) woodrats. Sarcocysts of *S. neotomafelis* were observed in 26 of 104 (25%) woodrats with more sarcocysts being found in the quadriceps muscle (26/26) (100%) compared with the myocardium (4/26) (15%). *S.*

neotomafelis cysts ranged from a few (3-4) to numerous (>50) per tissue sample. A few cysts were accompanied with a mild multifocal myositis. *Hepatazoon* cysts were observed in the liver of 21 (20%) woodrats compared to 17 (16%) that were positive with PCR of blood samples. Only six woodrats were positive for *Hepatozoon* sp. by PCR and histology. Two encysted flukes surrounded by fibrous capsules were found in the liver of one woodrat. Based on histology, *Besnoitia neotomofelis* was detected in the tissues of two woodrats (2%). A third woodrat, which was moribund at capture, had numerous grossly visible cysts present in the facial skin and throughout the musculature and subcutaneous layer of the body (Charles et al. 2011). Developmental stages (micro- and macrogametocytes and oocysts) of coccidia were found in the small and large intestine of seven (7%) woodrats.

Discussion

Southern plains woodrats are known to be hosts for a suite of parasites, some of which are of medical and veterinary importance (Packchanian 1942; Johnson 1966). Considerable work has been done to characterize the ectoparasitic fauna of southern plains woodrats, but to date, there is a paucity of reported information on the diversity and prevalence of parasites and vector-borne pathogens in this rodent species. In this current study, we report eight new parasite-host associations including one flea (*Orchopeas neotomae*), two trematodes (*Scaphiostomum* sp. and *Zonorchis* sp.), one stomach nematode (*Ascarops* sp.), an intestinal nematode (*N. neotoma*), and three protozoan parasites (*T. gondii*, *Babesia* sp., and coccidia). Additionally, we report *S. neotomafelis* infection in *N. micropus* for the first time in the US. Collectively, these data indicate that southern plains woodrats in Uvalde Co., Texas are hosts to several parasites and pathogens, some of which may cause disease in humans, woodrats, and other animals.

All ectoparasites recovered from the woodrats in this study have been previously recorded from this host with the exception of *O. neotomae* (Johnson 1966). This flea species feeds primarily on *Neotoma* spp., especially the Mexican woodrat (*N. mexicana*). The range of *N. mexicana* overlaps with that of *N. micropus* in New Mexico, far western Texas, and parts of Mexico but not in our study area. Similar to our findings, Stark (1958) found co-infestations of with *O. neotomae* and *O. sexdentatus* on the same host (Stark 1958). Both flea species are involved in the host-flea complex in the spread of *Yersinia pestis* in western United States (Anderson and Williams 1997).

Flies of the genus *Cuterebra* are very common in most temperate and tropical regions in the western hemisphere (Sabrosky 1986). Larvae of these flies infest the subcutis of lagomorphs and rodents (including *Neotoma* spp.). Although bots are relatively large compared to their hosts, they rarely cause mortality. Young animals are more prone to injury or increased susceptibility to predation if larval burdens are high. In this study two infested woodrats exhibited good body condition, but one juvenile woodrat that was infested with four bots ~2cm long likely died due to *Cuterebra* infestation. Sequences from our woodrat samples were most similar to *C. fontinella* and *C. grisea* (only two *Cuterebra* spp. in Genbank) which are both parasites of mice in the genus *Peromyscus* (Noel et al. 2004). Bot flies are common in woodrats, but unfortunately there are no sequences available for the many *Cuterebra* spp. reported from *Neotoma* spp. for comparison with the sequences we obtained (Baird 1979; Baird 1997; Wilson et al. 1997).

Several of the helminths detected in this study are common parasites of *Neotoma* spp. including *Trichuris* spp., *N. neotomae*, *Raillietina* sp., and *T. taeniaeformis*. Prevalence of *Trichuris* spp. in woodrats is typically very high; a previous study of *N. micropus* reported *T. muris* in all four woodrats examined (Johnson 1966). *T. muris* (likely now considered to be *T.*

neotomae) has also been reported from the eastern woodrat (*N. floridana*) from Oklahoma and *T. neotomae* has been reported from the dusky-footed woodrat (*N. fuscipes*) in California (Chandler 1945; Boren et al. 1993). Although this is the first report of *N. neotoma* (=*N. tortuosus*) (Hoberg et al. 1988) in *N. micropus*, it has been reported from numerous other woodrat species including *N. fuscipes*, the bushy-tailed woodrat (*N. cinerea*), the desert woodrat (*N. lepida*), *N. mexicana*, and *N. floridana* (Hall 1916; Tucker 1942; Miller and Schmidt 1982). Neither of these helminths has been associated with disease in woodrats.

Woodrats were hosts to two different species of tapeworms, *T. taeniaeformis* which were found as larvae in the liver and a *Raillietina* sp. that was found as adults in the small intestine. *T. taeniaeformis* has been reported from southern plains woodrats previously with 26% of 88 being positive (Johnson 1966). Strobilocerci of *T. taeniaeformis* are commonly found in the liver of woodrats, rabbits, squirrels, muskrats, bats, voles, other small rodents and occasionally humans and the adult form in felids which are definitive hosts (Johnson 1966; Theis and Schwab 1992; Fichet-Calvet, et al. 2003). Rarely does this parasite cause any significant lesions or disease in intermediate hosts; however, high numbers can cause infertility and hepatic neoplasia in some rodents (Lin et al. 1990; Irizarry-Rovira et al. 2007). Although *Raillietina* spp. are uncommon in woodrats, they have been reported in *N. cinerea*, *N. fuscipes*, and *N. lepida* (Linsdale and Tevis, 1951; Grundmann 1958; Miller and Schmidt 1982).

Prior to this study, no trematode species have been reported from *Neotoma* spp. We detected at least two species, *Scaphiostomum* sp. and *Zonorchis* sp., and a possible third species was found encysted in the liver of one woodrat that is not believed to be *Zonorchis* (typically found in the gall bladder, bile ducts or small intestine proximal to the bile duct opening) (McIntosh 1939; Santos et al. 2010). *Scaphiostomum* spp. are usually found in the pancreatic

duct of its definitive rodent hosts, including the white-ankled mouse (*Peromyscus pectoralis*) in Texas (Santos et al. 2010). The primary intermediate host is the terrestrial flamed disc snail, *Anguispira alternata*, and secondary intermediate terrestrial snail hosts include *Neohelix* (=*Triodopsis) albolaris* and *Haplotrema concavum* (Schell 1985). *Zonorchis* sp. has also been reported from the white-ankled mice in Texas, but this parasite utilizes the terrestrial snail *Polygyra texasiana* as an intermediate host (Schell 1985). The low prevalence of *Scaphiostomum* sp. and *Zonorchis* sp. is likely due to a low density of appropriate intermediate hosts or because these snails are rarely consumed by *N. micropus.*

A novel *Babesia* sp. was detected in a single woodrat from our 2008 collection. Unfortunately, no blood smears were made in 2008 so no morphologic data is available. Based on partial 18S rRNA gene sequence, this woodrat *Babesia* was most similar to *B. conradae*, a canine species from California (Kjemtrup et al. 2006). This represents the first report of a *Babesia* species in a *Neotoma* spp.; however, a different piroplasm, *Theileria youngi*, has been reported from *N. fuscipes* from California (Kjemtrup et al. 2001). Possible vectors include *I. woodi*, a common tick found in the current study and previous studies (Eads et al. 1952; Johnson 1966) and the less commonly found ticks, *Amblyomma inornatum, Dermacentor variabilis*, and *D. parumapertus* (Eads et al. 1952; Eads and Hightower 1956; Johnson 1966).

Based on histology and PCR testing of blood, *Hepatozoon* infections were common in this woodrat species. Recently, genetic characterization of *Hepatozoon* sp. from southern plains woodrats found it was related to a *Hepatozoon* sp. from *N. fuscipes* in California as well as other rodent *Hepatozoon* (Allen et al. 2011). Similar to a study of *Hepatazoon* in cotton rats (*Sigmodon hispidus*), meronts were present in the liver of our naturally infected woodrats (Johnson et al. 2007). Interestingly, *Hepatozoon* from *N. fuscipes* and *N. micropus* were

genetically similar to *Hepatozoon* detected in snakes and other rodents (Allen et al. 2011). Genetic characterization of *Hepatozoon* from snakes, as potential hosts, in southern Texas may clarify the natural history of this understudied group of parasites.

Three tissue cyst-forming coccidians, *S. neotomafelis, B. neotomofelis,* and *T. gondii*, were detected in woodrats in the current study and interestingly, all are suspected to utilize felids as definitive hosts. Although *S. neotomafelis* was common in the woodrats in the current study, this parasite had previously only been reported in *N. micropus* from Nuevo Leon, Mexico (Galaviz-Silva et al. 1991). Only sections of heart and quadriceps were examined in this present study and cysts were more prevalent in quadriceps muscles whereas in a previous study, cysts were more common in the masseter muscles and only rarely found in quadriceps muscles (Galaviz et al., 1991). Currently, the only suspected definitive host for *S. neotomafelis* is the domestic cat; however, the only study that has attempted to experimentally determine the life cycle depicts an oocyst passed in cat feces that is morphologically more similar to *Isospora* than *Sarcocystis* (Galaviz-Silva et al. 1991). However, we believe that felids could be the definitive host because a recent study found a *Sarcocystis*-infected woodrat co-infected with two other felid-transmitted protozoans, *Besnoitia neotomofelis* and *T. gondii* (Charles et al. 2011). Cysts of *B. neotomofelis* were detected in low numbers in two woodrats which had no lesions associated with infection; however, the finding of a single woodrat that was moribund due to *Besnoitia* infection indicates that *B. neotomofelis* can be a cause of clinical disease (Charles et al., 2011). Although *Besnoitia* spp. are typically considered nonpathogenic, *B. jellisoni*, a parasite of deer mice (*Peromyscus maniculatus*) and kangaroo rats (*Dipodomys ordii* and *D. microps*) can cause disease if parasite numbers become very high (Ernst et al. 1968; Chobotar et al. 1970). Similarly, *T. gondii* infection was rare in woodrats; only three had low antibody titers and a single

seronegative woodrat had viable *T. gondii* isolated from tissues (data not shown) (Dubey et al. in press). This woodrat *T. gondii*-isolate was genetically characterized as a type 12 lineage (Dubey et al. in press). The only previous study on woodrats failed to find any antibodies to *T. gondii* in *N. fuscipes* in California (Dabritz et al. 2008).

Our results provide evidence that southern plains woodrats in this locale have a high diversity of parasites and at least one of the parasites detected, *T. gondii* is zoonotic. A concurrent study also detected a high prevalence of *Trypanosoma cruzi*, another important zoonosis (Charles, unpublished data). Of interest is the absence of gross lesions consistent with *Leishmania mexicana* on any of our woodrats (data not shown) as this parasite has been reported in woodrats, including *N. micropus*, in Texas (McHugh et al., 1990; Grogl et al., 1991; McHugh et al., 2003). Additionally, several of the parasites detected (i.e., *B. neotomofelis, T. gondii*, and *Cuterebra*) can cause disease in woodrats. Additionally, the detection of a novel *Babesia,* which has unknown medical or veterinary importance, highlights the need for further work on parasites or vector-borne pathogens of these common, often peridomestic, rodents.

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Table 4.1. Oligonucleotide primers used in polymerase chain reaction assays.

Target organism*	Gene target	Primer	Primer sequence (5'-3')	Reference
Babesia/Hepatozoon (1°)	18S rRNA	3.1	CTCCTTCCTTTAAGTGATAAG	Yabsley et al. (2005)
Babesia/Hepatozoon (1°)	18S rRNA	5.1	CCTGGTTGATCCTGCCAGTAGT	Yabsley et al. (2005)
Babesia/Hepatozoon (2°)	18S rRNA	RLBH-F	GAGGTAGTGACAAGAAATAACAATA	Yabsley et al. (2005)
Babesia/Hepatozoon (2°)	18S rRNA	RLBH-R	TCTTCGATCCCCTAACTTTC	Yabsley et al. (2005)
Bartonella	16S–23S rRNA ITS region	325s	CTTCAGATGATGATCCCAAGCCTTTTGGG	Maggi et al. (2008)
Bartonella	16S–23S rRNA ITS region	1100as	GAACCGACGACCCCCTGCTTGCAAAGCA	Maggi et al. (2008)
Rickettsia (1°)	17kDa antigen	17k-3	TGTCTATCAATTCACAACTTGCC	Labruna et al. (2004)
Rickettsia (1°)	17kDa antigen	17k-5	GCTTTACAAAATTCTAAAAACCATATA	Labruna et al. (2004)
Rickettsia (2°)	17kDa antigen	17Kd1	GCTCTTGCAACTTCTATGTT	Labruna et al. (2004)
Rickettsia (2°)	17kDa antigen	17kD2	CATTGTTCGTCAGGTTGGCG	Labruna et al. (2004)
Cuterebra	Cytochrome oxidase I (COI)	C1-J-2183	CAACATTTATTTTGATTTTTTGG	Noël (2004)
		C1-N-2659	GCTAATCCAGTGAATAATGG	
		C2-J-3138	AGAGCTTCACCCTTAATAGAGCAA	
		C2-N-3661	CCACAAATTTCTGAACATTGACCA	

*1°, primers used in the primary amplification; 2°, primers used in secondary amplification of a nested PCR protocol.

Table 4.2. Ectoparasite infestation of 104 southern plains woodrats, *Neotoma micropus*, from Uvalde County, Texas.

Ectoparasites	**No. collected***	**No. infested (%)**	**Mean intensity**	**Range**
Fleas				
Orchopeas sexdentatus	33♂,73♀	40 (39)	2.7	1-13
O. neotomae	7♂	6 (10)	1.2	1-2
Ticks				
Ixodes woodi	3A♀, 5N	5 (5)	1.6	1-2
Ornithodoros turicata	1A	1 (1)	1.0	1
Mites				
Trombicula sp.	3L	2 (2)	1.5	1-2
Ornithonyssus (=Bdellonyssus) bacoti	36A	6 (6)	6.0	1-29
Bot-flies				
Cuterebra sp.	15L	7 (12)	2.1	1-4

*L, larva; N, nymphs; A, adults.

Table 4.3. Helminth parasites of 97 southern plains woodrats (*Neotoma micropus*) from Uvalde County, Texas

Parasite	No. positive (%)	No. collected	Mean intensity	Range
Nematoda				
Trichuris neotomae	78 (80.4)	*448A, 55I	6.4	1-53
Ascarops sp.	42 (43)	100A, 82I	4.3	1-17
Nematodirus neotoma	31 (32)	637	20.5	1-134
Filarial nematodes	7 (7)	n.a.	n.a.	n.a.
Dunnifilaria meningica	4 (4)	n.a.	n.a.	n.a.
Unidentified female spiurid	1 (1)	1	1.0	1
Cestoda				
Raillietina sp.	9 (9)	24	2.7	1-13
T. taeniformis cysts	8(8)	19	2.4	1-8
Trematoda				
Scaphiostomum sp.	1 (1)	1	1	0-1
Zonorchis sp.	1 (1)	3	3	0-3

*A, adults; I, immatures

Table 4.4. Bacterial and protozoan parasites of 104 southern plains woodrats (*Neotoma micropus*) from Uvalde County, Texas.

Organism	Diagnostic method	No. infected (%)
Rickettsia spp.	Polymerase Chain Reaction	0 (0)
Bartonella spp.	Polymerase Chain Reaction	0 (0)
Babesia sp.	Polymerase Chain Reaction	1 (1)
Hepatozoon sp.	Polymerase Chain Reaction	17 (16)
	Histology	21 (20)
Besnoitia neotomofelis	Gross examination and histology	3 (3)
Sarcocystis neotomafelis	Histology	26 (25)
Coccidian oocysts	Histology	7 (7)
Toxoplasma gondii	Serology	3 (5)

CHAPTER 5

BESNOITIOSIS IN A SOUTHERN PLAINS WOODRAT (*NEOTOMA MICROPUS*) FROM UVALDE, TEXAS[1]

[1]Charles, Roxanne A., Angela E. Ellis, J.P. Dubey, John C. Barnes and Michael J. Yabsley. Published in Journal of Parasitology. Reprinted with permission.

Abstract

Recently, *Besnoitia neotomofelis* was described from a southern plains woodrat (*Neotoma micropus*) from southern Texas. During May 2010, 1 of 55 southern plains woodrats trapped in Uvalde County, Texas was diagnosed with besnoitiosis. Grossly, the woodrat had bilateral swellings of the cheeks and numerous *Besnoitia* sp.-like cysts were observed in the tongue, facial region, musculature of the limbs, and subcutis of the dorsum and flanks. Little to no inflammation was noted around cysts. The cysts were morphologically similar to *B. neotomofelis* based on light and transmission electron microcopy. The sequence of the internal transcribed spacer region-1 (ITS-1) was identical to the type isolate of *B. neotomofelis*. Attempts to isolate *Besnoitia* sp. in laboratory mice failed; however, *Toxoplasma gondii* was isolated in a Swiss Webster mouse. This represents the first report of besnoitiosis caused by *B. neotomofelis* in a southern plains woodrat and the first concurrent *Besnoitia* and *T. gondii* infection in any host species.

Introduction

Species of *Besnoitia* are cyst-forming parasites that are members of the Sarcocystidae (Apicomplexa). There is a wide host range for *Besnoitia* species that includes mammals, birds, and reptiles. Common hosts include cattle, equids, goats, reindeer (known as caribou in North America [*Rangifer tarandus*]), opossums, rabbits, rodents, and lizards (Leighton and Gajadhar, 2001; Dubey et al., 2003). Currently, *Besnoitia* contains 10 validly described species, although there are likely numerous other species because many of these species have not been sufficiently morphologically, or molecularly characterized to determine species identification. The life cycles of most of these species are still poorly understood, although felids serve as definitive hosts for

at least 4 of the species, including *B. oryctofelisi, B. darlingi, B. wallacei*, and *B. neotomofelis* (Dubey and Yabsley, 2010).

In a previous study of *Besnoitia* from southern plains woodrats, a novel species (*B. neotomofelis*) was isolated from a single southern plains woodrat by inoculation of laboratory mice with tissue homogenates (Dubey and Yabsley, 2010). In that study, the positive woodrat was clinically normal at the time of capture and no cysts were observed grossly or histologically (Dubey and Yabsley, 2010). In the current report, we describe an unusual case of clinical besnoitiosis in a southern plains woodrat, caused by *B. neotomofelis* that had concurrent asymptomatic *Toxoplasma gondii* and *Sarcocystis* sp. infections.

Materials and Methods

In May 2010, 55 wood rats (17 juveniles and 38 adults) were trapped at 2 sites in Uvalde, Texas, during an ongoing study of *Trypanosoma cruzi* in southern Texas. Animals were captured using large Sherman traps (H.B. Sherman, Traps, Tallahassee, Florida) or small squirrel cage traps (Havahart, Lititz, Pennsylvania) with dried apricots as bait. Upon capture, animals were anesthetized with intramuscular injection of 100 mg/kg of ketamine (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa), weighed, and examined for ectoparasites. Whole blood was collected immediately by intracardiac puncture and animals were killed by cervical dislocation. Each rat was necropsied and samples of quadriceps muscle, tongue, diaphragm, liver, spleen, kidney, lung, heart, stomach, small and large intestines, cecum, adrenal glands, urinary bladder, and gonads were fixed in 10% neutral buffered formalin for routine histology. Tissues were embedded in paraffin, sectioned at 5µm, and stained with hematoxylin and eosin (H&E) for light microscopy examination. Sections of brain and muscle were submitted to the Athens Diagnostic Laboratory (Athens, Georgia) for immunohistochemical staining for *T. gondii*. For transmission

electron microscopy (TEM), tissues that were fixed in 10 % neutral buffered formalin were post-fixed in osmium and processed for TEM. For isolation of *Besnoitia* sp., samples of brain, tongue, and heart were homogenized and aliquots of homogenates (some digested in acidic pepsin) were inoculated subcutaneously into outbred Swiss Webster (SW) and gamma interferon knockout mice (Dubey et al., 2008); grossly visible cysts from buccal mucosa were fed to a laboratory-raised domestic cat. Serum from the woodrat was tested for antibodies to *T. gondii* by the modified agglutination test (MAT) as described by Dubey and Desmonts (1987).

For polymerase chain reaction, DNA was extracted from a single cyst using the DNeasy blood and tissue kit (Qiagen, Inc., Valencia, California) following the manufacturer's protocol. The internal transcribed spacer (ITS) -1 region was amplified with primers 15C and 13B as described (Bostrom et al., 2008) and amplicons were purified with a Gel Extraction Kit (Qiagen) and independently bi-directionally sequenced at The University of Georgia sequencing facility (Athens, Georgia).

Results

A single adult female woodrat (262 g) was found to be depressed and lethargic within the cage and had markedly swollen cheeks and muzzle (Fig. 5.1A.). The remaining 54 woodrats trapped from that site and a second site within the same county were bright, alert, and responsive. At necropsy, the fascia along the lateral aspects of the sinonasal area was reddened and edematous with numerous, firm, white, spherical cysts that were approximately ~0.5 mm in diameter (Fig. 5.1B). Additional cysts were observed in the musculature on the skull, tongue, nasal cavity, and inner ear. Cysts were distributed throughout the skin and musculature of the dorsum, flanks, and the thighs. Cysts were also found in linear chains within the musculature of the distal fore- and hind limbs (Fig. 5.1C). Within the visceral cavity, only 2 small cysts were

observed; both were located near the renal vein. Partial 18S rRNA, complete ITS-1, and partial 5.8S rRNA sequences from a single cyst were identical to sequences obtained from the type isolate of *B. neotomofelis* (Dubey and Yabsley, 2010). The sequences were submitted to GenBank with accession number HQ909085.

Histologically, cysts were morphologically consistent with *Besnotia* sp. and measured between 224 and 634 μm, with a mean diameter of 463 μm. The majority of cysts were embedded in collagen or skeletal muscle, although some cysts were found within endothelial lined spaces (blood and/or lymphatic vessels) (Figs. 5.1D, E). Tissue cysts contained numerous, closely packed, crescent-shaped zoites. Cysts had double contoured walls (5-15 μm) with a darkly eosinophilic inner layer and a thicker, paler eosinophilic outer layer covered by flattened, hyperchromatic nuclei. Results of the TEM showed that cyst walls consisted of 3 distinct layers as described in Dubey and Yabsley 2010.

Within the dermis and subcutis of the face, there were scattered foci of mild edema and small aggregates of neutrophils, lymphocytes, and rare plasma cells, and some cysts were surrounded by small numbers of neutrophils. One cluster of cysts in the superficial dermis was surrounded by homogeneous eosinophilic material resembling amyloid. A single cyst in the cheek had central necrosis with proliferation of bacteria. No inflammation was noted around cysts detected in the tongue or skeletal muscle. In a section of skeletal muscle, a cyst morphologically consistent with *Sarcocystis neotomafelis* was observed with no associated tissue reaction (Fig. 5.1F) (Galaviz-Silva et al., 1991).

Isolation attempts for *Besnoitia* sp. were compromised due to autolysis of samples during shipping to the lab (6 days before bioassay). The cat fed grossly visible cysts did not shed coccidian oocysts for 27 days after feeding tissues. Five SW and 4 gamma interferon gene

knockout mice inoculated with undigested muscle cysts did not become infected with *Besnoitia* or *T. gondii*. However, 1 SW mouse inoculated with pepsin digest of brain, heart, and tongue became infected with *T. gondii* (Dubey et al., in press). Genetic characterization of this *T. gondii* isolate is reported elsewhere (Dubey et al., in press). Serum from the woodrat was seropositive for *T. gondii* (titer 1:25).

Histologic lesions not attributable to *B. neotomofelis* were also noted. Diffuse, mild to moderate, lymphocytic proliferation was noted in the meninges and rare, loosely organized glial nodules were noted in the neuropil of the cerebrum. Immunohistochemical staining for *T. gondii* (that also reacts with *Besnoitia* antigens) failed to detect any protozoal antigens in the brain. In addition, the cerebellar meninges contained sections of adult and larval nematodes morphologically consistent with *Dunnifilaria meningica*, no inflammatory response was noted. In the liver, there were several random foci of necrosis and inflammation composed primarily of eosinophils and epithelioid macrophages with occasional neutrophils, lymphocytes, or plasma cells. No causative agent, e.g., migrating parasitic larvae, was identified for the liver lesions. In the lungs, a focal granuloma containing numerous fungal hyphae was identified.

Discussion

Historically, infections with *Besnoitia* spp. were considered to be nonpathogenic for the majority of hosts, although some *Besnoitia* species, e.g., *B. tarandi, B. bennetti,* and *B. besnoiti*, were recognized as important pathogens for their intermediate hosts. Infections with *B. besnoiti* of domestic cattle and wild bovids, *B. bennetti* of equids, *B. caprae* of caprids, and *B. tarandi* of reindeer (caribou) (*Rangifer tarandus*) can cause disease and economic losses due to the induction of poor body condition, edema, thickened skin, hair loss, blindness, vascular obstruction, severe inflammation of affected internal organs, and secondary infections (Leighton

and Gajadhar, 2001; Elsheikha et al., 2003). Recently, a series of clinical cases was noted in Virginia opossums (*Didelphis virginiana*) infected with *B. darlingi*, which has historically been considered nonpathogenic (Ellis et al., unpublished data).

Among the 3 rodent *Besnoitia* species (*B. jellisoni, B. wallacei*, and *B. neotomofelis*), clinical besnoitiosis has only previously been associated with *B. jellisoni. Besnoitia jellisoni* has been reported from deer mice (*Peromyscus maniculatus*) and kangaroo rats (*Dipodomys ordii* and *D. microps*) and, although most infected kangaroo rats did not exhibit any clinical signs in captivity (Ernst et al., 1968), disease has been reported in four naturally infected kangaroo rats (Chobotar et al., 1970). Similarly, clinical disease in woodrats infected with *B. neotomofelis* appears to be rare. The previous study of *B. neotomofelis* in woodrats from southern Texas only detected 1 of 42 being infected, and that woodrat had no gross or histologically visible cysts and was clinically normal (Dubey and Yabsley, 2010).

Interestingly, cysts of *B. neotomofelis* in this woodrat were significantly larger than the cysts described from experimentally infected laboratory mice and rats in Dubey and Yabsley (2010). In the laboratory mice and rats, the largest cyst observed was 210 μm compared to 634 μm in the present study (Dubey and Yabsley, 2010). Because *B. neotomofelis* was pathogenic for laboratory mice, the cysts may not have grown as large as they would have in an appropriate host and infections in laboratory rats only resulted in 2 small cysts (Dubey and Yabsley, 2010). Our inability to isolate *B. neotomofelis* in known strains of susceptible mice in the current study was likely due to autolysis of infected donor woodrat tissues; it appears that this species of *Besnoitia* is more susceptible to autolysis than *T. gondii*.

There is currently only 1 report of *S. neotomafelis*, which was also from the southern plains woodrat from Nuevo Leon, Mexico (Galaviz-Silva et al., 1991). Cysts of *S. neotomafelis*

were numerous, macroscopic, and found in numerous tissues, with most being found in the masseter muscles. In that first report, 29% of woodrats (37/129) had grossly visible cysts of *S. neotomafelis*, but no clinical signs or lesions were reported (Galaviz-Silva et al., 1991). In the current study, only 1 *S. neotomafelis* cyst was found in the skeletal muscles of the hind limb of our besnoitiosis woodrat.

It is currently unknown why this woodrat developed clinical besnoitiosis from the *B. neotomofelis* infection as in the only previous report of *B. neotomofelis*, the animal was clinically normal and no cysts were observed (Dubey and Yabsley, 2010). In the current study, no grossly visible cysts were observed in the other 54 woodrats and histologic examinations of numerous tissues from 28 woodrats were negative for *Besnoitia* cysts. This single clinically ill woodrat could have ingested an overwhelming number of oocysts or it could have been immunosuppressed for some unknown reason. The ill woodrat had a fungal granuloma in the lung suggesting it may have been immunosuppressed; however, if the woodrat was immunosuppressed, a subclinical *T. gondii* infection seems unlikely. Although speculative, the animal had a low serologic titer to *T. gondii*, so it is possible the infection was recently acquired.

Interestingly, the known definitive host for the 3 protozoal infections in this woodrat (*T. gondii, B. neotomofelis, and S. neotomafelis*) is the domestic cat (Galaviz-Silva et al. 1991; Dubey, 2009; Dubey and Yabsley, 2010). Feral domestic cats have been noted at the property where the 2 *B. neotomofelis* infections were found, and it is possible that wild felids could also serve as definitive hosts because bobcats (*Felis rufus*) and cougars (*Puma concolor*) are present in the region, although both wild felids are rare (Schmidly, 2004). The risk of exposure to cat feces is not likely uniform across a landscape because woodrats, in general, are asocial and maintain territories that may result in focal infections (Conditt and Ribble, 1997). This may

explain the low prevalence of *B. neotomofelis* noted in the current and previous study (Dubey and Yabsley, 2010) and why a particular woodrat may have been exposed to high numbers of parasites if a cat routinely defecated in this woodrat's territory.

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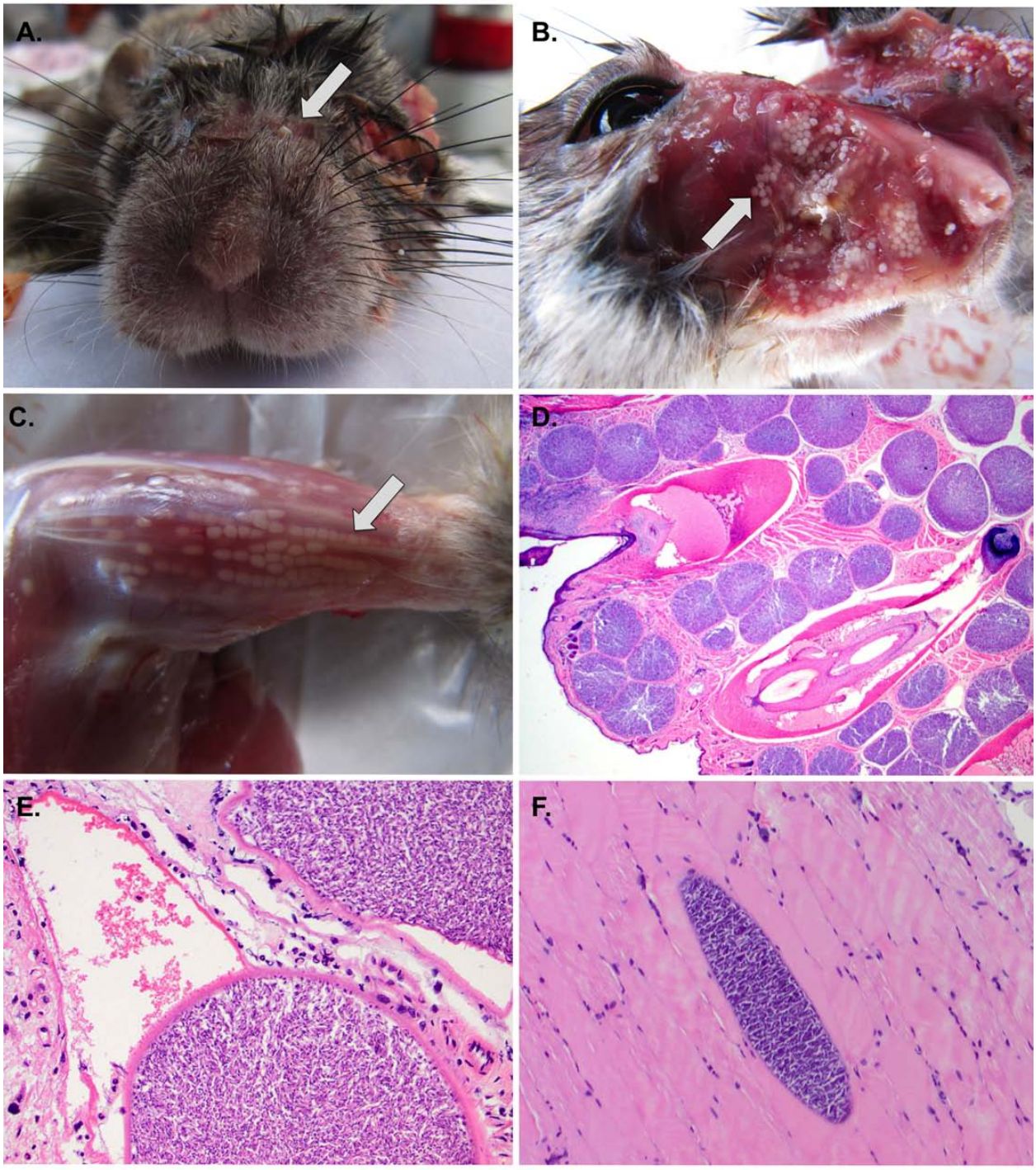
Figure 5.1. (A) External view of woodrat showing severe swelling of the face and a single superficial *Besnoitia* cyst (arrow). (B) Lateral view of the head with skin reflected. Exposed tissue is diffusely reddened and periocular tissue has a gelatinous appearance (edema). Numerous *B. neotomofelis* cysts are present in the muscles and fascia (arrow). (C) Rows of *B. neotomofelis* cysts present in the musculature of the lower leg (arrow). (D) Facial skin of woodrat. Numerous zoite filled cysts expand the dermis, 20x H&E stain. (E) Parts of 2 *B. notomofelis* cysts are visible. Cysts have a thick capsule and contain numerous banana- or teardrop-shaped zoites. The lower cyst is within a cystic space containing erythrocytes and fibrin (blood vessel), 200x, H&E stain. (F) A single *S. neotomafelis* sarcocyst in skeletal myocyte. The cyst has a thin (indistinct) wall and is filled with deeply basophilic zoites. No inflammation or degeneration is associated with the cyst, 100x, H&E stain.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CHAPTER 6

CONCLUSIONS

The ultimate goal of this thesis was to determine the importance of southern plains woodrats as reservoirs of *T. cruzi* and other parasites of veterinary and/or medical importance. Rodents are common hosts of *T. cruzi* in the Americas and at least 13 species of woodrats are known to be reservoirs (Burkholder et al., 1980; Navin et al., 1985; Packchanian, 1942; Roellig and Yabsley, 2010; Wood, 1952, 1962, 1975; Wood and Wood, 1961). While there are only seven reported autochthonous cases in the US, there is an increase in veterinary cases and an influx of potentially infected immigrants especially in southern states e.g., Texas. We hypothesized that woodrats would be hosts to a number of pathogens which may affect them, other animals, or people because they are common in peridomestic settings, share nesting sites with reduviid bugs and a number of other insects, arthropods and mammals, and are commonly infested with ectoparasites.

Study 1 (Chapter 3)

In this study, our data indicate that woodrats, similar to raccoons, skunks and other rodents, are reservoirs of *T. cruzi* in Uvalde County, Texas and that multiple diagnostic methods should be employed to acquire the best estimate of *T. cruzi* infection status in hosts. The other rodents reported for the first time to be infected with *T. cruzi* included a rock squirrel, black rat, two white-ankled mice, and a hispid cotton rat. In our study area, woodrats are as important or possibly more important as reservoirs compared with raccoons, skunks, and other rodents due to

high prevalence and infection with a genotype currently associated with human infections in the *US and Mexico. Genetic characterization data suggest that woodrats are infected with both TcI* and TcIV genotypes with similar frequency (Roellig and Yabsley, 2010) which is in contrast to raccoons, skunks, and other rodents were either primarily or exclusively infected with TcIV. We also report the first natural infection of a striped skunk with a TcI isolate.

Study 2 (Chapter 4)

In this study, we demonstrated that woodrats in Uvalde County, Texas were hosts to a high diversity of internal and external parasites. Of interest was the presence of a novel *Babesia* sp. in a single woodrat that was 94.4% similar to *B. conradae* found in domestic dogs in California. Also, three tissue cyst-forming coccidians, *S. neotomafelis, B. neotomofelis,* and *T. gondii* were found in one or more woodrats (all three were found in a single woodrat). At least one (*T. gondii*) is zoonotic and all three are suspected to utilize felids as definitive hosts (Galaviz-Silva et al., 1991).

Altogether we reported eight new parasite-host associations including one flea (*Orchopeas neotomae*), two trematodes (*Scaphiostomum* sp. and *Zonorchis* sp.), one stomach nematode (*Ascarops* sp.), an intestinal nematode (*N. neotoma*), and three protozoan parasites (*T. gondii*, *Babesia* sp., and coccidia). This is also the first report of *S. neotomafelis* infection in *N. micropus* in the US.

Study 3 (Chapter 5)

This was the first case report of clinical besnoitiosis in a southern plains woodrat in the United States. The morphology of cysts by light and electron microscopy combined with

sequencing of the internal transcribed spacer region-1 (ITS-1) confirmed infection with *Besnotia neotomofelis*. This parasite was recently first isolated from a clinically normal southern plains woodrat by inoculation of laboratory mice with tissue homogenates. (Dubey and Yabsley, 2010). Also of interest in this study was that the woodrat was concurrently infected with *Sarcocystis neotomafelis* and *Toxoplasma gondii*. This is the first report of these three cyst-forming genera in any single animal.

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