

Seroprevalence of *Trypanosoma cruzi* Among Eleven Potential Reservoir Species from Six States Across the Southern United States

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Abstract

Trypanosoma cruzi, the causative agent of Chagas' disease, is a substantial public health concern in Latin America. Although rare in humans and domestic animals in the United States, *T. cruzi* is commonly detected in some wildlife species, most commonly raccoons (*Procyon lotor*) and Virginia opossums (*Didelphis virginiana*). To increase our understanding of the reservoir host species range and geographic distribution, 11 species of mammals from six states spanning the known range of *T. cruzi* (Arizona, California, Florida, Georgia, Missouri, and Virginia) were tested for antibodies to *T. cruzi* using indirect immunofluorescent antibody testing. In addition, culture isolation attempts were conducted on a limited number of animals from Georgia and Florida. Evidence of *T. cruzi* was found in every state except California; however, low numbers of known reservoirs were tested in California. In general, the highest seroprevalence rates were found in raccoons (0–68%) and opossums (17–52%), but antibodies to *T. cruzi* were also detected in small numbers of striped skunks (*Mephitis mephitis*) from Arizona and Georgia, bobcats (*Lynx rufus*) from Georgia, two coyotes (*Canis latrans*) from Georgia and Virginia, and a ringtail (*Bassariscus astutus*) from Arizona. Culture-based prevalence rates for raccoons were significantly greater than those for opossums; however, seroprevalences of raccoons and opossums from several geographic locations in Georgia and Florida were not different, indicating that exposure rates of these two species are similar within these areas. For both raccoons and opossums, seroprevalence was significantly higher in females than in males. No difference was detected in seroprevalence between adults and juveniles and between animals caught in urban and rural locations. Our results indicate that *T. cruzi* prevalence varies by host species, host characteristics, and geographic region and provides data to guide future studies on the natural history of *T. cruzi* in the United States.

Key Words: Anaplasma—Ehrlichia—Trypanosome—Zoonosis.

Introduction

TRYPANOSOMA CRUZI, a hemoflagellate protozoan parasite, is the causative agent of American trypanosomiasis (Chagas' disease) in domestic animals and humans. *T. cruzi* is an important public health concern in Latin America, where 10–12 million people are estimated to be infected (Morel and Lazdins 2003). In North America, *T. cruzi* is commonly

detected in several species of mammalian wildlife, and is increasingly diagnosed in domestic dogs and exotic animals (Kasa et al. 1977, Jaime-Andrade et al. 1997, Meurs et al. 1998, Kjos et al. 2008). Autochthonous cases in humans are rare, with only six cases previously reported (Herwaldt et al. 2000, Dorn et al. 2007); however, serologic studies indicate that many autochthonous cases may be undiagnosed (Woody et al. 1965, Burkholder et al. 1980, Stramer et al. 2007, Bern et al. 2008).

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The two most commonly reported reservoirs in North America are the raccoon (*Procyon lotor*) and the Virginia opossum (*Didelphis virginiana*). In raccoons, published prevalence rates range from 1.5% in southwestern Georgia and northwestern Florida (McKeever et al. 1958) to 63% in Oklahoma (John and Hoppe 1986), with rates varying widely depending on the assay used (e.g., serology, culture, or both) and the geographic location. Reported prevalence rates for opossums have generally been lower, and range from 8% in North Carolina (Karsten et al. 1992) to 33% in southern Louisiana (Barr et al. 1991). Other wildlife species in the United States that are naturally infected with the parasite based on either serology or culture include the armadillo (*Dasypus novemcinctus*) (Yaeger 1988, Barr et al. 1991), badger (*Taxidea taxus*) (Burkholder et al. 1980), coyote (*Canis latrans*) and gray fox (*Urocyon cinereoargenteus*) (McKeever et al. 1958), striped skunk (*Mephitis mephitis*) (McKeever et al. 1958, Ryan et al. 1985), and various rodent species (Burkholder et al. 1980).

The majority of previous studies of *T. cruzi* in wildlife have focused on blood culture as the primary method for determining infection status, but this method has been shown to have a lower sensitivity than serologic testing (Jansen et al. 1985, Yabsley et al. 2001, Hall et al. 2007). Since culture of the parasite depends on high numbers of circulating parasites, animals in the chronic stage of infection, which are seropositive, are less likely to be culture positive. For example, Hall et al. (2007) tested 50 lemurs from St. Catherine's Island, GA, for *T. cruzi* using culture and serology, and found a 5% prevalence rate with culture and a 50% seroprevalence rate, and Yabsley et al. (2001) tested raccoons from Georgia using both serologic and culture techniques, and found a 30% prevalence rate using culture, but a 51% prevalence rate using serologic testing.

The aim of the current study was to determine the prevalence of *T. cruzi* in several species of mammals throughout the United States using serologic testing, and to further assess exposure rates of raccoons and opossums from several individual geographic locations in Georgia and Florida using both culture and serologic methods. Several demographic parameters of raccoons and opossums were also investigated in a subset of samples to assess any correlation with *T. cruzi* seroprevalence to determine if any broad-scale host-parasite relationships exist with this parasite.

Materials and Methods

Sample collection

In Georgia, Florida, and Missouri, animals were captured in box traps (Tomahawk Live Trap, Tomahawk, WI) baited with sardines or mackerel. Raccoons were anesthetized by intramuscular injection of either ketamine hydrochloride (25 mg/kg body weight; Aveco, Fort Dodge, IA) plus xylazine (0.25 mg/kg body weight; Mobay, Animal Health Division, Shawnee, KS), or tiletamine plus zolazepam (Telazol[®], 0.6 mg/kg body weight; Aveco). Opossums were anesthetized by intramuscular injection of tiletamine plus zolazepam. Approximately 10 mL of blood was collected via cardiac puncture from anesthetized animals (Georgia and Florida) or from the femoral vein (Missouri). Whole blood in ethylenediaminetetraacetic acid was collected for culture, and plasma or serum was used for serological testing. After blood collection, the Georgia and Florida animals were euthanized with sodium pentobarbital (Beuthanasia[®]-D Special; Schering-Plough

Animal Health, Omaha, NE) administered by intracardiac injection; the Missouri animals were released on-site after recovery from anesthesia. Additionally, serum or plasma samples from animals that had been previously collected for other studies (Arizona and Virginia) and stored at -20°C were tested. Because serum or plasma was not available from California animals, we conducted serologic testing on frozen whole blood (ethylenediaminetetraacetic acid). This technique was validated by testing frozen whole blood of animals from Georgia and Florida that had matching serum samples (both seronegative and seropositive animals tested, data not shown).

Demographic parameters, including age and sex, of captured raccoons and opossums were recorded in Georgia, Florida, and Missouri. Opossums and raccoons were classified as juveniles or adults based on weight, tooth wear, and development of reproductive organs (Grau et al. 1970, Kasparian et al. 2004). Only animals caught in Clarke County, GA, were used to assess land use effects (i.e., animals captured from urban vs. rural locations) on *T. cruzi* prevalence. Trapping locations within Clarke County were classified as urban or rural based on data from the Georgia Land Use Trends Project (Natural Resources Spatial Analysis Laboratory, Odum School of Ecology, University of Georgia, unpublished data).

Serology

Samples from 11 mammal species from six states (Arizona, California, Florida, Georgia, Missouri, and Virginia) were tested for antibodies to *T. cruzi* (Table 1) using the indirect immunofluorescent antibody (IFA) test as described by Yabsley et al. (2001) with the following modifications. Epimastigotes were grown in liver infusion tryptose medium, washed in phosphate-buffered saline (PBS), and placed onto each circle of a 12-well test slide (Fisher Scientific, Rome, GA). Slides were allowed to dry at room temperature and then fixed in acetone for 10 min. Samples were tested at a dilution of 1:40 made in PBS. The diluted sera were incubated on the test slides for 30 min at 37°C . After incubations, the slides were washed twice with PBS and then once with distilled water. A commercial fluorescein-labeled anti-species IgG antibody at a 1:50 dilution in PBS was then placed on the test slides and incubated for 30 min at 37°C . After incubation, the slides were washed again as described above. The last water wash included 1.65% Eriochrome Black T (Sigma, St. Louis, MO) that counterstained the epimastigotes red to allow for easier observation under fluorescent microscopy. Secondary antibodies used included a goat-anti-raccoon IgG (Kirkegaard and Perry Laboratories [KPL], Gaithersburg, MD); a goat anti-ferret IgG (KPL) for fishers, ringtails, striped skunks, and hooded skunks; a goat anti-dog IgG (KPL) for gray fox, red fox, and coyotes; a goat anti-pig for feral swine IgG (KPL); and goat anti-cat IgG (KPL) for bobcats. Opossum serologic testing followed the same procedure, except that slides were incubated first with serum samples, then a rabbit anti-opossum IgG (Bethyl Laboratories, Montgomery, TX), and then a fluorescein-labeled anti-rabbit IgG (KPL). A sample was positive for *T. cruzi* antibodies if the epimastigotes appeared green under fluorescent microscopy, or red with a green outline. Negative samples appeared red.

Culture

Only blood collected aseptically from live animals was used for *T. cruzi* culture attempts. Within 48 h of collection,

TABLE 1. RESULTS OF INDIRECT IMMUNOFLUORESCENT ANTIBODY TESTING OF MAMMALS FROM SIX STATES FOR *TRYPANOSOMA CRUZI* ANTIBODIES

State	Species	n	Seropositive (%)
Arizona	Striped skunk (<i>Mephitis mephitis</i>)	34	3 (9) ^a
	Raccoon (<i>Procyon lotor</i>)	5	1 (20) ^a
	Ringtail (<i>Bassariscus astutus</i>)	1	1 (100) ^a
California	Hooded skunk (<i>Mephitis macroura</i>)	1	0
	Fisher (<i>Martes pennanti</i>)	21	0
	Gray fox (<i>Urocyon cinereoargenteus</i>)	10	0
	Ringtail	10	0
	Striped skunk	6	0
Florida	Raccoon	3	0
	Raccoon	70	38 (54) ^a
Georgia	Opossum (<i>Didelphis virginiana</i>)	27	14 (52) ^a
	Raccoon	510	167 (33) ^a
Virginia	Opossum	421	118 (28) ^a
	Feral swine (<i>Sus scrofa</i>)	110	0
	Bobcat (<i>Felis rufus</i>)	62	2 (3.2) ^b
	Coyote (<i>Canis latrans</i>)	23	1 (4.4) ^b
	Gray fox	21	0
	Red fox (<i>Vulpes vulpes</i>)	5	0
	Striped skunk	3	1 (33) ^c
	Raccoon	108	74 (68)
	Coyote	26	1 (3.8) ^a
Missouri	Raccoon	12	0
	Opossum	6	1 (17) ^a
Virginia	Gray fox	1	0

Similar letters indicate no statistical differences between species within each state ($p < 0.05$).

buffy coats were collected from whole-blood samples and either (1) added to 9 mL of liver infusion tryptose medium and stored at 27°C or (2) inoculated on confluent layers of DH82 canine macrophage cells and maintained at 37°C as described (Hall et al. 2007). To minimize bacterial and fungal growth, 0.4 mL of penicillin (10,000 U/mL)–streptomycin (10 mg/mL) (Sigma) and 0.4 mL of 5-fluorocytosine (2.5 mg/mL; Sigma) were added to each culture. Cultures were monitored for growth of trypanosomes, and if no parasites were observed after 6–8 weeks, the samples were considered negative.

Statistical analysis

A chi-square analysis was used to assess significant differences between seroprevalence rates for different species, as well as differences between culture-based prevalence and seroprevalence. It was also used to assess if significant differences in seroprevalence were present due to age, sex, or land use for serologic data, as well as if differences in seroprevalence among different geographic areas.

Results

Serology

Results of IFA testing of 11 species of mammals from six states are shown in Table 1. Evidence of *T. cruzi* was found in every state except California. In general, the highest prevalence rates were found in raccoons followed by opossums (Table 1); however, antibodies to *T. cruzi* were also detected in small numbers of striped skunks from Arizona and Georgia, bobcats from Georgia, coyotes from Georgia and Virginia, and a ringtail from Arizona (Table 1). Antibodies to *T. cruzi* were not found in any animals from California, including

fishers (*Martes pennanti*), gray foxes, raccoons, ringtails, or striped skunks; in any gray foxes, red foxes, or feral swine from Georgia; or any gray foxes or raccoons from Virginia.

Culture

Of the 168 raccoons from Georgia and Florida tested by culture and serologic methods, 50 (30%) were culture positive and 70 (42%) were seropositive. Isolated trypanosomes were confirmed as *T. cruzi* by morphology and polymerase chain reaction sequence analysis (Roellig et al. 2008). No difference in raccoon prevalence was found using the two detection methods ($\chi^2 = 2.46$, $p = 0.117$). Of the 83 opossums tested, 11 (13%) were culture positive, and a significantly higher number (28, 34%) were seropositive ($\chi^2 = 6.04$, $p = 0.014$). Significantly more raccoons were infected with *T. cruzi* compared with opossums based on culture prevalence ($\chi^2 = 5.27$, $p = 0.027$); however, no difference was noted based on serologic results between raccoons and opossums ($\chi^2 = 0.66$, $p = 0.417$). Interestingly, we observed a marked difference in culture methods. Of the 10 animals that were tested for *T. cruzi* by the two culture methods, only 2 were positive for both techniques, while the remaining 8 were only positive by the DH82 method.

Exposure rates among raccoons and opossums in Georgia and Florida

Infection rates among raccoons and opossums from seven counties in Georgia and across four counties in Florida were not significantly different between the two species, indicating that these species have a similar exposure rate (Table 2). Significantly more raccoons and opossums from northern Florida (Leon and Wakulla counties) were seropositive compared to

TABLE 2. EXPOSURE RATES FOR *TRYPANOSOMA CRUZI* IN GEORGIA AND FLORIDA COUNTIES IN WHICH AT LEAST FIVE RACCOONS (*PROCYON LOTOR*) OR OPOSSUMS (*DIDELPHIS VIRGINIANA*) WERE TESTED

Location	IFA serology results No. of seropositive/no. of examined (% positive)	
	Raccoon	Virginia opossum
Baker County, GA	48/177 (27.1)	35/122 (28.7)
Chatham County, GA	46/105 (43.8)	3/7 (42.8)
Clarke County, GA	40/128 (31.3)	31/116 (26.7)
Franklin County, GA	2/7 (28.6)	2/6 (33.3)
Glynn County, GA	7/10 (70.0)	4/5 (80.0)
Thomas County, GA	8/22 (36.7)	39/113 (34.5)
Webster County, GA	1/6 (16.7)	1/9 (11.1)
Hendry County, FL	6/12 (50.0)	6/17 (35.3)
Leon County, FL	9/17 (52.9)	4/4 (100.0)
Wakulla County, FL	16/23 (69.6)	4/6 (66.7)

IFA, immunofluorescent antibody.

raccoons and opossums from the seven Georgia counties ($\chi^2 = 5.12$, $p = 0.024$ and $\chi^2 = 4.24$, $p = 0.039$, respectively).

Population parameters

Although greater numbers of adult animals were seropositive, no significant difference was noted between adult or juvenile raccoons or opossums from Georgia, Florida, or Missouri (Table 3). In contrast, significantly more females were seropositive than males among raccoons and opossums from Georgia and Florida. Although no significant difference in seroprevalence was observed between male and female raccoons from Missouri, the trend of more females being seropositive compared with males was evident. No significant differences were noted between raccoons and opossums from urban or rural areas of Clarke County, GA.

Discussion

This study presents a serologic survey of *T. cruzi* in several mammalian species from the southern United States. Evidence of *T. cruzi* was found in five of six states (Arizona, Florida, Georgia, Missouri, and Virginia) and 6 of 11 tested wild animal species. Differences in prevalence were noted across geographic region and host species.

The finding of *T. cruzi* in Arizona was not surprising; however, we did expect to find a greater number of seropositive animals. A previous study in Arizona detected *T. cruzi* in 4.7% of woodrats and mice (*Neotoma* spp. and *Peromyscus* spp.) using blood smears or xenodiagnosis (Wood 1952), and since serology has been shown to be more sensitive at detecting infections in wild animals (Yabsley et al. 2001), we expected a higher prevalence with serologic testing. The only species tested in relatively high numbers was the striped skunk, of which 9% were seropositive. Striped skunks had been shown to be experimentally susceptible to *T. cruzi* (Davis et al. 1980), and antibodies to *T. cruzi* had been detected in a single striped skunk from Los Angeles, CA (Ryan et al. 1985). The positive ringtail was not unexpected, due to their relatedness to other procyonids (e.g., raccoons, coati [*Nasua nasua*], and kinkajou [*Potos flavus*]) that are naturally infected with *T. cruzi* (Travi et al. 1994, Herrera et al. 2008). Also, ringtails use tree cavities and abandoned housing as den sites (Poglayen-Neuwall and Toweill 1988, Koepfli et al. 2007), which are common habitats for triatomine bug vectors and other potential reservoirs of *T. cruzi*. Additional work is needed to confirm if ringtails can serve as reservoirs for *T. cruzi*.

The lack of evidence of *T. cruzi* in California could be the result of a number of factors. The species most likely to possess antibodies to *T. cruzi*, raccoons and ringtails, were sampled in numbers that were likely too low to detect *T. cruzi* in the area. Additionally, the samples were collected from Humboldt County in northern California, which may be outside of the range for *T. cruzi*. The northernmost location where *T. cruzi* has been reported in California is in the San Francisco area (Navin et al. 1985), which is approximately

TABLE 3. EXPOSURE RATES FOR *TRYPANOSOMA CRUZI* IN RACCOONS (*PROCYON LOTOR*) AND VIRGINIA OPOSSUMS (*DIDELPHIS VIRGINIANA*) WITHIN SEVERAL POPULATION PARAMETERS

Location	Species	Population parameter	Total tested	Seropositive (%)
Georgia and Florida	Raccoon	Adult	133	53 (40) ^a
		Juvenile	30	8 (27) ^a
		Male	251	66 (27) ^a
		Female	116	47 (41) ^b
	Opossum	Adult	170	56 (33) ^a
		Juvenile	19	5 (27) ^a
		Male	137	29 (21) ^a
Clarke County, GA	Raccoon	Female	180	66 (37) ^b
		Urban	35	9 (26) ^a
	Opossum	Rural	89	29 (33) ^a
		Urban	35	9 (26) ^a
Missouri	Raccoon	Rural	95	21 (22) ^a
		Adult	85	64 (75) ^a
		Juvenile	23	10 (43) ^a
		Male	49	30 (61) ^a
		Female	59	44 (75) ^a

Similar letters indicate no statistical differences between parameters within each species and location ($p < 0.05$).

500 km south of Humboldt County. *Triatoma protracta*, a vector for *T. cruzi* in this area, is known to selectively feed on woodrats (*Neotoma* spp.) (Peterson et al. 2002); therefore, woodrats may be a more important reservoir for *T. cruzi* in this area than the species tested in the current study.

The prevalence of *T. cruzi* in wild canids from the southeastern United States appears to be low compared with previous studies in Texas. Two studies conducted in Texas showed that 12.8% and 14.2% of coyotes were seropositive by hemagglutination and IFA tests, respectively (Burkholder et al. 1980, Grogli et al. 1984). Based on blood culture, 1.7% of gray foxes from southwestern Georgia and northwestern Florida were found positive (McKeever et al. 1958), and recently, an IFA serologic survey of wild canids in South Carolina found 2 of 26 (8%) gray foxes positive for *T. cruzi* antibodies, but no antibodies in two coyotes tested (Rosypal et al. 2007). Although we only detected two seropositive coyotes and did not find any seropositive red foxes or gray foxes, the sample sizes were low, and more testing is needed to determine an accurate prevalence for these hosts in the southeastern United States.

All of the feral swine tested in the current study were seronegative despite raccoons on the same island had a high seroprevalence (57%). Previous studies have shown that swine are susceptible to infection with *T. cruzi*. The parasite was isolated from a single domestic pig from Mexico and from 4 of 105 (2.8%) domestic pigs in Brazil. A serologic study in Paraguay found that 2 of 20 domestic pigs were seropositive (Fujita et al. 1994, Salazar-Schettino et al. 1997, da Costa Valente 1999). Domestic pigs are also experimentally susceptible to infection with a North American raccoon strain of *T. cruzi* (Diamond and Rubin 1958).

Raccoons and opossums are considered to be the two major reservoir species in the United States. Depending on the diagnostic test used for identifying infected individuals, prevalence rates in raccoons have ranged from 1.5% to 63% (McKeever et al. 1958, Walton 1958, Schaffer et al. 1978, John and Hoppe 1986, Telford and Forrester 1991, Karsten et al. 1992, Pung et al. 1995, Pietrzak and Pung 1998, Yabsley and Noblet 2002, Hancock et al. 2005). Prevalences based on isolation attempts from blood or tissue samples typically are lower compared with those based on serology. The culture prevalence in raccoons in the current study (30%) is similar to previous rates from Georgia that range from 22% (Pung et al. 1995) to 43% (Pietrzak and Pung 1998). These culture rates are higher than previous studies in surrounding states that range from 14% in Alabama (Olsen et al. 1964) to 15% in North Carolina (Karsten et al. 1992), but lower than the 63% (6 of 8 raccoons) prevalence found in Oklahoma (John and Hoppe 1986). The seroprevalences noted in Georgia, Florida, and Missouri were similar to previous studies conducted in South Carolina (37–61%) and northern Virginia (16–41%) (Yabsley and Noblet 2002, Hancock et al. 2005). Although the reasons for the higher prevalence observed in Florida in the current study are unknown, it is possible that higher densities of triatomine bug vectors, higher densities of animals, or near year-round activity by the bugs or animals may contribute to increased transmission.

Previous studies on Virginia opossums have been limited to culture-based surveillance and have produced prevalences (8–33%) that are lower compared with raccoons (McKeever et al. 1958, Olsen et al. 1964, Barr et al. 1991, Karsten et al. 1992, Pung et al. 1995). In the current study, a similar culture-based

prevalence (13%) was noted that was significantly less than that detected in sympatric raccoons (30%). Importantly, however, we did not note a difference in the seroprevalence of sympatric raccoons and opossums, indicating that exposure rates among the two species are similar in a given study area. This apparent difference between serology and culture could be due to opossums having lower blood parasitemias at the time of sampling, which would lead to a decreased culture-based prevalence. This may be related to the differences in *T. cruzi* strains, as opossums are infected predominantly with *T. cruzi* I strains, while raccoons are infected predominantly with *T. cruzi* IIa (Clark and Pung 1994, Barnabe et al. 2001, Roellig et al. 2008), including the strains isolated in the current study (Roellig et al. 2008). These differences in parasitemias may also be due to immunologic factors that allow the opossums to clear the parasite from the bloodstream more quickly than other species. Experimental studies with North American host species and parasite strains are needed to investigate this dynamic.

Prevalence rates in female raccoons and opossums were significantly greater than in males. This is similar to rates found by Yabsley and Noblet (2002), who found a higher seroprevalence in females than in males, although the results were not significant. The higher rate of *T. cruzi* in females is possibly because of the denning activities in habitats that can lead to a higher contact rate with triatomine bugs. A study in the Yucatan, Mexico, also noted a trend for higher infection rates in females versus males, although the difference was not significant (Jimenez-Coello et al. 2008). In contrast, an experimental infection study conducted with *Calomys callosus* showed that males were more likely to develop parasitemias (Lourenço et al. 2008).

The similar prevalence rates in opossums and raccoons from urban and rural sites may be due to differences in transmission dynamics of different pathogens and the ecology of different vectors in urban and rural areas. Animals in urban settings are more likely to live in higher densities than animals in rural habitats (Prange et al. 2003), and if this results in higher contact rates, are possibly more likely to transmit pathogens directly (Wright and Gompper 2005). In the case of *T. cruzi*, however, this trend may be offset by a lower vector density in urban settings.

In summary, seroprevalence rates were highest in raccoons and opossums, which supports previous studies that these two hosts are commonly infected with *T. cruzi* (McKeever et al. 1958, Olsen et al. 1964, Burkholder et al. 1980, Grogli et al. 1984, John and Hoppe 1986, Karsten et al. 1992, Pung et al. 1995). Based on previous culture-based surveys, we believed that exposure rates among opossums would be lower compared with raccoons; however, data from this study indicate that exposure of these two species is similar in our tested geographic areas. Antibodies to *T. cruzi* were also detected for the first time in three species of hosts, Virginia opossums, ringtails, and bobcats. While previous culture-based work indicated that Virginia opossums were natural hosts, our data indicate that seroprevalence is much higher than culture-based prevalence. Evidence of *T. cruzi* infection for the first time in bobcats and ringtails suggests that they may also be natural hosts. Collectively, these data further our understanding of the natural history of this important zoonosis in the United States and indicate that *T. cruzi* prevalence varies by host species, host characteristics, and geographic region.

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No competing financial interests exist.

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