

Lack of *Trypanosoma cruzi* Infection in Urban Roof Rats (*Rattus rattus*) at a Texas Facility Housing Naturally Infected Nonhuman Primates

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The protozoan parasite *Trypanosoma cruzi* causes Chagas disease, uses kissing bugs as a vector, and is maintained in nature by a variety of wildlife reservoirs. Many natural cases of Chagas disease have been reported in NHP at facilities across the southern United States, where infected vectors and wildlife occur. Infection of NHP with *T. cruzi* can diminish their value as research models and lead to health problems and death. Identifying the modes of transmission and role of wildlife reservoirs in these facilities is therefore critical to guide interventions to reduce transmission. Here we investigated the role of roof rats (*Rattus rattus*), the most abundant nuisance species at a primate facility in San Antonio, in the maintenance and transmission of *T. cruzi*. The hearts and blood from the carcasses of the 145 rats collected underwent 2 independent PCR assays for detection of *T. cruzi* and other trypanosomes. The 145 hearts and 61 blood samples were all negative for *T. cruzi*. This population sample of 145 subjects would allow the detection of disease prevalence of 0.020 with a confidence level of 95%. The limited active vector surveillance efforts by our team combined with passive surveillance by facility personnel yielded no kissing bugs during the study period. Our results suggest that roof rats are unlikely to be important local reservoirs of *T. cruzi* at this facility. Further investigation of transmission dynamics across multiple years and more comprehensive vector surveillance is warranted.

Abbreviation: qPCR, quantitative PCR

Maintenance of biosecurity and prevention of disease transmission at NHP facilities involves intensive efforts to limit contact between primates and wildlife species. Rodent control, in particular, represents an ongoing challenge, especially for outdoor or indoor-outdoor facilities.²² Rodents can enter primate enclosures, consume and contaminate primate feed, and travel between enclosures and nearby sylvatic habitats. Primates with access to the outdoors are at increased risk of exposure to wildlife reservoirs of disease as well as to arthropod vectors of pathogens. The transmission of vector-borne and wildlife diseases, including West Nile virus, tularemia, and leptospirosis, to NHP at primate facilities have been reported.^{14,37,40}

Chagas disease is vector-borne, primarily affects humans and dogs, and is endemic throughout much of Latin America. Active transmission of the causative parasite, *Trypanosoma cruzi*, is increasingly recognized as an important public health issue in the southern United States. Entomologic surveillance has identified infected triatomine insect vectors (kissing bugs) across Texas.³⁸ *T. cruzi* is maintained in nature by diverse species of wildlife, which serve as reservoirs.³ In areas where the vectors and parasite are found, Chagas disease has emerged as a major concern in NHP facilities. At least 14 reports of *T. cruzi* infection of NHP in the United States have been published, and all of the affected primates originated from southern states.¹⁰ Texas is home to several NHP facilities, including 1 of the 7 national primate research centers, and sporadic natural cases of Chagas

disease in these NHP have been reported for decades in areas where kissing bugs are established.^{16,17,45} Although reports of infected NHP continue to increase with increased testing, few centers currently conduct routine comprehensive surveillance. Infection of NHP with *T. cruzi* can diminish their value as appropriate models in research and can lead to health problems and death, resulting in significant scientific and economic losses. An undiagnosed infection in a NHP enrolled in a research study might potentially confound results of that study. Primates housed with outdoor access are at risk of encountering kissing bugs, and transmission can occur either through contamination of a bite wound or mucous membrane with feces from the bug after blood-feeding or through direct ingestion of the bug by the primates.³⁵ Although the pathologic manifestations of Chagas disease in primates have been well described,^{1,5,46} the specific details of transmission and the role of wildlife reservoirs in these facilities are relatively unknown.

Identifying reservoirs is crucial to devising effective interventions in a complex multihost system, such as Chagas disease.⁴⁴ Southern plains woodrats have repeatedly been implicated as important wildlife reservoirs of *T. cruzi* in the United States.^{6,12} Other species of rodents, such as urban rats, have been investigated less thoroughly in this country, although they have been shown to harbor *T. cruzi* in highly endemic areas of Latin America.^{13,15,19,25,32,36} A recent survey of potential *T. cruzi* reservoirs in Texas found an infection prevalence of 34% in woodrats (*Neotoma micropus*), 75% in striped skunks (*Mephitis mephitis*), 60% in raccoons (*Procyon lotor*), and 18% in other rodents, including a single infected black or roof rat (*R. rattus*) and 2 house mice (*Mus musculus*).⁶ We investigated the presence and *T. cruzi* infection status in kissing bugs and roof rats,

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the most abundant nuisance wildlife species, at a NHP facility with endemic Chagas disease.

Materials and Methods

NHP facility. The Southwest National Primate Research Center, located at the Texas Biomedical Research Institute (San Antonio, TX), houses approximately 2500 NHP, including baboons, chimpanzees, and 2 species of macaques housed in indoor and outdoor cages, as well as common marmosets housed exclusively in indoor cages. The 200-acre property is partially surrounded by dense brushy vegetation with a small dry creek and is bordered by 3 major highways (Figure 1). Chagas disease was first detected in primates at this facility in 1984¹⁶ and has since been well-characterized.^{1,17,27,45,46} Roof rats (*Rattus rattus*; also known as ship rats, black rats, and house rats) are the most predominant rodent pest species identified by pest-control personnel at the facility.

Collection of rats. Through collaboration with the center's pest-control service, we obtained roof rat carcasses that were collected as part of routine pest-control activities from May through July 2015 and October through November 2015. These rats were trapped in snap traps or were found dead, presumably after ingestion of poison baits from bait boxes within the facility. Rats were collected across the facility, which was divided into 4 general zones (Figure 1); the pest-control service focused their control efforts on areas with known high rat activity during the study period. Rats were stored at -20°C for as long as 3 wk before transfer to Texas A&M University. We dissected the carcasses under Biosafety Level 2 laboratory conditions and recorded the species, sex, and postmortem condition. Postmortem condition was scored according to a 5-point scale, with a score of 1 representing minimal autolysis and progressing to score of 5 for marked decomposition. Heart and clotted blood from within the ventricles were collected from animals in adequate postmortem condition. The use of these rats collected for pest control was exempted from oversight by the IACUC at Texas A&M University and the Texas Biomedical Research Institute.

***T. cruzi* detection.** The DNA was extracted (EZNA Tissue DNA Kit; Omega Bio-Tek, Norcross, GA) from heart and blood samples according to the manufacturer's protocol but with an overnight lysis period. The extracted DNA was evaluated by using 2 independent PCR protocols. For the specific detection of *T. cruzi*, a 166-bp segment of the *T. cruzi* 195-bp repetitive satellite DNA was amplified by using a probe-based quantitative PCR (qPCR) assay with Cruzi 1 and 2 primers and the 6-carboxyfluorescein (FAM)-labeled probe Cruzi 3 as described³⁴ but with an initial denaturation time of 3 min. This assay has previously been shown to be a best-performing method in an international PCR study,³⁹ and is sensitive and specific for all strain types of *T. cruzi*, including TcI, TcIV,³⁹ and TcII,²⁶ the strain types found in the United States. On the basis of internal laboratory validations, the cutoff for positive samples was determined to be a quantification cycle value of 32 (or less). We also performed a nested traditional PCR assay by using genus-level primers targeting a fragment of the 18S RNA-encoding gene of *Trypanosoma* spp.²⁹ on each sample to allow for sequencing of positive results and potential detection of other species of trypanosomes. This primer set has been used to detect and characterize novel trypanosomes in a variety of species as well as other known trypanosomes including *T. rangeli*, *T. dionisii*, and all strain types of *T. cruzi*.^{7,24,30,31} DNA extractions, primary and secondary amplifications, and product analyses were performed in separate dedicated laboratory areas. A negative control was

included in each set of DNA extractions, and one or more water negative controls were included as contamination controls in every PCR run. The DNA from *T. cruzi* Sylvio X10 clone 4 (American Type Culture Collection, Manassas, VA), which is strain type TcI, served as a positive control. For the qPCR assay, positive and negative controls always gave expected results. For the nested PCR assay, when either the positive or negative control did not perform as expected, the entire plate was rerun, and the expected results were always obtained on the second attempt.

In addition, because of concerns about inhibition of PCR amplification, 10% of the negative rat samples ($n = 15$), selected across a variety of autolysis scores and dates of extraction, were 'spiked' with a low concentration (dilution, $1:10^6$) of *T. cruzi*-positive control DNA and then analyzed by qPCR assay, which also included negative water controls and a positive control template DNA the same concentration as that of the spiked samples.

Vector surveillance. Active nighttime kissing-bug surveillance was performed during 2 different visits to the facility in summer 2015 by using active searches and stationary white cloth sheets with dry ice and UV lights, methods which have successfully been used by us to collect kissing bugs in other areas across Texas. Surveillance was conducted between 2100 and 2400 for one night during each visit by a 4-person team. Four stations with lights, sheets, and dry ice were set up in an area between a sylvatic habitat and a building housing rhesus macaques, where animals have seroconverted in years past (area B, Figure 1), and the stations and immediate vicinity were actively checked for bugs 3 to 4 times each hour. Between checks of the stations, team members patrolled the facility (areas A, B, and D; Figure 1) with flashlights to actively search walls and sidewalks for bugs. For passive surveillance, after providing an informational lecture about Chagas disease and distributing outreach materials at the start of the study period, we enlisted the help of facility personnel. In addition, during October, the facility's pest control operators, acting on their own initiative, erected 4 ft \times 4 ft white-glue boards under fluorescent lights nightly along the perimeter fence facing the sylvatic habitat and checked for insects each morning; these were not actively monitored overnight.

Analysis of sample size. We calculated the detectable level of parasite prevalence by using the equation for sample size to detect disease in a large (infinite) population:

$$n = \ln(\alpha) / \ln(q),$$

where n is the required sample size, q is $1 -$ the minimal expected prevalence, and α is 0.05 (for a 95% confidence level).⁹

Results

In total, 152 roof rats were collected over the 5-mo study period (Table 1). Rats were collected from 4 main areas spread across the facility (areas A through D), within and around cages of all the species of primates and in food storage areas (Figure 1). Of the 152 rats collected, 69 (45.4%) were male, 75 (49.3%) were female, and the sex of 6 rats (6.9%) could not be determined. In addition, 16 of the 152 rats (18.4%) were classified as juveniles based on immaturity of external genitalia, and the rest were adults. Distribution of the degree of autolysis was as follows: score of 1 (minimal autolysis), 2.6%; 2, 23%; 3, 35.5%; 4, 15.1%; and 5 (maximal autolysis), 23.7%. A total of 145 of the 152 roof rats were tested; the remaining 7 carcasses were too autolyzed to determine sex and identify organs. Heart tissues

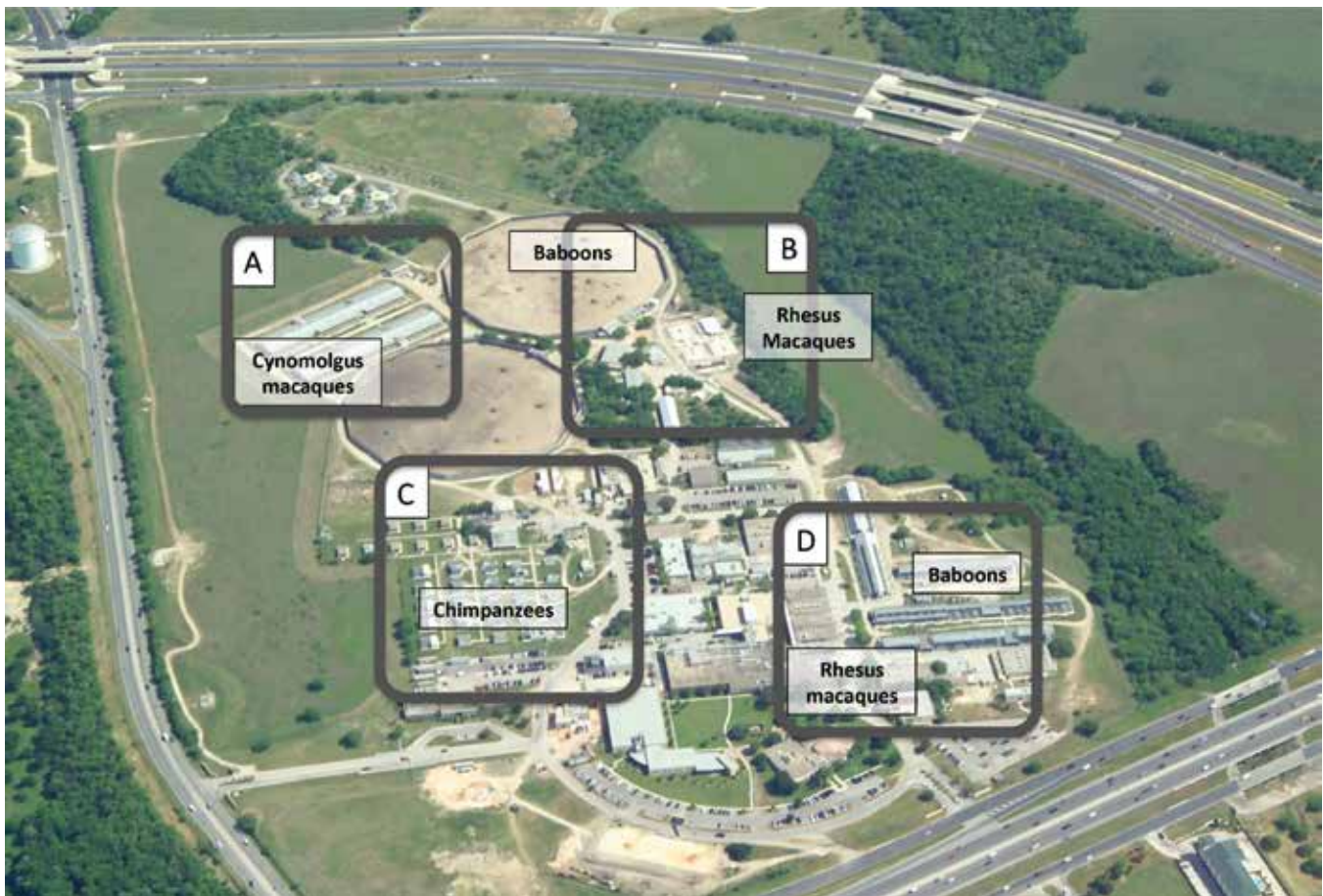


Figure 1. Aerial photograph of the Southwest National Primate Research Center (San Antonio, TX), showing its sylvatic habitat and the surrounding highway systems and bordering urban areas. Boxes represent unique sampling areas, and labels indicate the NHP species housed in each area.

Table 1. Demographics and *T. cruzi* PCR results of the of rats collected

	n	Sex			Site of collection				
		M	F	U	A	B	C	D	U
May	22	10	10	2	0	3	1	12	6
June	51	24	23	4	0	12	1	35	3
July	20	7	13	0	2	5	0	12	1
October	39	15	22	2	0	13	0	26	0
November	20	13	7	0	0	3	0	17	0
Total	152	69	75	8	2	36	2	102	10

U, unknown

Regardless of date or site of sample collection, all samples were negative for *T. cruzi*.

were collected from all 145 tested rats, and clotted blood was collected from 61.

None of the 145 rat hearts or 61 blood samples tested was positive for *T. cruzi* according to conventional or qualitative PCR analysis. We were able to detect *T. cruzi* in all 15 of the spiked samples, and the quantification threshold values were approximately equal to that of the positive control containing the same concentration of *T. cruzi* DNA, whereas the negative control was negative; these findings demonstrate a lack of PCR inhibition. This sample size of 145 subjects affords the detection of a disease prevalence of 0.020 with a confidence level of 95%.

We did not collect any kissing bugs during a combined total of 7 h of vector surveillance activities between the 2 nights that we visited the facility in June and July, although several other species of bugs were observed. Facility personnel noted no kissing bugs onsite throughout the duration of the study, although 3 bugs of other species suspected to be kissing bugs were collected.

Discussion

Our inability to detect *T. cruzi* DNA in a sample of 145 rats indicates that the prevalence of *T. cruzi* infection in roof rats at this facility is low (less than 2%) or 0%, suggesting that this species may not serve as an important wildlife reservoir of *T. cruzi* at this time. Furthermore, neither our active vector surveillance nor passive surveillance by facility personnel and pest managers yielded any kissing bugs from the site, but our active surveillance efforts were limited, primarily due to security constraints by the facility. In contrast, our statewide kissing-bug citizen submission program received hundreds of kissing bugs from the greater San Antonio area during the same time period.^{8,42} Although it seems most likely that the infection of adult primates at this facility results from contact with kissing bug vectors that our sampling failed to detect, alternative modes of transmission have not been investigated fully. For example, *T. cruzi* has been identified by PCR analysis in blood-sucking lice of the suborder Anoplura at the same primate facility, but transmission of the parasite by lice remains to be demonstrated.²

Previously published evaluations of *R. rattus* for *T. cruzi* infection in endemic areas of Latin America have found an infection prevalence ranging from 5% to 57% by using PCR assay, microscopy, or culture^{13,15,19,25,32,36} and seropositive rates up to 73%,²⁵ but there is little published on the infection prevalence of this species in the United States. Through PCR analysis, one study⁶ detected infection in a single *R. rattus* from Uvalde County, Texas, but this was the only member of this species tested in the study. Interestingly, Old World rats such as *R. rattus* are the natural hosts of the nonpathogenic trypanosome *Trypanosoma conorhini* and are implicated in the spread of this parasite and its associated vector, *Triatoma rubrofasciata*, around the world, most likely through transport in shipping vessels.^{11,20} *T. cruzi* might be spread in a similar fashion,¹¹ but thus far, infection of rats with *T. conorhini* in the continental United States has not been reported, and reports of *T. rubrofasciata* are limited to Florida and Hawaii.³

The lack of apparent infection in rats in our study may reflect that this species is not important in the local transmission ecology of *T. cruzi* or may result from limitations to our study design. First, we timed our study from May through July to coincide with the period of peak adult kissing bug dispersal activity, as previously documented for our study region in Texas,^{8,33} and from October through November, when infection is expected to be established in reservoirs. However, the timing of our survey may have provided only the ability to detect infection in rats that resulted from active transmission during the current study year and not previous transmission seasons. In the wild, roof rats have an average lifespan of about a year, with an annual mortality rate of 91% to 97%,²⁸ so it is likely that very few of our sampled rats were alive during the previous year's period of kissing-bug activity and parasite transmission. Thus, the lack of apparent infection in rats might reflect an overall low year for transmission due to unmeasured biotic or abiotic factors. Second, the interval between death and preservation of carcasses varied among specimens and could have been as long as 2 d in some cases. At the time of heart collection, 40% of rat carcasses were scored as 4 or greater on the 5-point autolysis scale (moderate–marked to marked autolysis). However, this condition is unlikely to have significantly affected the outcome of testing because the qualitative PCR assay we used targets a very short fragment of DNA and thus is relatively resistant to effects of sample degradation. A final limitation of the study is that our active bug-surveillance efforts were limited, and future efforts should include additional active trapping as well as excavation of woody debris and other potential kissing-bug harborage sites along the perimeter of the facility across an extended time frame.

Kissing bugs have been collected from around the outdoor cages of this facility in past years,¹⁶ but since 2011, pest control efforts have been intensified, including measures such as reducing debris and bug harborage sites, mowing a wide perimeter around outdoor enclosures, sealing of outside doors, and applying diatomaceous earth around the perimeter. Anecdotally, personnel have reported reductions in bug activity overall and in the incidence of new *T. cruzi* infections of primates as a result of these measures. However, animals are not routinely tested for *T. cruzi* at this facility until they are sold or enrolled in a study, so although we know seropositive animals are present as a result of prior studies, we do not know whether, in fact, bug activity and the incidence of infections have been reduced. Therefore, in the absence of systematic data on incidence of seroconversions, it is not possible to ascertain what effect these

increased vector control measures have had on the incidence of *T. cruzi* at this facility.

A reservoir is defined as one or more epidemiologically connected populations or environments in which the pathogen can be maintained permanently and from which infection is transmitted to the defined target population.¹⁸ Therefore, given our results, roof rats are unlikely to serve as important as local reservoirs of *T. cruzi* at this facility. Increased bug and rodent control efforts may have reduced the frequency of transmission, but other mammals likely are serving as wild reservoirs of *T. cruzi*, given the facility's location in San Antonio, a known hotspot for Chagas disease transmission, with a high number of infected vectors and canine and human cases.^{8,23,38,41,43} The facility is partially surrounded by a brushy area, with a dry creek on one side, and facility personnel have reported the presence of several mesomammals, including skunks, raccoons, opossums, and armadillos, all of which have been implicated as important reservoirs of *T. cruzi* in the United States,^{3,4,6} in addition to rabbits and squirrels, the reservoir competency of which is largely unknown. A primate facility in North Carolina isolated *T. cruzi* from raccoons on its property and from an opossum in the surrounding area after the detection of *T. cruzi* in a squirrel monkey.²¹ Multiple wild species as well as infected NHPs more likely serve as the local reservoirs of *T. cruzi*. Efforts to characterize the reservoir community should include a multidisciplinary approach to data collection and analysis, with interventions that simultaneously answer questions about reservoir importance and provide direct benefits in control of the parasite.⁴⁴ Future work should include expanded efforts to trap and test additional species of mammals, as well as increased efforts to confirm vector presence.

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