Comparative Medicine
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Vol 73, No 3
June 2023
Pages 229–241

*Original Research*

# Exclusion of Horizontal and Vertical Transmission as Major Sources of *Trypanosoma cruzi* Infections in a Breeding Colony of Rhesus Macaques (*Macaca mulatta*)

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**The vector-borne protozoal parasite *Trypanosoma cruzi* causes Chagas disease in humans and animals. This parasite is endemic to the southern United States where outdoor-housed NHP at biomedical facilities are at risk of infection. In addition to the direct morbidity caused by *T. cruzi*, infected animals are of limited biomedical research use because infections can produce confounding pathophysiologic changes even in animals with no clinical disease. In part due to concerns for direct *T. cruzi* transmission between animals, infected NHP at some institutions have been culled, removed, or otherwise isolated from uninfected animal populations. However, data that document horizontal or vertical transmission in captive NHP in the United States are not available. To evaluate the potential for inter-animal transmission and to identify environmental factors that affect the distribution of new infections in NHPs, we conducted a retrospective epidemiologic study of a rhesus macaque (*Macaca mulatta*) breeding colony in south Texas. We used archived biologic samples and husbandry records to identify the time and location of macaque seroconversion. These data were used to perform a spatial analysis of how geographic location and animal associations affected the spread of disease and to infer the importance of horizontal or vertical routes of transmission. The majority of *T. cruzi* infections were spatially clustered, suggesting that environmental factors promoted vector exposure in various areas of the facility. Although we cannot not rule out horizontal transmission, our data suggest that horizontal transmission was not a critical route for spread for the disease. Vertical transmission was not a contributing factor in this colony. In conclusion, our findings suggest that local triatome vectors were the major source of *T. cruzi* infections in captive macaques in our colony. Therefore, limiting contact with vectors, rather than segregation of infected macaques, is a key strategy for disease prevention at institutions that house macaques outdoors in the southern United States.**

**Abbreviations:** DTU, discrete typing unit; TAM, *T. cruzi*-associated monkey

**DOI:** 10.30802/AALAS-CM-23-000005

## Introduction

Chagas disease (also known as American trypanosomiasis) is caused by the vector-borne protozoan parasite *Trypanosoma cruzi*. This parasite is endemic throughout much of the Americas, including the southern United States.[40] The most common route of *T. cruzi* transmission is host exposure to the feces of infected *Triatoma* spp. insects, the primary vectors of the parasite,[6,34,35] through transcutaneous inoculation, contamination of mucous membranes, ingestion of contaminated food sources, or ingestion of the entire insect.[31] In the southern United States, a robust enzootic cycle of *T. cruzi* transmission exists between native triatomine species and a variety of wild mammalian hosts. Spillover from this sylvatic cycle results in infection of humans and domestic animals such as dogs.[30] In addition to vector-borne transmission, both horizontal and vertical routes of *T. cruzi* transmission have been documented in humans;[45] in humans, infection can occur through blood transfusions and organ transplantations, and some evidence suggests that transmission may also occur through sexual contact.[3,27,51] Human vertical (congenital) transmission of *T. cruzi* between mother and child occurs naturally in approximately 5% to 10% of births from *T. cruzi* infected mothers in endemic areas,[38] and at a rate of 1% to 5% in the United States.[23,47]

*Trypanosoma cruzi* is classified genetically into 7 discrete tying units (DTU): TcI, TcII, TcIII, TcIV, TcV, TcVI, and TcBat. Each DTU has unique epidemiologic characteristics including differences in distribution, insect vectors, host species, and clinical forms of disease.[39,54] While all 7 DTU are endemic within South America, TcI and TcIV, and recently TcII, are the only DTU that have been detected in triatomine insect and wildlife populations in the southern United States.[7,16,17,21,30]

In the southern United States, naturally acquired *T. cruzi* infections have been documented in captive NHP since the 1970s, and contemporary studies of biomedical research

*Submitted: 02 Feb 2023. Revision requested: 26 Feb 2023. Accepted: 08 Mar 2023.*
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facilities have documented a 2% to 10% prevalence in some NHP colonies.[7,14,17,30-32] Many NHP facilities are located in rural areas surrounded by natural habitats that support wildlife and sylvatic *T. cruzi* transmission.[31] Furthermore, large numbers of NHP in the southern United States are housed in open-air enclosures (for example, corncrib cages, corrals, and indoor–outdoor buildings) and thus may have contact with *T. cruzi*-infected insect vectors.

*T. cruzi*-infected NHP can present with a variety of clinical conditions that are comparable to those described for humans. In both humans and NHP, *T. cruzi*-infections are typically characterized into acute, indeterminate, and chronic phases.[39,50] The acute phase can be characterized by mild, nonspecific illness persisting for days to weeks, although more severe acute manifestations have been reported for both humans and NHP.[8-10] After the acute phase of infection, most infected individuals enter the indeterminant phase during which no clinical signs are present. While many humans and NHP in the indeterminate phase have no further disease progression and remain asymptomatic for life, a percentage of infected individuals do eventually develop chronic Chagas disease, which is characterized by heart failure[1,8,9,12,53] and, to a lesser extent, gastrointestinal disease[26] in both humans[5,6,39] and NHP.[14,20] Beyond the morbidity and mortality associated with some cases of Chagas disease, *T. cruzi* infections are also problematic for research monkeys due to their potential to produce confounding background cardiac lesions or altered immunologic function, even in animals with no appreciable signs of clinical disease.[13,41,43,50,52] Furthermore, and similar to what has been described for humans, several NHP studies have documented the reactivation of dormant *T. cruzi* infections secondary to SIV infections and drug-induced immune suppression.[14,20,42,46]

Considering the health and study-utility issues associated with *T. cruzi* infections in NHP, concentrated efforts have been undertaken by biomedical research facilities over the last decade to minimize natural *T. cruzi* infections in NHP colonies. These efforts include the development of molecular and diagnostic screening assays and the use of novel husbandry measures. While literature is not available to document the variety and extent of the husbandry measures being used at different NHP facilities in the United States, several different measures are being employed. Some of these husbandry measures are designed to limit insect vector exposure based on the known epidemiologic characteristics of the disease. Examples of these husbandry measures include the increased use of indoor NHP housing and the reduction of vegetation surrounding NHP habitats. Other measures include the isolation of infected individuals from other colony animals, and the removal or culling of infected individuals. Although many factors can play into the decision to remove or cull *T. cruzi* infected animals from colonies (for example, occupational health issues for personnel), 2 concerns that have been voiced in regard to breeding colonies are that *T. cruzi* infections might decrease the overall fecundity of breeding animals, and that *T. cruzi* may spread between colony animals through horizontal or vertical transmission. A recent study involving the same rhesus macaque (*Macaca mulatta*) breeding colony used in the current study found no significant difference in reproductive outcomes of infected and uninfected colony animals over a 5-y period.[33] Further, while naturally occurring horizontal transmission has not been reported in the NHP literature, concerns over the possibility of horizontal transmission in NHP colonies remain because direct blood exchange, and potentially sexual contact, have been identified as modes of transmission in humans and animal models.[3,27,37,45,51] While vertical transmission has been well documented in some human populations,[48] we found only a single documented case of vertical transmission in NHP: a report of dam-to-infant *T. cruzi* transmission in laboratory-housed, wild-caught squirrel monkey.[22]

Although anecdotal observations have suggested that vertical and horizontal transmission are uncommon in the rhesus macaque breeding colony we describe here, the exact contributions of these modes of transmission to the incidence rate of infection had never been fully examined for this colony or for any research NHP colony in the southern United States. Likewise, no published data define specific environmental factor(s) might be responsible for the different incidence rates of *T. cruzi* infection seen within or between NHP facilities in the United States. To address this issue and to provide data that might be generally useful in the management of outdoor-housed NHP colonies, we undertook the formal epidemiologic study described in this report. The 3 hypotheses for this study were as follows: (1) environmental factors influence the incidence and distribution of *T. cruzi* infections in outdoor-housed NHP breeding colonies from the southern United States; (2) vertical transmission is not a primary contributing factor for the spread of *T. cruzi* in outdoor-housed NHP in the southern United States; and (3) horizontal transmission is not a primary contributing factor for the spread of *T. cruzi* in outdoor-housed NHP in the southern United States. We used archived samples and data collected over a 20-y period from a rhesus macaque breeding colony comprised of approximately 1000 animal and located in south Texas, together with an environmental analysis, to determine the relationship of physical proximity and relatedness associations among animals to the detection of new infections. These relationships were used to infer the importance horizontal or vertical transmission of the parasite.

## Materials and Methods

**Breeding colony.** This study used data collected over 20 y (1999 to 2018) from an Indian-origin, rhesus macaque breeding colony. The breeding colony was housed at the AAALAC-accredited NHP facilities at The Keeling Center for Comparative Medicine and Research (University of Texas, MD Anderson Cancer Center, Bastrop, TX). The annual colony census over this period ranged between approximately 750 to 1100 animals. The average female:male ratio of the colony through this same time was approximately 1.9:1. The breeding colony has been a closed colony since 1983; 1982 was the last year animals born outside the colony were acquired for use in the breeding colony. Since 1991, the colony has been documented through serologic means to be SPF for *Macacine alphaherpesvirus 1*, SIV, STLV, and simian retroviruses 1, 2, and 5.

Throughout this 20-y period, the breeding groups typically consisted of 5 to 10 adult females and one adult male. These groups were routinely reorganized every 4 to 5 y in efforts to promote the genetic diversity of the breeding colony as a whole. As per the standard husbandry protocols of the Center, infants remained in their breeding groups until weaning at approximately 7 to 9 mo of age, at which time they were placed in a larger group of other weanling macaques. These weanling animals remained housed together throughout their entire juvenile stage of development or until they were sold. In general, the female juvenile macaques that had not been sold were placed into their first breeding groups at 3 to 4 y of age, and male juvenile macaques that had not been sold colony were placed into their first breeding groups at 5 to 7 y of age.

Starting at weaning age, each macaque received an annual physical examination sometime between late winter and spring with routine blood work and serologic screening. Residual serum from blood collected during these annual physical examinations was archived in −80 °C storage. All routine husbandry, health monitoring, and serum archiving protocols were approved by the IACUC of the University of Texas–MD Anderson Cancer Center and followed the NHP standards established by the *Guide for the Care and Use of Laboratory Animals*.[15]

**Breeding colony facilities.** The breeding colony was housed in a 381-acre, partially wooded campus in Bastrop County in central Texas. The majority of the animals included in this study resided within a chain-linked–fenced, 3.6-acre area identified as the 'main rhesus compound' (Figure 1). Housing within the main rhesus compound included 8 corncrib housing structures and 8 animal buildings, with each of these buildings partitioned into 10 to 12 individual rooms around a central corridor. A second fenced area approximately 0.33 km north of the main rhesus compound was also used to house rhesus macaques prior to early 2018. This 0.56-acre 'north rhesus compound' contained 2 additional 12-room animal buildings identical to those of the main rhesus compound. Corncrib structures and rooms in the animal buildings housed either a group of juveniles, a single breeding group, or a single-sex group of adults awaiting placement into breeding groups. From early spring through late autumn each year, all building rooms and corncrib structures had open-air access to the environment. During the colder periods of each year, the buildings and corn crib structures had large fiberglass panels affixed to their outer walls to shelter the animals from colder temperatures.

Over the 20 y encompassed by this study, the environmental features of the north rhesus compound remained essentially unchanged other than the continued growth of mature trees. In contrast, the main rhesus compound underwent several significant environmental alterations over this same period. The earliest of these environmental changes occurred between late 2001 and the middle of 2002, with the removal of approximately 6 to 10 mature trees and the construction of Building 3 in the main compound (Figure 1). Between late Summer 2011 and Spring 2012, the facility undertook intensive fire mitigation activities in response to wildfires that threatened the campus during Summer 2011. These activities included the cutting back mature trees and removal of most of the ladder fuel (young trees, shrubs, and leaf litter) from the wooded area directly south of the main compound. In addition, in 2012, a laundry and locker building was constructed on the west side of the main rhesus compound. Finally, in Fall 2016, several ornamental plant beds were removed from the main rhesus compound due to concern that they may provide habitat to triatomine bugs.

**Serodiagnostic assays for identification of seropositive animals and year of infection.** Two commercially available serodiagnostic approaches were used to characterize the *T. cruzi* serostatus of each animal included in the study. The first involved a 2-step process that used a suspension microarray (Macaque Chagas Multiplexed Fluorometric ImmunoAssay, Charles River Laboratories, Wilmington, MA) followed by ELISA testing, as previously reported.[31,33] Any suspected seropositive serum samples were then tested using a second method. The second test was a rapid, benchtop screening assay (Chagas Stat-Pak Assay; Chembio Diagnostic Systems, Medford, NY).[33] This assay is a single-use immunochromatographic screening test for the detection of *T. cruzi* antibodies in human blood, serum, or plasma. The assay was used off-label in the macaques, with tests run as previously reported.[33] The presence of any line in the reading area, regardless of its strength, was considered to be a positive result.

As previously reported, mass serosurveillance of breeding colony animals for *T. cruzi* antibodies was first undertaken in 2013 and was performed a second time in 2015, encompassing approximately 66% and 85% of the resident colony animals respectively.[33] Although the animals included in the 2013 and 2015 colony surveys were initially tested using a proprietary inhouse indirect ELISA, rather than the commercial suspension microarray, the 2013 and 2015 serum samples that had been archived from these same animals were later assayed using a multiplexed fluorometric immunoassay platform. Archived serum samples were also used to determine the *T. cruzi* serostatus of all the remaining breeding colony animals present at the facility between 2012 and 2015. From 2016 through 2018, *T. cruzi* serosurveillance was performed on all breeding colony animals annually as part of their standard health examinations. Collectively, these efforts resulted in the serologic screening of 1899 of the 2166 animals that were housed at the Center between 2012 and 2018. No archived serum samples were retained from the remaining 267 animals prior to their departure from the colony.

In addition to the *T. cruzi* seropositive animals identified in the 2012 to 2018 colony surveys, 24 animals that died or were sold from the breeding colony prior to 2012 had also been identified as seropositive for *T. cruzi*. Archived serum samples had previously been tested for *T. cruzi* seroconversion for the following purposes: to identify the seroprevalence of *T. cruzi* in the 2003 rhesus macaque breeding colony; to investigate the seroprevalence of *T. cruzi* in association with other disease states in the rhesus macaques; and to support the diagnosis of Chagas disease in necropsied rhesus with histologic lesions suggestive of chronic *T. cruzi* infection.

Once animals were identified as *T. cruzi* seropositive, the earliest year of seropositivity was determined for each animal, when possible, by sequential screening of the archived serum samples in reverse chronologic order. Specifically, serologic testing was performed on progressively older samples for each seropositive animal until the first seronegative result was obtained. Once the earliest year of seropositivity was identified for each animal, the year of *T. cruzi* infection of each animal was designated to be 1 y prior to its earliest seropositive serum result. Considering the phenology of the local kissing bug species (specifically, that adults are most active and disperses from June through August),[16,18,44] and the timing of annual blood sampling (January through April), we conjecture that most animals in the colony initially became infected with *T. cruzi* sometime between the late spring and summer but that these infections were not typically detected until the winter or early spring of the following year.

**PCR assessment of seropositive breeding colony animals.** Between 2016 and early 2017, PCR analysis was performed on DNA extracted from whole blood samples collected from all *T. cruzi* seropositive breeding colony animals that were housed at the facility during that period.[31] The goal of these efforts was to quantify the *T. cruzi* DNA in the blood and to determine the DTU of these infections. Since early 2017, additional PCR testing of select seropositive animals in the breeding colony was also done to support of other ongoing studies. DNA extraction and PCR analysis were performed as previously described.[31] The DTU types that were identified through these PCR analyses were used in this study to investigate horizontal transmission.

**Identification of the housing location and cage mates for seropositive animals.** Animal husbandry records documenting the month-to-month location of all animals in the breeding colony were available for October 1998 through January 2011

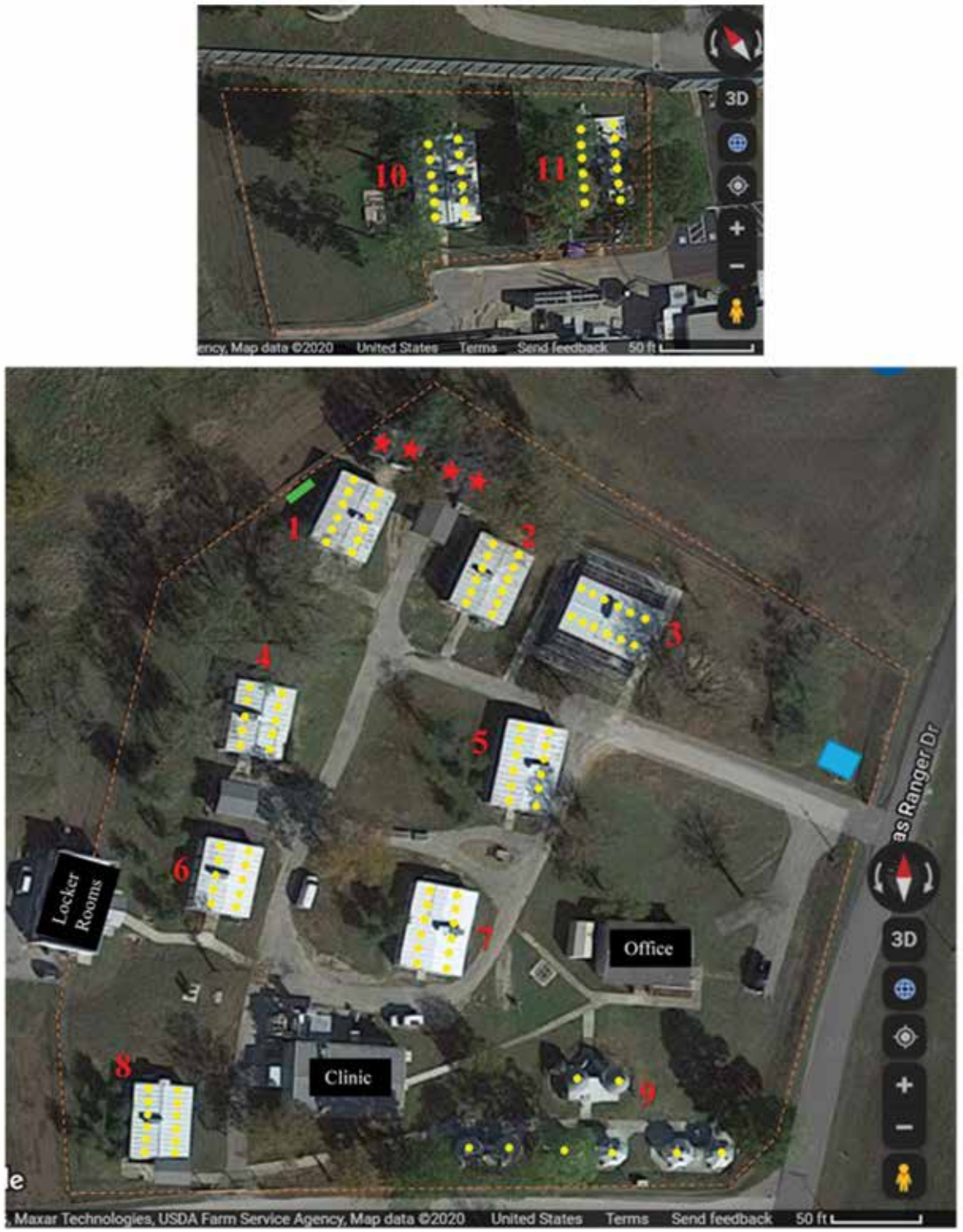


**Figure 1.** Satellite image (2018) of a portion of the facility, illustrating the 2 rhesus macaque colony compounds, with animal building numbers labeled in red. The main rhesus compound (lower image) contains Buildings 1 through 8, the 8 corncrib structures (collectively referred to as 'Building 9'), a veterinary clinic, an administrative office, and a laundry–locker facility. The north rhesus compound (upper image) contains Buildings 10 and 11. Yellow circles denote the center of each corncrib structure and the individual rooms in each animal building. Red stars indicate the locations of 4 elevated storage sheds north of Buildings 1 and 2. The summer storage site for the fiberglass winterizing panels between 2011 and 2018 is denoted by the green box directly west of Building 1. The previous location of a planter box of interest is denoted by the blue box in the northeast corner of the main rhesus compound. The fenced boundary of each compound is denoted with dashed orange lines. Photo credit: Map data © 2020 Google, Maxar Technologies.

and January 2012 through December 2018. These records, in conjunction with the results of the serologic testing described above, were used to identify the housing location of each seropositive animal during the late spring and summer on their year of infection, when they would have been most likely to encounter triatomine vectors. The records were also used to identify all animals that were housed with any seropositive animals on or after April 1st of the year of infection. Collectively,

we have called the cage mates to these *T. cruzi* seropositive animals—which included breeding partners, same-sex social partners, and offspring—'*T. cruzi*-associated monkeys' (TAM).

After we identified the TAM for all seropositive animals, we attempted to determine the serostatus of each TAM. The serostatus of the TAM that had remained part of the breeding colony until 2012 or later was already known. The serostatus of the TAM that had died or been sold from the colony prior to 2012 was established through serologic testing of the last archived serum sample collected from each animal just before its departure from the colony. Any TAM that was identified as seropositive underwent additional serologic testing, as described above, to establish its earliest year of seropositivity. The housing location of each seropositive TAM in their year of infection and all of the cage mates (secondary TAM) to these initial TAM were identified using animal records. The serostatus of these secondary TAM was then established through colony records or additional testing as needed.

**Evaluation of environmental influence on the incidence of *T. cruzi* infections.** We conducted a spatial analysis to identify any specific buildings or rooms in that consistently had higher ('hot spots') or lower ('cold spots') incidences of *T. cruzi* infections than the colony in general. To accomplish this goal, we mapped the geographic location of all seropositive animals for which we knew the year of infection. We documented the exact locations of animals with incident infections during a 5-mo period (April to August) of the year in which each animal was identified to have become infected. In addition, the location that housed the newly infected animal was also assigned a numeric 'room score' of 1 to 5, with the room score representing the number of months that the newly infected animal had lived in that location during April through August.

Two master maps of the colony were created for the tabulation of room scores covering the periods of 1999 to 2010 and 2012 to 2017. The division of the room scores into these 2 time periods was done because (1) facility husbandry records between February and December 2011 had previously been lost during a data transfer and were not available; (2) the serologic status of the colony as a whole was well-established for the period between 2012 to 2018, whereas the only serologic information available for the colony prior to 2011 was collected as part of a few small studies through the retrospective testing of the known seropositive animals and their TAM; and (3) a large number of environmental alterations occurred within and around the main rhesus compound in 2011 through 2012, as described above. The $x$ and $y$ global-positioning coordinates of each individual building room or corncrib structure underwent spatial hot-spot data analysis through Geographic Information Systems by using ArcGIS 10.3.1 Spatial Statistics toolbox (ESRI, Redlands, CA).

We examined storage sheds and trees as environmental elements that might promote the presence of triatomines. Four elevated storage sheds are located just north of Buildings 1 and 2 in the main rhesus compound (Figure 1). *T. cruzi*-infected triatomine bugs had previously been identified in and around these sheds with the assistance of the Texas A&M University community science program. Trees were included because triatomines have been documented to live in the bark of trees[4,28] and often inhabit rodent and mesomammal nests associated with woody or leafy ground cover.[19,49] To evaluate the environmental influence of the storage sheds on the incidence of *T. cruzi* infections in the colony, a weighted metric termed 'cumulative shed power' was assigned to all animal rooms within 20 m of a shed. To calculate the shed power for a given animal room, the floor area of each shed (in square meters) was used as a proxy for its potential to harbor triatomine bugs, and the closest measured distance between the shed and animal room (in meters) was used to account for the likelihood of an interaction event between the insects and the monkeys. Division of the floor area by the distance between the shed and animal room resulted in a shed power for that one room from that one shed. For animal rooms that were within 20 m of multiple sheds, each of the shed powers was calculated and then added together to find the cumulative shed power associated with each room by using the formula

$$\text{shed power} = \sum\left(\left[\text{area (m)}\right] / \left[\text{distance (m)}\right]\right)^{\text{shed1}-\text{n}},$$

where $n$ is the farthest shed found within 20 m. A similar approach was used to evaluate the environmental influence of trees on the incidence of *T. cruzi* infections. Here a weighted metric termed 'cumulative tree power' was assigned to all animal rooms within 20 m of a tree. To calculate the tree power for a given animal room, the circumference of each tree (in centimeters) measured at 1 m off the ground was used as a proxy for its potential to provide habitat to wildlife hosts or triatomine bugs, and the closest measured distance between the tree and animal room (in meters) was used to account for the likelihood of an interaction event between the insects and the monkeys. Division of the circumference of the tree by the distance between the shed and animal room resulted in a tree power for that one room from that one tree. For animal rooms that were within 20 m of multiple trees, each of the tree powers was calculated and then added together to find the cumulative tree power associated with each room using the formula

$$\text{tree power} = \sum\left(\left[\text{circumference (cm)}\right] / \left[\text{distance (m)}\right]\right)^{\text{tree1}-\text{n}},$$

where $n$ is the farthest tree found within 20 m.

The distance of 20 m as the outer limit of influence for these environmental features was based on a study that explored the daily movement events of 2 triatomine species common to the facility, *Triatoma gerstaeckeri* and *T. sanguisuga*; that study documented daily triatomine movement events ranging that ranged 1 to 20 m, with an average of 3.8 m.[29]

**Investigations into the possibility of horizontal and vertical transmission.** We looked for time and space links between the seropositive animals and their TAM that could be suggestive of vertical or horizontal transmission of *T. cruzi*. Where possible, all infants born to seropositive dams between 1999 and 2018 were examined for evidence of seroconversion during their first 2-y of life. For the purposes of investigating horizontal transmission, any seropositive TAM that was identified to have had its earliest year of seropositivity occur while housed with a seropositive animal or within 2-y of cohousing was considered 'linked' to that seropositive animal (i.e., linked TAM). The 2-y period for vertical and horizontal transmission detection was used to account for any acute infections that may have occurred between a TAM and a seropositive animal just prior to their separation, given that infected animals may not develop *T. cruzi* antibodies by the time of their first annual serologic exam after the separation. Although this 2-y allowance is arguably be too generous, it ensured inclusion of all seropositive animals for whom direct transmission of the parasite could have occurred. Linked TAM then underwent further analyses using DTU typing comparisons, where available, to help assess the possibility of horizontal transmission (Figure 2). The logic behind this approach was that if horizontal transmission was responsible for a TAM seroconversion, then both the linked TAM and its associated

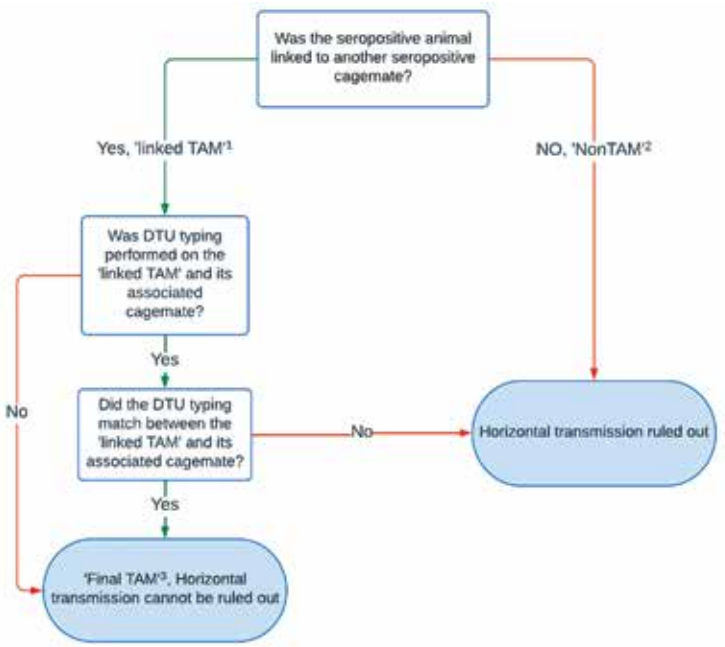


**Figure 2.** Flow diagram of criteria used for determining whether horizontal transmission remained a potential route of transmission for individual seropositive animals.

seropositive animal should have *T. cruzi* infections comprised of the same DTU. Horizontal transmission was ruled out as a cause of infection for seropositive animals that were not linked to another seropositive animal before its own seroconversion (i.e., nonTAM) and was likewise ruled out for linked TAM found to have *T. cruzi* infections comprised of different DTU than their associated seropositive animal. Conversely, horizontal transmission could not be ruled out as a source of infection for linked TAM that did not have DTU testing, or for linked TAM with *T. cruzi* DTU that did not matched their associated seropositive animal. Linked TAM for whom horizontal transmission could not be ruled out are referred to as 'Final-TAM'.

**Statistical methods.** VassarStats was used to perform a z-ratio for the significance of the difference between 2 independent proportions, the number of seropositive animals that seroconverted during 2012 through 2018 after exposure to other seropositive animals, and the number of seropositive animals that seroconverted between 2012 and 2018 without previous exposure to seropositive animals.[36] Google Maps (Google, Menlo Park, CA) was used to determine the $x$ and $y$ coordinates of housing rooms for spatial autocorrelation analysis. These coordinates, the number of seropositive/seronegative NHP, and the total number of cumulative months that seropositive animals occupied individual rooms were used for spatial data analysis to identify statistically significant hot spots and cold spots.

A local Moran I measure of spatial autocorrelation, known as Local Indicator of Spatial Association (LISA), was used to distinguish clusters of cases (i.e., hot spots).[2] With the use of a Moran scatterplot, interpretation of the significance of spatial clusters becomes possible; these are designated as 'high-high' and 'low-low' clusters. Spatial outliers are designated as 'high-low' and 'low-high.'

Another class of local spatial autocorrelation was performed by using Getis–Ord statistics in which spatial outliers are not considered. When using Getis–Ord statistics, a value larger than the mean suggests a high-high cluster or a hot spot, whereas a value smaller than the mean indicates a low-low cluster or cold spot.[25]

## Results

**Seropositive breeding colony animals.** A total of 80 breeding colony rhesus macaques housed at our institution during 1999 through 2018 were identified to be *T. cruzi* seropositive (Table 1). Notably, all 80 animals were seropositive on both commercially available testing methods. Prior to the start of this study, 75 of these 80 monkeys had already been identified to be seropositive through colony serosurveillance. The 5 remaining seropositive animals were identified from serologic testing of TAM. Of the 80 seropositive macaques included in this study, the year of infection was determined for 74 animals (Table 1) and the caging location during year of infection was determined for 71 animals (Table 2). The year of infection could not be accurately determined for 6 animals due to the lack of consistent archiving of serum samples prior to 2000. However, 4 of these 6 monkeys were seropositive as early as 2000, and the other 2 monkeys were seropositive as early as 1989. Because of a loss of husbandry records for 2011, the caging location at year of infection could not be accurately determined for 3 animals that seroconverted

**Table 1.** Seropositive breeding colony animals

| | 1999–2018 | 2012–2018 |
|---|---|---|
| Total no. seropositive animals | 80[a] | 53 |
| Age (y) at infection (mean, median, range) | 11.7, 12.0, 3–24 | 11.3, 10.5, 3–24 |
| Female:male ratio | 59:21 (2.8:1)[b] | 38:18 (2.1:1)[b] |

[a]Year of infection was determined for 74 seropositive animals. The mean, median, and age range were calculated for the 74 animals that became seropositive during 1999 through 2018.
[b]Animal ratio (odds ratio)

**Table 2.** Data regarding *T. cruzi*-associated monkeys (TAM) and nonTAM

| | 1999–2018 | 2012–2018 |
|---|---|---|
| Total no. of TAM identified | 688 | 497 |
| Total no. of TAM tested | 624 (90.6%) | 449 (90.3%) |
| TAM seropositivity (no. positive / no. tested × 100%) | 34 of 624 (5.4%) | 30 of 449 (6.7%) |
| Seropositive study animals identified as linked TAM[a] | 34 of 71 (47.9%) | 1450 of 1669 (86.9%) |
| Seropositive study animals identified as nonTAM[b] | 37 of 71 (52.1%) | 23 of 1450 (1.6%) |

[a]80 animals were determined to be seropositive for *T. cruzi* antibodies in this study. However, caging location during year of infection—and therefore linked TAM or nonTAM status—could be determined for only 71 animals. These values represent the number of animals that seroconverted after having been previously associated with another seropositive animal.
[b]80 animals were determined to be seropositive for *T. cruzi* antibodies in this study. However, caging location during year of infection—and therefore linked TAM or nonTAM status—could be determined for only 71 animals. These values represent the number of animals that seroconverted prior to having been associated with another seropositive animal.

in that year. Although data from these 9 animals could not be used for some aspects of the study (e.g., mean age at infection, linked TAM and nonTAM comparisons), the TAM associated with these 9 animals were included as part of the study from 1999 onward if inclusion was supported by the data. Given that serostatus for the entire breeding colony was well characterized during 2012 through 2018, additional analyses were performed using just the 53 rhesus macaques that seroconverted during this period (Table 1).

**PCR-positive breeding colony animals.** Of the 80 seropositive rhesus macaques included in this study, 52 had previously undergone PCR testing for *T. cruzi* as part of other ongoing studies at the Center (Figure 3). Some of these animals had been identified to be PCR positive for *T. cruzi* from a single test, although most animals required multiple blood collections and testing (2 to 4 assays per animal) to identify the infections. Ultimately, 50 of the 52 seropositive animals were identified as PCR positive for *T. cruzi* (mean, 1.9 PCR tests performed per positive result). DTU typing of these infections was achieved for 44 of the 50 PCR-positive monkeys; 24 animals were identified as TcI, 17 as TcIV, and 3 animals as mixed (TcI and TcIV) infections. For the 30 seropositive macaques of unknown PCR status, 28 had never been PCR-tested because whole-blood samples were not available for analysis at the time of their serodiagnosis. The 2 remaining macaques of unknown PCR status had been PCR-tested previously but had never been identified as PCR positive for the parasite (that is, lacked parasitemia) at the time of their PCR testing.

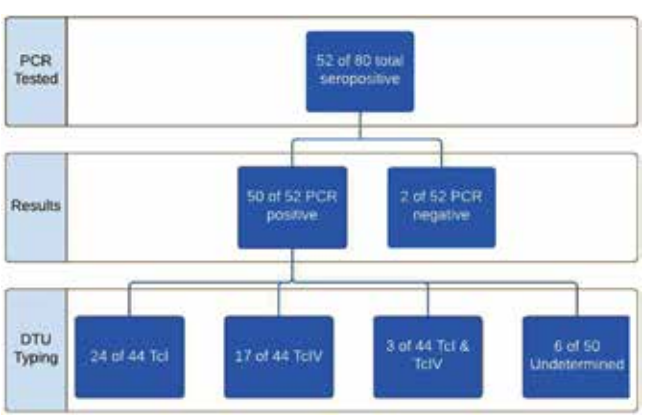


**Figure 3.** Previously acquired PCR results and DTU typing determinations for seropositive animals.

***T. cruzi*-associated monkeys.** In total, 688 TAM were identified; these animals were cohoused with the 80 seropositive macaques present in the colony between 1999 and 2018. This number included 197 infants born to seropositive dams and 54 secondary TAM associated with the 5 animals that were newly identified to be seropositive through the TAM testing in this study. In total, archived serum was available for serologic testing from 624 of the 688 TAM (91%). Analysis of the serologic data from these 624 TAM in conjunction with the colony husbandry records revealed several important findings (Table 2). First, of the 71 animals for which the caging location at year of infection was determined, 34 had been linked to at least one seropositive monkey immediately before their own seroconversion. These animals were classified as 'linked TAM.' Second, among the 71 animals for which the caging location was known for the year of infection, 37 had seroconverted before their association with another seropositive monkey. Serologic testing was performed on all of the cagemates to these 37 seropositive monkeys for the year in which each animal seroconverted and on all of the cagemates to these monkeys for the 2 y before each animal's seroconversion. Because none of these cage mates were seropositive, these 37 monkeys were classified as nonTAM.

To compare the overall prevalence of seroconversion between TAM and nonTAM throughout the colony, we used the subset of serodata collected between 2012 and 2018 (Table 2) because the serostatus of the entire breeding colony was well characterized for this time. Of the 688 total TAM identified for this study, 497 TAM were present at the facility between 2012 and 2018. Archived serum samples were available for 449 of the 497 TAM (90%), of which 30 (7%) were *T. cruzi* seropositive. Likewise, serologic screening was performed on 1450 of the 1669 nonTAM (87%) housed at the Center between 2012 and 2018; 23 (2%) of these animals were *T. cruzi* seropositive. In comparing these 2 seroprevalence rates, the occurrence of seroconversion in TAM was significantly greater than the occurrence of seroconversion in nonTAM for the time period of 2012 through 2018 (z-ratio, 5.73; one-tailed probability, <0.001).

**Investigations into the influence of the environment on *T. cruzi* infections.** Spatial analysis identified areas in the animal compounds in which significant seroconversion hot spots and cold spots occurred (Figure 4). The location data were available for 71 of the 74 animals for which a year of infection had been determined; 18 were from 1999– to 2010 and 53 were from 2012–2017. Results from the 1999–2010 data identified several rooms and corncrib structures as hot spots in the main rhesus compound (Figure 4 A). Results from the 2012–2017 data identified several rooms as hot spots and others as cold spots in the main rhesus compound (Figure 4 B). All of the rooms in the north rhesus compound were determined to be 'nonsignificant' for both time periods examined (data not shown).

Data obtained from the cumulative shed power calculations are presented in Figure 5. Buildings 1 and 2 were the only buildings to have sheds within 20 m and were the only buildings in either animal compound to have cumulative shed power values assigned to their rooms. The 5 rooms with the highest


**Figure 4.** Spatial hot-spot analysis for *T. cruzi* seroconversion in the main rhesus compound. (A) Data obtained from analysis of animals that became infected during 1999 through 2010. (B) Data obtained from analysis of animals that became infected during 2012 through 2017. Each colored dot represents an individual room or corncrib structure. Red and orange dots represent animal housing sites with a significantly higher incidence of *T. cruzi* seroconversion than the colony in general. Blue and gray dots represent animal housing sites with a significantly lower incidence of *T. cruzi* seroconversion than the colony in general. Yellow dots represent animal housing with an incidence of *T. cruzi* considered similar to that of the colony overall. The yellow stars on the lower portion of each figure highlight the wooded area that underwent intensive fire mitigation efforts in 2011–2012. Photo credit: Map data © 2014 Google image edited to remove red cars in parking lots.

cumulative shed power values for Buildings 1 and 2 were also the same 5 rooms identified as hot spots on spatial analysis of the 1999–2010 animal data (Figure 4 A).

The cumulative tree power calculations used 106 trees in the 2 compounds. The cumulative tree power values and the incidence of seroconversion were not correlated. In addition, 13 of the 15 rooms with the highest cumulative tree power values, and 11 of the 15 rooms with the lowest cumulative tree power values were identified as not significant on both hot-spot spatial analyses.


**Figure 5.** Cumulative shed power values assigned to rooms in Buildings 1 and 2 in the main rhesus compound. The 4 black boxes at the top of the image represent the 4 sheds. The 2, 12-room buildings are represented in the bottom half of the illustration. The illustration provides the approximate size and location of each shed relative to the 2 animal buildings. The number located in each of the building rooms is the cumulative shed power value assigned to each room. The 5 rooms with the largest cumulative shed power values are highlighted in red.

**Investigations into the possibility of vertical transmission.** Of the 59 seropositive female macaques identified in this study, 54 were of breeding age and produced offspring. After seroconversion, these 54 dams collectively produced a total of 196 live infants between 1999 and 2018. Of these 196 infants, 137 were either still present in the colony or had archived serum samples available for testing. The first 2 postweaning serum samples available from each of these 137 monkeys were analyzed and identified to be *T. cruzi* seronegative by both of the diagnostic tests used for this study.

**Investigations into the possibility of horizontal transmission.** We evaluated 4 different animal-to-animal associations for data suggestive of horizontal *T. cruzi* transmission in the breeding colony (Table 3). Because each cohousing event between a seropositive animal and a TAM represented a potential opportunity for horizontal transmission, we first arranged the animals into their known associations and then determined the total number of cohousing events that occurred between the 80 seropositive animals and the 624 TAM for which we had serologic data (Table 3, 'TAM events'). Many of the seropositive animals had been cohoused with more than one group of TAM as a result of the routine realignment of breeding groups that occurred every few years. In total, 925 TAM events were identified to have occurred during 1999 through 2018.

We next performed a more detailed review of the 34 known seropositive linked TAM to determine whether they could have been infected through horizontal transmission. Based on available DTU data, 9 of the linked TAM and their associated seropositive animals did not have a matching DTU and therefore did not represent infections derived from horizontal transmission (Table 3). However, horizontal transmission could not be ruled out for 25 final TAM.

**Table 3.** Criteria used to assess horizontal transmission potential between animals.

| Association explored | No. of seropositive animals | No. of TAM | No. of TAM events[a] | No. of linked TAM[b] | Matched DTU[c] (No. of linked TAM) | Nonmatched DTU[d] (No. of linked TAM) | Final TAM[e] (No. of linked TAM [% of TAM events]) |
|---|---|---|---|---|---|---|---|
| Male-to-male | 5 males | 23 males | 28 | 1 | 0 | 0 | 1 (3.6%) |
| Male-to-female | 21 males | 208 females | 232 | 5 | 1 | 2 | 3 (1.3%) |
| Female-to-male | 59 females | 66 males | 135 | 7 | 2 | 2 | 5 (3.7%) |
| Female-to-female | 59 females | 327 females | 530 | 21 | 5 | 5 | 16 (3.0%) |

[a]TAM events are the total number of unique cohousing events between seropositive animals and their TAM.
[b]linked TAM are TAM that seroconverted when cohoused with a seropositive animal or that seroconverted within 2 y of leaving shared housing with a seropositive animal.
[c]Matched DTU are linked TAM for which samples were available for PCR comparison and for which the DTU of infection matched between the TAM and its associated seropositive animal. Matched DTU animals were retained in the final TAM counts.
[d]Nonmatched DTU are linked TAM for which samples were available for PCR comparison and for which the DTU of infection did not match between the TAM and its associated seropositive animal. Nonmatched DTU animals were excluded from the final TAM counts.
[e]Final TAM are the seropositive TAM for which horizontal transmission could not be excluded as a possible route of infection. Final TAM included all linked TAM for which no PCR evaluation was performed and all linked TAM from the Matched DTU column.

## Discussion

Natural infections with *T. cruzi* occur in humans through direct insect contact, horizontal transmission (i.e., blood transfusion, organ transplantation, sexual contact) and vertical (i.e., congenital) transmission. This study was designed use direct and indirect methods to quantify the contribution of each of these routes of infection in a breeding colony of research rhesus macaques. During 1999 through 2018, a total of 80 animals in this colony were determined to be seropositive. Of these animals, 34 had been a TAM to another seropositive animal before its own seroconversion (linked TAM), 37 monkeys had never been associated with any seropositive animal before its own seroconversion (nonTAM), and 9 monkeys could not be definitively classified as linked TAM or nonTAM (undefined). Lacking another reasonable explanation for the infections in the 37 seropositive nonTAM, we presume that they contracted *T. cruzi* through exposure to triatomine insects in the environment.

Analysis of serostatus data collected during 2012 through 2018 revealed that TAM had a statistically significantly higher incidence of infection than did nonTAM. Although this finding could suggest monkey–monkey transmission of the parasite, an equally likely explanation is that environmental factors increased monkey exposure to infected insect vectors. To explore these 2 possibilities, we first analyzed the role of environmental factors of the incidence of disease. We identified the geographic location of 71 monkeys at the time of their seroconversion and performed 2 separate spatial analyses for the time periods 1999–2010 and 2012–2018. The 2 analyses identified distinct variations in the number of new infections (hot and cold spots) across the campus (Figure 4 A and B). Furthermore, these analyses found that many of the hot spots varied between the 2 time periods examined for the study. Investigations into the areas immediately surrounding these hot and cold spots identified several environmental factors (described later) that may have contributed to the differences in new infections throughout the colony. Although the reasoning regarding how each of these factors could have contributed to a hot spot of infection is logical, it is nonetheless hypothetical, given that we have no direct evidence supporting an increase in the number of *T. cruzi*-infected insects in these areas.

The most notable finding related to the influence of environmental factors on incidence is arguably the difference in the distribution of hot spots for the corncrib caging structures (collectively, Building 9) between the 2 spatial analyses. In the 1999–2010 spatial analysis, 6 of the 8 corncrib structures were identified as hot spots, whereas the 2012 to 2017 spatial analysis did not identify any corncrib structure as a hot spot. We suspect that this shift in the incidence rate of new infections occurred as a direct result of the fire mitigation efforts undertaken by the Center during 2011 through 2012. Prior to these efforts, thick undergrowth and ladder fuel extended to the edge of the road directly opposite the corncrib structures (approximately 12 m from the nearest corncrib structure). After the completion of the fire mitigation work in 2012, the undergrowth and ladder fuel have consistently been kept more than 35 m away from the nearest corncrib structure (Figure 4 A and B). In addition to differences in hot spot clusters between the 2 spatial analyses, the 1999–2010 analysis also implicates undergrowth and ladder fuel as potential environmental factors that influence the incidence of infection at the facility. The corncribs with the highest hot-spot confidence levels (99%) in Figure 4 A are also the 4 corncribs nearest to the wooded area. Furthermore, directly north of these 4 corncribs are 2 other corncribs that have slightly lower hot-spot confidence levels (90%), suggesting a lower likelihood of infection with greater distance from the wooded area. Finally, the 2 eastern-most corncrib structures, which have always been over 40 m from any wooded area, were not identified as hot spots in either spatial analysis. This last finding suggests that the incidence of infection at this facility is not wholly dependent on the type of caging structure (building room compared with corncrib) used to house animals.

As a second example, the elevated sheds in the northwest corner of the main rhesus compound are associated with geographic hot spots of seroconversion (Figures 4 A and 5). Specifically, the rooms in Buildings 1 and 2 with hot spots on the 1999–2010 spatial map and the rooms with the highest cumulative shed power correlate almost perfectly. This finding is what might be expected if these sheds were the sole environmental factor involved in increasing the incidence of infection for the local area. However, having found variations in the hot-spot locations in Buildings 1 and 2 between 1999–2010 and 2012–2017, we realize that other, previously unrecognized, environmental factors that differed between the 2 time periods analyzed for this area of the campus. Through additional investigation, we learned that the large fiberglass panels used to winterize the buildings and corncrib structures had been stored on wooden pallets approximately 10 to 15 m west of Building 1 from April through October every year between 2011 and 2018 (Figure 1). Each year the area under and around these pallets accumulated dense weeds and abundant leaf litter from late spring to early

autumn—the same time of year in which triatomine bugs are active at the Center. We also discovered that prior to the fire mitigation efforts of 2011, the panels had been stored approximately 35 to 40 m north of Building 1. Given these findings and the pattern of distribution for new infections, we suspect that the fiberglass panel storage site contributed to the higher incidence rate of infection for Building 1 during the 2012–2017 period.

A third difference in the incidence of infections between the 2 analyses was the southeast corner room of Building 3, which was a hot spot only in the 2012–2017 spatial analysis (Figure 4). This hot spot is due to 3 new infections that occurred in 2015 and 2016. Given the relative isolation of this room from other clusters of infection, this hot spot might represent an anomaly of the data set, could suggest horizontal transmission for 2 of the 3 new cases, or could be the result of environmentally derived infections from triatomine bugs living in some environmental feature that was present in the area during this 2-y period. One environmental feature that has been identified as a potential source for this hot spot is a large planter box that was located east of Building 3 (Figure 1). Suspicion over this feature stems from the findings that the planter box had a dense ground cover of leaf litter at the time of its removal in late 2016 and no additional seroconversions were found in that breeding group or room between 2017 and 2022.

In addition to these associations between the hot spots of infection and environmental features that likely harbored infected insect vectors, 2 other pieces of information from the study supported the role of the environment on the incidence of *T. cruzi* infections in the colony. First, most of the hot spots consisted of multiple groups of animals housed in units that either highly limited (building rooms) or completely prevented (corncrib structures) direct physical contact between groups of animals. Second, most of these hot spots persisted over long periods of time. This persistence occurred despite the fact that, on average, each of the 80 seropositive study animals underwent 1.5 social group rearrangements (which often entailed a room or building change) and an additional 2.6 room or building moves of the entire groups after seroconversion. This information is notable because both findings are consistent with what might be expected if an environmental effect, but not direct transmission of the parasite, was the primary driving force on incident infections in hotspots at the Center.

The prevalence of infections and presence of mature trees were not correlated in either period. Although triatomine insects are known to use leaf litter as a habitat, we conjecture that no correlation was found because the well-maintained landscaping in the compound resulted in very little leaf litter associated with these trees.

To study the potential contribution of horizontal transmission to the incidence of *T. cruzi* infections in the colony, we evaluated the husbandry records of the 71 seropositive animals for which the caging location during the year of infection was known. This analysis identified 34 animals as linked TAM (Table 3, linked TAM). We then compared the DTU of infections between each Linked TAM and its associated seropositive animal and ruled out direct transmission as the cause of infection for 9 TAM (Table 3, nonmatched DTU). As with the 37 nonTAM described earlier, because we had no other reasonable explanation for the infections in these 9 nonmatched linked TAMS, we presume that they contracted *T. cruzi* through exposure to triatomine insects from the environment. Ultimately we identified 25 linked TAM for which horizontal route of transmission could not be ruled out as the cause of disease (Table 3, final TAM). Final TAM accounted 35% (25 of 71) of the seropositive animals with a defined linked TAM or nonTAM status. If all 9 of the undefined animals are assumed to have seroconverted due to horizontal transmission, then final TAM would be 43% (34 of 80) of all seropositive colony animals. This value represents the maximum possible contribution of horizontal transmission to the incidence of *T. cruzi* infection in the colony.

However, several study findings and various aspects of the study design suggest this maximum-possible-contribution value overestimates the true incidence of horizontal transmission in the colony. First, our spatial analysis data suggest that most hot spots of infection on the campus are associated with specific environmental elements; therefore geographic clustering of infected animals is to be expected. Without the comparative genomic analyses of the parasites from linked TAM and their associated seropositive cohorts, the origin (direct or environmental) of most linked TAM infections could not be definitively determined. Because anecdotal evidence obtained prior to the start of the current study suggested that most of the colony infections were derived from the environment, we designed this study to maximize our detection of all potential cases of horizontal transmission. We achieved this goal by: (1) designating any animal that seroconverted within 2 y of leaving shared housing with another seropositive animal as a linked TAM and (2) including all linked TAM (except those with nonmatched DTU) in the final TAM counts. With regard to the second point, only 17 of the 34 of the linked TAM had DTU data available for comparison testing, and 9 of 17 of these were excluded from the final TAM count because the DTU did not match between the pair. If a similar ratio was present in the remaining 17 linked TAM (for which no DTU data was available), another 8 or 9 animals might also have been removed from the final TAM count. Second, as part of our maximum-possible-contribution calculation, we assumed that all 9 undefined animals seroconverted as a result of horizontal transmission. However, the 25 final TAM represented only 35% of the 71 animals that were able to be characterized as linked TAM or nonTAM. Therefore, if similar ratios existed among the 9 undefined seropositive animals, then 5 or 6 of them might have become infected through environmental exposure and should have been characterized as nonTAM.

To identify the maximum frequency with which seropositive animals might infect their TAM through horizontal transmission, we first identified 925 unique instances in which TAM were cohoused with the 80 seropositive animals (Figure 3; 'TAM Events'). We then undertook an analysis in which we assumed that all 25 Final-TAM and all 9 Undefined animals seroconverted due to horizontal transmission. Through these calculations we determined that, at most, 3.7% (34 of 925) of the TAM Events could have led to seroconversion of a TAM through horizontal transmission. This finding, although likely hyperbolic in its assumptions of horizontal transmission, suggests that the exposure of an uninfected TAM to a seropositive animal does not routinely result in seroconversion. In further support of this, we identified several TAM in this study that never seroconverted despite having had multiple seropositive cagemates over many years. The 2 animals that best exemplified this point were a female animal housed with 4 seropositive females and 2 seropositive males over a cumulative period of 18 y, and a male animal housed with 9 different seropositive females over a cumulative time period of 12 y.

To assess whether any specific animal associations might correlate with an greater likelihood of horizontal transmission, we performed additional analyses, again assuming that all 25 final TAM were infected through horizontal transmission

(Table 3). The seropositive animal-to-TAM associations and the maximum percentage of TAM events that may have resulted in horizontal transmission of *T. cruzi* for each association were as follows: male-to-male (3.6%), male-to-female (1.3%), female-to-male (3.7%), and female-to-female (3.0%). Breeding and fighting with wounding are the 2 most obvious animal interactions that could allow horizontal transmission to occur; we therefore determined whether these analyses implicated either of these activities as risk factors for infection. However, no single animal relationship truly dominated the data, nor was any direct correlation detected between individual adult animal associations and wounding or breeding. Specifically, if wounding was the primary method of horizontal transmission for the colony, we reasoned that female-to-female association would have higher transmission than the other 3 associations. This expectation was based on the fact that more than 90% of the wounding at this facility occurs as a result of fighting between female cagemates in breeding groups. However this analysis instead revealed that the female-to-female association was the second lowest percentage identified. In a similar manner, we reasoned that if sexual transmission was the primary method of horizontal transmission, then percentages would be higher in the female-to-male and male-to-female associations as compared with the other 2 adult animal associations. This expectation was based on the results of previous studies, which identified *T. cruzi* in the seminal fluid of seropositive men and documented sexual transmission of *T. cruzi* in male and female mice.[3,37] However, although the female-to-male percentages were slightly higher than male-to-male and female-to-female associations, the male-to-female association had the lowest percentage of all 4 associations. Although these analyses did not provide support for either breeding or wounding as the primary contributor to horizontal transmission at our facility, horizontal transmission between colony animals cannot be ruled out because similar results could be expected if contributions from breeding and wounding were similar.

In summary, our study provided no evidence that directly confirmed that a contribution of horizontal transmission to the incidence of *T. cruzi* infections in this colony. More specifically, given that all of the potential cases of horizontal transmission identified here also could have occurred as a result of environmental exposure to infected insect vectors, we can only state that this study has identified a group of animals for which horizontal transmission could not be ruled out. However, if horizontal transmission is indeed occurring between colony animals, 3 conclusions can be made in light of the collective findings of this study. First, the majority of *T. cruzi* infections in this colony are not derived from horizontal transmission. Second, horizontal transmission is not an efficient mode of transmission between the colony animals in general. Third, if horizontal transmission occurs, neither breeding nor wounding are its sole route.

For our investigation of vertical (congenital) transmission, we performed serologic testing of 137 infants born to seropositive dams. None of these infants were seropositive at any the time point between weaning (7 to 9 mo old) and 2 y of age. Although the human literature has reported vertical transmission of *T. cruzi* in certain populations for decades, recently data show that only a few of the genetic types (i.e., DTU) of the parasite are readily capable of infection through vertical transmission. Specifically, vertical transmission of the parasite occurs commonly in women infected with the TcII, TcV, and TcVI DTU.[11] In contrast, vertical transmission is only occasionally identified in people harboring TcI infections and only a single case report identified DTU TcIV.[3,24,46,47] This information is useful in understanding the apparent absence of vertical transmission in the colony, given that the NHP at this facility have only ever been documented to harbor TcI and TcIV infections.[31]

Limitations of this study are that it is a retrospective records review and relied on contemporary testing of stored serum by using tests that were not validated for frozen serum that has been stored for years. Another limitation is that because only subsets of colony animals were screened serologically for *T. cruzi* prior to 2012 (as detailed in the Methods section), some seropositive animals that were only present between 1999 and 2011 may not have been identified for this study. Accordingly, the true number of infected animals in the colony between 1999 and 2011 is likely greater than what we have reported here.

In conclusion, our findings suggest that geographic hotspots exist for *T. cruzi* infections in monkeys, and the surrounding habitats should be considered in efforts for integrated vector control at biomedical facilities housing NHP outdoors in the southern United States. Although we cannot rule out the role of horizontal transmission as a contributing factor for *T. cruzi* infections in rhesus macaques, we obtained clear evidence to suggest that environmental exposures are the leading contributor to infection in at least one colony in the southern United States. Future research studies focused on minimizing insect exposure and interrupting spillover from the sylvatic cycle are likely to provide valuable insight for the control of *T. cruzi* infections at United States NHP facilities. Additional studies into the horizontal transmission of the disease are likewise warranted, and we recommend that future investigations include a genomic analyses of the parasites isolated from animals to provide stronger evidence of linkage between seropositive animals and their TAM. Finally, because the current study yielded no evidence of vertical transmission of the parasite, the *T. cruzi*-infected dams in this colony remain an active part of the breeding colony. However, because the likelihood of vertical transmission of *T. cruzi* varies among different DTU of the parasite, future studies into vertical transmission at other domestic NHP colonies are warranted.

## Acknowledgments

We thank the faculty and staff of the Michale E Keeling Center for Comparative Medicine and Research at the MD Anderson Cancer Center University of Texas, specifically Ms Amanda Smith and Ms Cindy Barron for their assistance in collecting colony data and Ms Brenda Webb, Ms Paula Bulak, Ms Bridget Burris, and Dr Leqin Yan for procuring and testing serologic samples. In addition, we thank Dr Tracy Vemulapalli for her assistance with funds allocation and editorial contributions.

This project was funded in part by the Department of Veterinary Pathobiology at Texas A&M University and made possible in part by the American College of Laboratory Animal Medicine under Grant Number 1805379.

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