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Original Research

Reproductive Outcomes in Rhesus Macaques (*Macaca mulatta*) with Naturally-acquired *Trypanosoma cruzi* Infection

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Chagas disease is a zoonotic vector-borne disease caused by infection with the protozoan parasite *Trypanosoma cruzi*. *T. cruzi* is found in Latin America and the Southern United States, where it infects many species, including humans and nonhuman primates (NHPs). NHPs are susceptible to natural infection and can develop clinical symptoms consistent with human disease, including Chagasic cardiomyopathy, gastrointestinal disease and transplacental transmission, leading to congenital infection. Due to evidence of Chagas transmission in Texas, this study hypothesized *T. cruzi* infection was present in a closed, outdoor-housed breeding colony of rhesus macaques (*Macaca mulatta*) located at a biomedical research facility in Central Texas. In addition, we questioned whether seropositive female rhesus macaques might experience reproductive complications consistent with maternal-fetal Chagas disease. The seroprevalence of *T. cruzi* infection in the colony was assessed using an Enzyme Linked Immunosorbant Assay (ELISA) to detect antibodies against Tc24 antigen as a screening assay, and a commercially available immunochromatographic test (Chagas Stat Pak) as a confirmatory assay. Retrospective serologic analysis was performed to confirm the status of all *T. cruzi*-infected animals between the years 2012 to 2016. The medical history of all seropositive and seronegative breeding females within the colony from 2012 to 2016 was reviewed to determine each animals' level of reproductive fitness. The percentage of *T. cruzi*-seropositive animals ranged from 6.7% to 9.7% in adult animals and 0% to 0.44% in juveniles or weanling animals, depending on the year. An overall 3.9% seroprevalence of *T. cruzi* infection was found in the total population. No significant differences in any measure of reproductive outcomes were identified between seropositive and seronegative females from 2012 to 2016. The lack of significant adverse reproductive outcomes reported here may help inform future management decisions regarding seropositive female rhesus macaques within breeding colonies.

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Chagas disease (American trypanosomiasis), categorized as a neglected tropical disease by the World Health Organization, is caused by infection with the kinetoplastid protozoan parasite *Trypanosoma cruzi*. Transmission of *T. cruzi* to humans and other susceptible mammalian hosts are typically by blood-feeding triatomine insects in the Reduviidae family, commonly known as "kissing bugs." Chagas disease is endemic in 21 countries in Latin America, affecting 6 to 8 million people with approximately 12,000 associated deaths per year worldwide.[52] Further, around 13% of the entire Latin American population, disproportionately those in the lowest socioeconomic regions, are considered to be at risk for infection.[37] Transmission to susceptible hosts is most commonly through contact with *T. cruzi* trypomastigotes in the feces of infected reduvid bugs via a blood-feeding bite wound, contact with mucous membranes, or ingestion.[4] Other major documented means of transmission in humans include blood transfusion, organ transplantation, and mother to fetus (called congenital or vertical transmission). A recent meta-analysis of published studies of congenital transmission found an approximately 5% rate of congenital transmission to infants born to *T. cruzi* infected mothers.[31] In humans, *T. cruzi* infection is well documented to cause an acute phase of infection that is usually asymptomatic but in rare cases, can result in death from severe myocarditis or meningoencephalitis or both.[44] Chronic disease (cardiac, digestive, or cardiodigestive) develops in 30% to 40% of people infected, usually 10 to 30 y after the initial infection.[44] Cardiac manifestations are the most common clinical signs of chronic infection, characterized by electrocardiogram (ECG) abnormalities, cardiac failure, thromboembolism, and sudden death.[39,43,44] Studies in asymptomatic chronically infected pregnant women have shown that they have an increased risk of early-term births, low-birth weight and stillbirths.[31] However, this is still controversial because other studies have not demonstrated an association between pregnancy outcome and seropositivity status.

T. cruzi and the triatomine insects that transmit the parasites are widespread throughout Latin America and the southern regions of North America. Newer evidence indicates that human transmission also occurs in Texas, and possibly neighboring states.[18-23,30,48] The sylvatic transmission cycles are complex

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involving multiple genetic strains and numerous wildlife hosts.[5,26,28] In addition to humans, *T. cruzi* has been shown to infect over 200 species of mammals, including domesticated dogs and New World and Old World NHPs.[24] This complexity presents major challenges to Chagas disease control and prevention efforts. Conversely, the wide range of host susceptibility presents opportunities to gain insight into disease transmission and pathogenesis from naturally or experimentally infected animals. Natural *T. cruzi* infection in outdoor-housed NHPs has been previously reported.[11,16,34,42] Baboons living in Texas (*Papio hamadryas*) and cynomolgus macaques (*Macaca fascicularis*) have been reported to be infected naturally with *T. cruzi*.[41] One study found a prevalence rate of 8.5% in a Texas colony of outdoor-housed cynomolgus macaques.[42] A recent investigation in Texas of potential *T. cruzi* reservoirs found an infection prevalence of 75% in striped skunks (*Mephitis mephitis*), 60% in raccoons (*Procyon lotor*), 34% in woodrats (*Neotoma micropus*), and 18% in other rodents, including a single infected black or roof rat (*Rattus rattus*) and 2 house mice (*Mus musculus*).[10]

As with humans and dogs, NHP *T. cruzi* infection can manifest with both acute and chronic forms, with subsets of those infected developing cardiomyopathy, or much less commonly, gastrointestinal issues.[6,9,40] Much less is known about how acute or chronic *T. cruzi* infection may affect the reproductive system or reproductive outcomes in NHPs. Vertical transmission of *T.cruzi* in NHPs could potentially occur during all stages of the disease, similar to humans; however, robust evidence of vertical transmission in NHPs is currently lacking.[24] Similarly, evidence of more frequent adverse reproductive outcomes from seropositive NHPs relative to seronegatives is also lacking.[24] One recent report in baboons[24] shows no significant differences in reproductive outcomes between a relatively small cohort of seronegative and seropositive baboons (*Papio hamadryas* spp.); this same report[24] described a single case of *T. cruzi* amastigotes in the placenta after stillbirth in a cynomolgus macaque (*Macaca fascicularis*). Due to their phylogenetic relatedness and concomitant physiologic and anatomic similarities with humans, NHPs naturally infected with *T. cruzi* represent a valuable model to study reproduction[50] and the possible associated adverse reproductive outcomes that may result from *T. cruzi* infection.[40]

The Michale E Keeling Center for Comparative Medicine and Research (KCCMR), a research campus of The University of Texas MD Anderson Cancer Center located in Bastrop, TX, houses a closed breeding colony of SPF (SPF), Indian-origin rhesus macaques (*Macaca mulatta*). This colony previously had 3 confirmed cases that were seropositive for *T. cruzi* between the years 2010 and 2012. The presence of the 3 confirmed cases of *T. cruzi* infection, one with cardiac disease and 2 with a potential decrease in reproductive fitness, prompted a further assessment of the colony to determine the overall seroprevalence of *T. cruzi* infection.

This paper focuses on tracking *T. cruzi* seroprevalence and reproductive outcomes in a cohort of naturally-infected rhesus macaques (*Macaca mulatta*) over a 5-y period at KCCMR. Due to various lines of evidence of Chagas transmission in wildlife reservoirs and outdoor-housed NHPs in Texas, we predicted that colony-wide seromonitoring would identify *T. cruzi* infection in greater numbers beyond the 3 initial cases identified 2010 to 2012. In addition, we questioned whether seropositive females in this colony might have an increased frequency of adverse reproductive outcomes consistent with Chagas disease, compared with seronegative females. The objectives of the current study were to: (1) document the seroprevalence of *T. cruzi* within the KCCMR rhesus macaque breeding colony, and (2) retrospectively evaluate total breeding events and reproductive outcomes in seropositive and seronegative females over a 5 y period (2012 to 2016).

Materials and Methods

Breeding Colony. Animal care and husbandry before, during, and after treatments conformed to practices established by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), *The Guide for the Care and Use of Laboratory Animals*,[33] and the Animal Welfare Act.[1] All procedures conformed to and were approved by MD Anderson IACUC guidelines. Veterinary care was provided as required. The colony of Indian-origin rhesus macaques (*Macaca mulatta*) at KCCMR is a closed breeding colony, which is SPF (SPF) for Macacine herpesvirus-1 (Herpes B), Simian retroviruses (SRV-1, SRV-2, SIV, and STLV-1), and *Mycobacterium tuberculosis* complex. All animals are socially housed in shaded, temperature-regulated indoor-outdoor enclosures with numerous barrels, perches, swings, and various feeding puzzles and substrates to mimic natural foraging and feeding behaviors. Standard monkey chow, ad libitum water, and novel food enrichment items are provided daily.

The SPF rhesus macaque breeding colony at the KCCMR in Bastrop, TX was founded in 1975, and has been a closed colony since approximately 1985. KCCMR is located in a rural area on 381 acres with numerous trees around the perimeter of the colony. The total population is historically between 850 to 1000 with approximately 300 breeding females each year in harem breeding groups (a single male for 3 to 10 females) in indoor/outdoor, temperature-regulated buildings. Stable breeding groups are maintained year-round, but the typical breeding season occurs from November until February. A representative age distribution of seronegative and seropositive breeding females within the colony from 2016 is shown in Figure 1. Females are typically initially placed in breeding groups at 4 y of age, males typically at 5 to 6 y. Animals older than 3 y of age are classified as adults, while those 3 y of age or younger are classified as juveniles or weanlings. Since first-time female breeders (4 y olds) have expected lower pregnancy rates compared with experienced breeder females (older than 4 y old), they were excluded from comparisons with seropositive females.

Evaluation of *T. cruzi* seroprevalence. Serologic screening for *T. cruzi* was performed on blood samples collected at the animals' annual physicals that occurred in 2013, 2015, and 2016. For the years 2013 and 2015, only a subset of the colony was sampled. For 2016, the entire colony was sampled. Archived serum samples from 2012 and 2014 were processed for all of the animals diagnosed as *T. cruzi* positive in 2013 and 2015, respectively. This serum processing was performed to confirm the serostatus of all the *T. cruzi*-infected animals throughout the 2013 to 2016 period-of-interest.

All Chagas-affected animals in this study were diagnosed as positive for *T. cruzi* infection through a 2-step serological testing process. The initial step was a screening of serum through an indirect ELISA using a recombinant purified protein, specifically the Tc24 antigen. The Tc24 antigen is a calcium-binding flagellar protein and has been used as a research tool to screen for *T. cruzi* infection.[3] Serum was diluted to a 1:1600 dilution and Tc24 specific IgG was detected by indirect ELISA as previously described, using a horseradish peroxidase-conjugated mouse antirhesus/cynomolgous IgG1 as the secondary antibody.[3] Suspected positive serum samples were then tested using the Chagas Stat PAK (Chagas STATPAK, Chembio Diagnostic Systems, Medford, NY) following the manufacturer instructions (Figure 2). Serum

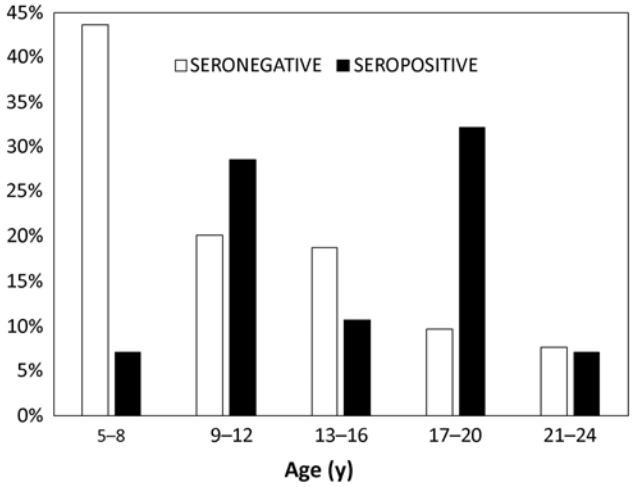

Figure 1. Representative age distribution of seropositive and seronegative breeding females within the colony.

Year	Testing
2013	Tc24 ELISA + Chagas StatPak
2015	Tc24 ELISA + Chagas StatPak
2016	MFIA + 2nd MFIA + ELISA

Figure 2. Chagas disease clinical testing summary.

samples collected from the entire colony during the 2016 annual physical examinations, including those identified as suspected positives during prior years, were tested inhouse using a multiplexed fluorometric ImmunoAssay (MFIA) (Charles River Chagas MFIA beads system). Suspect positive samples identified from the 2016 annual physical screening were sent to Charles River Laboratories (CRL) for testing using a second MFIA and confirmatory ELISA. (Figure 2)

Review of breeding history. The medical records of all adult female breeding animals from 2012 to 2016 were reviewed to assess reproductive fitness. Specific parameters that were assessed included failure to become pregnant, miscarriages, stillbirths, and live births. Animals were categorized as having normal reproductive fitness if each breeding season they were confirmed pregnant, and gave birth to a live, healthy infant. Animals were categorized as having reduced reproductive fitness if they experienced a failed pregnancy (no birth), miscarriage, or stillbirth in a given breeding season.

Statistical analysis. Individual reproductive fitness parameters of confirmed seropositive females and seronegative females were compared for each breeding season evaluating using a Z-test comparing proportions (Microsoft Excel 2013). *P* values less than or equal to 0.05 were considered significant.

Results

Seroprevalence in the breeding colony. During the 2013 annual physical examinations, a total of 572 animals were serologically screened including a total of 340 adults (291 females and 49 males) and 232 juveniles/ weanlings (128 females and 104 males). Of those animals screened, 25 animals (24 adults and 1 juveniles/ weanlings) were identified as *T. cruzi* seropositive (Figure 3 and Table 1). From this initial seroprevalence survey, 4.7% of the sample population was seropositive, with 7.1% and 0.4% seroprevalence in adults and juveniles/ weanlings, respectively (Table 1). During the 2015 annual physical examinations, a total of 792 animals, including 410 adults (236 females and

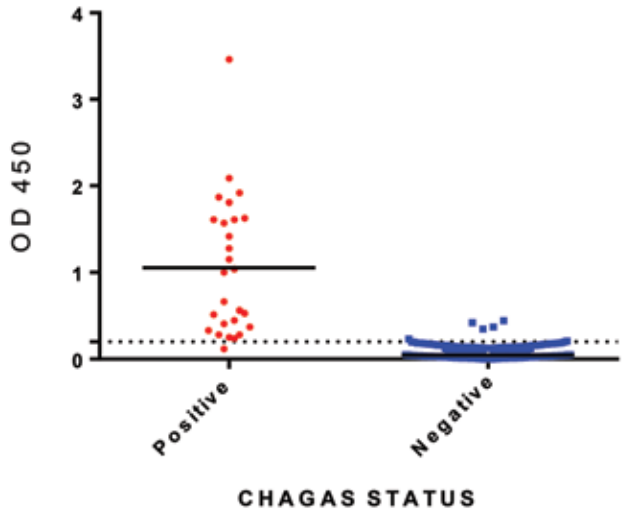

Figure 3. Representative Tc24 specific serum total IgG measured by indirect ELISA. Serum from each animal obtained during the annual physical was diluted 1:1600 and analyzed for Tc24 specific total IgG by indirect ELISA. Of the 572 samples analyzed, 26 were considered positive while 546 were considered negative.

74 males) and 378 juveniles or weanlings (185 females and 193 males) were screened. Of those animals screened, 23 animals (all adults) were identified as *T. cruzi* seropositive (Table 1). From this second survey, 2.9% of the sample population was seropositive, with 5.9% in the adult population and 0% in the juvenile/ weanling population sampled. During the 2016 annual physical examinations, a total of 1005 animals were screened including 452 adults (367 females and 85 males) and 543 juveniles/ weanlings (283 females and 260 males). Of those animals, 41 adults and 0 juveniles/weanlings were identified as *T. cruzi* seropositive (Table 1). From this survey, 4.1% of the sample population was seropositive, with 9.1% of adults seropositive and 0% of the juveniles/ weanlings being seropositive (Table 1). Over the entire testing period from 2013 to 2016, 1,191 unique individual animals were screened and 46 individual animals were identified as seropositive for an overall seroprevalence of 3.9% (Table 1). Of the 46 individual animals that were found to be positive over the 3 testing years, test results from 2 or more testing years were available for 32 individual animals (Table 2). As shown in Table 2, 30 of the 32 individual animals remained positive in subsequent years after initially testing positive during the evaluation period. Two individual animals, animal no. 8 and animal no. 18, tested positive in 2013 and had discordant results in 2016.

Reproductive Outcomes. None of the seropositive first time breeding females were 4 y of age or younger. Females that had experienced at least one breeding season were typically between 5 and 24 y of age, with the largest proportion of seronegative females ranging from 5 to 8 y (Figure 1), and the largest proportion of seropositive females ranging from 9 to 20 y. The average age of seronegative females is 10.9 y and the average age of seropositive females is 14.5 y (Table 3).

Figure 4 shows reproductive outcomes for seropositive and seronegative breeding females 5 y of age and older between 2012 to 16. Overall pregnancy rates between seropositive and seronegative animals were 67.4% and 69.8% respectively (Figure 4). Of the 1021 total seronegative pregnancies, 932 were live births (91.3%); of the 62 total seropositive animal pregnancies, 56 were live births (90.3%; Figure 4). The 62 total pregnancies from seropositive females included 3 stillbirths (4.8%), 2 abortions (3.2%), and no in utero deaths (Figure 2). The 1,021 total

Table 1. Seroprevalence in the sample population by year

Annual physical year	Seropositive female adults	Seropositive male adults	All seropositive adults	Seropositive female juveniles/ weanlings	Seropositive male juveniles/weanlings	All seropositive juveniles/ weanlings	Overall seroprevalence
2013	6.2% (18/291)	12.2% (6/49)	7.1% (24/340)	0% (0/128)	1.0% (1/104)	0.4% (1/232)	4.7% (27/ 572)
2015	6.3% (21/336)	4.1% (3/74)	5.9% (24/410)	0% (0/185)	0% (0/193)	0% (0/378)	2.9% (23/792)
2016	8.2% (30/367)	12.9% (11/85)	9.1% (41/452)	0% (0/283)	0% (0/260)	0% (0/543)	4.1% (41/ 1005)
Cumulative seroprevalence 2013–2016							3.9% (46/1191)

Serum samples from individual rhesus macaques were tested each year for antibodies against *T. cruzi*. A subset of the total population was tested in 2013 and 2015; the entire colony was tested in 2016. Data is presented as the percent seropositive and the number positive/ total number tested for each category. Cumulative seroprevalence based on total number of individual seropositive and total individuals tested between 2013-2016.

Table 2. Seropositive test results for individual animals over multiple years

Animal no.	Age (y) in 2016	Sex	2013	2015	2016
1	23	Female	+	+	+
2	23	Female	+	+	+
3	23	Female	+	+	+
4	25	Female	+	+	N/T
5	22	Female	+	+	+
6	22	Male	+	N/T	+
7	22	Male	+	N/T	+
8	22	Female	+	+	discordant
9	21	Female	+	+	+
10	20	Female	—	+	+
11	20	Female	+	+	+
12	20	Female	+	+	+
13	19	Female	+	+	+
14	19	Female	+	+	+
15	19	Female	+	+	+
16	19	Female	+	+	N/T
17	18	Female	+	+	+
18	18	Female	+	N/T	discordant
19	17	Male	+	+	+
20	17	Female	—	+	+
21	15	Female	+	+	+
22	15	Male	—	+	+
23	15	Female	+	+	+
24	15	Female	N/T	+	+
25	11	Male	+	+	+
26	11	Female	+	+	+
27	11	Female	—	+	+
28	11	Male	—	+	+
29	11	Female	+	+	+
30	11	Female	N/T	+	+
31	10	Female	—	+	+
32	8	Female	—	+	+

Test results of serum samples from seropositive individuals with 2 more tests over the testing period were compared. Discordant indicates animals that tested positive on the MFIA assay, but negative on the confirmatory ELISA. NT denotes the sample was not tested during that test period.

pregnancies from seronegative females included 32 stillbirths (3.1%), 37 abortions (3.6%), and 20 in utero deaths (2.0%) (Figure 4). There were no statistically significant ($P > 0.05$) differences in any reproductive fitness parameters between seronegative and seropositive females in any given year.

Table 3. Average age of breeding females

	Age (y)	
	Seronegative	Seropositive
Mean	10.9	14.5
Std Dev	5.2	4.5
Range	5–24	5–23

Representative average age of seropositive and seronegative breeding females within the colony.

A common understanding among those working with breeding colonies of NHPs is that females introduced to a breeding group for the first time will have lower pregnancy rates than females who have previously been bred and/or produced offspring. However, robust documentation of this phenomenon is lacking in the scientific literature. The present study facilitated such a comparison and provides additional justification for limiting the comparisons of seronegative and seropositive females to over 4 y of age. Figure 5 shows that overall pregnancy rate of *seronegative* 4 y old first-time breeders was significantly lower (57.0%) than that of *seronegative* females greater than 4 y old (69.8%) during 2012 to 2016 ($P < 0.01$). However, analysis by individual breeding year shows statistical differences in pregnancy rate only in years 2014 and 2015 (Figure 5).

Discussion

Natural *T.cruzi* infection of captive NHPs is increasingly recognized in biomedical research facilities across the southern United States. In our study, across the 3 y where testing was performed we found an overall seroprevalence of *T.cruzi* infection of 3.9%, including adults, juveniles and weanlings. In this study, the overall seroprevalence ranged from 6.7% to 9.7% across all adult age ranges; however, only 4 juvenile animals were identified to be seropositive for infection, with a seroprevalence ranging from 0% to 0.4% depending on the year of testing. This finding suggests that increased age results in increased risk infection, which is most likely directly related to the greater incidence of exposure to infected insect vectors over time. While the bulk of the infected adult animals were within the 17 to 20 y age range (the second-oldest age group in the study), there was no clear cut pattern to the distribution to suggest age is the primary factor for infection of adult animal. These data are comparable to seroprevalence studies in other NHP colonies in Texas, where the reported seroprevalence ranged from 2.0% to 22.5%.[2,51] Further, this trend of higher seroprevalence in older animals as seen between the juvenile/weanling and adult animals at KCCMR is similar to an outdoor housed colony of baboons in San Antonio, TX, where the seroprevalence was highest, in animals 15 y of age and older.[2] Seropositive female

Breeding year	Breeding female subpopulations	Breeding females	Pregnant females	Pregnant (%)	Live births	Live births (%)	Stillbirths	Stillbirths (%)	Abortions	Abortions (%)	*In utero* deaths	*In utero* deaths (%)
2012	Seronegative	315	210	66.67	190	90.48	8	3.81	11	5.24	1	0.48
	SEROPOSITIVE	12	9	75.00	7	77.78	1	11.11	1	11.11	0	0.00
2013	Seronegative	287	204	71.08	180	88.24	9	4.41	9	4.41	6	2.94
	SEROPOSITIVE	14	12	85.71	11	91.67	1	8.33	0	0.00	0	0.00
2014	Seronegative	277	210	75.81	199	94.76	8	3.81	2	0.95	1	0.48
	SEROPOSITIVE	20	15	75.00	15	100	0	0.00	0	0.00	0	0.00
2015	Seronegative	289	211	73.01	194	91.94	4	1.90	4	1.90	9	4.27
	SEROPOSITIVE	22	13	59.09	11	84.62	1	7.69	0	0.00	0	0.00
2016	Seronegative	294	186	63.27	169	90.86	3	1.61	11	5.91	3	1.61
	SEROPOSITIVE	24	13	54.17	12	92.31	0	0.00	1	7.69	0	0.00
		Total breeding events	Total pregnancies	Cumulative pregnancy (%)	Total live birth events	Cumulative live births (%)	Total stillbirth events	Cumulative stillbirths (%)	Total abortion events	Cumulative abortion (%)	Total *in utero* Death Events	Cumulative *In utero* deaths (%)
Total breeding events 2012–16	Seronegative	1462	1021	69.84	932	91.28	32	3.13	37	3.62	20	1.96
	SEROPOSITIVE	92	62	67.39	56	90.32	3	4.84	2	3.23	0	0.00

Figure 4. Measures of reproductive outcomes of seronegative and seropositive females 5 y of age and older from the 2012 through 2016 breeding seasons. No statistical differences were seen. *P* values ≤ 0.05 were considered statistically significant.

Breeding year	SERONEGATIVE breeding female subpopulations (y)	Breeding females	Pregnant females	Pregnant (%)	Live births	Live births (%)	Stillbirths	Stillbirths (%)	Abortions	Abortions (%)	*In utero* deaths	*In utero* deaths (%)
2012	> 4	315	210	66.67	190	90.48	8	3.81	11	5.24	1	0.48
	4	22	13	59.09	9	69.23[a]	2	15.38	2	15.38	0	0.00
2013	> 4	287	204	71.08	180	88.24	9	4.41	9	4.41	6	2.94
	4	25	17	68.00	15	88.24	1	5.88	1	5.88	0	0.00
2014	> 4	277	210	75.81	199	94.76	8	3.81	2	0.95	1	0.48
	4	28	16	57.14[a]	16	100	0	0.00	0	0.00	0	0.00
2015	> 4	289	211	73.01	194	91.94	4	1.90	4	1.90	9	4.27
	4	32	13	40.63[c]	13	100	0	0.00	0	0.00	0	0.00
2016	> 4	294	186	63.27	169	90.86	3	1.61	11	5.91	3	1.61
	4	21	14	66.67	13	92.86	1	7.14	0	0.00	0	0.00
		Total breeding events	Total pregnancies	Cumulative pregnancy (%)	Total live birth events	Cumulative live births (%)	Total stillbirth events	Cumulative stillbirths (%)	Total abortion events	Cumulative abortion (%)	Total *in utero* death events	Cumulative *in utero* deaths (%)
Total breeding events 2012–16	> 4	1462	1021	69.84	932	91.28	32	3.13	37	3.62	20	1.96
	4	128	73	57.03[b]	66	90.41	4	5.48	3	4.11	0	0.00

Figure 5. Reproductive outcomes for 2012 to 2016 breeding seasons of seronegative first-time breeder females 4 years of age compared to seronegative experienced breeding females 4 y of age and older. *P* values ≤ 0.05 were considered statistically significant. *a* = $P < 0.05$; *b* = $P < 0.01$; *c* = $P < 0.001$.

and male animals were both detected in this colony with comparable seroprevalence rates. However, larger numbers of adult males would need to be evaluated to determine if any sex differences in infection rates are evident in this colony. Risk factors that may contribute to the infection rates of NHPs may include the outdoor environment surrounding the animal enclosures and the age of the animal. The KCCMR NHP housing structures allow the animals to have contact with the environment and there are numerous shade trees within the facility, which may provide habitats for infected insect vectors and wildlife reservoirs. Some anecdotal evidence suggests that more seropositive animals have been located in housing units directly adjacent to trees but further studies will be necessary to confirm or refute this possibility. Ingestion of triatomine vectors is another possible route for *T. cruzi* infection; rhesus macaques in the KCCMR colony have been observed ingesting these bugs. Oral transmission of *T. cruzi* is a confirmed important route of transmission for humans, usually from consumption of beverages made from fruit contaminated with infected triatomines.[29] In addition, one study of blood sucking lice collected from the hair and skin of *T. cruzi* infected baboons found that the lice were PCR positive, thus consumption of other blood feeding insects during grooming behaviors is another possible mode of oral transmission in NHPs.[2] However, there has been no evidence of lice or any other ectoparasites present within the KCCMR colony.

Our data showed no statistically significant difference in reproductive outcomes when comparing seropositive and seronegative rhesus macaque breeding females (Figure 4). Overall pregnancy rates of the 5 y period evaluated were comparable, at approximately 70% (Figure 4). Further, the cumulative live births were high, at approximately 90% (Figure 4). This is consistent with a previous retrospective study of female baboons seropositive or seronegative for *T. cruzi*, where even with history of fetal loss in both groups, there were no differences in menstrual cycle parameters and the number of fetal losses.[24] Very little is confirmed in the literature about the effects of *T. cruzi* on human fertility.[8] One longitudinal study showed fertility being similar in seropositive and seronegative Chilean women.[49] In animal studies, acute infection with TcVI during gestation strongly reduced mouse fertility,[32] whereas earlier rat studies did not observe any effect.[14] In humans, it has been postulated that reinfection or acute infection during pregnancy could be associated with human pregnancy loss. In Texas, documented seasonal triatomine activity generally occurs from late April through October, peaking in June and July.[12,53] While NHPs in this colony could be exposed to triatomines during this time, the typical breeding season is from November through February, indicating very little overlap with peak triatomine activity and less chance for acute infection from vectorial transmission during pregnancy in breeding females, which could impact

breeding fitness. An additional consideration is the very low seroprevalence found in juveniles/weanlings, which suggests they have low exposure and that the likelihood of congenital infection is low. Overall, these data show that despite significant seroprevalence for *T. cruzi* infection within this colony, the fact that there is little to no seroprevalence in juvenile and weanling animals suggests that congenital transmission is low. Therefore, seropositive breeding animals do not necessarily have to be removed from the colony. Thus, while measures should be taken to prevent new infections in the colony, consistent screening for seropositivity and careful monitoring for reproductive fitness and overall health may serve to preserve the seropositive animals as a valuable breeding resource while limiting further transmission.

Previous studies identifying the *T. cruzi* strains that circulate in domestic and wildlife animal species in the US identified the distinct typing units (DTUs) TcI and TcIV in several states, including Texas[13,45] Both TcI and TcIV have specifically been detected in nonhuman primates at this facility.[28] Previous testing of wildlife at KCCMR identified raccoons, opossums and skunks as *T. cruzi* infected reservoirs maintaining the sylvatic cycle of transmission.[27] Furthermore, this same study identified 3 species of triatomines from the facility in close proximity to the NHP enclosures and these bugs were identified to have a 17% incidence of *T. cruzi* infection.[28] Further, *T. cruzi* strains representing DTUs, I, II, III, V and VI have been reported in human cases of congenital transmission throughout South America, with TcV being reported in the majority of cases in Argentina, Bolivia, Southern Brazil and Paraguay[8] Another study of Argentina, Honduras and Mexico found that the majority of congenital transmission cases were strains other than DTU TcI.[7] Considering these data showing that the majority of reported cases of congenital transmission involve DTUs other than TcI and TcIV, and that DTUs TcI and TcIV have been shown to circulate in NHPs, wildlife and triatomine vectors in Texas, the likelihood of congenital transmission in this breeding colony is low. Further investigation to specifically test newborns of seropositive dams would be necessary to confirm this. Such studies could compliment evaluation of the specific DTUs found in human cases in the region, to confirm that strains found in wildlife, vectors and NHPs are representative of those that infect humans.

Although this current study showed no indication of reproductive complications caused by Chagas disease, it is imperative to annually test animals for *T. cruzi* to characterize length of infection status and monitor breeding efficiency. The index cases of *T. cruzi* infection were detected in this colony between 2010 and 2012. Without retrospective data before 2010, it cannot be ruled out that the colony had lower seroprevalence, or may have been seronegative, prior to 2010. Thus, while no difference in reproductive health was detected in this study over the 5 y period, it is still possible it may become evident in the future. An additional important consideration is the impact of subclinical *T. cruzi* infection on research studies, both within Texas research facilities as well as other facilities in regions where *T. cruzi* transmission does not occur. Most NHPs used in research are purpose bred in the US, so they avoid the importation of pathogens from their native countries. Knowledge about the infection status of these research animals is very important with regard to colony health, the outcome of the scientific studies that use these NHPs, and the safety of both husbandry and laboratory personnel. However, many receiving institutions do not test for *T.cruzi* upon receipt, therefore the subclinical effects of *T. cruzi* may play a significant effect on the research results, unbeknownst to the investigator. One study reported that a rhesus monkey had to be removed from an experimental SIV study due to reactivation of *T. cruzi* infection. The animal developed no clinical symptoms, but chagasic myocarditis was detected by histology.[36] Two geriatric rhesus macaques housed in the Pacific Northwest with signs of cardiac disease upon postmortem examination were found to be infected with *T. cruzi*, likely acquired more than a decade earlier when they had lived in facilities in South Texas.[15] Another study described a cynomolgous macaque who developed significant clinical anemia after receiving cardiac transplantation from a *T. cruzi* infected donor, ultimately resulting in removal of that animal from the study.[46] An additional study described 2 pigtail macaques housed in Georgia that became infected with *T. cruzi* after receiving blood transfusions from a *T. cruzi* infected donor that had been housed in a facility in Louisiana[17] These studies highlight the importance of screening NHPs for *T. cruzi* prior to their use on research studies, or as blood and tissue donors for research studies so as not to confound vital research data. This is important not only for research facilities in the Southern US where *T. cruzi* is known to circulate in wildlife populations but also in distant locations as many NHPs are obtained from breeding and import facilities located in enzootic areas and animals may become infected while living in those regions.

One limitation of this study is the difference in testing methods used across multiple years to detect *T. cruzi* infection in this colony. In the 2013 time period and the 2015 time period serum samples collected during the annual physicals were initially screened using the Tc24 antigen ELISA. This Tc24 antigen, which was produced at National Tropical School of Medicine at Baylor College of Medicine (NTSM), is a calcium-binding flagellar protein that has been shown to identify naturally infected humans by serology.[35] We developed an ELISA based on this antigen as a research tool to screen for infection in experimentally and naturally infected animals. This research procedure was readily available and efficient, consequently this was used as our initial screening method. However, as the Tc24 antigen has homologues in other members of the Trypanosomatidae family,[38] a commercially available *T. cruzi* specific diagnostic test, the Chagas Stat Pak (Chembio Diagnostic Systems) was used to confirm any suspect positives identified on the Tc24 ELISA. During the 2016 testing period, a Multiplexed Fluorometric ImmunoAssay (MFIA) produced commercially by Charles River Laboratories (CRL) was used as a screening test to screen the entire colony at KCCMR. Any samples found to be positive on the initial screening was sent to CRL to repeat the MFIA test in their diagnostic laboratories, followed by a confirmatory ELISA on any samples that were found to be positive by MFIA. In addition to the different testing strategies used, only a subset of the colony was tested in 2013 and 2015. While we were unable to obtain test results from all seropositive animals over the entire testing period, 30 of 32 seropositive animals with test results over multiple testing years repeatedly tested positive (Table 2). The fact that 2 animals showed discordant results, particularly since they were found positive when tested using the Tc24 ELISA and Chembio Stat Pak in 2013 and the CRL MFIA and ELISA in 2016, is not surprising as studies have shown that sensitivity and specificity of different commercially available diagnostic tests can vary.[47] Prospectively, screening of the entire colony using a single testing strategy will be used to continue monitoring seroprevalence and to calculate transmission rates.

Upon initially determining the seroprevalence of *T. cruzi* infection in the colony in 2013, KCCMR initiated strategies to repurpose seropositive animals to research projects at other facilities as appropriate. This led to a slight decrease in

seroprevalence between 2013 and 2015, even though small numbers of monkeys became newly-infected each year. By the end of 2015, however, it became apparent that the seropositive animals were able to produce viable, uninfected offspring and these animals were then intentionally retained as breeders in the colony and also for use in Chagas-related research projects. This change in management practice led to an apparent increase in the seroprevalence rates identified in 2016. While these colony management strategies could have an impact on overall seroprevalence within the colony, the fact that small numbers of animals become newly-infected each year indicates that additional strategies are necessary to decrease active transmission of *T. cruzi*, such as environmental management to decrease the number of triatomines in the area.

We demonstrated with this study that the seroprevalence of *T. cruzi* infection in this captive rhesus macaque colony is comparable to the seroprevalence of other NHP colonies in Texas. There were no differences in reproductive outcomes between seropositive and seronegative breeding female rhesus macaques. However, it is prudent to initiate measures to prevent and reduce any incidence of new infection through pesticide programs, management of shrubbery, setting traps around perimeter of research facility to reduce exposure to wildlife, and supervising of cleanliness of food preparation areas and animal living quarters. In addition, routine screening for *T. cruzi* should be incorporated into general health monitoring to prospectively evaluate the incidence of infection. Finally, seropositive animals should be monitored at least annually for clinical signs concomitant with chronic Chagas disease, namely cardiac disease which can be monitored with ECGs and echocardiograms. Acute disseminated Chagas disease could also be a rare possibility in endemic areas, particularly in pregnant or otherwise immunosuppressed outdoor-housed macaques.

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