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***Trypanosoma cruzi* in non-human primates with a history of stillbirths: a retrospective study (*Papio hamadryas* spp.) and case report (*Macaca fascicularis*)**

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Abstract

Background—Congenital transmission of *Trypanosoma cruzi* has been described in humans and experimental work has been conducted with mice, but not with non-human primates (NHPs).

Methods—We conducted a retrospective study of female baboons (*Papio hamadryas* spp.) naturally seropositive or seronegative for *T. cruzi* with history of fetal loss, and we report a stillbirth in a cynomolgus macaque (*Macaca fascicularis*) with placental *T. cruzi* amastigotes.

Results—There were no differences in menstrual cycle parameters and the number of fetal losses between seropositive and seronegative baboons with history of fetal loss. The amount of parasite DNA detected using quantitative polymerase chain reaction (Q-PCR) in *M. fascicularis* placenta was within the range detected in infected baboon tissues.

Conclusions—There is no evidence that chronic maternal *T. cruzi* infection causes fetal loss in baboons. Q-PCR is a useful diagnostic tool to study archived NHP placentas.

Keywords

Baboon; macaque; placental lesions; reproductive performance; serology; trypanosomiasis

Introduction

Chagas disease (also known as American trypanosomiasis) affects 16–18 million people in Central and South America and 0.6% of pregnant women in some regions in the United States [14, 15]. The cause of this disease, the protozoan parasite (*Trypanosoma cruzi*), is usually transmitted by various species of triatomines, but blood transfusion is also a significant source of infection. These routes of transmission have become less important; however, as insect control and blood screening programs have been implemented in endemic countries, and congenital (vertical) transmission is now more common in some areas of Latin America than is infection caused by vector transmission [2, 6, 17, 23].

Chagas disease is characterized by acute, intermediate, and chronic stages of infection. Transmission of *T. cruzi* from mother to fetus can occur during all stages of the disease [23, 37, 43, 52]. Maternal–fetal transmission cannot be prevented because the most common therapeutic agents used to treat *T. cruzi* infection, nifurtimox, and benznidazole, cannot be used in pregnant women due to their high toxicity [22]. No chemoprophylaxis administered to seropositive pregnant women has been shown to prevent transplacental passage of the parasite [28].

Congenital Chagas disease has been associated with premature labor, stillbirth, abortion, and placentitis [5], but the evidence for the influence of *T. cruzi* antibody presence on the incidence of stillbirth and abortion is controversial. Although some authors have found an increased risk of abortion or premature birth in seropositive mothers, other studies have not demonstrated an association between serologic status and pregnancy outcome [6, 8, 14, 26].

Non-human primates such as the baboon and macaque are excellent models for research in both reproduction [25] and infection with *T. cruzi* [34]. To date, the effect of maternal *T. cruzi* infection on pregnancy outcome has been studied in mice, but not in NHPs [9, 26, 50, 52].

The goals of the present study were to investigate whether maternal seropositivity for *T. cruzi* in baboons (*P. hamadryas* spp.) influences the frequency of stillbirths/abortions and to examine maternal age, weight, menstrual function, parity, placental histopathology, and inflammatory markers in seropositive and seronegative baboons in this group. We also analyzed an individual case of stillbirth in a cynomolgus macaque (*Macaca fascicularis*) with *T. cruzi*-related placentitis in the absence of fetal infection. The amount of *T. cruzi* DNA in the macaque placenta (archived formalin-fixed, paraffin-embedded tissue) was quantified using a quantitative polymerase chain reaction (Q-PCR) assay.

Materials and methods

Animals

The NHPs used in this study were members of an AAALAC International accredited colony managed by the Southwest National Primate Research Center (SNPRC) at the Southwest Foundation for Biomedical Research (SFBR) in San Antonio, TX, USA. The primates were housed in metal and concrete indoor/outdoor enclosures, fed commercial NHP diets, and offered water *ad libitum*. All procedures were performed in accordance with guidelines of the SFBR Institutional Animal Care and Use Committee.

Serology

Seropositivity to *T. cruzi* was assessed using an *in vitro* enzyme immunoassay (EIA) for the qualitative detection of antibody to *T. cruzi* (Abbott Laboratories, Abbott Park, IL, USA). This assay does not cross-react with the non-pathogenic trypanosome *Trypanosoma rangeli*, which is transmitted by some of the same vectors as *T. cruzi* [40]. Additionally, most of the baboons were assessed for seropositivity on serial samples with two or more tests at each time point (ranging from 2 to 15 tests per animal over 15 years). These additional assays, all of which detect IgG, were as follows: enzyme-linked immunosorbent assay (ELISA; using *T. cruzi* antibody; IVD Research, Inc., Carlsbad, CA, USA, and Bio-Manguinhos, Ministry of Health, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil), Indirect immunofluorescence assay (IFA; using immobilized *T. cruzi*, Bio-Manguinhos), Immuno-chromatographic assay (ICA or Chagas Stat-Pak™ Assay; using *T. cruzi* recombinant antigens, Chembio Diagnostic Systems, Medford, NY, USA). All five tests gave highly concordant results. In most cases a window of time during which seroconversion occurred was determined.

Retrospective study: design and reproductive and clinical history

This study was a retrospective analysis of weight and reproductive histories of 84 female baboons having at least one incidence of fetal loss and having been evaluated by serological testing for presence of *T. cruzi* antibodies. Serum for antibody testing was selected from SNPRC blood and tissue bank based on the dates prior to or at the time of the first reproductive event (as generally at the age of 7 years old). We randomly selected animals for *T. cruzi* testing from a population of baboons with a history of fetal loss. Weights of the baboons were recorded between the ages of 9 and 11 years, at nonpregnant time points. Turgescence scores were recorded every 3–7 days, and the animals were observed daily for indications of pregnancy or pregnancy loss.

Case report

A 12-year-old *M. fascicularis* naturally infected with *T. cruzi* was presented with stillbirth at term. Gross examination revealed a macerated stillborn fetus with uninflated lungs. This female had delivered one full-term healthy offspring vaginally before the stillbirth and has delivered three healthy offspring after the reported case.

Pathology

Three placentas from seronegative dams and one from a seropositive dam were available for examination from the group of 84 stillborn baboons. Additionally, two placentas, one from a *M. fascicularis* seronegative for *T. cruzi* and the other from a *M. fascicularis* seropositive for *T. cruzi*, were examined. All tissues were fixed in 10% neutral buffered formalin for a minimum of 48 hours, processed conventionally, embedded in paraffin, cut at 5 μ m, and stained with hematoxylin and eosin. To evaluate placental inflammatory responses available baboon and macaque placental tissues were additionally stained with CD45RO (Clone UCHL-1), natural killer cell (CD 57, Clone NK-1) and macrophage (CD68, Clone KP-1) (Dakopatts, Copenhagen, Denmark) antibodies as described elsewhere [1]. The number of positive stained cells was calculated at 40 $\times$ magnification in 16 randomly chosen fields of view and presented as number of positive stained cells per square millimeter of tissue on the slide.

Trypanosoma cruzi DNA extraction and detection in archived tissues

Tissue collection—Placental tissue was collected from the *T. cruzi* seronegative and seropositive *M. fascicularis*. Archived esophageal and myocardial tissues from four seropositive and four seronegative male baboons were used as positive and negative controls for the PCR reaction. Protozoal amastigotes were detected histologically in the myocardium, but not in esophageal sections of the seropositive male baboons.

DNA extraction—Tissue was excised from the paraffin blocks using sterile scalpel blades. Approximately 25 mg tissue was placed into 1.2 ml of xylene, vortexed, and centrifuged at $8000 \times g$ for 5 minutes at room temperature. Supernatant was removed by pipetting; the pellet was washed twice in 1.2 ml of absolute ethanol. DNA extraction was performed according to the manufacturer's instructions using a Qiagen (Valencia, CA, USA) tissue DNA extraction kit. DNA concentration was measured in DNA dilution with 10 mM Tris, pH 8.5. The yield of DNA extracted ranged from 9.78 to 37 ng/ μ l.

Quantitative PCR—Quantitative PCR was performed with primers specific for *T. cruzi* minicircle S36/S35 [12], using 2 $\times$ Quantitec sybr green master mix (Qiagen) with 30 μ M of each primer in the reaction in ABI PRISM 800 system (Applied Biosystems, Foster City, CA, USA). A standard curve for quantification was generated from the dilution of *T. cruzi* DNA (American Type Culture Collection, Manassas, VA, USA). The specificity of the

product was verified by performing PCR with the same primer set using HOT Star Tag DNA polymerase (Qiagen) and subsequent product sequencing. Melting curves were generated and analyzed at the end of Q-PCR cycles.

Statistical analysis

Records of menstrual cycle (dates of vaginal bleedings and degree of turgescence) were randomly distributed to three blinded observers to avoid bias. Statistical analysis was performed using statistica 6.0 (StatSoft, Inc., 2001, Tulsa, Oklahoma, USA). Data are presented as mean $\pm$ SEM. Comparison of means from independent samples was conducted using Student's *t*-test adjusted by variance. The relative amount of *T. cruzi* DNA was compared using ANOVA with a Fisher *post hoc* test. We considered *P*-values below 0.05 to be statistically significant.

Results

Trypanosoma cruzi serology among animals with a history of fetal loss

Of 84 serologically tested female baboons, 13 were seropositive and 71 were seronegative (Table 1). There were no differences between seropositive and seronegative animals regarding parity and menstrual cycle characteristics; however, the mean weight of the animals in the seropositive group was higher than that of those in the seronegative group (Table 2).

Pathology

There were no differences in placental structure, hemorrhage, fibrin deposits, or inflammation between the placentas from the three seronegative and one seropositive baboon stillbirths. The analyses of immunohistochemistry for CD45RO, CD57, and CD68 positive cells revealed that the numbers of positive cells in placenta from the seropositive animal were 5, 27, and 67 per mm² of tissue, respectively. In placentas from the seronegative animals the relative amount of positive cells ranged between 22–485, 22–459 and 136–366 per mm², respectively (Fig. 1).

Case report

Histologically there was a severe, multifocal necrosuppurative placentitis with intracellular protozoal amastigotes morphologically consistent with *T. cruzi* (Fig. 2A). Lesions and organisms were not observed in the fetus. A placenta was collected after stillbirth from a *T. cruzi*-negative *M. fascicularis* for comparison (Fig. 2E). The number of cells expressing CD45RO and CD57 markers in the infected placenta (Fig. 2B,C) was 260 and 84 per mm², respectively; the number of cells expressing these markers was lower in the placenta from non-infected animal (Fig. 2F,G) (42 and 5 per mm², respectively). The number of cells expressing CD68 in the infected placenta was 485 per mm² compared with 747 cells/mm² in the non-infected placenta (Fig. 2D,H). The analyses of PCR data revealed the relative amount of *T. cruzi* DNA in placenta as 2.09. This amount was consistent with the relative amount of *T. cruzi* DNA in the tissues of the group of seropositive baboons with histologically visualized parasites and was significantly higher compared with the seropositive animals without parasites and to seronegative animals (Fig. 3).

Discussion

In the southern United States, infection with *T. cruzi* and resulting seroconversion has been demonstrated in a variety of wild animals and domesticated dogs [4, 24, 27, 48, 54]. The acute and chronic stages of Chagas disease have been studied in several NHP species including the rhesus macaque and the baboon [7, 33, 35, 47, 56–58] (Table 3). Chagas

disease was first observed in a colony baboon at the SFBR in San Antonio, Texas in 1986 [21]. The outdoor housing of animals results in natural infection through exposure to the triatomine insect vector. In primates it is presumed that transmission is mainly through ingestion of infected insects [49].

There have been a few reports of Chagas disease in humans in the United States and there has been concern about the safety of the blood supply as donors from endemic areas migrate to the United States [5, 11, 29, 38, 46].

The seroprevalence in pregnant women in endemic areas has been shown to be as high as 81% and congenital transmission can occur in 2–21% of cases [2, 3, 8, 19, 20, 51, 55]. Why transmission from mother to fetus is not more common is still being studied. Torrico et al. [52] found that transmission rates were decreased in older women with higher parity and that when parasites were not transmitted to the fetus there was no effect on outcome of gestation or health of newborns in seropositive mothers. Our retrospective study did not find any influence of maternal age on the number of stillbirths. Moreover, the indirect indices of maternal hormonal status in our study – length of menstrual cycle and periovulatory phase – in both groups were not different between seropositive and seronegative animals and were within published values for baboons [34]. Our study was performed on a selected population of baboons with at least one incident of fetal loss and may not mirror the situation in the overall colony. Further analyses are underway at the SNPRC.

The surprising finding – higher body weight in seropositive animals with fetal loss compared with seronegative ones – could be explained partly, that by increased macrophage infiltration into the omental tissues in infected humans [12].

Lujan et al. [30] showed that chorionic villi from normal human placentas do not allow penetration or sustained infection by *T. cruzi* *in vitro*. This finding is in agreement with the documented histologic presence of parasites in the placenta of newborns in which congenital transmission did not occur, and is consistent with the case presented here [56]. Fretes and de Fabro [18] showed that two of six placentas from women seropositive for *T. cruzi* contained occlusion of the villi blood vessels with hyalinization of their walls. We did not observe this feature, possibly due to our restricted number of samples.

The features of placental infection – lymphocyte infiltration and villitis in our case – were similar to those described for human placental reaction to the parasite [1, 13, 17]; however, contrary to the observations in women, we did not find increased inflammation in placentas from the seropositive baboons when compared with the placentas of seronegative animals; and the number of placental macrophages was not increased in the infected placenta of the *M. fascicularis*, compared with the placenta from non-infected animal. The absence of signs of fetal *T. cruzi* infection in the presence of an infected placenta in the reported case may be due to chronic infection and minimal body parasite invasion, as supported by the quantitative PCR data. The data presented in this study are also in agreement with our previous report regarding absence of seropositive offspring in chronically infected baboons [53].

In summary, there was no difference in fertility, menstrual cycle parameters and number of stillbirth/ abortions, between seropositive and seronegative baboons seronegative for *T. cruzi* in the population of animals with at least one incident of fetal loss. Placental lesions in the presence of *T. cruzi* amastigotes in the decidua (*M. fascicularis*) were similar to those reported in humans except the placental macrophage reaction (with no difference compared with the placenta from seronegative animal). Applied here for the first time in NHPs, Q-PCR of *T. cruzi* DNA is a useful diagnostic tool to study the presence of *T. cruzi* in archived NHP placentas.

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Fig. 1.

Photomicrographs of placentas from baboons seronegative (A–D) and seropositive (E–H) for *Trypanosoma cruzi*. Hematoxylin and eosin (A, E); CD45RO (B, F), CD57 (C, G), and CD68 (D, H) immunohistochemistry. Note the absence of immunostaining of CD45RO (F) and CD57 (G) in the placenta from the seropositive dam.

Fig. 2.

Photomicrographs of *Macaca fascicularis* placenta with intracellular protozoal amastigotes (A, arrow) and placentitis caused by *Trypanosoma cruzi* (A–D) and control placenta (E–H) from seronegative *M. fascicularis*. Hematoxylin and eosin (A, E); CD45RO (B, F), CD57 (C, G), and CD68 (D, H) immunohistochemistry. Note the intracellular protozoal amastigotes in the basal plate in the infected placenta (A, arrow) and the decreased number of CD45RO positive cells (F) and the absence of immunostaining of CD57 (G) antibodies in the control placenta, as indicated by brown stain.

Fig. 3. Relative amount of *Trypanosoma cruzi* DNA per total DNA in tissues from seropositive (with and without histologic identification of amastigotes) and seronegative baboons (*Papio* spp.) (n = 3–4 in each group).

Table 1

List of the non-human primates used in this study and the assays that were performed

	Total number of animals	Study performed	Histology	IHC	Q-PCR	
			Number of animals	Number of animals	Placenta (n)	Esophagus and/or myocardial tissue (n)
Female baboons (<i>Papio</i> spp.) with history of fetal loss						
Seropositive for <i>Trypanosoma cruzi</i>	13	Retrospective study and placental analyses	1	1	NA	NA
Seronegative for <i>T. cruzi</i>	71	Retrospective study and placental analyses	3	2	NA	NA
Male baboons (<i>Papio</i> spp.)						
Seropositive for <i>T. cruzi</i>	4	Tissue analyses	4	NA	NA	4
Seronegative for <i>T. cruzi</i>	4	Tissue analyses	4	NA	NA	4
<i>Macaca fascicularis</i> with stillbirth						
<i>T. cruzi</i> infected	1	Case report, placental evaluation	1	1	1	NA
Seronegative for <i>T. cruzi</i>	1	Placental evaluation	1	1	NA	NA

IHC, immunohistochemistry; Q-PCR, quantitative polymerase chain reaction; NA, not applicable.

Table 2

Weight, cycle length, and parity comparison between seropositive and seronegative baboons with a history of fetal loss

	Seronegative (n = 71)	Seropositive (n = 13)	<i>P</i> -value
Weight (kg)	18.36 ± 0.42	21.35 ± 0.80	0.01
Number of stillbirths	1.41 ± 0.10	1.92 ± 0.40	0.23
Number of vaginal deliveries	5.45 ± 0.39	4.23 ± 0.69	0.14
Number of cesarean sections	0.59 ± 0.14	0.69 ± 0.31	0.77
Parity	7.41 ± 0.39	6.86 ± 0.73	0.56
Length of periovulatory phase (days)	7.5 ± 0.53	8.1 ± 0.5	0.51
Length of menstrual cycle (days)	35.7 ± 1.2	35.1 ± 0.8	0.75

Data are presented as mean ± SEM. The *P*-value was calculated with Student's *t*-test, corrected for variances.

Table 3
Selected literature review of *Trypanosoma cruzi* infection reports in non-human primates (NHPs)

Species	<i>T. cruzi</i> strain	Study design	Results	Conclusions	Reference
<i>Cebus apella</i>	Ypsilon	Prospective experimental, acute phase	ECG abnormalities in 86% of monkeys; antibody titer highest at 47 days; parasitemia was detectable between days 4 and 96; all monkeys had fever (average of 22 days); all monkeys showed weight loss throughout the study	<i>Cebus apella</i> is a good model for acute <i>T. cruzi</i> infection due to similarities to acute disease in humans, as indicated by serology, ECG alterations and parasitemia	[45]
<i>Cebus apella</i>	Ypsilon	Prospective experimental, acute and chronic phases	ECG abnormalities were seen in all monkeys (most severe with RA strain), none or slight pathology was seen in acute animals, concentric hypertrophy and aneurism of apex was seen in chronic monkeys, myocarditis (lymphoplasmacytic) was seen in all monkeys, colonic inflammation was seen in 50% of chronic monkeys	Direct parasitemia in acute phase; serologic response; ECG abnormalities and histopathology in <i>T. cruzi</i> -infected <i>Cebus apella</i> monkeys are similar to what has been reported in humans	[44]
<i>Saimiri sciureus</i>	NA	Case report, congenital transmission	<i>T. cruzi</i> -like trypanosomes were seen in blood smears from mother and offspring, serology was inconclusive, no lesions noted on necropsy or histopathology; no ECG changes were seen	Infection was acquired transplacentally; frequency of congenital transmission in NHPs is unknown, as is the ratio of chagasic placentitis and fetal deaths to live births	[16]
<i>Saimiri sciureus</i>	Brazil	Prospective experimental, acute phase	IgM peaked at 6–15 weeks, IgG peaked at 6–23 weeks, ECG changes were seen in 50% of monkeys	Experimental Chagas' disease induces cardiomyopathy and measurable humoral and cellular immune responses in the squirrel monkey those are similar to that which occur in infected humans	[41]
<i>Saimiri sciureus</i>	NA	<i>T. cruzi</i> detection method comparison	6.1 % were positive by Giemsa-stained slide examination; 10.4% were positive by ELISA serology; 26.5% were positive by PCR	PCR has a clear advantage over microscopy and ELISA	[39]
<i>Macaca mulatta</i>	Colombo	ECG study in experimental chronically infected animals	ECG and echocardiography abnormal patterns in seven animals are not frequently observed in human chronic Chagas' disease, however they are highly relevant when detected	Rhesus monkeys is an experimental model for acute, indeterminate, and cardiac chronic Chagas' disease	[10]
<i>Macaca mulatta</i>	Peru	Prospective experimental, acute phase	No difference in course of infection in vaccinated vs. unvaccinated (all developed parasitemia, all	Acute phase of <i>T. cruzi</i> infection in rhesus monkeys is similar to that occurring in humans with Chagas' disease; vaccination was	[47]

Species	<i>T. cruzi</i> strain	Study design	Results	Conclusions	Reference
<i>Macaca mulatta</i>	Peru	Prospective experimental, chronic phase	were IgM and IgG positive) One monkey died at day 166 with megasophagus	not protective Rhesus monkeys seem to be a reasonable model for the course of infection in man, although they may be more susceptible than humans	[32]
<i>Macaca mulatta</i>	Peru	Prospective experimental, chronic phase	All animals were xenodiagnosis positive throughout infection; ECG abnormalities were not found	The ECG abnormalities found in humans with gray chagasic heart disease were not found in the monkeys	[35]
<i>Leontopithecus rosalia</i>	NA	Prevalence, capture of animals in natural conditions for testing and release	45% of seropositive animals had abnormal ECGs	ECG abnormalities seen in <i>L. rosalia</i> are typical of other species including humans; high parasitemias did not correlate with ECG abnormalities; <i>T. cruzi</i> did not cause extensive health problems in the majority of wild golden lion tamarins	[36]
<i>Aotus trivirgatus</i> <i>Cebuella pyraeae</i> <i>Cebus</i> spp., <i>Saimiri sciureus</i> <i>Ateles</i> spp., <i>Lagothrix I</i> <i>agotrichia</i> , <i>Saguinus</i> spp.	NA	Prevalence study by animal dealer of monkeys in Colombia	Overall infection rate was 15.5%; of those examined by blood culture 30.6% were positive; anastigotes were found in the heart muscle of three <i>Cebus</i> monkeys; xenodiagnosis was positive in two <i>Saimiri sciureus</i> monkeys	North Colombia has the lowest (1.3%) and Southwest Colombia the highest (25.0%) <i>T. cruzi</i> infection rates	[31]
<i>Macaca silenus</i> , <i>Lemur catta</i> black and white ruffed lemur	NA	Prevalence study in free ranging primates released on St Catherine's Island, GA, USA	7 of 11 macaques were blood culture positive; 1 of 19 <i>Lemur catta</i> were blood culture positive; 0 of 10 black and white ruffed lemurs were blood culture positive	Animals were captive-bred, so most likely were infected on the island; Old World primates are not resistant to infection	[42]
<i>Papio</i> spp.	NA	ECG study in naturally infected animals	17 seropositive and 13 seronegative captive baboons Chagasic heart disease was present in 24% of naturally infected baboons. Echo evidence of systolic dysfunction was found in four seropositives and in none of the controls	Most NHPs, irrespective of their serology, have diffuse, non-specific ECG changes not necessarily diagnostic of myocardial disease, two-dimensional echo should be added to their non-invasive assessment	[57]
<i>Papio cynocephalus</i>	NA	Case report	Four month old baboon found dead in an outdoor gang cage; at necropsy had severe diffuse myocarditis; histopathology showed numerous intracellular amastigote forms of <i>T. cruzi</i> dam was seronegative	Lack of seropositivity in dam and two cage mates indicates this is a solitary case of natural exposure, presumed via ingestion of a <i>T. cruzi</i> -infected triatomine bug	[21]

ECG, electrocardiogram; NA, not applicable.