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BLOOD-SUCKING LICE MAY DISSEMINATE *Trypanosoma cruzi* INFECTION IN BABOONS

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SUMMARY

Trypanosoma cruzi (*Schyzotrypanum*, Chagas, 1909), and Chagas disease are endemic in captive-reared baboons at the Southwest Foundation for Biomedical Research, San Antonio, Texas. We obtained PCR amplification products from DNA extracted from sucking lice collected from the hair and skin of *T. cruzi*-infected baboons, with specific nested sets of primers for the protozoan kinetoplast DNA, and nuclear DNA. These products were hybridized to their complementary internal sequences. Selected sequences were cloned and sequencing established the presence of *T. cruzi* nuclear DNA, and minicircle kDNA. Competitive PCR with a kDNA set of primers determined the quantity of approximately 23.9 ± 18.2 *T. cruzi* per louse. This finding suggests that the louse may be a vector incidentally contributing to the dissemination of *T. cruzi* infection in the baboon colony.

KEYWORDS: Baboons; *Papio hamadrias;* Lice*; Pedicinus obtusus*; *Trypanosoma cruzi* infection.

INTRODUCTION

American trypanosomiasis is a zoonanthroponosis caused by the kinetoplastid protozoan *Trypanosoma cruzi*. The infection leads to Chagas disease, a chronic consumptive ailment affecting the heart and the digestive tube of susceptible hosts[25]. Wild mammals dwelling on the American continents serve as *T. cruzi* reservoirs that maintain the parasite in nature[12]. The geographic distribution of *T. cruzi* infection coincides with that of its vector, blood-feeding reduviid bugs of the subfamily Triatominae that are found in tropical and subtropical regions, within latitudes 42 °N to 43 °S. When the protozoan infection gets established in the reduviid host it persists for the entire 1-year life of the host. The triatomids are nocturnal, hiding in the daytime and emerging at night when they attack the sleeping prey. The sylvatic species may fly from their diurnal hiding places to feed on a host located some distance away[22]. Contamination of a host with metacyclic forms of the parasite in the feces of the reduviids has been associated with the human disease[25]. Serological evidences have shown the prevalence of *T. cruzi* infections ranges from 0.43 per cent in Georgia, USA[7], to 74.2 per cent in rural areas of Santiago del Estero, Argentina[6]. It has been estimated that 18 million people are chronically infected with *T. cruzi*, and 35 million are at risk of contracting the infection[31].

Transplacental transmission of *T. cruzi* may occur in 2.5% of human fetuses of infected mothers[2,3,9,20]. Breast-feeding has been associated with transmission of *T. cruzi* from infected mothers to their infants, but further studies are needed to determine the epidemiologic importance of this route of transmission[15]. *T. cruzi* has been considered the most infective blood protozoan, and several cases of accidental transmission to laboratory workers have been reported[4]. In addition, oral transmission of *T. cruzi* is well documented, and is often related to consumption of insectivorous and carnivorous mammals[25].

The Anoplura (or sucking lice) live on the surface of the host. To our knowledge, the possibility that sucking lice of the family Pediculidae Linaeus, 1758, comprising the genera *Pedicinus*, *Pediculus* and *Phthirus*, are capable of transmiting of *T. cruzi* has not previously been investigated. We have tested this hypothesis in a baboon (*Papio hamadryas*, Cercopithecidae) colony[16], which is maintained in large open-air pens in San Antonio, Texas[19]. In this colony, serological data indicate *T. cruzi* infection in 9.4% of 2-to-3-year-olds, 14.0% of 7-to-10-year-olds, and 22.5% of baboons that are 15 years old or older (unpublished data). In this study we show that sucking lice, *Pedicinus obtusus*, captured from nine baboons reared in the colony, yielded PCR amplification products of *T. cruzi* kinetoplast (kDNA) and nuclear (nDNA) DNA with specific primers. Cloning and sequencing the PCR products confirmed the presence of the parasite DNA in the lice.

MATERIALS AND METHODS

Baboons: This investigation was conducted with animals from a breeding colony of approximately 3,300 baboons, whose members have been useful animal models in many areas of biomedical research[29,30]. An earlier observation of natural death of an infant baboon[8] infected with *T. cruzi* and a more recent observation of three baboons that died spontaneously and exhibited nests of amastigote forms of *T. cruzi* in

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host cells and lesions consistent with Chagas heart disease (unpublished data) prompted us to search *T. cruzi* infection in a cohort of 30 baboons. The enzyme-linked immunosorbent assay, indirect hemagglutination, and indirect immunofluorescence assays[28] were used to determine the presence of specific antibodies in the serum. These positive assays are regarded as evidence of *T. cruzi* infection[31]. In adition, 2 mL blood samples drawn from six baboons were seeded in 10 mL of liver infusion tryptose medium. Aliquots of each culture tube were examined monthly for eight months.

Collection of lice and extraction of DNA: We collected *P. obtusus* (Fig. 1) from the hair and skin of severely parasitized baboons in the colony. Bulk samples of lice from each baboon was pooled, fixed in 70% ethanol and subjected to DNA extraction[1,21]. DNA was extracted from the blood, and from the heart of baboons that died of Chagas disease. DNA was also extracted from epimastigote forms of Berenice *T. cruzi* grown in axenic medium[27] and from macrophage line P388DI-IL1 grown in Dulbecco's Minimum Essential Medium supplemented with 20% fetal calf serum, 100 µg/mL streptomycin and 100 IU penicillin[26].

Fig. 1 - Adult *Pedicinus obtusus*, the Anoplura sucking lice.

Polymerase chain reaction (PCR): DNA was analyzed by PCR with specific primers for the constant minicircle kDNA region[1,21,23], and for highly repetitive sequence[18] of nDNA of *T. cruzi*. The kDNA primer set (S35, 5'-ATAATGTACGGG(T/G)GAGATGC-3' and S36, 5'-GGTTCGATTGGGGTTGGTG-3') annealing to the constant region of minicircles yields a 330 bp product and its catamer of 660 bp[23]. The nDNA primer set (PON1, 5'-TGGCTTGGAGGAGTTATTGT-3' and PON2, 5'-AGGAGTGACGGTTGATCAGT-3') amplifies a 250 bp fragment[18]. A DNA thermal cycler (MJ Research, Watertown, MA) was used for 30-32 cycles as follows: PON1/2, 94 °C for 2 min, 58 °C for 1 min, and 72 °C for 1 min; S35/36, 94 °C for 2 min, 64 °C for 1 min, and 72 °C for 1 min. The reactions were run with 100 ng of lice target template and with 50 pg of DNA from *T. cruzi* culture forms. Each reaction was done in 25 µL aliquots containing 2.5 U of Taq polymerase (Perkin Elmer, Cetus Norwalk), 0.2 mM dNTPs, 50 mM Tris-HCl (pH 9.0 at 20 °C), 1.5 mM $MgCl_2$, 200 mM ammonium sulfate, and 5 µM each primer. A 10 µL aliquot from each PCR reaction was subjected to a 1% agarose gel eletrophoresis. Negative controls (water, healthy baboon DNA, and P388D1-IL1 DNA) and positive control (*T. cruzi* DNA) were always included to detect DNA contamination and ensure that the PCR worked efficiently.

Competition assays: After the presence of *T. cruzi* in sucking lice was confirmed in nine bulk samples, we ran competitive PCR assays with S35/36 primers set to quantify the number of parasites in these samples[5]. These assays consisted of a mixture of an unknown quantity of *T. cruzi* template minicircle DNA with serial dilutions of a known quantity of competitor DNA (pT7 Blue vector containing the 280 pb kDNA fragment). The competitor fragment binds the same primers and yields a product that is distinguishable from the sample template (280 versus. 330 bp). The equivalency point corresponds to the tube with equimolar concentrations of template and competitor PCR products in the reaction mix. The determination of equivalency points was made by visual comparison in 1.5% wide range agarose/0.5% standard agarose gel. In all PCR positive samples, using the known amount of the competitor DNA in the reaction, the unknown quantity of the template was calculated, under the asssumption that there were 10,000 minicircles/parasite and, thus 15 fg of 330 bp template/parasite[5].

Hybridization, cloning and sequencing: The PCR amplification products were transferred by capillarity to a nylon membrane. Prehybridization and hybridization were performed in 6X SSC, 5X Denhardt's, 0.5% SDS and 100 µg/mL salmon DNA solution. The membranes were prehybridized for 4 hr, and then were hybridized for 12 hr with internal oligonucleotide sequences for different amplified fragments. For S35/36 products[23], the sequence was S67 5'-GGTTTTGGGAGGGG(CG)-(G/C)-(T/G)TC-3'. For PON1/2 products[18], the sequence was PON3 5'-CCGGCCTGTGTCTGCGGC-3'. These oligonucleotides were radiolabelled with [γ ^{32}P]-dATP (3000 CimMol) using the polynucleotide kinase method following the manufacturer's recomendation (Life Technologies). After the membrane was washed once for 5 min with 1X SSC and 0.1% SDS and twice for 30 min each with 0.2X SSC and 0.1% SDS, it was autoradiographed for variable periods of time.

The amplification products obtained with specific kDNA primers were cloned into PCRII vector, and several clones were selected and subjected to sequencing using an *f*-mol DNA cycle sequencing system (Promega). The sequences were submitted to PDU, GBU, GenBank and EMBL BLAST analyses.

RESULTS

Phenotypic and genotypic markers of *Trypanosoma cruzi* infection: Phenotypic and genotypic markers that indicate the presence of *T. cruzi* infection in the baboon colony reared in outdoor cages at the Southwest Foundation for Biomedical Research, San Antonio, Texas, are shown in Table 1. The results of three serologic assays covalidated the PCR amplification products from target template *T. cruzi* DNA extracted from blood or tissue samples of 30 baboons. Furthermore, the culture of blood from three out of six baboons yielded parasitic forms of *T. cruzi* that were inoculated in BALB/c mice. Culture of macerated louse tissue was not performed because samples were received in 70% ethanol. The animals showed trypomastigote forms in the blood and amastigote forms of *T. cruzi* encysted in tissue cells (data not shown).

Then, we collected lice from the skin of those three baboons that showed parasitemia and from six others showing positive serologic tests and clinical evidence of the infection.

Detection of *Trypanosoma cruzi* from *Pedicinus obtusus*: Here we tested the hypothesis that the blood sucking lice *P. obtusus* may play some role in disseminating the *T. cruzi* among members of the baboon colony. In the absence of direct demonstration of live *T. cruzi* in lice, the evidence supporting this hypothesis is derived from PCR amplification of sequences from lice target template DNA with specific nested sets of *T. cruzi* kDNA[23], and nDNA[18] primers. Table 2 summarizes results of experiments showing that lice captured from the hair, and from the skin of baboons with Chagas disease are infected with the *T. cruzi*. This evidence was given by PCR with nested sets of primers annealing to the protozoan kDNA, and nDNA templates. In the control experiment, 20 lice collected from the hair, and from the skin from a healthy baboon reared in a cage far apart from that having *T. cruzi* infected baboons, and that yielded negative PCR for the protozoan infection.

Table 1
Phenotypic and genotypic markers of *Trypanosoma cruzi* identified in baboon tissue

		Phenotype*	PCR Genotype¶	
	No. of baboons	No. of baboons with specific antibodies	No. of baboons with kDNA	No. of baboons with nDNA
Positive	20	20	17	17
Negative	10	0	1	0

* Enzyme-linked immunosorbent assay, indirect hemagglutination, and indirect immunofluorescence assays identified specific antibodies against *T. cruzi* antigens. The presence of at least two positive assays for each of 20 baboon samples is regarded as an evidence of infection; ¶ PCR amplification products from target template DNA extracted from blood or tissue samples of 30 baboons, using specific sets of kDNA and nDNA primers.

Figure 2A shows PCR amplification products with specific kDNA primers. The amplification products hybridized with an internal kDNA probe (Fig. 2B). Sequencing of the PCR amplification product showed typical constant and hypervariable kDNA minicircle regions. This sequence is deposited in GenBank (accession number AF114153). Confirmation of the presence of *T. cruzi* DNA in the target template extracted from bulk samples of lice (Table 2) was obtained by PCR with a PON1/2 nested set of primers (Fig. 3A). The amplification product of 250 bp hybridized with its complementary internal sequence, thus showing its specificity (Fig. 3B). This amplification of the parasite nDNA suggested the presence of living *T. cruzi* in the sucking louse.

Quantitation of living *Trypanosoma cruzi* in lice: Therefore, we performed competitive, quantitative PCR with a kDNA-nested set of primers to determine the number of parasites in each louse[5]. To determine the sensitivity of this quantitative procedure we used concentrations of *T. cruzi* DNA corresponding to 1, 10 and 100 parasites. Figure 4A shows

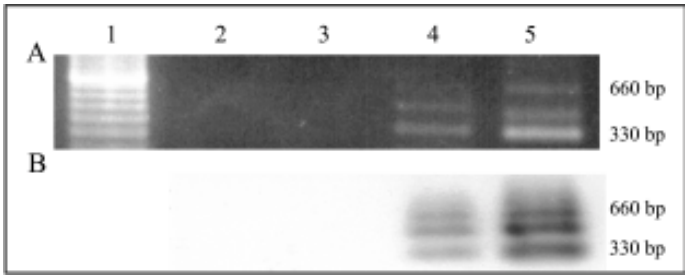

Fig. 2 - A: PCR amplification of sequences of minicircles of kDNA from *Trypanosoma cruzi* with specific S35/36 primers. Lane 1, 100 bp molecular weight marker; Lane 2, water; Lane 3, P388D1-IL1 DNA (negative control); Lane 4, sucking lice DNA; Lane 5, *T. cruzi* DNA. **B**: Southern hybridization with an internal fragment of DNA complementary to the PCR amplification products. Note the 330 bp fragment and its catamer in Lanes 4 and 5 (see methods).

Table 2
Quantitative PCR detection of *Trypanosoma cruzi* from *Pedicinus obtusus* captured on baboons' hair, and skin showing positive phenotypic, and genotypic markers for the protozoan infection

Animal Id.	Age	Sex	PCR§ kDNA	PCR§ nDNA	Lice (total/bulk)	Quantitative PCR§ *T. cruzi*/louse
PCA14660	10 mo	F	+	+	4	20
PCX14499	11 mo	M	+	+	7	2.5
PCX14650	11 mo	M	+	+	2	40
PCX14011	12 mo	F	+	+	4	10
PCA14190	17 mo	F	+	+	4	13.3
PCA13988	17 mo	F	+	+	5	1
PCX1X3758	18 years	M	+	+	2	46
PCA1X2576	20 years	M	+	+	2	42
PCA1X2891	20 years	M	+	+	2	41
13640*	Unknown	-	Neg	Neg	20	Neg

* Twenty lice were captured from hair, and skin from a baboon, which had shown negative phenotypic, and genotypic markers for the *T. cruzi* infection; § DNA was extracted from baboon's tissue, and from bulks of lice, and used as template for PCR amplification with primer sets annealing to specific *T. cruzi* kDNA (SK35/36), and nDNA (PON1/2), as described in methods, and in legend for Figure 4B.

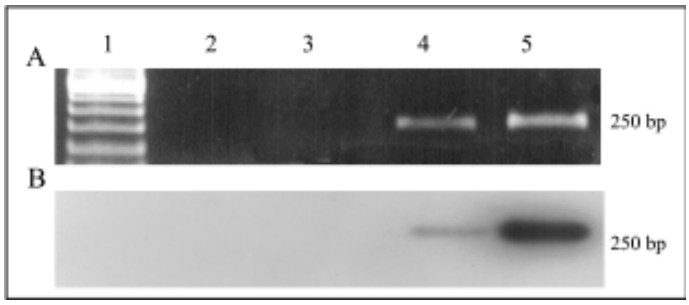

Fig. 3 - A: PCR amplification of sequences of nDNA from *Trypanosoma cruzi* with specific PON1/2 primers. Lane 1, 100 bp molecular weight marker; Lane 2, water; Lane 3, P388D1-IL1 DNA (negative control); Lane 4, sucking lice DNA; Lane 5, *T. cruzi* DNA. **B**: Southern hybridization with an internal fragment of DNA complementary to the PCR amplification product. Note the 250 bp bands in lanes 4 and 5. (See methods).

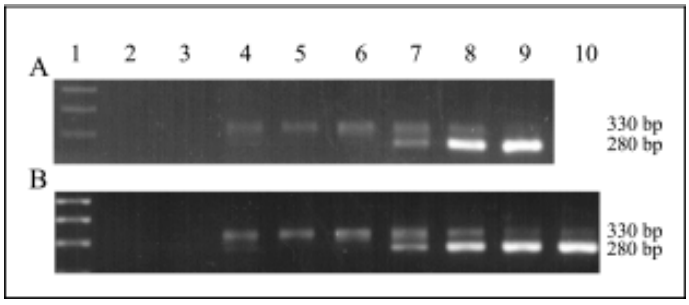

Fig. 4 - A: Quantitation of *T. cruzi* DNA by competitive PCR. Lane 1, 100 bp molecular weight marker; Lane 2, water; Lane 3, P388D1-IL1 DNA (negative control); Lane 4, *T. cruzi* DNA equivalent to 1 parasite. Lanes 5-to-8, *T. cruzi* DNA equivalent to 1 parasite and different quantities of competitor DNA; Lane 5, 0.15 fg; Lane 6, 1.5 fg; Lane 7, 15 fg; Lane 8, 150 fg. Note the 330 and 280 bp bands, whose point of equivalence corresponded to 15 fg of competitor DNA that represent circa 1 parasite. **B**: Quantitation of *T. cruzi* DNA in sucking lice by competitive PCR. Lane 1, molecular weight marker; Lane 2, water (negative control); Lane 3 P388D1-IL1 DNA; Lane 4, *T. cruzi* DNA equivalent to 1 parasite. Lanes 5-to-10, 1/50 aliquots of DNA extracted from five sucking lice and various quantities of competitor DNA: Lane 5, 0.15 fg; Lane 6, 0.75 fg; Lane 7, 1.5 fg; Lane 8, 15 fg; Lane 9, 150 fg; Lane 10 1500 fg. The point of equivalency between the 330 and the 280 bp bands was at 1.5 fg (Lane 7) of competitor DNA, which is approximately 1/10 of the amount of DNA in 1 parasite. Since each reaction represents 1/50 of the total DNA sample, it was calculated that approximately five parasites were present in five sucking lice.

the result of a standard competitive PCR to detect the DNA equivalent of a single parasite. Then, we determined the quantity of parasite DNA in 100 ng of DNA extracted from bulk samples of lice. Interestingly, the quantity of 23.9 ± 18.2 *T. cruzi* per louse was found. A typical experiment carried out with lice captured from the hair, and from the skin of a 17 month old baboon (Id PCA13988, Table 2) is shown in Figure 4B. The zone of equivalency corresponded to 1.5 fg of competitor DNA. Because this quantity of DNA corresponds to 1/10 parasite[5], and because the reaction contained 1/50 of the DNA sample, we calculate that there were approximately five *T. cruzi* in this bulk sample of five lice tested.

DISCUSSION

We have demonstrated for the first time the contamination of blood-sucking lice of the family Pediculidae with *T. cruzi*. However, the only evidence of the protozoan in blood sucking lice was obtained by PCR amplification of *T. cruzi* kDNA and nDNA sequences in target templates with specific nested set of primers[18, 23]. We believe that the sequence of a minicircle kDNA belongs to a living *T. cruzi*, instead of being simply kDNA integrated in the genome of blood cells[26] sucked in by the louse, because nDNA sequence was also amplified from the lice target template and there is no published evidence that *T. cruzi* nDNA integrates in the genome of a host. These findings suggest, therefore, that sucking lice are capable of transmiting living *T. cruzi* infection among baboons, either by contamination of mucosal surfaces, conjunctiva and skin wounds with its intestinal waste, or by the oral route.

The insects belonging to the family Pediculidae are host specific. They are usually transmitted by direct contact. Of interest, it has been shown that allo grooming by Old World monkeys is an altruistic behavior that removes external parasites from others. In particular, grooming of infants by mothers, which appears to be an important component of maternal care, would contribute to the dissemination of the *T. cruzi* infection, because the mothers pick up lice and eggs of lice and eat them[24]. It has been reported that insectivorous and carnivorous mammals become contaminated with *T. cruzi* through the oral route[25]. The demonstration of *T. cruzi* kDNA and nDNA in lice captured from baboons supports the hypothesis that contamination of nonhuman primates with the protozoan may contribute to the endemicity of Chagas disease in the colony. The estimated number of *T. cruzi* in blood sucking lice (23.9 ± 18.2 parasite per louse) suggests that this protozoan does not multiply in the intestine of the bug. However, dissemination of the infection by lice contaminated with *T. cruzi* is highly plausible, because the infection can be readly acquired through the oral route[13,14,17]. In this regard, it has been shown that reducing the number of the parasites in the inocula may retard but does not prevent the late course of experimental Chagas disease[13]. A single *T. cruzi* trypomastigote form is sufficient to establish an infection to produce widespread, fatal disease[14]. Although mechanical transmission of *T. cruzi* has been considered by several authors[4,8,13-15,17], its epidemiological importance remains to be determined.

The sucking lice that occur on human beings comprise the species *Pediculus humanus*, the body louse and head louse, and *Phitirus pubis*, the crab louse. These lice occur only on man and not on other hosts. The demonstration of PCR amplification products of *T. cruzi* sequences, with specific nDNA[18], and kDNA[23] primers, from template DNA obtained from sucking lice of the suborder Anoplura, which infest nonhuman primates, suggests that other sucking lice (*P. humanus*, and *P. pubis*) also may transmit the infection. However, the hypothetical epidemiological importance of sucking lice in the dissemination of the *T. cruzi* infections in human beings may be difficult to demonstrate, in the absence of allo grooming and ingestion of external parasites.

The primary vector for transmission of *T. cruzi* in this outdoor, baboon colony in San Antonio, TX, is presumed to be hemiptera, reduviid bugs of the family Triatominae. Several species of triatomids found in Southern United States (*Triatoma rubrofasciata*, *T. sanguesuga*) have been related to transmission of Chagas disease[22]. These species have been implicated in an outbreak of acute Chagas disease in a Rhesus monkey colony at Brooks Air Force Base, also in San Antonio[10]. Interestingly, baboon caretakers and researchers carrying on long term scientific work with baboons are aware that these flying insects occasionally are seen at night at the outdoor cages.

We believe that the *T. cruzi* infections in the colony were initiated by reduviid bugs, which possibly continued to transmit them, regardless of the lack of information with respect to the presence of the main vector

of Chagas disease in the baboon colony. However, our results suggest that *T. cruzi* infection may be further disseminated in the colony by sucking lice of the suborder Anoplura. We propose that the blood-sucking lice *P. obtusus* infesting Old World monkeys do transmit *T. cruzi* infection, as indicated by the presence of specific kDNA and nDNA sequences, probably acquired during feeding on an infected baboon.

RESUMO

Piolhos hematófagos podem disseminar infecção pelo *Trypanosoma cruzi* em babuínos

As infecções pelo *Trypanosoma cruzi* e a doença de Chagas são endêmicas em babuínos (*Papio hamadryas*) reproduzidos em cativeiro na Southwest Foundation for Biomedical Research, em Santo Antonio, Texas. Nós obtivemos produtos de amplificação por PCR do DNA extraído de piolhos colhidos do cabelo e da pele de babuínos chagásicos, com primers aneladores específicos para DNAs nuclear e de cinetoplasto do protozoário. Esses produtos foram hibridizados com suas respectivas seqüências internas complementares. Seqüências selecionadas foram clonadas e o sequenciamento demonstrou a presença de DNA nuclear de *T. cruzi*, e de minicírculo de kDNA. A PCR competitiva com primers de kDNA determinou a quantidade de aproximadamente 23.9 ± 18.2 *T. cruzi* por piolho. Este achado sugere que o piolho pode ser um vetor contribuindo para a disseminação de *T. cruzi* na colônia de babuínos.

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