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Short Communication

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Survival and Transstadial Persistence of *Trypanosoma cruzi* in the bed bug (Hemiptera: Cimicidae)

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Abstract

Bed bug populations are increasing around the world at an alarming rate and have become a major public health concern. The appearance of bed bug populations in areas where Chagas disease is endemic raises questions about the role of these insects in the transmission of *Trypanosoma cruzi*, the etiological agent of the disease. In a series of laboratory evaluations, bed bug adults and nymphs were experimentally fed with *T. cruzi*-infected blood to assess the ability of *T. cruzi* to survive inside the bed bug and throughout the insect's molting process. Live *T. cruzi* were observed in gut contents of experimentally infected bed bug adults via light microscopy and the identity of the parasite was confirmed via polymerase chain reaction analysis. *T. cruzi* persisted at least 97-d postinfection in adult bed bugs. Nymphal stage bed bugs that were infected with *T. cruzi* maintained the parasite after molting, indicating that transstadial passage of *T. cruzi* in bed bugs took place. This report provides further evidence of acquisition, maintenance, and for the first time, transstadial persistence of *T. cruzi* in bed bugs.

Key words: Chagas disease, disease transmission, vector competence, persistence, transstadial transmission

The resurgence of a blood sucking insect, such as the bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), raises concerns about the role that these insects may have in transmitting disease-causing microorganisms. Bed bugs have been found harboring and maintaining more than 40 human pathogens including the disease agents that cause leishmaniasis, rickettsiosis, pasteurellosis, tularemia, Q fever, plague, relapsing fever, leprosy, brucellosis, and American trypanosomiasis (Burton 1963). However, transmission of disease agents by bed bugs is still discussed (Goddard and deShazo 2009). In the last decades, most of the research in this area has been devoted to determining the vector competence of bed bugs for pathogens of public health importance, including the human immunodeficiency virus (HIV) (Jupp and Lyons 1987, Webb et al. 1989), hepatitis B virus (HBV) (Jupp and S. McElligott 1979, Blow et al. 2001), *Bartonella* spp. (Rhizobiales: Bartonellaceae) (Leulmi et al. 2015), and *Trypanosoma cruzi* Chagas (Trypanosmoatida: Trypanosomatidae) (Salazar et al. 2015). In vitro and in vivo studies with HIV demonstrated that transmission by bed bug bites was unlikely to occur under natural conditions (Jupp and Lyons 1987, Webb et al. 1989). Bed bugs that were experimentally infected with HBV excreted viral particles in feces for at least 2 mo (Jupp and S. McElligott 1979, Blow et al. 2001). Since bed bugs often defecate on the skin of the host after feeding (Salazar et al. 2015), deposition of HBV particles in feces represents a possible source of HBV infection to humans (Blow et al. 2001). In experimental infections of bed bugs with *Bartonella quintana* (Schmincke) Brenner, the etiologic agent of trench fever, viable organisms can also be released in feces for at least 2 wk (Leulmi et al. 2015). Early studies in South America that looked at natural and experimental infections of bed bugs with *T. cruzi* reported on the potential involvement of these insects in *T. cruzi* transmission in areas where Chagas disease is endemic (Jörg and Natula 1982, Jörg 1992). Recent reports on emerging bed bug populations in Venezuela (Reyes-Lugo and Rodríguez-Acosta 2002), Colombia (Posso et al. 2011), Argentina (Vassena 2016), and Brazil (Bernardes et al. 2015) have triggered the interest of researchers to evaluate the vector competence of bed bugs for transmitting *T. cruzi*. Recently, experimental studies demonstrated that bed bugs can potentially be vectors of *T. cruzi* to mammals (Salazar et al. 2015). Here, we expanded these studies to determine the survivability of *T. cruzi* in bed bug gut contents and establish whether the parasite is transmitted transstadially.

Materials and Methods

Bed Bugs

Bed bug adults and nymphs were obtained from a colony that was maintained in an environmental chamber at 25°C, 50 ± 5% relative humidity, and a 12:12 (L:D) h photoperiod. This colony was originally established from bed bugs collected in Fort-Dix, NJ, in 1973. Insects were regularly fed in the laboratory through a parafilm membrane feeder with defibrinated rabbit blood that was heated to 37°C using a circulating water bath (Montes et al. 2002). Unfed insects were experimentally infected 7 d after emergence in a dark room at ambient temperature between 22 and 25°C.

Trypanosome Culture and Experimental Infection

The *T. cruzi* (Y strain) used in this study was cultured in Grace's Insect Medium and suspended in phosphate buffer solution and was obtained from the Centers for Disease Control and Prevention (CDC, Frank Steurer). Citrated human blood (BioreclamationIVT, Baltimore, MD) was inoculated with *T. cruzi* on the seventh day of growth to have a final concentration of 1×10^5/parasites/ml of blood. Both infected and uninfected blood was warmed to 37°C with a water bath circulator system and offered to the insects in a feeding membrane system similar to the one used by Montes et al. (2002). The system consisted of several custom-made water-jacketed glass feeders with a synthetic membrane (Nescofilm, Alfresa Pharma Corporation, Osaka, Japan) stretched across the bottom through which the insects fed. Bed bugs were placed into 2 oz (60 ml) clear round wide mouth jars. The bottoms of the jars were removed and replaced with a plankton mesh (mesh size 300 µm, BioQuip Products, Rancho Dominguez, CA), which was attached to the jar using methylene chloride to melt the plastic. Bugs were provided with strips of file folder paper of the same height as the jar to allow them to climb to the mesh and reach the blood. Bed bugs were allowed to feed for 20 min and only fully engorged bugs were used. All infected and uninfected bugs were maintained under the same conditions as the colonies. Prior to initiation of the infection, the bed bugs and their gut contents were shown to be free of *T. cruzi* using a polymerase chain reaction (PCR). All work for this study was conducted in a biosafety level 2 laboratory, and this experiment was approved by the Institutional Biosafety Committee at New Mexico State University (approval number # 1210RANA0201).

A group of adult bed bugs was infected as above, surface sterilized with 0.05% sodium hypochlorite, and 70% ethanol as previously described (Zurek et al. 2000). Always four insects were analyzed by PCR at 2 h, 1, 2, 3, 7, 9, and 14 d after infection for evidence of *T. cruzi* DNA. DNA extraction of whole bed bugs was performed using DNeasy Blood and Tissue extraction kit (QIAGEN, Valencia, CA). Each bed bug was macerated and processed individually during the entire detection process. The DNA collected from each sample was standardized to 100 ng/ml. Detection of *T. cruzi* was performed using PCR. In order to detect the presence of *T. cruzi* in this assay, we used TCA1 (5′-AGGCAGGACGGCAGCGGTGC-3′) and TCA2 (5′-GGTGRCCGCGTGAGAGTGAG-3′) primers (Integrated DNA Technologies, Coralville, IA), which targeted the repetitive element TclRE (Virreira et al. 2003). The PCRs were performed in 20-µl reactions containing 13.2 µl of UltraPure Distilled Water (Invitrogen, Waltham, MA), 0.4 µl of Platinum Taq DNA Polymerase (Invitrogen) 0.2 µl of forward and reverse primers (each at 1 ng/µl) and 1 µl of extracted bed bug DNA (from a bed bug fed either uninfected or *T. cruzi* infected blood). In every group of PCRs, positive and negative controls were used. For the positive controls, DNA that was extracted from the pure culture of *T. cruzi* or a customized TCA gBlock Gene Fragment (Integrated DNA Technologies) replaced the insect DNA in a PCR well. For the negative controls, UltraPure Distilled Water replaced the insect DNA. Target amplification was performed under the following conditions: a hot start cycle of 95°C for 90 s, followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 70°C for 15 s, and extension at 70°C for 15 s. Finally, the PCR was completed by an additional cycle of 70°C for 5 min to allow the products to completely extend. All PCR products were separated in electrophoresis gel composed of 2% agarose in a 1× SB buffer solution and visualized using ultraviolet light with Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA). Amplicons from four positively infected samples and both positive controls were sent to an independent laboratory (Sequetech, Mountain View, CA) for sequencing to verify their identity. Sequencing results were aligned using Geneious version 9 integrated DNA sequence analysis package (Kearse et al. 2012) in order to confirm that the detected amplicons were the same strain of *T. cruzi* used for inoculation.

To create cohorts of infected and uninfected adult male bed bugs, we exposed one group of insects to *T. cruzi* in human blood and the other to human blood without *T. cruzi*. Fed bugs were separated and held at 25°C and 50 ± 5% RH. The bed bugs from both groups were fed once a week using uninfected human blood until the end of the experiment. We extracted the gut contents from one to three bed bugs at variable intervals until day 97 following the technique used by Jörg and Natula (1982). For microscopic observation, gut contents were diluted with saline solution and compressed between a slide and a cover slip. The entire gut sample was examined for the presence of the *T. cruzi* at 40× magnification. We considered a sample to be positive when at least one form of the parasite displaying typical tumbling behavior was observed (Uppaluri et al. 2011, Salazar et al. 2015).

Two groups of 30 third-instar nymphs were fed with human blood or *T. cruzi* infected blood and maintained under the same conditions as the colonies. They molted regularly within approximately 4–5 d into fourth-instar nymphs. Four nymphs of both groups were surface sterilized as above and homogenized in 20 µl of saline solution. We then examined samples microscopically for the presence of *T. cruzi*. We recorded the total number of infected and uninfected insects. The remaining cohorts of molted nymphs were fed two more times 6–7 d apart on uninfected blood to obtain fifth-instar nymphs and adults. Three to four newly molted fifth-instar nymphs were either macerated and microscopically examined as above or whole body was analyzed by PCR for evidence of *T. cruzi* DNA. The gut contents of eight emerged adults (four males and four females) were extracted and examined microscopically to observe live forms of *T. cruzi*. Each time a group of bed bugs fed infected blood was analyzed; two to eight uninfected specimens were also evaluated.

Results and Discussion

A flurry of research has demonstrated in the last decade that many human pathogens are naturally associated with bed bugs, can be experimentally transferred to bed bugs via blood meals, and can remain in the insect digestive system or be excreted in feces. Recently, the possibility of transmission of *T. cruzi* by bed bugs has drawn the attention of researchers and public health authorities due to the appearance of populations of bed bugs in areas endemic for Chagas disease. To complement studies on vector competence of bed bugs for this disease, we experimentally infected bed bug adults with *T. cruzi* using an artificial model of infection. We recovered, DNA of *T. cruzi* from all bed bugs fed *T. cruzi* infected blood up to 14-d postinfection (Table 1). Groups of bed bugs fed on uninfected blood (four specimens analyzed each day) were PCR negative. The DNA sequences of the PCR amplicons from the *T. cruzi* culture used for inoculation was 100% identical to the *T. cruzi* recovered from sampled bed bugs and several *T. cruzi* sequences present in GenBank (EU825831.1, EU825812.1, EU825810.1, EU825825.1, and others). These data confirm the identity of *T. cruzi* recovered from infected bugs and show that parasite DNA can be recovered at least 2 wk after the uptake of an infected bloodmeal.

The gut contents of experimentally infected male bed bugs were also examined microscopically over a 97-d period. Overall, we observed live forms of the parasite in 97% (29/30) of gut samples from *T. cruzi*-exposed groups (Table 1). No live forms of *T. cruzi*

Table 1. Results of gut content microscopy and whole-body PCR of male adult bed bugs fed human blood that was infected with *T. cruzi*

Time postfeeding (d)	PCR of whole body (no. positive per total)	Microscopy of gut contents	
		Fed on blood without *T. cruzi*[a] (no. positive per total)	Fed on blood with *T. cruzi*[a] (no. positive per total)
1	4/4	0/3	2/2
2	4/4	0/2	3/3
3	4/4	—	—
5	—	0/1	2/2
6	—	—	2/2
7	4/4	—	—
8	—	0/2	2/3
9	4/4	—	—
10	—	0/3	2/2
12	—	0/1	3/3
14	4/4	—	—
30	—	0/2	3/3
35	—	0/1	1/1
37	—	—	2/2
42	—	0/2	2/2
49	—	0/1	2/2
68	—	—	1/1
97	—	—	2/2

Dash (—) represents days where no examination was conducted.

[a]A group of male adult bed bugs was fed blood either infected with *T. cruzi* (1×10^5/parasites/ml of blood) or uninfected at day 0. Both groups were evaluated at specific days, while the remaining living insects were fed once a week until examination. A male adult ingests approximately 6.0 µl of blood; therefore, it is estimated that 600 trypanosomes were ingested by each male.

were detected in bed bugs that were fed on uninfected blood (0/18) (Table 1). According to these results, bed bugs can acquire *T. cruzi*, maintain it, and potentially transfer it to humans via feces. This way of transmission was experimentally proven in mice which acquired the *T. cruzi* infection from feces of infected bed bugs (Salazar et al. 2015). Several components of the immune system of blood feeding insects are activated after ingestion of an infected bloodmeal affecting the survivability of microorganisms (Garcia et al. 2010a). In triatomines, a sudden reduction in the number of infective stages of T. *cruzi* (trypomastigotes) in the gut is evident after fresh blood ingestion which is associated with the antiparasitic activity of the complement system, digestive enzymes, hemolysins, and agglutinins (Garcia et al. 2007, Ferreira et al. 2016). Along with the insect's defensive mechanisms, symbionts and nonsymbionts microbial communities contained in the digestive tract of triatomines have also been recognized as potential players in modulating vector competence for parasite transmission (da Mota et al. 2012). There is limited information regarding defensive mechanisms of the digestive systems of bed bugs that determine the viability of ingested microorganisms. However, intrinsic physiological mechanisms that occur in the gut content of cimicid bugs after feeding might explain the viability of *T. cruzi* in our bed bugs. In *C. lectularius* (Vaughan and Azad 1993) and the related *Cimex hemipterus* L. (Hemiptera: Cimicidae) (Azevedo et al. 2009), a perimicrovillar membranes is readily formed in the bug's midgut after blood intake. This membrane surrounds the bloodmeal and delays its digestion by the insect's digestive enzymes for 6–18 h after blood intake (Vaughan and Azad 1993). The time lag before digestive breakdown in the insect midgut and the availability of a nutrient rich environment might also favor the survival of the protozoan and its development into forms that migrate later to the intestine and rectum (Vaughan and Azad 1993, Kollien and Schaub 2000). Further studies on interactions between *T. cruzi*, immunological and biochemical mechanisms of bed bug's digestive system, and their intestinal microbial community might provide new insights into basic mechanisms that explain the survival and persistence of *T. cruzi* in bed bugs and other insect hosts.

Although we did not quantify life stages of *T. cruzi* in gut contents, evidence suggests that *T. cruzi* can develop into effective stages and be maintained in bed bugs (Chang and Chao 1999). Parasite development was demonstrated by Jörg (1982), who observed *T. cruzi* evolutive forms colonizing the midgut of bed bugs 30-d postinfection. High numbers of epimastigotes and infective trypomastigotes were also observed in gut contents of experimentally infected *C. lectularius* with peaks of these forms occurring 2 wk after feeding (Chang and Chao 1999). Interestingly, these authors observed more parasite forms in bed bugs than in two natural hosts of *T. cruzi*, *Rhodnius prolixus* Stål (Hemiptera: Reduviidae) and *Triatoma infestans* Klug (Hemiptera: Reduviidae) (Chang and Chao 1999). According to these results, the bed bug's digestive system is a hospitable environment for the survival and development of *T. cruzi*. We detected live forms of *Trypanosoma* in gut contents of bed bugs until day 97, the last day we dissected insect guts (Table 1). Longer survival time of *T. cruzi* in gut contents (for up to 320 d) was reported in bed bugs that were experimentally infected with a wild strain of *T. cruzi* (Jörg and Natula 1982, Jörg 1992). In contrast, parasite survival diminished in bed bugs that were infected with a *T. cruzi* strain cultured in the laboratory for many years (Chang and Chao 1999). In this regard, as reported with triatomines, *T. cruzi* kept too long in culture may affect the course of their infections in insect hosts, and this might explain the reduced persistence of *T. cruzi* in some studies (Garcia et al. 2010b).

We have molecular and microscopic evidence that *T. cruzi* transstadially persists in bed bugs. Overall, we observed live forms of *T. cruzi* in 93% (14/15) of molted insects from the third-instar nymph group initially exposed to *T. cruzi* (Table 2). After infecting nymphs with *T. cruzi* at the third nymphal stage, we examined a

Table 2. Microscopy and molecular detection of *T. cruzi* in whole bodies or gut content of molted bed bugs infected with *T. cruzi* as third-instar nymphs

Group[a]	Stage	Treatment	Gut microscopy	PCR
+	Third instar[b]	Fed blood with *T. cruzi*	—	—
–	Third instar	Fed blood without *T. cruzi*	—	—
+	Fourth instar	Fed blood without *T. cruzi*	4/4	—
–	Fourth instar	Fed blood without *T. cruzi*	0/4	—
+	Fifth instar	Fed blood without *T. cruzi*	2/3	3/4
–	Fifth Instar	Fed blood without *T. cruzi*	0/2	0/2
+	Adults	—	8/8	—
–	Adults	—	0/8	—

[a]A group of third-instar nymphs was fed blood either infected with *T. cruzi* (1×10^5/parasites/ml of blood) (+) or uninfected (–). Cohorts of insects were evaluated after each molting, while the remaining living insects were fed a clean blood meal to induce molting into the next life stage.

[b]A third-instar nymph ingests approximately 2.2 µl of blood; therefore, it is estimated that 220 trypanosomes were ingested by each nymph.

cohort of whole bodies of four newly molted fourth-instar nymphs and all had live *T. cruzi* in their alimentary canal (Table 2). In addition, from a cohort of newly molted fifth-instar nymphs, two out of three nymphs had microscopic evidence of the parasite, whereas three out of four of these nymphs were positive by PCR. In microscopy of homogenates of eight adults, all contained live forms of the parasite (Table 2). These results confirm that *T. cruzi* survives molting, a frequent biological event in bed bugs (Usinger 1966).

With this evidence, it can be argued that survivorship of *T. cruzi* through nymphal stages until adulthood, a stage that offers suitable conditions for development of infective stages (Jörg and Natula 1982, Jörg 1992, Chang and Chao 1999), and long persistence of *T. cruzi* in the insect may have important epidemiological implications for Chagas diseases. Because bed bugs can live for at least a year (Usinger 1966), adults and nymphs infected with *T. cruzi* may be a source of infection to hosts for long periods. Our evaluations complement competence studies on bed bugs as vectors of Chagas disease. Parasitological and quantitative analysis in situ, with help of mathematical models, will improve our knowledge of the potential role of bed bugs in transmitting *T. cruzi* through a host population.

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