

Attempt to Artificially Infect *Cimex lectularius* (Hemiptera: Cimicidae) with *Bartonella henselae* (Alphaproteobacteria: Bartonellaceae)¹

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Abstract Bed bugs (Hemiptera: Cimicidae) are common, hematophagous ectoparasites of humans and other animals and are experiencing an international resurgence. Cimicids have been suspected in the transmission of many disease agents, including *Bartonella* species; however, disease transmission of any kind has not yet been confirmed in natural disease cycles. *Bartonella* spp. are transmitted by a variety of arthropods, including fleas, lice, and sand flies, but the potential role of bed bugs in transmission remains unknown. In this study, we used an artificial membrane to feed rabbit blood, either infected or uninfected with *Bartonella henselae* Regnery et al. (Alphaproteobacteria: Bartonellaceae) to two groups of adult *Cimex lectularius* L. After 2 wks, the presence of *B. henselae* in the gut and salivary glands of bugs was assessed via PCR and transmission electron microscopy (TEM), respectively. Although 4 of 10 bed bug guts showed evidence of *B. henselae*, we were unable to visually detect *B. henselae* in any of the salivary gland TEM images.

Key Words *Bartonella*, bed bugs, cat scratch disease, vector-borne disease, transmission electron microscopy

Bed bugs (Hemiptera: Cimicidae) are obligate hematophagous ectoparasites of humans and animals. Two species, namely, *Cimex lectularius* L. and *Cimex hemipterus* F., are commonly associated with humans and are of increasing concern due to a rise in reports of bed bug infestations in multiple countries, particularly in residential homes, hotels, retirement homes, and more (Bencheton et al. 2011, Delaunay 2012, Doggett and Russell 2009, Durand et al. 2012, Hwang et al. 2005, Ralph et al. 2013). A variety of factors have contributed to the rise in bed bug populations, such as an increase in pesticide resistance, increased global travel, and a decreased ability of the public to identify and thus control bed bugs (Doggett and Russell 2009, Doggett et al. 2012, Harlan 2007, Kweka et al. 2009,

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Lilly et al. 2016, Potter 2006, Romero et al. 2007, Tawatsin et al. 2011). As humans are encountering bed bugs with increasing frequency, it is important to understand possible threats to public health posed by bed bugs, including their potential competency as biological vectors of disease (Doggett et al. 2012, Goddard and deShazo 2009, Leulmi et al. 2015).

The transmission of disease agents by bed bugs is controversial and incompletely understood. Bed bugs have been implicated in the biological transmission of many diseases, including human immunodeficiency virus and hepatitis B, but the transmission of either disease has not been confirmed in natural or laboratory settings (Blow et al. 2001, Burton 1963, Delaunay et al. 2011, Goddard and deShazo 2009, Jupp and Lyons 1987, Silverman et al. 2001, Vall Mayans et al. 1994, Webb et al. 1989). Pathogens such as *Rickettsia parkeri* Lackman et al., *Trypanosoma cruzi* Chagas, and *Bartonella quintana* (Schmincke) Brenner et al. can be maintained in the gut of bed bugs for up to 2 wks, and the transmission of *T. cruzi* to mice has been demonstrated in a laboratory setting (Salazar et al. 2015). However, the role of these pathogens in natural disease cycles remains unknown (Blakely et al. 2018, Goddard et al. 2012, Leulmi et al. 2015, Salazar et al. 2015). Bed bugs have not been confirmed in the biological transmission of any pathogens to humans, but proactive investigations into their potential to transmit diseases mechanically or biologically are needed to anticipate any looming effects to public health.

Bartonella is a genus of Gram-negative, rod-shaped bacteria that have been isolated from a wide array of mammals (Angelakis and Raoult 2014, Jacomo et al. 2002). These intracellular bacteria are unique in their ability to cause a prolonged bacteremia in their hosts with few, if any, symptoms (Jacomo et al. 2002). Several species, including *Bartonella bacilliformis* Strong et al., *B. quintana*, and *B. henselae* have been implicated in human diseases, such as Carrión disease, trench fever, and cat scratch disease, respectively. Different *Bartonella* species are known to be transmitted by a variety of arthropods, including fleas, lice, and sand flies. *Bartonella henselae*, the causative agent of cat scratch disease, is traditionally thought to be transmitted to humans through scratches delivered by feral or domestic cats. However, an increasing body of literature suggests that the disease ecology of *B. henselae* is more complex, with recent reports implicating various tick species in the bacterial disease cycle (Bai et al. 2015, Bouhsira et al. 2013b, Chang et al. 2001, Cotté et al. 2008, Matsumoto et al. 2008, Mokhtar and Tay 2011, Podsiadly et al. 2007, Sanogo et al. 2003, Wechtaisong et al. 2020, Wechtaisong et al. 2021).

In this study, we investigated the potential of *C. lectularius* to attain *B. henselae*, by attempting to (a) detect the DNA of the bacteria in the guts of bed bugs by performing PCR 2 wks after an infectious bloodmeal and (b) visualize the bacteria in the salivary glands using transmission electron microscopy (TEM) after feeding bed bugs blood spiked artificially with *B. henselae*. This work aimed to further understand the role of *C. lectularius* in the *B. henselae* disease cycle, if any.

Materials and Methods

***Bartonella henselae* culture and growth.** *Bartonella henselae* strain San Antonio 2 267HO04, a human clinical isolate, was obtained from Ed Breitschwedt of

Table 1. Bed bug cohorts and time of treatments applied. In cohort 1, bed bugs were fed uninfected blood and PCR was performed on the guts. In cohort 2, bed bugs were fed uninfected blood and TEM was performed on their salivary glands. In cohort 3, bed bugs were fed blood spiked artificially with *B. henselae* and after 2 wks, salivary glands were removed for TEM analyses, and PCR was performed on the guts.

Treatment Applied to Bed Bugs	Bed Bug Cohort 1, Fall 2020	Bed Bug Cohort 2, Spring 2021	Bed Bug Cohort 3, Fall 2021
No. fed (no. infected with <i>B. henselae</i>)	13 (0)	10 (0)	10 (10)
PCR performed on guts	3	0	10
No. TEM attempted*	0	10	10
TEM of paired salivary glands	n/a**	10	7†
TEM of pronotum	n/a	n/a	1

* No *B. henselae* detected in any TEM sample.

** n/a, TEM was not performed.

† Sample biomass was not sufficient to perform TEM.

North Carolina State University (Raleigh, NC) in a frozen saline stock solution and placed in a -80°C freezer until use. The bacteria were grown on sheep blood agar plates. Following inoculation with *B. henselae*, plates were maintained at 37°C in 5% CO_2 in the dark. For bed bug infection assays (described below), bacteria were collected after 7 d of growth and suspended in sterile phosphate-buffered saline (pH = 7.4; Thermo Fisher Scientific, USA). The bacteria were then diluted with phosphate-buffered saline to approximately 1×10^6 bacteria per ml, with the concentration determined by serial dilution and counting colony-forming units as per Liu et al. (2014).

Bed bug colony maintenance and infection assay. Bed bugs (Harold Harlan strain) were maintained in the lab at $24 \pm 1^{\circ}\text{C}$ and 50–55% relative humidity on a 12:12-h light:dark photo regime at the Tulane National Primate Research Institute in Covington, LA. The following three cohorts of insects, described in further detail below, were assayed: (a) one group fed uninfected blood and analyzed using PCR, (b) one group fed uninfected blood and analyzed using TEM, and (c) one group fed blood spiked artificially with *B. henselae* and analyzed using both PCR and TEM (Table 1).

Bed bugs were fed on defibrinated rabbit blood obtained from Hemostat Laboratories (Dixon, CA) using an artificial membrane system, as described by Goddard et al. (2012). After the infection assay, bed bugs were maintained in the lab for 2 wks at room temperature ($\sim 23^{\circ}\text{C}$). Males and females were not separated, and 30 bed bugs were used in total across all cohorts, which are described as follows: 13 received uninfected rabbit blood (cohorts 1 and 2) and 17 received rabbit blood spiked artificially with *B. henselae* (cohort 3). Blood was spiked with

Table 2. PCR primer sequences used in nested PCR.

Nested PCR Level	Direction	Sequence
External	Forward	5'-ATT ATG CAA TTG CTA TAG GTT TAG AAG CTG A-3'
	Reverse	5'-CAA AGT GAA TTC AAT TTT ATG CTT ATT ATC TAC AG-3'
Internal	Forward	5'-ACG ATC CTG CAG CAA ATG CTC CGA TAA CA-3'
	Reverse	5'-CAG AAA TTT TCC AAG CCC CTG CTA CCA CAT CT-3'

approximately 1×10^6 bacteria per ml, which was the concentration determined by serial dilution and counting colony-forming units. The first cohort was analyzed using PCR in fall 2020, and the second cohort was analyzed in spring 2021. The third cohort was analyzed in fall 2021. After the 2-wk waiting period, 20 first-instar nymphs were found in the vials of cohort 3, which were removed, pooled, frozen, and included in the PCR analyses.

DNA isolation and PCR. Following methods described in previous experiments (Jupp et al. 1991, Ogston et al. 1979), bed bug DNA from cohorts 1 and 3 was isolated 2 wks postfeeding from guts using the Qiagen DNeasy Blood and Tissue Kit. Note that salivary glands were removed prior to DNA extraction for later analyses using TEM (see below). Briefly, guts were individually homogenized through manual grinding with a mortar and pestle. Following homogenization, we followed the manufacturer's protocol "DNA Isolation from Tissue Samples" and then proceeded with a precipitation of total DNA. DNA was resuspended in Tris-EDTA buffer. Optical density 260/280 readings were obtained using a Nanopore spectrophotometer.

A nested PCR of bed bug DNA was performed to amplify *B. henselae* DNA in bed bug guts after salivary gland removal. Nested PCR consists of two subsequent rounds of PCR, with the product of the first round being used in the second round to increase likelihood of *B. henselae* DNA amplification. The amplification conditions were as follows: external PCR was performed at 98°C for 30 s; followed by 98°C for 10 s, 63°C for 30 s, and 72°C for 18 s for 30 cycles; and 72°C for 2 min. Similarly, internal PCR was performed at 98°C for 30 s; 98°C for 10 s, 63°C for 30 s, and 72°C for 18 s for 30 cycles; and 72°C for 2 min. Primer concentration was 0.4 mM, and primer sequences, specific to *B. henselae*, are listed in Table 2. Distilled deionized water was used as a negative control in the PCR reaction, and *B. henselae* DNA was used as a positive control. The resulting amplicons were confirmed by gel electrophoresis in a 1.5% agarose gel. The external PCR amplicon was 605 base pairs, and the internal PCR amplicon was 204 base pairs. To avoid contamination, 70% ethyl alcohol and DNase Away was used.

Transmission electron microscopy. TEM offers visualization of the salivary gland at the cellular level, allowing the identification of molecular structures and, in this case, presumably artificially introduced bacteria. Two weeks after insects fed

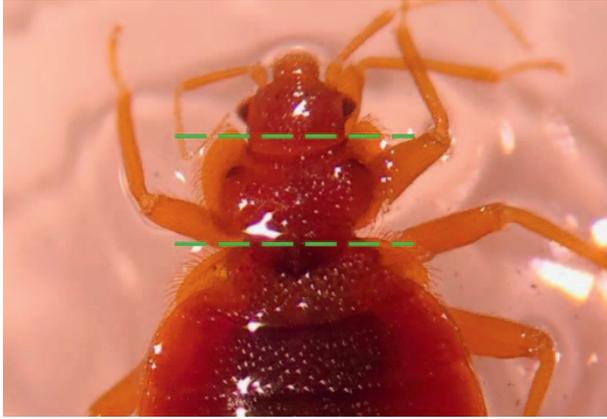


Fig. 1. Dorsal view of a bed bug. Green, dashed lines depict where cuts were made to extract the pronotum, which was transferred into a fixative to perform TEM.

on either uninfected blood or blood spiked artificially with *B. henselae*, salivary glands of bed bugs were dissected by first placing the insects on double-sided sticky tape in the bottom of a petri dish filled with 2.5% glutaraldehyde and CaCl_2 in 0.1 M sodium cacodylate buffer, the primary fixative in the TEM process. Once salivary glands were located, they were removed using fine forceps and placed into the previously mentioned primary fixative for 2 h. Salivary glands from 10 bed bugs (cohort 1) and 7 bed bugs (cohort 3) were pooled in one tube of primary fixative (5–8 glands per tube). For three insects (cohort 3), the salivary gland was not dissected and removed but rather the whole bed bug pronotum was removed using a small scalpel (Fine Science Tools, Foster City, CA) and suspended in fixative (Fig. 1).

The samples underwent a graded ethanol dehydration series and a gradual infiltration with propylene oxide and finally with epoxy resin. Samples were embedded in epoxy resin and then light microscopy was conducted to locate the salivary gland in the pronotum sample. Once it was located, TEM was performed. Ultrathin sections were stained with 1% uranyl acetate and analyzed using a TEM (JEOL 1230 120kV; JEOL USA) at the Institute for Imaging and Analytical Technologies at Mississippi State University. About 10–15 of these ultrathin sections were placed on a grid, and multiple grid cells were examined, resulting in over 30 fields of view. Given the precision of TEM, we viewed one of three possible pronotum samples and selected one from a bed bug whose gut was positive for *B. henselae* DNA. By observing previously published TEM images of *B. henselae*, the morphology of the bacteria could be recognized (Kordick and Breitschwerdt 1995). Similarly, *C. lectularius* salivary gland morphology was compared to previously published TEM images of *C. hemipterus* (Serrão et al. 2008). To identify *B. henselae* in the salivary glands, we were looking for similarly shaped structures as seen in previous electron microscopy micrographs.

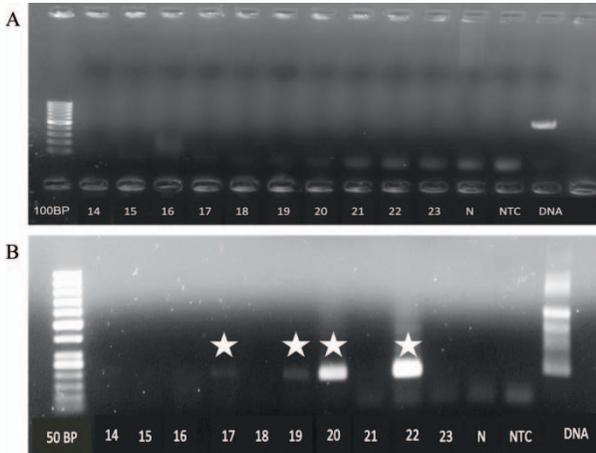


Fig. 2. Image of gel electrophoresis, depicting results from the first round of PCR (A) and the second round of PCR (B) conducted on bed bugs fed blood artificially spiked with *B. henselae*. NTC means no template control, or negative control; DNA is *B. henselae* DNA, the positive control; N is nymph DNA. Numbers 14–23 denote guts of individual bed bugs fed blood spiked artificially with *B. henselae*, with the salivary glands removed. (A) No samples were positive in the first round of PCR for *B. henselae* DNA. (B) Four samples (lanes 17, 19, 20, and 22) denote samples positive for *B. henselae* DNA, which are shown with stars above the lane.

Results

Two weeks after the blood meal, DNA was isolated from the guts of bed bugs (after removal of the salivary glands), and a nested PCR was performed to ascertain the presence or absence of *B. henselae* DNA. Of the 13 bed bugs fed with uninfected blood and assayed with PCR, none were positive for *B. henselae*.

In the first round of PCR on guts of bed bugs fed blood spiked artificially with *B. henselae*, none tested positive for *B. henselae* (Fig. 2A). In the second (nested) round of PCR, however, 2 samples showed bright bands, indicating strong amplification of the PCR product (Fig. 2B). Two additional samples had dimmer bands, indicating less amplification of the PCR product (Fig. 2B). In addition, DNA of 20 pooled nymphs was also isolated, and PCR was performed to determine the presence of *B. henselae* DNA; no samples were positive in either round of PCR.

The TEM image of uninfected bed bugs revealed salivary glands consistent with previously published literature (Serrão et al. 2008), without any visually detectable foreign bodies (Fig. 3). The use of TEM on bed bugs fed blood spiked artificially with *B. henselae* (the samples contained entire pronotums) similarly showed a salivary gland without visually detectable foreign bodies, consistent with previously published literature (Serrão et al. 2008). In summary, no structures resembling bacteria were visually detected in any TEM image (Fig. 4).

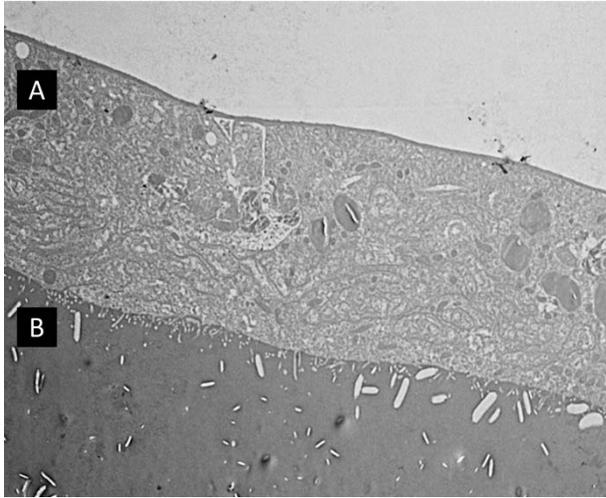


Fig. 3. Image from a TEM of salivary gland tissue (A) and salivary gland lumen (B) dissected from a single bed bug fed uninfected blood.

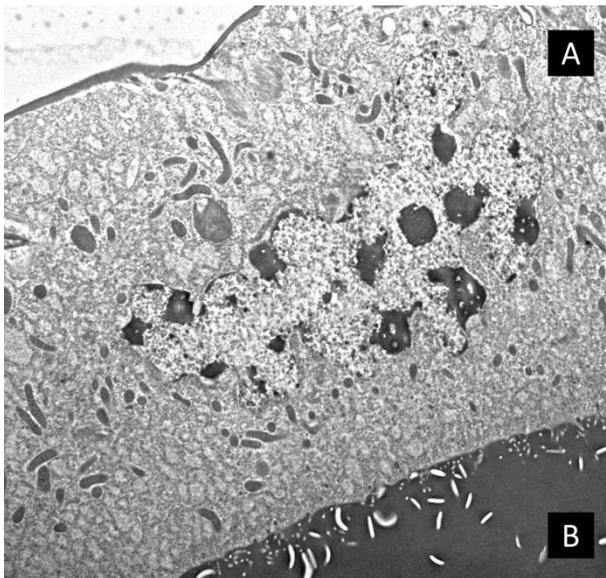


Fig. 4. Image from a TEM of salivary gland tissue (A) and salivary gland lumen (B) of a single bed bug fed blood spiked artificially with *B. henselae*.

Discussion

Bartonella henselae was not detected in salivary glands of bugs fed blood spiked artificially with *B. henselae*. However, there may be other explanations as to why we did not visualize *B. henselae* in the salivary glands. Although care was taken to visualize a representative subsection of approximately 30 fields of view, it is possible that our samples were from areas of the salivary gland without *B. henselae* infection. It is also possible that the inoculum was too low to infect bed bug salivary glands or that not enough or too much time elapsed between feeding and analysis. That is, *B. henselae* may have not yet traveled back to the salivary glands or had been cleared by the insect's immune system. A lack of movement from the gut to the salivary glands could be due to limited bacterial motility, as electron microscopy has detected twitching motility but no flagella on *B. henselae* (Diddi et al. 2013). Lastly, it is possible that *B. henselae* is transmitted fecally, as it is in *Ctenocephalides felis* (Bouche), the cat flea (Bouhsira et al. 2013a, Bouhsira et al. 2013b, Finkelstein et al. 2002, Higgins et al. 1996). Indeed, Finkelstein et al (2002), showed that *B. henselae* can persist in cat flea feces for 3–4 d post-infectious blood meal, increasing in concentration over that period. A related species, *B. quintana*, can be maintained and detectable in bed bug feces for up to 19 d post-infectious blood meal (Leulmi et al. 2015).

Although the PCR data showed evidence of bacterial DNA in multiple bed bug samples, this finding does not imply that the DNA belonged to intact, viable organisms. The DNA may have been left from partially digested bacteria after the blood meal. Additional studies are needed to determine whether DNA found in the guts—or potentially elsewhere in bed bugs or their feces—is viable and/or infectious, including via immunofluorescence assays and culturing.

Cimex lectularius is undergoing a major resurgence, with the frequency of occurrence and range expansion leading to increasing infestations in multiple countries (Doggett et al. 2012, Potter 2006). Thus, it is important to understand the full extent of the clinical and epidemiological implications of living in close association with these hematophagous insects. The direct and diverse effects of bed bugs are well documented, with clinical manifestations including, but not limited to, small cutaneous lesions induced by bites and decreased mental health of individuals living in infested environments (Goddard and deShazo 2009, Goddard and de Shazo 2012). Although we did not detect *B. henselae* in the salivary glands of bed bugs fed blood spiked artificially with *B. henselae*, the role of bed bugs as mechanical and/or biological vectors in disease transmission remains equivocal.

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