Am. J. Trop. Med. Hyg., 100(6), 2019, pp. 1407–1412
doi:10.4269/ajtmh.18-0804

Testing the Competence of *Cimex lectularius* Bed Bugs for the Transmission of *Borrelia recurrentis*, the Agent of Relapsing Fever

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Abstract. In recent years, bed bugs have reappeared in greater numbers, more frequently, and are biting humans in many new geographic areas. Infestations by these hematophagous insects are rapidly increasing worldwide. *Borrelia recurrentis*, a spirochete bacterium, is the etiologic agent of louse-borne relapsing fever. The known vectors are body lice, *Pediculus humanus humanus*. However, previous studies have suggested that bed bugs might also be able to transmit this bacterium. Adult *Cimex lectularius* were artificially infected with a blood meal mixed with bacterial suspension of *B. recurrentis*. They were subsequently fed with pathogen-free human blood until the end of the experiment. Bed bugs and feces were collected every 5 days to evaluate the capacity of bed bugs to acquire and excrete viable *B. recurrentis* using molecular biology, cultures, fluorescein diacetate and immunofluorescence assays. The feces collected on the day 5 and 10 postinfection contained viable bacteria. Immunofluorescence analysis of exposed bed bugs showed the presence of *B. recurrentis* in the digestive tract, even in bed bugs collected on day 20 after infection. Like human body lice, bed bugs can acquire, maintain, and excrete viable *B. recurrentis* that might infect humans through skin lesions. This preliminary work suggests that bed bugs might be competent vectors of *B. recurrentis*. Because bed bugs and body lice may share the same ecological niches, the role of bed bugs in transmitting recurrent fevers deserves further study.

INTRODUCTION

Bed bugs are hematophagous insects that have lived alongside humans for thousands of years. They include two cosmopolitan species, *Cimex lectularius* and *Cimex hemipterus* (Hemiptera: Cimicidae),[1] and both sexes feed on blood. Their life cycle includes five stages from eggs to immatures (nymphs) and then adults. The females lay about five eggs daily throughout their adult lives (up to 500 eggs in life time). Eggs hatch within 4–12 days into first instar nymph. Each stage takes a single 10–20-minute human blood meal every 3–5 days before molting to the next stage.[2,3] They can live for 12 months without feeding and even longer in colder environments.[4] Since 1990s in the United States, Canada, and Australia and 2000s in Europe, bed bugs have reappeared in greater numbers and more frequently.[5] An increase in the frequency of international travel, immigration, changes in pest management methods, and resistance to insecticides may have contributed to this resurgence.[6]

Bed bug bites lead to systemic skin reactions and local reactions.[7] The vectorial potential of these arthropods, in other words their ability to transmit pathogens to humans, has long been suspected but has never been clearly proven. Although many viruses, bacteria or parasites have been suspected in the past, It has been suggested that bed bugs may be vectors of the agents of plague,[8,9] tuberculosis, leprosy,[7,10] filariasis,[11] smallpox,[10] Chagas disease,[12] and yellow fever.[1] The possibility of HIV and hepatitis B virus being transmitted by bed bugs has also been explored, but it was concluded that such transmission is unlikely to occur in bed bugs under natural conditions.[13,14] Some species have been detected in bed bug's feces such as *Brucella melitensis*, *Coxiella burnetii*, *Francisella tularensis*, *Salmonella typhi*, and *Leishmania donovani*.[1] More recently, a study has demonstrated that bed bugs can acquire *Bartonella quintana*, the agent of louse-borne trench fever, and that they eliminate viable organisms in their feces.[15]

Relapsing fevers include vector-borne diseases caused by spirochete bacteria of the genus *Borrelia*, transmitted by lice and soft ticks.[16,17] *Borrelia recurrentis* is known to be the agent of louse-borne relapsing fever (LBRF), which is endemic in Africa, particularly in Ethiopia, and which has been reported in southern Sudan and Rwanda.[18–21] It is transmitted by lice from the human body and is found in areas where people are infected by this arthropod, that is, those living in poor conditions, or during famine and wars in developing countries.[22] *Borrelia recurrentis* is known to be transmitted to humans by skin lesions, by crushing the lice or by contamination with infected feces on scraping lesions. It has been shown that the infested louse excretes *B. recurrentis* in its feces, but it has no effect on lice, which may explain the speed at which an epidemic can develop in parasitized human populations.[17] After incubation for between 4 and 8 days, the disease presents with a period of high fever, associated with pain, rash, and jaundice. This first episode lasts for an average of 6 days. Similar episodes may recur every 2 weeks (with between one and five possible recurrences). Hemorrhagic, neurological, hepatic, and cardiovascular complications may occur. In the absence of treatment, death occurs in 40% of untreated cases. The reference treatment is doxycycline.[22,23]

Infestations by body lice and louse-borne infections are generally associated with reduced social and hygienic conditions caused by civil unrest and economic instability, which explains their reappearance throughout the world.[24] Some of these epidemiological and ecological conditions also promote the development of bed bugs and their contact with humans. In archives on the history of borreliosis, authors cited the detection of cases of relapsing fever in camps and hospitals. In addition, they reported that the complete disappearance of this disease followed the simultaneous destruction of lice and bed bugs.[25,26] However, investigators reported that bed bugs infected with *B. recurrentis* were not infectious for splenectomized squirrels.[27]

Our objective was to further investigate the acquisition of *B. recurrentis* by *C. lectularius* and to demonstrate viability and infectiousness of borreliae in bed bug feces. To do so, we used an experimental model of artificial infection of bed bugs using

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four different approaches: quantitative polymerase chain reaction (qPCR), culture, immunofluorescence, and fluorescein diacetate for the first time for these bacteria.

MATERIALS AND METHODS

Ethical statement. Human blood was obtained from the "Etablissement Français du Sang" (EFS, the French national blood bank) accredited by the Institutional Animal Care of Institut Hospitalo-Universitaire (IHU) Méditerranée Infection.

Adult BALB/c mice obtained from the Charles River Laboratories (Saint-Germain-Nuelles, France) were housed in a specific facility and fed sterile food and water ad libitum. They were inoculated using the intraperitoneal route with feces from bed bugs artificially infected or not infected with *B. recurrentis*.

Mice were handled according to the rules of French Decree No. 2013-118, February 7, 2013, and the experimental protocol (reference APAFIS #01071.02) was approved by the "C2EA-14" Ethics Committee of Aix-Marseilles University, France, and the French Ministry of National Education, Higher Education and Research.

Bacterial strain. *Borrelia recurrentis* strain A1 (American Type Culture Collection 700241), which was isolated from the blood of an Ethiopian patient,[17] was cultured in vitro in 9 mL of Barbour-Stoner-Kelly (BSK-H) medium (Sigma-Aldrich, St. Louis, MO) and 1 mL of rabbit serum (Sigma-Aldrich), 100 µL of rifampicin, and 100 µL of Amphotericin B solution (Sigma-Aldrich). Ten milliliters of the culture medium containing the bacterium was centrifuged at 5,283 *g* for 10 minutes and the pellet was then resuspended in phosphate-buffered saline (PBS), pH 7.2 (BioMerieux, Craponne, France). The concentration was determined by counting the number of bacteria present in 10 µL of the bacterial suspension using a dark-field microscope (Nikon, Champigny sur Marne, France).[17]

Procedure for rearing bed bugs. Bed bugs (*C. lectularius*) were maintained by our team in our laboratory, obtained from a *Cimex* commercial supplier (cimexstore.co.uk; London, England). The breeding containers were placed in the laboratory incubator at 60% humidity and 22°C, the same conditions were used for adults and nymphs.[15] The bed bugs were fed once a week with human blood obtained from the EFS. Two milliliters of blood were placed in a Hemotek artificial feeder machine (Hemotek 5W1; Discovery Workshops, Accrington, UK) covered by Parafilm M (Sigma-Aldrich) that was stretched to twice its length and width.[28]

Infection of bed bugs with *B. recurrentis*. Two separate trials (Trial 1 and Trial 2) were performed using adults *C. lectularius* from the same colony and same age. For each trial, two groups of bed bugs were formed: a control group of 50 bed bugs, and an exposed group of 160 bed bugs. The exposed group was fed with 3 mL of human blood mixed with 200 µL of bacterial suspension of *B. recurrentis*, and the control group was fed with 3 mL of human blood mixed with 200 µL of PBS, pH 7.2 (BioMerieux). Two hundred microliters of blood mixed with bacteria was tested to ensure the presence of *B. recurrentis* using *Borrelia*-specific qPCR by targeting the internal transcribed spacer (ITS).[29] One hundred microliters of inoculum were cultured to ensure the viability of bacteria. All bed bug groups were fed with uninfected blood every 5 days, starting on the fifth day postinfection (dpi) until the end of the experiment.

A third artificial infection of bed bugs (Trial 3) was made to make a video of *Borrelia* living in the feces of bed bugs. A total of 30 *C. lectularius* were fed with 3 mL of human blood mixed with 200 µL of bacterial suspension. The feces were collected 10 days after the infection and 10 µL of infected feces was observed at ×100 magnification, using DMI 6000 microscope (Leica Microsystems, Nanterre, France). Videos were steered by ImageJ software (NIH, Bethesda, MD).

Sampling strategy. A first collection of 10 viable bed bugs and feces from each group (from *B. recurrentis* exposed group and from the control group) was made 24 hours postinfection. Then, 30 viable bed bugs and their feces were collected every 5 days until day 30 postinfection. The feces were collected from a sheet of paper placed at the bottom of the tubes containing the bed bugs and diluted in PBS.

DNA extraction and qPCR detection of *B. recurrentis* on bed bugs and feces. After each collection, the bed bugs were washed individually with 70% ethanol and rinsed twice with distilled water. Each bed bug was dissected using a sterile scalpel. Half of the abdomen was used for real-time PCR detection of the presence of *B. recurrentis*. These samples were incubated overnight at 56°C in 180 µL of G2 buffer (Qiagen, Hilden, Germany) (30 mM Tris-Cl, 30 mM ethylenediaminetetraacetic acid (EDTA), 5% Tween 20, 0.5% Triton X-100, and 800 mM GuHCl) and 20 µL of proteinase K (Qiagen) (activity of 600 mAU/mL solution or 40 mAU/mg of protein). The other half was stored at –20°C.

After lysis of the bed bug samples, DNA extraction was performed on 200 µL of each samples and 200 µL of feces in the automatic extractor EZ1 (BioRobot® EZ1; Qiagen, Tokyo, Japan).[15] The presence of *B. recurrentis* DNA in these bed bugs was assessed by *Borrelia*-specific qPCR by targeting the ITS.[29]

Giemsa staining of the salivary glands of bed bugs. In Trial 2, and for each exposed and control group, the salivary glands were dissected carefully by tearing the head of the bed bugs using a needle under a microscope, every 3 days to evaluate the presence of *B. recurrentis*, from day 1 to day 30 postinfection. The salivary glands were fixed directly on the slide and stained for 5–10 minutes. Staining was tested on a smear of *B. recurrentis* culture sample as a positive control.

Culture of feces. For Trial 1 and Trial 2, 200 µL of the feces and PBS mix of exposed and control bed bugs of each collection was filtered to eliminate a maximum but to allow the passage of borreliae of bacterial contaminant using 0.8-mm filters (Millipore, Sigma-Aldrich) and suspended in 4 mL of BSK-H medium and 500 µL of rabbit serum, 50 µL of rifampicin, and 50 µL of Amphotericin B solution.[17]

Immunofluorescence assay. For both trials, two exposed bed bugs and two control bed bugs were fixed on Carnoy's fixative (chloroform:ethanol:glacial acetic acid, 6:3:1) and embedded in paraffin. An immunofluorescence assay was performed on 3-µm-thick sections of fixed bed bugs. Following deparaffinisation, each sample was incubated with a rabbit anti-*B. recurrentis* strain polyclonal antibody diluted to a concentration of 1:250 in PBS.[30] The pure strain of *B. recurrentis* was used as positive control and the uninfected bed bugs as negative control.

Viability test with fluorescein diacetate assay (FDA). To test the viability of *B. recurrentis* in the feces, a FDA (Sigma-Aldrich) was used to measure enzyme activity produced by bacteria in a sample. Ten milligram of FDA was dissolved in dimethyl sulfoxide (Sigma-Aldrich) at a concentration of 10 mg/mL and was used to assess the viability of *B. recurrentis* on feces. Plates were incubated in dark at 4°C for 15 minutes and observed at ×100 magnification, fluorescence at 518 nm, and

excitation at 494 nm using DMI6000 fluorescent microscope (Leica Microsystems). The images were viewed using Metamorph software (Molecular Devices Corp., Sunnyvale, CA).[31] The pure strain of *B. recurrentis* was used as positive control and the feces of uninfected bed bugs as negative control.

Inoculation of feces to mice. Two hundred microliters of qPCR-positive feces were inoculated intraperitoneally into female BALB/c mice (7-week-old) obtained from Charles River Laboratories. One mouse was inoculated on day 5 and 1 at day 10. Blood and organ specimens (brain, liver, spleen, and lung) were taken 24 hours after inoculation. Samples were incubated overnight at 56°C in 180 µL of G2 buffer (30 mM Tris-Cl, 30 mM EDTA; 5% Tween 20, 0.5% Triton X-100; and 800 mM GuHCl) and 20 µL of proteinase K (activity of 600 mAU/mL solution or 40 mAU/mg of protein). After lysis, DNA extraction was performed on 200 µL of each sample in the EZ1 automatic extractor (BioRobot EZ1; Qiagen). The presence of *B. recurrentis* DNA was assessed by *Borrelia*-specific qPCR by targeting the ITS.[29]

RESULTS

Acquisition of *B. recurrentis* by bed bugs. Throughout the follow-up period of the two trials, almost all the bed bugs feed in each group. The mortality rate was not significantly different between the two groups of exposed (30/160 for Trial 1 and 36/160 for Trial 2) and control (6/50 for Trial 1 and 15/50 for Trial 2) bed bugs (*P*-value 0.1).

In Trial 1, the qPCR detected the presence of the DNA *Borrelia* in 42 of the 130 exposed bed bugs (32.30% infection rate), with threshold cycle (Ct) values ranging from 29.72 to 35.31, whereas in Trial 2, 50 of the 124 exposed bed bugs (40.32% infection rate) with Ct values ranging from 30.19 to 35.85. All control bed bugs tested negative.

***Borrelia recurrentis* in the bodies of bed bugs.** Immunofluorescence analysis of exposed bed bugs from the Trial 1 showed the presence of *B. recurrentis* in the digestive tract and legs, even in bed bugs collected on day 20 after infection for both trials (Figure 1). The bed bugs used as controls did not emit any fluorescence. In Trial 1, the salivary glands of the positive bed bugs in the qPCR were examined, but no *Borrelia* was detected. Furthermore, no *Borrelia* was observed in bed bugs that tested negative or in controls.

Detection of *B. recurrentis* in feces. During the first 4 dpi of Trial 1, the feces tested negative by qPCR. The feces collected on days 5 and 10 postinfection were positive, with a Ct of 35.20 and 34.17 respectively, and with a Ct of 35.56 and 33.11 for the second trial. After day 10 postinfection, all bed bugs tested negative until the end of the trial. Uninfected bed bug feces, which were used as a negative control, tested negative by qPCR.

On days 5 and 10 after infection in Trial 1, a large number of motile *B. recurrentis* was detected 7 days after cultivation under dark-field microscope from bed bug feces after filtration on a 0.8-mm filter. The two samples of feces collected on days 5 and 10 postinfection were FDA positive. The viable bacteria emitted green fluorescence, and *Borrelia* was distinguished by its elongated shape similar to the positive control prepared with the pure *B. recurrentis* strain (Figure 2). Using Giemsa-stained smears, and for the two samples, bacteria were clearly visible in their distinguished form (Figure 3). These results were confirmed in Trial 2.

In Trial 1, the inoculation of infected (qPCR positive) and control feces collected on day 5 postinfection had no effect on the first inoculated mouse, and all blood smears performed every 3 days were negative. However, after inoculation of the feces collected on day 10 postinfection to another mouse, all the mouse's organs were positive for *B. recurrentis* by qPCR, including the brain (Ct 35.08), lungs (Ct 31.75), liver (Ct 27.49), and spleen (Ct 27.49). In Trial 2, blood and organs collected from the euthanized mouse inoculated with feces collected on day 10 postinfection were positive, with the following Ct: 35.14 for the blood, 31.58 for the brain, 35.17 for the liver, and 34.60 for the spleen.

In addition, the video made of the feces sample from Trial 3, taken on day 10 after infection, showed the presence of *B. recurrentis*, characterized by its spiral shape and motile character (Video link: https://www.youtube.com/watch?v=beg3Xz3gnbA).

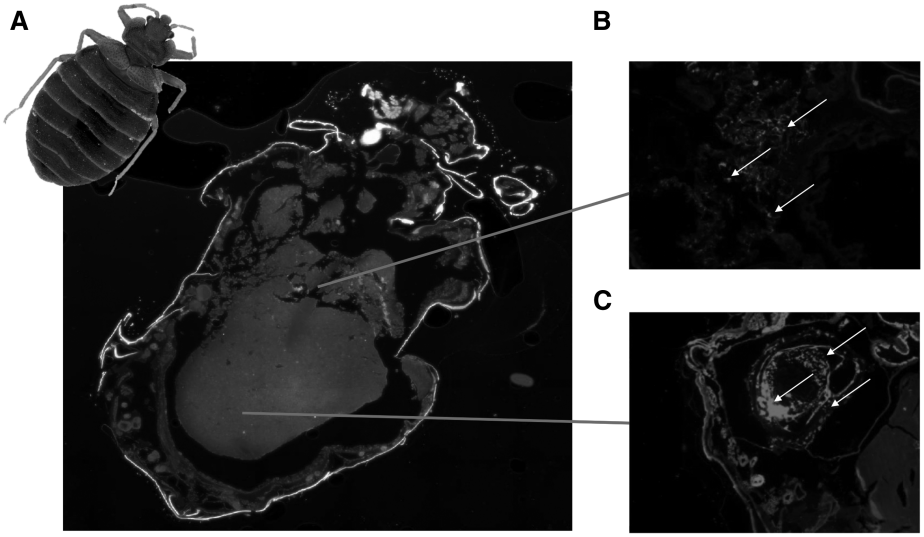

FIGURE 1. (**A**) Localization of *Borrelia recurrentis* inside the body of infected *Cimex lectularius*. Because of the bed bug's dimensions, several photographs were taken, and the complete body was reconstituted by digital overlay. (**B** and **C**) White arrows indicate the fluorescence emitted by *B. recurrentis* in the digestive tract of the infected bed bugs (immunofluorescence staining and confocal microscopy; original magnification, ×100). This figure appears in color at www.ajtmh.org.

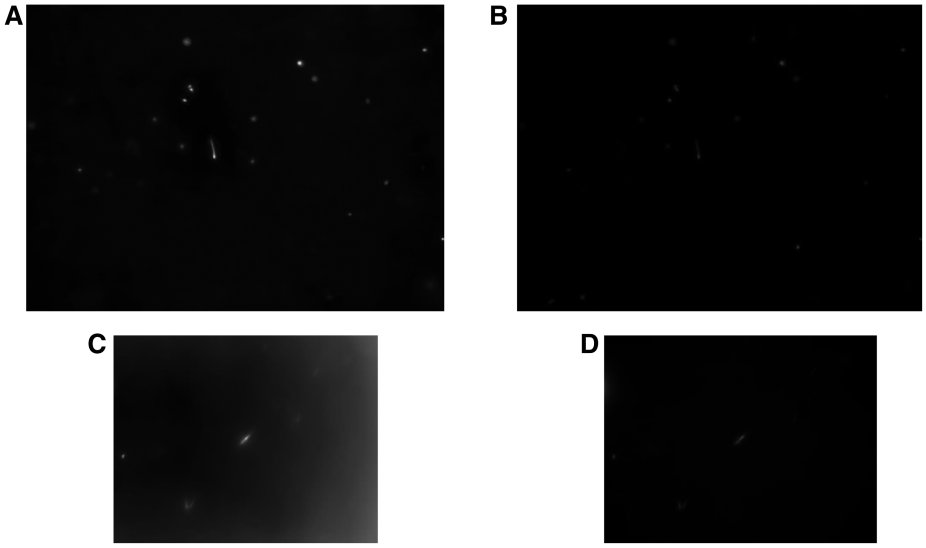

FIGURE 2. (**A** and **C**) Representative images of *Borrelia recurrentis* feces on day 5 postinfection and the positive control, respectively, observed with a fluorescence microscope equipped with a Spot slider color camera using fluorescein diacetate assay stain. (**B** and **D**) Representative images of *B. recurrentis* feces on day 5 postinfection and the positive control, respectively, observed with a fluorescence microscope equipped with a Spot slider color camera using 4',6-diamidino-2-phenylindole. This figure appears in color at www.ajtmh.org.

DISCUSSION

In this work, we demonstrated the acquisition and elimination of live *B. recurrentis*, the agent of LBRF, by bed bugs. The mortality rate of infected bugs was low and was not statistically different from that of the control bed bugs, suggesting that *B. recurrentis* is not pathogenic for *C. lectularius*, such as for lice. The bacteria can survive in bed bugs until day 20 postinfection, located in the midgut and hindgut of the bed bugs' digestive tract.

Various approaches were used in this study to detect the acquisition and elimination of live *B. recurrentis* in *C. lectularius*, because molecular tools cannot establish the viability of the bacteria in the insect's body or feces. Here, the qPCR-positive samples were cultivated in BSK-H medium, and they were positive after 1 week. In addition, the FDA test was performed on the same samples and for the same purpose. This assay has already been tested on *Borrelia burgdorferi*,[32] but this is the first time that it has been developed on *B. recurrentis*. The emission of green fluorescence confirms

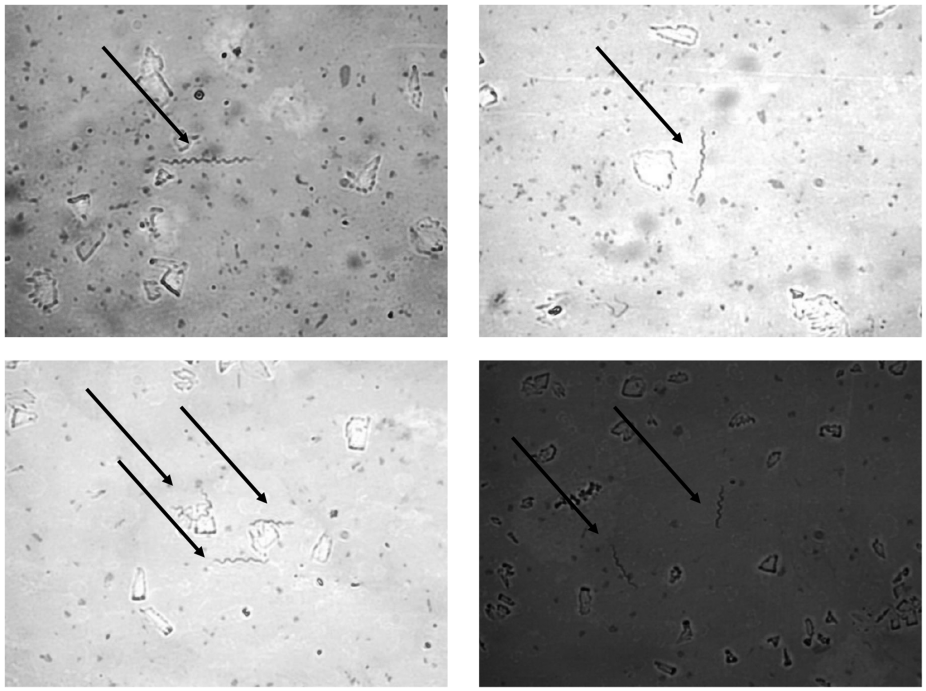

FIGURE 3. Giemsa-stained smear feces collected on day 10 postinfection showing spirochetes. *Borrelia recurrentis* bacteria are indicated by black arrows. This figure appears in color at www.ajtmh.org.

the viability of the bacteria. Indeed, only living cells are able to actively transform nonfluorescent FDA into green fluorescein after enzymatic activity (intracellular hydrolysis), considered to be a sign of viability.[33] Finally, when mice were inoculated with infected samples collected on day 10 after exposure, all organs were infected, including the blood, brain, spleen, and lungs. Inoculation of the feces collected on day 5 postinfection did not cause any infection to mice, which could be explained by a lower quantity of bacteria in the feces on day 5 postinfection, or by the fact that a small number of mice were used. It should be recalled that our goal was not to make a mouse model of infection but to obtain another piece of evidence that the *Borrelia* eliminated in the bed bug feces were alive.

The immunofluorescence assay showed the location of *B. recurrentis* in the body of the bed bug. The bacteria are disseminated in the digestive tract and the hemolymph, although invasion of the bacteria to the hemolymph could be due to the manipulation and the fixation process of the tissues. However, we did not find any bacteria in the salivary glands using Giemsa staining, which makes the theory of transmission by bite unlikely.

All these data support the fact that bed bugs exposed to *B. recurrentis* can acquire the bacterium and eliminate it alive in their feces, suggesting that bed bugs are potential vectors of *B. recurrentis*.

However, the vectorial competence of an arthropod is the ability to acquire a pathogen, spread and/or develop it, and transmit it to a vertebrate host. The vectorial capacity is the result of the vectorial competence and the bio-ecology of the vector (abundance, longevity, trophic preferences, etc.) over which environmental factors act.[34] Bed bug vectorial competence is an element of vectorial capacity and corresponds to the bed bug's intrinsic ability to become infected, to keep the pathogen alive, and to transmit it. In our experiments, we only addressed the vectorial competence of *C. lectularius* to transmit *B. recurrentis*.

The worldwide distribution of bed bugs according to their vectorial capacity, their hematophagous character, and their intimate association with humans has attracted the interest of the medical community in recent years. An increasing number of infestations have been reported in America, Australia, Asia, and Africa[1,15] in various environments, including private houses, apartments, military camps, and refugee camps.

Since the end of the First World War, relapsing fever has been a major public health problem. Currently, its spread is increasing and several cases have been detected in Europe.[35] In 2015, 15 cases of LBRF were reported among refugees in Bavaria, Germany. We assumed that they probably acquired this infection in their country of origin or during their migration.[36] In the same year, four cases of asylum seekers in Switzerland and two cases in Finland showed symptoms of LBRF, and sequencing of 16S rRNA gene proved the presence of *B. recurrentis* in blood samples of patients.[37] In Italy, in 2015, the first case of imported LBRF diagnosed in northern Italy in a Somali refugee was described.[35] In the same year, three other cases of LBRF were reported in three different Italian cities. These patients had migrated from Somalia and arrived in Italy after having traveled to several African countries and crossed the Mediterranean Sea.[38]

Lice and bed bugs, a very harmful duo for human health, develop in particular circumstances (wars, poverty, promiscuity, and captivity) and can share the same habitats. Migrants arriving with *B. recurrentis* infection transmitted by lice may come into contact with bed bugs when they arrive in areas with poor living conditions in Europe or elsewhere in nonendemic areas. This means that the bacteria may encounter local potential vectors, such as bed bugs. Therefore, even in the absence of body lice, there may be a risk of relapsing fever related to the presence of bed bugs. The diagnosis of relapsing fever, prevention of transmission, and entomological monitoring of bed bugs in refugee and migrant camps are crucial steps in preventing this epidemic.

CONCLUSION/SIGNIFICANCE

The present study shows that bed bugs can acquire, maintain, and excrete viable *B. recurrentis*, suggesting that bed bugs might be competent vectors of *B. recurrentis*. We did not test whether artificially infected bed bugs can transmit *B. recurrentis* to an animal (mouse or rabbit). This is one limitation of the study, but the currently known transmission of *B. recurrentis* by body lice is by excretion in the feces of viable *B. recurrentis*, which then infects humans through skin lesions. Although the biological role of bed bugs in the transmission of *B. recurrentis* under natural conditions has yet to be confirmed, the present work highlights the need to reconsider monitoring of these arthropods for the transmission of human pathogens, including *B. recurrentis*. Because bed bugs and body lice may share the same ecological niches, entomological monitoring of both arthropods is needed. Bed bugs might naturally acquire *B. recurrentis* by feeding on humans infected by body lice bites. The role of bed bugs in transmitting relapsing fevers could be underestimated. Our concern is for infected patients, including migrants from endemic areas, who may come into contact with bed bugs.

Received October 5, 2018. Accepted for publication February 19, 2019.

Published online April 1, 2019.

Acknowledgments: We thank Nathalie Duclos (IHU Méditerannée Infection, Marseille) for providing the *Borrelia recurrentis* strain, and Jean-Pierre Baudoin for his help in making the spirochete video.

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