Quantitative Analysis of Proliferation and Excretion of Bartonella quintana in Body Lice, Pediculus humanus L.

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Abstract. Although body louse is a well-known vector of trench fever, the growth kinetics of Bartonella quintana in body lice has not been fully understood. We performed a quantitative analysis of bacterial multiplication rate. B. quintana started proliferation in body lice 4 days after ingestion and was constantly excreted in the feces for at least 3 weeks. The number of bacteria in feces reached the maximum 10^7/louse per day on Day 15. The doubling time of B. quintana estimated from logistic regression formula was 21.3 hours. Scanning electron microscopy showed the presence of bacterial masses in feces. Immunofluorescent study using specific monoclonal antibody confirmed identification of B. quintana. Such an explosive multiplication rate and active excretion of B. quintana from the body lice could be related to epidemics of trench fever in developed countries.

INTRODUCTION

Trench fever is a historical disease where it is caused great epidemic outbreaks during World Wars I and II.1,2 Subsequently, its outbreaks have been by improvement of sanitary conditions, control of body lice by insecticides, and dissemination of effective antibiotic treatments. Since the 1990s, however, the re-emergence of trench fever has been reported in refugee camps and prisons in developing countries of Africa.3 In addition, trench fever can also be seen among specific populations in urban areas of developed countries, such as homeless people and drug addicts,4–6 and is referred to as “urban trench fever.”7

The causative agent of trench fever is Bartonella quintana, which is a short, pleomorphic, Gram-negative bacterium thought to be transmitted through the feces of infected body lice to human beings by scratching of the skin.1 The human body louse, Pediculus humanus, is known to be the vector of B. quintana,3 but there are few studies that describe details of the growth kinetics and transmission of the bacteria. By using an animal model, Fournier and colleagues performed an experimental infection of rabbit-adapted body lice with B. quintana that expressed green fluorescent protein.9 They showed B. quintana being excreted in the feces and that life span and survival rate of body lice were not significantly influenced by B. quintana infection.9 However, their study was not sufficient to understand the proliferation dynamics in the body lice and persistent excretion of B. quintana in the feces.

Recently Kasai and colleagues10 developed an artificial blood feeding system using human whole blood, and the blood-fed adult body lice were able to be kept alive for at least 3 weeks in the laboratory.

In this study, a membrane feeding system was used to provide infectious blood meal and we report herein the proliferation dynamics of B. quintana in body lice and the massive excretion of B. quintana in louse feces.

MATERIALS AND METHODS

Body lice. The laboratory strain of body lice (NIID strain) used in this study originated from Sapporo, Hokkaido, Japan, in 1954 and has been maintained on human blood every day for > 50 years.11 The body lice of this strain can fully engorge with blood within 20 min and is adapted to only once feeding per day.10 Adult female lice 20 days post-hatching were used in the experiments. Lice were proven to be free from B. quintana by using the polymerase chain reaction (PCR) method with the primers described below. The average amount of blood sucked by a louse was estimated by weight method from 20 lice. Five lice were sampled for DNA extraction before feeding and put in individual 1.5-mL plastic tubes on the days of sampling. Every day, 10 lice were randomly selected and put in a 20-mL plastic tube with a small piece of cloth to collect the feces. Both samples were stored at −80°C and thawed at 0°C before using.

Bacterial strain and culture conditions. All procedures involving experimental infections of lice were conducted in a biosafety level 2 (BSL2) room at the National Institute of Infectious Diseases, Tokyo, Japan. The strain of B. quintana (Hassani strain), originally colonized from blood samples from homeless patients in Marseille, France, was used. The bacteria were cultured using selective media on Columbia sheep blood agar plate (5%, BioMerieux, Marcy l’Etoile, France) at 37°C under a 5% CO₂ atmosphere, as described previously.8

On Day 10 post-plating, the bacteria were harvested by adding 4 mL of phosphate-buffered saline (PBS, Dulbecco, 0.1 M, pH 7.2), and 50 μL of the bacterial suspension was mixed with 1 mL of whole human blood and used for an experimental infection.

For the purpose of estimating the initial concentration of bacteria as colony forming units (CFU) per microliter, the bacterial suspension was diluted from 10⁴ to 10⁷ times and 10 μL of each dilution was cultured on the blood agar plates.

Cultures of B. quintana from feces of experimentally infected body lice on the 11 days after infection were also carried out by plating homogenized feces in PBS on the blood agar plates. After colonies formed on the plate, a second culture was performed and identification of bacterial species...
from the colonies was carried out by sequencing PCR-amplified product.

Membrane feeding system and louse infection with *B. quintana*. The artificial membrane feeding method was performed according to Kasai. Human whole blood was prepared by adding an anti-coagulant, sodium citrate (final concentration, 0.3%), followed by freezing at $-80\, ^\circ\mathrm{C}$ until use. Parafilm (American Can Co., Neemah, WI) was stretched to 4-fold (= 16-fold of the original area) and put on the top of a glass Petri dish (90 mm in diameter). A circular cotton pad (30 mm in diameter and 2 mm in thickness) was put on the Parafilm. One milliliter of blood was absorbed onto the cotton and covered with another sheet of stretched Parafilm. The circumference of the cotton pad was attached securely to ensure that the blood-containing cotton was sealed. The edge of the blood pad was clipped with a razor, placed on the bottom of another Petri dish, and incubated on a heating block at 37$\, ^\circ\mathrm{C}$.

After the infective blood meal, lice were fed non-infective human blood meals for 3 weeks post-infection and kept in the incubator at 30$\, ^\circ\mathrm{C}$ and 60% humidity.

Isolation of genomic DNA. Genomic DNA was isolated using a SepaGene kit (Sanko Chemicals, Tokyo, Japan). Each louse was homogenized in 100 $\mu\mathrm{L}$ of the homogenization buffer (Reagent I; Tris-HCl buffer, pH 7.9) using a pestle for 30 sec in a 1.5-mL plastic tube. Feces in the 20-mL plastic tube were collected by centrifugation at 1500 $\times$ g for 10 min and then mixed with 200 $\mu\mathrm{L}$ of the homogenization buffer. The subsequent DNA extraction proceeded by the manufacturer’s instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR). The qRT-PCR was performed with a SYBR-green detection system using iCycler (Bio-Rad, Tokyo, Japan). Specific primers were designed based on the gene sequence of the intergenic spacer (ITS) region of *B. quintana*: BO-ITS-Forward, GCCGCTTTCCGTTCTTCTTTC and BO-ITS-Reverse: AGTGTTCTTCTTAAGTCCCAGG. Body louse sodium-channel gene-specific primers (L-SC-Forward, ACGATGATGAGATGAGATGAGG and L-SC-Reverse, CAAGAGGAGTAGAGCCCAAATC) were also designed to correct the efficacy of DNA extraction and standardize the number of *B. quintana*. The qRT-PCR conditions were as follows: the reaction mixtures were first kept at 95$\, ^\circ\mathrm{C}$ for 10 min, then put through 50 cycles of 95$\, ^\circ\mathrm{C}$ for 15 sec and 60$\, ^\circ\mathrm{C}$ for 60 sec. The number of *B. quintana* in each sample was calculated on the basis of DNA copy number of...
B. quintana. Standard curves were obtained using serial dilutions of purified PCR products obtained with the above primer sets, covering five template doses and yielding correlation coefficients of at least 0.98 in all experiments. Each standard value was determined in triplicate in every experiment. After 50 cycles of the PCR reaction, samples were run for the dissociation protocol, and we confirmed that only a single melting peak was obtained for all samples, showing no amplification of the non-target DNA. The number of amplified DNA copies were converted into the number of bacteria (1 attomole = 3.01 x 10^7 cells). The average number of bacteria per louse was calculated from the data of 5 lice with standard error.

Preparation of feces of body lice for scanning electron microscopy. The feces from 10 body lice after 17-day infection were selected. Fecal samples were washed 3 times with 0.1 M PBS for 30 min and washed with 0.1 M PBS 3 times. After dehydration through an ascending alcohol series, the specimens were freeze-dried (model ES-2030, Hitachi, Tokyo, Japan) using tert-butyl ethanol, coated with osmium, and examined with a scanning electron microscope (model S-5200; Hitachi).

Preparation of feces of body lice for immuno-fluorescence assay. The feces from 10 body lice collected on Day 16 post-infection were fixed with 100% methanol for 10 min and dried at room temperature. The samples were then incubated with 10 μL of mouse anti-B. quintana monoclonal antibody (IgG subclass 1) at a dilution of 1:10 in 0.1 M PBS for 30 min at 25°C. This antibody was established by Tsuguo Sasaki (not published) by immunizing whole bacterial lysate to BALB/c mice and fusing with myeloma cells (SP2/0-Ag14). Hybridomas specifically reacting with cell surface antigens of B. quintana were selected. Fecal samples were washed 3 times with 0.1 M PBS by flash centrifugation for 10 sec and incubated with 100 μL of fluorescein isothiocyanate (FITC)-conjugated goat Fc specific anti-mouse IgG (Funakoshi Inc., Tokyo, Japan) at a dilution of 1:150 in 0.1 M PBS for 30 min. After 3 final washes with 0.1 M PBS by flash centrifugation for 10 sec and being mounted on slides with glycerol solution half-diluted by PBS, they were observed by fluorescence microscopy (BX51, Olympus, Tokyo, Japan). Images were recorded by CCD camera (QICAM Fast cooled mono, 12 bit, QImaging, Burnaby, Canada). A negative control sample from the feces of non-infected body lice was prepared in the same manner as for the infected sample.

RESULTS

Proliferation of B. quintana in the body lice and excretion in their feces. A total of 84 lice were distinguished as blood-engorged by changing of their abdominal color (Figure 1A). From Day 1 to Day 21 post-infection, a total number of 60 lice were sampled and their DNA was extracted to estimate numbers of bacteria. The average number of B. quintana in 1 body louse decreased until Day 3 post-infection to a minimum number of bacteria. The number of B. quintana increased ∼60,000 times when compared with the minimum number of bacteria. The number of B. quintana excreted in feces increased gradually until Day 15 (Figure 2). The maximum bacterial excretion in the feces was observed on Day 15 at 10 × 10^6 bacteria per louse. Logistic regressions were calculated from the data on Days 3–17 (Figure 2) to determine the excretion dynamics of B. quintana in the feces as it is associated with the level of bacteria in body lice. The doubling time of bacteria calculated from logistic regression was 21.3 hrs, which corresponds to the characteristically slow growth of B. quintana on blood agar plates. The average amount of blood sucked by one louse was estimated as 2.4 μL by the weight method; thus the estimated number of B. quintana in the blood of the experimental infection was ∼8.5 x 10^8 cell/μL.

The number of colonies formed from the 50-μL bacterial suspension was also counted 10 days post-plating, and the estimated concentration of B. quintana in the infection meal was 2.00 × 10^5 CFU/μL. From these results, we estimated that 4.21 cells were inoculated per louse. From this number after conversion and the results of other papers, the initial dose of B. quintana taken by louse was similar to the bacteremia level in patients with urban trench fever.13

By plating of dissolved feces from infected body lice, translucent small colonies were successfully formed on the blood agar on Day 14 post-plating. Three colonies were chosen for the second culture, and 17 days afterward the bacteria were harvested from blood agars by adding 4 mL of PBS for DNA extraction. DNA of bacteria from 3 plates were amplified by PCR using B. quintana-specific primer, described previously, and sequencing analysis of the amplified DNA products showed that all of them contained the identical sequence with B. quintana.

Electron-microscopic observation of feces from infected lice. Scanning electron microscopy identified a massive number of short-rod bacteria (1 μm) scattered on the inner and outer surfaces of fecal masses (Figure 1C). In the enlarged
view (Figure 1D), excreted bacteria were observed to be in a mesh-like structure entwined with each other, which is called a “biofilm.” There were great differences in the structure of biofilm of *B. quintana*. In some parts of feces, immature biofilm structures were observed (Figure 1C, in the box), while some parts showed more mature biofilm structures of *B. quintana* (Figure 1D).

**Microscopic observation of immunostained feces from infected lice.** Immunofluorescence staining of feces from infected lice using monoclonal antibodies against bacterial lysate of *B. quintana* demonstrated a strong positive reaction on the outer and inner surface of feces (Figure 1B). No specific fluorescence was observed in the fecal sample from non-infected lice prepared in the same manner. Both the immunologic data and electron-microscopic examination of the feces of infected lice (Figure 1C) confirmed infection of *B. quintana*.

**DISCUSSION**

The re-emergence of trench fever is a serious public health problem that warrants attention from public health authorities and additional investigations. Proliferation of *B. quintana* was confirmed by molecular, morphologic, and immunologic methods. The great number of bacteria in the feces detected by the qRT-PCR methods suggested that an outbreak of trench fever could occur in an urban environment where festation with body lice may be increasingly common.

In this study, we also provide the findings of the mesh-like structure of *B. quintana* in the fecal masses of infected body lice. In our detailed examination by scanning electron microscopy, *B. quintana* attached to the exopolysaccharide (EPS)-like matrix and fecal materials (Figure 1D). It is generally considered that biofilms are characterized by an EPS matrix created by the bacteria, and this EPS matrix plays a vital role in the survival of bacteria, providing ecological advantages to the organisms. This biofilm-like structure may contribute to the storage of *B. quintana* in a fecal environment.

The culture of *B. quintana* in the feces from infected body lice suggests that its infectivity remains in a fecal environment for a long time. *B. quintana* infection has been reported to be related to poverty, alcoholism, and homelessness in developed countries; however, some studies showed a high prevalence of anti-*B. quintana* antibodies in the healthy population. Massive excretion of infective *B. quintana* in the feces of heavily lice-infested people may be the cause of accidental infection in crowded public places in developed countries. Although cross-reactivity of human antibodies to *B. quintana* is reported, it would be necessary to carry out more-detailed epidemiologic studies, particularly focusing on healthy people.

Experimental infections of louse-borne diseases have been performed by preliminary artificial membrane feeding or rectal inoculation of the pathogen into body lice. Currently a rabbit model is commonly used and has been adapted for not only louse-borne diseases but also for other pathogens. However, animal models are not suitable for quantification of bacterial proliferation, because after intravenous injection of the pathogens the number of bacteria may be diminished by the influence of the immune system of the host animal. When the *B. quintana* is injected into a rabbit, the bacteria may be rapidly cleared by cellular defense systems and the initial concentration cannot be estimated accurately. Our artificial membrane feeding system has great advantages for quantitative studies of bacterial proliferation in body lice and for potential adaptation to experimental infection by other pathogens.

Sites of proliferation in body lice are not fully understood, although Kostrzewski describes extracellular proliferation of *B. quintana* in the midgut of body lice. Further detailed observations would be needed to determine whether *B. quintana* proliferates in the epithelial cells of the louse intestine or in the lumen of the gut.

In this paper, we have provided the first quantitative description of *B. quintana* proliferation in body lice and excretion in the feces. We hope that our results provide some explanation for trench fever outbreaks among not only homeless populations but also ordinary people in developed countries.

Our results suggest that control of body lice may be an essential step toward urban trench fever epidemics among indigent people, and proper management of louse feces containing huge numbers of *B. quintana*, which seems able to survive for a long time, should be considered by medical staff and health workers in health and welfare sections of local governments to avoid accidental infection themselves by trench fever.

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