QUANTITATIVE COMPETITIVE PCR AS A TECHNIQUE FOR EXPLORING FLEA–YERSINA PESTIS DYNAMICS

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Abstract. We used a quantitative competitive polymerase chain reaction assay to quantify Yersinia pestis loads in fleas and bacteremia levels in mice that were used as sources of infectious blood meals for feeding the fleas. Xenopsylla cheopis, the Oriental rat flea, achieved higher infection rates, developed greater bacterial loads, and became infectious more rapidly than Oropsylla montana, a ground squirrel flea. Both flea species required about 10⁶ Y. pestis cells per flea to be able to transmit to mice. Most fleas that achieved these levels, however, were incapable of transmitting. Our results suggest that at the time of flea feeding, host blood must contain ≥10⁷ bacteria/ml to result in detectable Y. pestis infections in these fleas, and ≥10⁸ bacteria/ml to cause infection levels sufficient for both species to eventually become capable of transmitting Y. pestis to uninfected mice. Yersinia pestis colonies primarily developed in the midguts of O. montana, whereas infections in X. cheopis often developed simultaneously in the proventriculus and the midgut. These findings were visually confirmed by infecting fleas with a strain of Y. pestis that had been transformed with the green fluorescent protein gene.

INTRODUCTION

The etiological agent of plague, Yersinia pestis, is typically transmitted between mammalian hosts by various species of rodent fleas. Adult fleas become infected while feeding on a bacteremic host and support development of the bacteria within their alimentary tracts. Transmission occurs only after bacteria in the flea gut have multiplied sufficiently to form a large mass of bacteria and clotted blood, or "block," which occludes the proventriculus. After block formation, subsequent feeding attempts cause blood to backwash off the block and introduce bacteria into the bite wound.

Identification of infected fleas allows researchers and public health officials to easily determine the presence of plague in an area and take appropriate preventative actions. Several polymerase chain reaction (PCR) assays have been developed recently to identify Y. pestis in different materials, including fleas, by targeting various chromosomal and extra-chromosomal genes for amplification. The use of the PCR assay for this purpose has been shown to be more rapid and sensitive than mouse inoculation, which was the previous gold standard for identifying Y. pestis in fleas.

Measuring quantities of bacteria in infected fleas may allow researchers to identify not only infected fleas, but also those containing sufficient bacteria to be blocked and presumably infectious. Previously, the quantity of bacteria in fleas has been estimated by culturing triturated flea material on blood agar plates. This method has not proven to be very reliable, as plates are often overgrown with contamination. Recently, a quantitative competitive PCR (QC-PCR) assay was developed to estimate the number of bacteria in individual fleas by quantifying the amount of target DNA present. The QC-PCR assay uses a DNA competitor, which, by deletion or insertion, is a shortened or lengthened mutation of the target DNA and is, therefore, amplified by the same primers as the target DNA. Standards are created by adding known amounts of Y. pestis and a constant amount of competitor, or vice versa, to the PCR mix. Target and competitor amplicons are of different sizes and can be distinguished by electrophoresis. The log ratio between the amounts of target and competitor DNA, as measured by band density, is then used to produce a standard line equation by linear regression. Log ratios derived from samples can then be inserted into the line equation to give an accurate quantity of target DNA in the sample.

In a previous study using QC-PCR, Hinnebusch and others demonstrated that fleas must be infected with 10⁷ Y. pestis cells to appear blocked when viewed under a microscope and, therefore, presumably capable of transmission. These researchers proposed that testing fleas collected from rodent epizootics, namely those involving prairie dogs, would allow public health officials to determine blocking rates in these fleas, and possibly the stage of the epizootic from which the fleas were collected. In this paper, we used a modified version of the QC-PCR developed by Hinnebusch and others to quantify bacteria in laboratory-infected fleas and to determine whether QC-PCR is useful as a research tool for following Y. pestis infections in two different species of fleas, Xenopsylla cheopis and Oropsylla montana. Xenopsylla cheopis, the Oriental rat flea, is an Old World flea that is considered the premier vector for Y. pestis. Oropsylla montana, a New World flea found on certain ground squirrels, is a competent vector, but, as previous studies have shown, is not nearly as efficient in transmitting plague as X. cheopis. We also used QC-PCR to determine what host bacteremia levels are required to reliably infect fleas. Finally, we comment on the utility of using QC-PCR as a public health tool to determine the status of rodent epizootics by determining the rates of blocked fleas.

MATERIALS AND METHODS

Yersinia pestis strains. Yersinia pestis strain CO963188 was used for all laboratory flea infections. CO963188 is a fully virulent strain (LD₅₀ of 10–100 cfu). The avirulent strain A1122 was grown in brain-heart infusion (BHI) broth at 28°C and harvested during log phase growth. Serial ten-
Fold dilutions were then smeared on blood-agar plates to determine the number of colony-forming units, according to standard methods.

**Fleas.** In order to evaluate the utility of QC-PCR to identify blocked fleas, we infected two species of fleas, *X. cheopis* and *O. montana*. Laboratory colonies of *X. cheopis* were obtained originally from a laboratory colony maintained at Rocky Mountain Laboratories in Hamilton, Montana, and the colonies of *O. montana* were established from fleas originally collected in Bernalillo County, New Mexico.

**Laboratory infection of fleas.** *Oropsylla montana* and *X. cheopis* were infected by feeding on mice with various bacteremia levels of *Y. pestis* CO963188. Mice were inoculated with $10^5$–$10^7$ *Y. pestis* cells and observed for signs of illness or presence of the characteristic bipolar *Y. pestis* cells on a thin blood smear stained with Wright’s stain. Fleas were starved for 5–14 days prior to infectious feeding and kept in small acrylic flea cages covered with nylon mesh (Flea Data Inc., Freeville NY). Flea cages were placed on the shaved abdomens of bacteremic mice for 30 to 60 min to allow fleas to feed to repletion. Blood was then taken from the anesthetized mice, via cardiac puncture, for later bacterial quantification, and the mice were killed. Fleas were removed from the cages, put into clear plastic resealable bags, and examined by fluorescence-labeled antibody that was reactive against the F1 antigen of *Y. pestis*. Isolation of uninfected mice. Following each maintenance feeding, the fleas had transmitted *Y. pestis* to develop a high bacterial load. Fleas were held in acrylic cages and maintained for up to 8 weeks, at 21°C and 80% relative humidity. For the duration of the experiment, the fleas held in the acrylic cages were fed semi-weekly on the abdomens of bacteremic mice for 30 to 60 min to allow the fleas to feed to repletion. Blood was then taken from the anesthetized mice, via cardiac puncture, for later bacterial quantification, and the mice were killed. Fleas were removed from the cages, put into clear plastic resealable bags, and examined under a stereomicroscope to determine which fleas had taken a complete or nearly complete blood meal (visualization of distended gut full of fresh blood). Those that had taken a full blood meal were returned to individual flea cages and maintained for up to 8 weeks, at 21°C and 80% relative humidity. For the duration of the experiment, the fleas held in the acrylic cages were fed semi-weekly on the abdomens of infected mice. Following each maintenance feeding, the fleas were removed from cages and microscopically examined for evidence of blocking (presence of fresh blood anterior to the proventriculus). The mice used for each maintenance feeding were held for 21 days to determine whether the feeding fleas had transmitted *Y. pestis* to these animals.

Tissues from mice that died during the 21-day period were examined by fluorescent antibody assay using fluorescein isothiocyanate-labeled antibody that was reactive against the F1 antigen of *Y. pestis*. Liver and spleen tissues were also taken for bacterial culture. Flea cages were checked daily, and dead fleas were removed and held at −70°C for later testing. Live fleas were also selectively removed from cages at various time points and frozen (−70°C) for later testing. All experiments using mice were done according to a protocol approved by the Center for Disease Control and Prevention’s Division of Vector-Borne Infectious Disease’s Animal Care and Use Committee (AUC #97-09-008-AM).

**Preparation of fleas for PCR and QC-PCR.** Fleas were prepared for PCR as previously described. Briefly, individual fleas were triturated in 100 μL of BHI broth in 1.5-mL microcentrifuge tubes with sterile sea sand and disposable pestles. Suspensions were heated at 95°C for 10 min and immediately centrifuged for 10 sec at maximum speed (15,600 X g) to pellet flea tissue and sand. Supernatants were assayed by *pla* PCR within 10 min and stored at −70°C until subsequent testing by QC-PCR.

**Preparation of flea alimentary tracts for PCR.** To further analyze the development of *Y. pestis* in *X. cheopis* and *O. montana*, fleas were fed on bacteremic mice with at least $10^5$ *Y. pestis* cells/mL in the blood. Fleas were held in acrylic cages (up to 25 fleas per cage) and maintained by semi-weekly feedings on mice until Day 21 post-feeding, allowing enough time for the flea to develop a high bacterial load. Fleas still alive on Day 21 were frozen at −70°C. Individual fleas were dissected under a dissecting microscope (Olympus SZ11, Long Beach CA), at 30X magnification, to remove the foregut and the midgut. The midgut was then separated from the foregut (containing the proventriculus), by using the edge of a sterile 25.5-gauge syringe needle; the two portions of the gut were placed in separate 1.5-mL microcentrifuge tubes containing 50 μL of BHI broth. Tubes were then heated to 95°C for 10 min to break up flea tissue and bacteria cells. These samples were then tested by *pla* PCR, and if found to be *pla* PCR-positive, quantitated by QC-PCR.

**PCR and QC-PCR amplification targets.** Primer sequences for the initial PCR screening assay (used to distinguish infected from non-infected fleas) were the same as those used previously and were derived from the published sequence data for the plasminogen activator gene (*pla*). Primer Yp1 (5’-ACCTACTTCTCCGTGAGAAC-3’) corresponds to nucleotides 971–990 and primer Yp2 (5’-CTTGGATGTTGAGCTTCCTA-3’) corresponds to nucleotides 1431–1450. Amplification with primers Yp1 and Yp2 produced a 478 bp product.

**Amplification with primers Yp1 and Yp2 produced a 478 bp product.** Primer sequences for the QC-PCR assay were designed to target the chromosomal ferric iron uptake regulation (*fur*) gene and are the same as those described by Hinnebusch and others. Preparations of competitor template, pFURYD, is described previously. The methodology for the *pla* PCR was described previously. Briefly, for each *pla* PCR assay, 2.5 μL of original flea preparation was combined with 0.25 μL of each primer (Yp1 and Yp2; 30 pmol of each primer/microliter), 0.25 μL of 50 mM MgCl₂, and 21.75 μL of deionized, distilled water in a 0.65 mL tube containing a Ready-To-Go PCR bead (Amersham Pharmacia Biotech, Piscataway NJ). Positive controls used 2.5 μL of A1122 strain of *Y. pestis* in BHI broth (4.8 × 10⁵ *Y. pestis* cells/mL BHI broth). Negative controls used 2.5 μL of sterile BHI broth. Finally, two drops of sterile mineral oil were added to each tube.

**Quantitative competitive-polymerase chain reaction.** Bacterial loads of flea preparations that were positive by *pla* PCR were quantitated by QC-PCR. The QC-PCR methodology used was a modification of the protocol described by Hinnebusch and others. The protocol is essentially the same as described above for the *pla* PCR with the additional step of adding the competitor template. The final mixture for PCR amplification contained 2.5 μL of original flea preparation: 0.25 μL each of competitor template, primers Ypfur1 and Ypfur2 (30 pmol of each primer/microliter), and 50 μM MgCl₂; and 21.50 μL of deionized, distilled water in a 0.65-mL tube containing a Ready-To-Go PCR bead (Amersham). Preparation of competitor template, pFURYD, is described previously.
in Hinnebusch and others.\textsuperscript{10} pFURYD is a pUC plasmid containing the \textit{Y. pestis fur} gene with a 65-bp interior deletion. Suspensions of pFURYD were obtained by transformation (Electroporator 2510, Eppendorf, Hamburg, Germany) with electrocompetent cells (Epicuran Coli XL1-Blue, Cedar Creek, TX). For preparation of standards, 2.5 \( \mu \)L of 10-fold dilutions of a known amount of A1122 \textit{Y. pestis} bacteria were prepared in separate tubes. Negative controls used 2.5 \( \mu \)L of sterile BHI broth.

**PCR amplification and analysis.** Amplifications for the \textit{pla} PCR screening assay and the QC-PCR were done in a Minicycler thermocycler (MJ Research, Watertown, MS) by using the following amplification program: initial denaturation at 95\(^\circ\)C for 5 min, followed by 35 cycles of denaturing at 95\(^\circ\)C for 1 min, annealing at 51\(^\circ\)C (\textit{pla} PCR) or 55\(^\circ\)C (QC-PCR) for 1 min, and primer extension at 72\(^\circ\)C for 2 min. After the last cycle, extension conditions were continued at 72\(^\circ\)C for 10 min. Samples were analyzed by electrophoresis on 2\% agarose gels with ethidium bromide, according to standard methods.

**Quantitation of \textit{Y. pestis} in flea samples.** Following electrophoresis, stained gels were digitized with a UV gel camera (Gel Doc 1000, Bio-Rad Laboratories, Hercules, CA) and the relative densities of the 328-bp and 263-bp bands were analyzed by using Molecular Analyst/PC Software (version 1.4, Bio-Rad Laboratories). The area under the peaks, from profile analysis of each 328-bp and 263-bp band, was calculated. The area under the 263-bp peak was normalized to the 328-bp peak by multiplying it by 1.247 (328/263).

The log of the ratio of the measurement for the 328-bp peak to the 263-bp peak for each of the standards was plotted against the log number of \textit{Y. pestis} added to the standard samples. A standard curve was computed with statistical software (SPSS for Windows, version 6.1.4, Chicago, IL) using linear regression analysis. The reliability of this assay was verified by calculating the fit of the densitometric values for samples containing triturated flea material, spiked with known amounts of \textit{Y. pestis}, to our standard line equation. Each assay was run with 3 to 4 standards (Figure 1). Only those assays which had standards that provided a line equation (\( y = mx + b \)) with an \( R^2 \) of 0.95 or greater were used. The standard curve was used to determine the quantity of target DNA for individual test samples by using the log of the ratio of the area from the 328-bp peak to the area from the 263-bp peak as the \( y \) value and solving for \( x \), the log number of \textit{Y. pestis} cells.

**Quantitation of \textit{Y. pestis} in mouse blood.** Bacteremic mice, used to infect fleas, were bled immediately after flea feeding. DNA was removed from whole mouse blood by using QIAamp DNA Mini Kit (Qiagen, Valencia, CA), and tested by QC-PCR in the same fashion as mentioned above for testing flea samples. This method was evaluated by testing whole blood spiked with known amounts of A1122 \textit{Y. pestis} and found to be accurate within 10\%, based on comparisons of control samples containing similar samples of A1122, but no blood (data not shown).

**Preparation of \textit{Y. pestis} cells expressing green fluorescent protein (GFP).** \textit{Yersinia pestis} CO963188 was grown in 30 mL BHI broth for 3 days or until the OD\(_{562}\) = 0.6 to 0.8. Cells were washed once with cold distilled water and then several times with cold water containing 10\% glycerol, with each succeeding wash using half the volume of the previous wash. This resulted in a final suspension of 1 mL. Next, 50 \( \mu \)L of the washed cell suspension was incubated with 1 \( \mu \)L of commercially prepared GFP gene plasmid (pEGFP, Clontech, Palo Alto, CA) for 20 min on ice. This suspension was then placed in a pre-chilled 0.2-mm cuvette (Eppendorf, Hamburg, Germany) and electroporated (Gene Pulser, Bio-Rad Laboratories) at 2.5 kV, 25 \( \mu \)F, 200 ohm with a time constant of 4.0 msec. Transformed cells were allowed to recover for 1 hr in 300 \( \mu \)L SOC medium at 30\(^\circ\)C.
Recovered cells were plated on blood-agar plates containing 100 µg/mL ampicillin and allowed to grow for 48 hr until colonies were observed. Colonies that appeared green under UV light were selected and replated on clean ampicillin plates. These transformed cells were stored at −70°C in BHI or Luria broth with 10% glycerol and 100 µg/mL carbenicillin. Flea infection with *Y. pestis:*pEGFP was accomplished with an artificial membrane feeder, as previously described, using a mouse skin membrane. Whole mouse blood, spiked with 10⁶ *Y. pestis:*pEGFP/mL was added to a small chamber that was covered by a mouse skin-membrane and heated by a water-jacket, to 37°C. The acrylic flea cages were then placed on the membrane, and fleas were allowed to feed for 1 hr. Fleas were maintained as above and were sampled on Days 7 and 21 after infection. The alimentary tracts of these fleas were removed as above, mounted in 0.85% saline on a glass slide, and observed under 400X on a Zeiss UV microscope (AxioSkop, Carl Zeiss, Inc., Thornwood, NY) and photographed with a 35-mm Contax 167MT camera (Kyocera, Irwindale, CA).

RESULTS

A total of 260 *X. cheopis* and 421 *O. montana* were infected in the laboratory by feeding on mice with varying degrees of bacteremia. Of the fleas fed on bacteremic mice with greater than 10⁶ bacteria/mL of blood, 70.5% (67/95) of the *X. cheopis* and 51.5% (101/196) of the *O. montana* were found to be *pla* PCR positive at the time of death. Based on standard curve calculations, 10.4% of the 67 infected *X. cheopis* had less than 10⁶ *Y. pestis* bacteria per flea, 23.9% had 10⁶ to 10⁷ *Y. pestis*, and 65.7% had greater than 10⁷ *Y. pestis* per flea (Figure 2). Of the 101 infected *O. montana, 35.6% had less than 10⁵ *Y. pestis*, 40.6% had 10⁵ to 10⁹ *Y. pestis*, and 23.8% had greater than 10⁹ *Y. pestis* per flea (Figure 2).

Analysis of surviving fleas. When infection rates for live fleas fed on mice with varying bacteremia were investigated on Day 10 after feeding, *X. cheopis* had higher infection rates than *O. montana* (Figure 3). Infection rates of greater than 50% were not obtained unless fleas of both species had fed on mice with bacteremia levels of 10⁸ bacteria/ml or higher. *Xenopsylla cheopis* also had greater mean bacterial loads by Day 10 post-feeding than *O. montana* (Figure 4). Only *X. cheopis* that had fed on blood with 10⁶ *Y. pestis* cells/ml of blood or higher were able to reach the “transmission threshold” of 10⁶ *Y. pestis*/flea by Day 10 (Figure 4).

Of the fleas that had successfully taken a full blood meal from a mouse with a bacteremia of greater than 10⁹ *Y. pestis* bacteria/mL of blood, only 15.8% (15/95) of the *X. cheopis* and 2.0% (4/196) of the *O. montana* succeeded in transmitting plague to mice (Table 1). *Xenopsylla cheopis* had a mean extrinsic incubation period (EIP) of 16 days whereas the EIP was 23 days for *O. montana* (Table 1). Eleven of the 15 *X. cheopis* and all four *O. montana* that transmitted *Y. pestis* to mice had originally fed on mice that had bacteremias of ≥10⁶ *Y. pestis*/mL of blood. The other four *X. cheopis* that transmitted *Y. pestis* had fed on mouse blood containing 10⁷ bacteria/mL. When blocking was defined as being able to see fresh blood only anterior to the proventriculus of a recently fed flea, transmission rates did not correlate with blocking rates. According to these visual criteria, nine *X. cheopis* were identified as blocked, only 2 of which were able to transmit plague bacteria. One other *X. cheopis* that appeared to be “partially blocked” (flesh blood was seen anterior and posterior to the proventriculus) successfully transmitted *Y. pestis*. None of the *O. montana* that...
transmitted *Y. pestis* to mice, was visually identified as blocked or partially blocked. The mean bacteria load of all *X. cheopis* that transmitted to mice was $10^{7.2}$ (range: $10^{6.8}$–$10^{7.6}$) s.d.; $10^{7.0}$, compared to $10^{6.7}$ (range: $10^{6.1}$–$10^{7.1}$) s.d.; $10^{6.7}$ for the four *O. montana* that transmitted. One of the four *O. montana* that transmitted *Y. pestis* to mice had only $10^{6.0}$ bacteria, but this flea transmitted on Day 4, two weeks earlier than the next earliest transmission by an *O. montana*. Mice died within a mean of 3.2 days (range = 2–5 days) when infected by *X. cheopis*, whereas death occurred within a mean of 2.8 days (range = 2.5–3 days) when infected by *O. montana*.

**Analysis of dead fleas.** Individual fleas were collected on the day of death for at least 6 weeks following original infection. Infection rates, as measured by PCR results, in *X. cheopis* remained high throughout the 6-week period, as compared to *O. montana* infection rates (Figures 5 and 6). All eight *X. cheopis* tested during the first week after infection remained infected and were found to have reached the $10^6$ bacteria/flea threshold thought to be required for reliable transmission. By week six, 40% (10/25) of the *X. cheopis* remained infected, 7 of which had greater than or equal to $10^6$ bacteria. At least 25% of *X. cheopis* tested from each time period had reached the $10^6$ threshold.

**Analysis of alimentary tract.** When the midgut and the proventriculus were removed and tested separately, *X. cheopis* was more likely (13/16, 81.3%) than *O. montana* (2/12, 16.7%) to have a proventriculus infection concurrent with a midgut infection. Only 3/16 (18.7%) *X. cheopis* had infected midguts only, as opposed to 10/12 (83.3%) of infected *O. montana*. At no time was the proventriculus positive by PCR when the midgut was negative. Fleas of both species were found to have apparently uninfected proventriculi and bacterial loads of $\geq 10^8$ in the midgut only.

Visual observations of the colonization of the flea alimentary tract by plague bacteria were made by using *Y. pestis* transformed with the green fluorescent protein gene. These observations were similar to the above data in that both species were found to have infections solely in the midgut, often with bacterial loads greater than $10^7$; however, only *X. cheopis* fleas were found to have colonies of *Y. pestis* in the spines of the proventriculi (Figure 7). Fleas sampled on Days 7 and 21 post-infection yielded similar results, with Day 21 fleas having higher overall bacterial loads than those sampled on Day 7 post-infection.

**DISCUSSION**

Our results indicate that at least $10^6$ *Y. pestis*/flea are required for *X. cheopis* or *O. montana* to transmit bacteria to
Figure 7. Ultraviolet micrographs of flea alimentary tracts infected with Yersinia pestis::pEGFP. Oropsylla montana: A, B (Day 7 post-infection), C, D (Day 21 post-infection): A, midgut infection ($10^{4.7}$ Y. pestis); B, midgut infection ($10^{5.8}$ Y. pestis); C, midgut infection ($10^{4.4}$ Y. pestis); D, midgut infection ($10^{4.7}$ Y. pestis). Xenopsylla cheopis: E, F (Day 7 post-infection), G, H (Day 21 post-infection): E, proventriculus infection ($10^{4.8}$ Y. pestis) and midgut infection ($10^6$ Y. pestis); F, midgut infection ($10^{4.7}$ Y. pestis); G, proventriculus infection ($10^{4.3}$ Y. pestis) and midgut infection ($10^{4.9}$ Y. pestis); H, proventriculus infection ($10^{4.7}$ Y. pestis) and midgut infection ($10^{5.4}$ Y. pestis). (Arrows point to proventriculus).
a mouse or become visibly blocked. This is in accordance with the findings of Hinnebusch and others, who reported that *X. cheopis* required at least \(10^5\) *Y. pestis* cells for fleas to appear blocked under microscopic observation. Presumably, this is because a threshold of at least \(10^6\) bacteria must be present to result in a large enough mass of bacteria and clotted blood to occlude the proventriculus. However, for both species tested, it was clear that the majority of fleas infected with \(10^6\) bacteria or more did not transmit before dying or being killed at the end of the experiment (6 weeks after feeding). Obtaining a QC-PCR value of \(10^6\) or greater, therefore, does not necessarily indicate the flea’s ability to block and transmit, but only its potential to transmit. The fact that visual observations of blocking did not correlate with transmission rates has also been previously reported. It is likely that some fleas that were able to transmit had a block, or a partial block, which could not be observed. The single *O. montana*, which transmitted on Day 4 post-infection, had only \(10^5\) *Y. pestis* in its midgut. This was too early for a block to have developed, suggesting that mechanical transmission may have occurred. It is also possible that the un-blocked flea regurgitated a small amount of infectious material from its gut, as has been reported to occur rarely among other species. Burroughs also observed a single *O. montana* that transmitted on Day 4 post-infection, but he did not propose a mechanism for this transmission. A much greater percentage of the *O. montana* cleared themselves of infection before death than *X. cheopis*, a finding reported by several other researchers.

The QC-PCR assay allowed us to easily quantify bacteremia levels in mice used to infect fleas. We found that at least \(10^5\)–\(10^6\) *Y. pestis* cells/mL of blood must be present in the blood of host mice to achieve high rates of infection in fleas and the bacterial loads necessary to result in blocking of the flea vector. Mice with these levels of bacteremia appeared severely ill and frequently died during the flea feeding process. This finding has been alluded to previously by researchers who determined that infected laboratory mice had to be moribund, and near death, to have a high enough bacteremia level to infect fleas. Previous studies suggest that fleas imbibe up to 0.5 µL of blood during each feeding, depending on sex and species. If an individual flea ingests 0.5 µL of blood with \(10^5\)–\(10^6\) bacteria/mL, it will be infected with approximately \(10^5\)–\(10^6\) bacteria immediately after feeding. Ingesting smaller quantities of *Y. pestis* results in inconsistent infection and/or inability to transmit at a later time. An earlier method of quantitation, using colony counts from ground flea material, found that only \(10^5\) *Y. pestis* cells/mL of blood were needed to infect fleas; however, no data were shown to as subsequent infection and transmission rates for fleas fed on blood containing this number of bacteria.

Our findings may have significant implications for the ecology of plague. If such a high bacteremia level is required for rodent blood to be infectious for feeding fleas, it is unlikely that the rodent will survive its infection. Other researchers believe that fleas are unlikely to become infected by feeding on chronically infected or resistant hosts, or hosts with mild, sub-acute infections. Our findings agree with these hypotheses and seem to contradict later studies that look to the importance of resistant or chronically infected rodent reservoirs, which may have a low level of bacteremia over a long period of time. It would seem that a low level of bacteria circulating in the blood would not allow for sufficient numbers of bacteria to be ingested by fleas, which would prevent these fleas from later becoming infectious. It is, however, possible that repeated feedings on animals with low bacteremias could result in fleas occasionally becoming infected, but this needs to be further investigated, perhaps by using the techniques described in this study.

To determine why fleas often harbor such high numbers of bacteria and yet remain incapable of transmission, we tested foregut and midgut regions of infected fleas in QC-PCR assays. The results clearly showed that *Y. pestis* colonies can appear, initially, either in the midgut alone or in both the proventriculus and the midgut simultaneously. These results are similar to earlier reports that stated that plague bacteria will colonize in various locations, depending on the species of flea. It has been reported, through visual observations, that *X. cheopis* is an excellent vector because it will rapidly form a block in the proventriculus, whereas other sylvatic flea species are relatively poor vectors since colonization of *Y. pestis* initially occurs in the midgut, and only later spreads to the point where the proventriculus becomes occluded. Our data concurred with these findings, and showed that *O. montana* falls into the latter category, explaining its inferiority to *X. cheopis* as a vector. We also showed, however, that the midgut can be the primary site of colonization in *X. cheopis*. This might explain why many *X. cheopis* could become infected while simultaneously feeding on the same animal and yet vary greatly with respect to the time required to become infectious. The fact that bacterial colonization may occur only in the midgut of certain fleas could have important implications for the progression of epizootics, perhaps prolonging them, as fleas would become blocked and infectious over widely varying intervals of time. Delayed blocking may also result in fleas harboring bacteria from one transmission season to another, allowing these insects to serve as important reservoirs of *Y. pestis* infection.

The transformation of *Y. pestis* with the green fluorescent protein gene allowed us to visualize the colonization of bacteria in the alimentary tract of fleas, and provided supporting evidence for the results of our QC-PCR studies. Previous attempts had been made to visually monitor plague colonization by observing the growth of dark masses in the foregut and midgut, but visual observation may be unreliable because uninfected fleas will also have similar dark masses. Again, infection was seen either solely in the midgut or in both the midgut and proventriculus. *Oropsylla montana* did not develop a proventriculus infection until after several weeks of midgut infection. The QC-PCR data for those fleas with only midgut infections showed that fleas often harbor \(>10^6\) *Y. pestis* bacteria and can feed freely, as indicated by fresh blood in the midgut, with little potential for transmission. *Yersinia pestis* was observed in certain *X. cheopis* in the proventricular spines and in large masses located in the posterior of the midgut by Day 7 post-infection, which agrees with the observations of others. This was not observed in *O. montana*, where bacteria were found during the first week only in the midgut of those fleas infected with *Y. pestis*:eGFP, agreeing with earlier suggestions that certain species, including *O. montana*, are unable to support the de-
development of Y. pestis in the proventriculus early in the course of infection. Such fleas become blocked only after the midgut colonies coalesce and become large enough to occlude the proventriculus, which would explain why O. montana requires much more time to become blocked than X. cheopis.

The ability to determine whether an infected flea has reached the thresholds necessary for blocking would allow investigators to determine the rates of blocking in field-collected fleas, and possibly identify whether an ongoing epizootic is in its earlier stages or has peaked. It should also provide a better estimate of human risk, as only blocked fleas are likely to transmit. Our data, however, indicate that the QC-PCR technique cannot be used alone to determine whether an individual flea is blocked and, therefore, capable of transmission. Previously, fleas collected from epizootics had been tested in pools of up to 25 fleas. If most flea pools from a given epizootic tested positive for Y. pestis, it could be assumed that a large majority of these fleas were infected or even infectious. By analyzing individual field-collected fleas, we found that fleas collected from rodent burrows during different stages of epizootics usually have infection rates of 15% or less. Oftentimes, when a plague epizootic has eliminated most of the prairie dogs in a colony, the infection rate will be close to, or equal to, zero because the fleas that are collected are most likely newly emerged and, therefore, uninfected.

In a previous study, Quan and others used bacterial culture as a method for both identifying infected fleas and roughly estimating bacterial loads in fleas collected weekly during a California vole (Microtus californicus) epizootic. The highest infection rate (11%) was identified in fleas collected during the first week of the study, with decreasing rates every week thereafter. These researchers’ rough estimates of bacteria loads indicated that a large majority of infected fleas had “thousands to millions” of bacteria throughout the duration of the epizootic; however, relatively few infected fleas could be found. These researchers were unable to estimate the number of blocked or infectious fleas because they did not do transmission studies and reported that visually observing a mass of “bacteria” in the proventriculus was an unreliable means of determining whether a flea was infected, let alone blocked—an observation that has been reported by other researchers. We, too, have found that visually observing a dark mass in the proventriculus is not a good indicator of a flea’s ability to transmit. The low number of potentially infectious fleas present during epizootics suggests that it would be difficult to 1) collect enough infected fleas to obtain reliable data on the proportion of fleas that harbor sufficient bacteria to be potentially infectious, or 2) provide information for prevention and control activities that would be of greater use than simply identifying infected fleas in a given area.

Blocking rates, infection rates, and extrinsic incubation periods have been previously documented with widely varying results for X. cheopis and O. montana. These variations are likely attributed to differences in rearing conditions, feeding mechanisms, bacterial strain differences, and flea strains. The methods presented in this study should be more easily standardized than other methods for infecting fleas, determining infection rates and bacterial loads in fleas, and monitoring bacteremia levels in rodent hosts.

In conclusion, the QC-PCR was found to be useful in studying the dynamics of Y. pestis infections in fleas and determining bacteremia levels in rodent hosts. This technique should be especially useful for vector competence studies with other species of fleas, as well as for analyzing pathogen-vector relationships for other arthropod-associated agents. GFP-transformed pathogens, such as the ones used in our study, as well as ones used in other studies, also provide a unique means in visualizing these dynamics.

Acknowledgments: We thank May Chu for advice and manuscript review. We thank Leon Carter, Brook M. Yockey, Zenda L. Berrada, and Todd S. Deppe for their assistance in performing diagnostic serology tests and culture identification.

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REFERENCES


