ESTIMATION OF VECTOR INFECTIVITY RATES FOR PLAGUE BY MEANS OF A STANDARD CURVE-BASED COMPETITIVE POLYMERASE CHAIN REACTION METHOD TO QUANTIFY \textsc{Yersinia pestis} IN FLEAS

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Abstract. The prevalence of infectivity within a vector population is a critical factor in arthropod-borne disease epidemiology but it is difficult to estimate. In the case of bubonic plague, infective flea vectors contain large numbers of \textit{Yersinia pestis} within a bacterial mass that blocks the flea’s foregut, and only such blocked fleas are important for biologic transmission. A bacterial quantitation method could therefore be used to assess the prevalence of plague-infective (blocked) fleas in a population. We developed a standard, curve-based, competitive polymerase chain reaction (PCR) procedure to quantify \textit{Y. pestis} in individual fleas. The quantitative PCR (Q-PCR) method equaled a colony count reference method in accuracy and precision when evaluated using mock samples and laboratory-infected fleas. The Q-PCR was more reliable than colony count, however, for field-collected fleas and for blocked fleas collected after their death. In a sample of fleas collected from a prairie dog colony in the aftermath of a plague epizootic, 48% were infected but less than 2% contained numbers of \textit{Y. pestis} indicative of blockage. The method provides a means to monitor plague epizootics and associated risks of flea-borne transmission to humans, and is applicable to the study of other vector-borne diseases.

\textit{Yersinia pestis}, the bacterial agent of bubonic plague, is primarily a parasite of wild rodents that persists in permanent, discrete enzootic foci throughout the world. For example, since 1970 wild rodent plague has been documented in 15 western states, an area comprising about 40% of the continental United States. \textit{Yersinia pestis} is transmitted from mammal to mammal primarily by the bites of infected fleas, and most human cases of plague in the United States result from incidental contact with wild rodents or their fleas, i.e., plague is a zoonosis. More than 200 species of rodents and at least 80 species of fleas have been implicated in maintaining cycles of plague throughout the world.

The worldwide rodent plague foci constitute a vast enzootic reservoir that is an ever-present threat to human health, as history has demonstrated. The disease exhibits a pattern of periodic eruptive epizootics that result in rapid amplification and geographic spread followed by regression to focal areas. The risk to humans can increase in the aftermath of such epizootics, particularly if the disease crosses over into urban rat populations; therefore, public health officials monitor plague in susceptible wild rodent populations. This surveillance usually entails serologic detection of plague-specific antibodies from domestic dogs, other carnivores, and wild rodents, investigation of precipitous die-offs among plague-susceptible colonial rodents such as prairie dogs, and inoculation of suspect mammal tissue or pooled flea triturates into experimental animals and subsequent isolation of \textit{Y. pestis}.

As an option to these time-consuming standard methods, we recently developed a sensitive polymerase chain reaction (PCR) method to rapidly detect \textit{Y. pestis} in fleas. Using the insect host to monitor prevalence of plague offers some inherent advantages. Fleas are relatively easy to collect and are more convenient to handle than mammalian tissue. The course of \textit{Y. pestis} infection in the flea that leads to transmission suggests that additional epidemiologic information could be gained by examining fleas. Infective or blocked fleas (capable of biologic transmission) contain large numbers of \textit{Y. pestis} in a bacterial mass that blocks the flea digestive tract. Quantifying the bacterial load in plague-positive fleas would thus indicate the prevalence of blocked fleas. Vectorial capacity, an important parameter for predicting the course of a vector-borne disease in a population, could then be estimated.

There is at present, however, no satisfactory way to quantify plague bacilli in an infected flea. The classic method of triturating a flea in liquid culture medium, making dilutions, and determining colony-forming units is not practical for field-collected fleas unless they are frozen while still alive, because dead fleas dehydrate and the bacteria they contain lose viability. Contaminating microorganisms present in field-collected fleas also often overgrow the culture plates. Here we describe a quantitative PCR (Q-PCR) method to quantitate \textit{Y. pestis} in individual fleas, thus providing a way to estimate the percentage of blocked fleas in a population. This method, in conjunction with the previously described qualitative PCR method, provide new investigative tools for plague surveillance and epidemiology. In particular, the ability to easily monitor the increase and decrease of the incidence of the plague bacillus within stable colonies of rodents via their fleas makes feasible the development of predictive mathematical models of plague epizootics.

MATERIALS AND METHODS

\textit{Yersinia pestis} strains and PCR amplification target. The \textit{Y. pestis} PCR target was the chromosomal ferric iron uptake regulation (\textit{fur}) gene. The PCR primers Ypfu1 (5’-GAAGTGTGGCAAATCTGG-3’) and Ypfur2 (5’-AGTGACCGTATAATACAGGC-3’) correspond to nucleotides 70–90 and 377–397, respectively, of the \textit{fur} gene sequence reported by Staggs and Perry and yield a 328-basepair (bp) fragment in the PCR of \textit{Y. pestis} DNA. \textit{Yersinia pestis} strains 195/P (wild type), 195/P-2, and KIM6+ cultured in brain-heart infusion (BHI) broth at 28°C were used.
in this study. The KIM6+ strain lacks the 70-kb Y. pestis plasmid and is therefore nonvirent for mammals, but infects fleas normally. The 195/P-2 strain lacks the 10 kb and the 70-kb Y. pestis plasmids.

**Preparation of PCR competitor.** The competitor template was a deletion derivative of recombinant plasmid pFURY0.6 (provided by Robert Perry, University of Kentucky, Lexington, KY) that contains the cloned Y. pestis fur gene. To make the deletion, pFURY0.6 was digested with restriction endonucleases *Esp* 1 and *Sfu* I. Plasmid pFURY0.6 contains unique recognition sites for these two enzymes that are located 65 bp apart within the fur gene sequence internal to the PCR primer binding sites. Digestion with these two enzymes therefore removed a 65-bp segment from the fur gene, leaving the rest of the plasmid otherwise intact. Following digestion, blunt ends were produced with Klenow enzyme. The modified plasmid DNA was then re-circularized by ligation and used to transform *Escherichia coli* DH5α. The resultant plasmid was named pFURYD. The PCR with primers Yp fur1 and Yp fur2 using pFURYD as template resulted in a product estimated to be 263 bp, the predicted 65 bp shorter than the 328-bp PCR product from pFURY0.6 or *Y. pestis* genomic DNA. Plasmid pFURYD was linearized with Hind III, purified by phenol-chloroform extraction and ethanol precipitation, and quantified by UV spectrophotometry.

**Flea samples.** Individual Oriental rat fleas (*Xenopsylla cheopis*) from a laboratory colony maintained as previously described, or field-collected prairie dog fleas (*Oropsylla hirsuta*) were placed into separate 1.5-mL microcentrifuge tubes containing 20 μL of a 0.1-mm glass sand slurry in 1% bovine serum albumin. Each flea was thoroughly triturated in the BHI broth with a heat-sealed disposable plastic micropipet tip. *Xenopsylla cheopis* fleas that had fed only on uninfected mice were used for standard and negative control samples. Fifty microliters of BHI broth containing 105 or 106 *Y. pestis* 195/P-2 cells was added to the four samples used as standards. These cells were from a stationary-phase culture that had been quantitated by direct count using a Petroff-Haussner bacterial counting chamber and phase microscopy, diluted to 2 × 105 cells/ml in BHI broth, and frozen in 100-μL aliquots. For each Q-PCR run, a single aliquot was thawed and a dilution series was made in BHI broth for use as standards. Sterile BHI broth (50 μL) was added to all other flea samples. Tubes containing the flea suspensions were immediately placed in a 95°C heat block for 10 min to lyse the cells. The tubes were next centrifuged for 10 sec at maximum speed in a microcentrifuge to pellet the flea tissue debris and glass sand. Sample tubes were stored at 4°C until Q-PCR was performed, usually within 15 min.

**Quantitative PCR protocol.** The hot start PCR protocol was essentially as previously described, with the additional step of adding a known amount of the competitor template to each sample. A 2× bottom-layer reagent mixture (10 mM Tris, pH 8.3, 3 mM MgCl2, 400 μM of each of the four deoxyribonucleoside triphosphates, 0.6 μM of primers Yp fur1 and Yp fur2) and an upper-layer dilution buffer (10 mM Tris, pH 8.3, 100 mM KCl) were prepared and stored at −20°C until ready for use. A separate 0.5-ml PCR tube (GeneAmp; Perkin-Elmer, Norwalk, CT) was labeled for each flea sample. A total of 50 μL of the 2× bottom-layer reagent was dispensed into each tube and a single wax bead (AmpliWax PCR Gem 100; Perkin Elmer) was added. The tubes were put in an 80°C heating block for 5 min to melt the wax and then placed at room temperature for 5 min to allow the wax to form a solid barrier on top of the bottom layer reagent. Five microliters of AmpliTaq DNA polymerase (5 U/μL; Perkin-Elmer) diluted 1:10 in upper-layer dilution buffer was added to the top of the wax barrier in each PCR tube, followed by 43 μL of upper-layer dilution buffer containing 104 molecules of pFURYD competitor and 2 μL of each flea-BHI broth preparation.

The PCR amplifications were conducted in a Perkin-Elmer thermal cycler by using the following program. For cycles 1 and 2, template denaturation was at 95°C for 2 min (this also melted the wax barrier, allowing the upper and lower layers to combine), primer annealing was at 55°C for 1 min, and primer extension was at 72°C for 1 min. Cycles 3–35 were identical except that denaturation steps were 1 min. After the last cycle, primer extension was continued for 10 min at 72°C. The reactions were held at 8°C until further analysis.

**Quantitation of *Y. pestis* per flea.** Thirty microliters of the 100-μl reaction mixtures was subjected to electrophoresis in 2% NuSieve GTG-0.5% SeaKem GTG agarose gels (FMC BioProducts, Rockland, ME) at 2 V/cm in TBE buffer (90 mM Tris, pH 8, 90 mM borate, 2 mM EDTA) containing 0.1 mg of ethidium bromide/ml. Following electrophoresis, the stained gel was photographed using Polaroid (Cambridge, MA) 55 film. The negative was scanned with an enhanced UltroScan XL Laser Densitometer (model 2222-020; Pharmacia LKB Biotechnology, Piscataway, NJ) using the GelScan XL software package (version 2.1; Pharmacia). The areas under the peaks corresponding to the 328-bp and 263-bp ethidium bromide–stained bands were recorded. Since the amount of ethidium bromide incorporated by a DNA fragment is directly proportional to its length, the signal derived from the shorter fragment was normalized to the larger fragment by multiplying it by 1.247 (328/263).

To make a standard curve, the log of the ratio of the *Y. pestis*-derived 328-bp signal to the competitor-derived 263-bp signal was plotted against the log number of *Y. pestis* cells. A standard colony count method. *Yersinia pestis* 195/P-2 cells in a fresh stationary-phase culture in BHI broth were first quantitated by using a Petroff-Haussner counting chamber. A series of dilutions in BHI broth was made, and 50 μL of BHI broth containing known numbers of cells was added to uninfected flea triturates. Prior to heating the samples for Q-PCR, a portion was removed, diluted, spread onto BHI agar plates, and incubated at 28°C for four
days to determine the number of colony-forming units. The Q-PCR was then conducted on the samples. Results obtained by Q-PCR and by colony count were compared with each other and to the reference direct count.

**Laboratory-infected unblocked and blocked flea samples.** *Xenopsylla cheopis* fleas were infected using a membrane feeder apparatus with blood containing approximately $5 \times 10^8$ *Y. pestis* KIM$6+$/ml. This concentration of bacteria in the blood meal has been shown to result in stable infection of a majority of fleas. Fleas that had taken an infectious blood meal were maintained at $20^\circ C$, 76% relative humidity and subsequently fed on uninfected mice two days and six days after infection. Seven days after infection (prior to development of blockage), surviving female fleas were frozen at $-70^\circ C$ until analysis by Q-PCR and colony count.

Known blocked flea samples were from a separate cohort of *X. cheopis* fleas that had been infected by feeding on a mouse that had approximately $4.3 \times 10^8$ *Y. pestis* 195/Pl/ml of peripheral blood at the time of flea feeding. These naturally infected fleas were maintained as above and fed twice a week on uninfected mice. Immediately after each feeding period, the fleas were individually examined under a dissecting microscope for blockage, indicated by the presence of fresh blood in the esophagus but not in the midgut. Blocked fleas were segregated and later analyzed by Q-PCR and colony count. Because blocked fleas typically contained greater than $10^6$ bacteria, the Q-PCR standard curve was prepared with $10^4$–$10^7$ *Y. pestis* standards, and $10^4$ copies of the competitor were added to all samples.

**Field-collected flea samples.** *Oropsylla hirsuta* fleas were collected from a prairie dog (*Cynomys ludovicianus*) colony in Larimer County, Colorado. Fleas were collected in June 1994 from burrows by using a white flannel square attached to the end of a wire cable. A plague epizootic associated with a human case had exterminated the colony in the months prior to collection, and many of the flea specimens were dead and dessicated. Dead and living fleas were placed together in 2% NaCl containing 0.001% Tween 80, transported at ambient temperature, and stored at $-20^\circ C$ 48 hr later. Prior to analysis in August 1994, fleas were thawed, identified, and surface-sterilized by washing them once with sterile saline, twice with 3% H$_2$O$_2$, for 3 min, and twice with sterile distilled H$_2$O. Individual fleas were then triturated as described above, and assayed by both colony count and a sterile distilled H$_2$O. Individual *Y. pestis* were then triturated as described above, and assayed by both colony count and a sterile distilled H$_2$O.

**RESULTS**

In this study we used a competitive PCR method to quantify *Y. pestis* in its flea vector. In the competitive Q-PCR, a DNA fragment containing the same primer template sequences as the target competes for primer binding and amplification. Since the target to be quantified and the competitor are coamplified in the same tube and share the same primer binding sites (and in fact are identical in sequence except for a small deletion in the competitor), they are presumably amplified with equal efficiency. Any variable effecting PCR amplification, for instance inhibitors present in fleas or their blood meal, has the same effect on both. To quantitate the target, the ratio of target to competitor products is measured. Regardless of whether the PCR gives a high or a low yield, the ratio of the two products depends only on their initial ratios and is independent of the number of PCR cycles. In equimolar competitive Q-PCR, increasing known amounts of the competitor are added to replicate samples of the DNA target to be quantitated, and separate PCRs are carried out. Because this requires multiple reactions for each sample, it is impractical for assaying large numbers of fleas. We used instead the modification of Zachar and others based on comparing the results of individual samples to a single set of standard samples, all of which contain a fixed amount of the competitor. The PCR target was the chromosomal *Y. pestis* fur gene, so quantifying this target would quantitate genome equivalents, and by extension, numbers of bacteria present in the fleas. The competitor was a linearized recombinant plasmid that contained a cloned version of the fur gene from which a 65-bp segment had been deleted.

**Evaluation of Q-PCR method.** Each assay included four standards, in which $10^3$, $10^4$, $10^5$, or $10^6$ *Y. pestis* cells were added to an uninfected flea triturate. A constant amount of competitor, predetermined empirically to result in a detectable product in each of the four standard reactions, was added to all flea samples. After the PCR, the 328-bp product derived from the *Y. pestis* chromosomal target and the 263-bp product derived from the competitor were visualized by gel electrophoresis and staining with ethidium bromide. As expected, the intensity of the 263-bp competitor band was inversely proportional to the number of *Y. pestis* target cells present in the standards (Figure 1A). For each of the four standards, the intensities of the target- and competitor-derived bands were quantitated by densitometry and the log of their ratio was plotted against the log number of *Y. pestis* cells present. From this plot a standard curve (Figure 1B) was computed by linear regression analysis. To calculate the number of *Y. pestis* in the unknown flea samples, the log of the ratio of the two coamplified bands was used in the linear equation of the standard curve to solve for the log number of *Y. pestis*. In 30 separate assays over a 13-month period, the plotted standard values were always nearly colinear, with $R^2$ values ranging from 0.93 to 1.0 (mean = 0.98, SD = 0.02) and slopes ranging from 0.71 to 1.21 (mean = 0.93, SD = 0.14). These results indicated that with sensitive densitometry to measure faint bands, the standard curve was highly reproducible and linear over three orders of magnitude. Changing the Mg$^{++}$ concentration or decreasing the number of PCR cycles from 35 to 25 did not change the relative yield of the two products, although absolute yields were affected. The use of frozen aliquots of a stock cell suspension to prepare the standards and incorporation of the competitor into a master mixture made the Q-PCR no more cumbersome than routine PCR. The standard curve-based competitive Q-PCR is similar in principle to radioimmunoassay (RIA) and ELISA procedures in which a constant amount of labeled ligand is added to standard and unknown samples and competes for binding with native ligand present in the samples.

To explicitly evaluate the reliability of the method, the Q-PCR was compared with a standard colony count method. Random error (precision) was ascertained by quantifying *Y.
FIGURE 1. A, ethidium bromide–stained agarose gel showing quantitative polymerase chain reaction (Q-PCR) results for four standard samples (uninfected laboratory-reared Xenopsylla cheopis rat fleas to which the indicated number of Yersinia pestis cells was added), a negative control (Neg. Cont.) sample (uninfected rat flea), and six laboratory-infected rat fleas (labeled 1–6). Arrows at left indicate the 328-basepair (bp) and 263-bp bands that are derived from the Y. pestis and the competitor templates, respectively. B, standard curve generated from densitometry analysis of the electrophoretic pattern of the four standard samples shown in A. For each of the four standards, the logarithm of the ratio of the Y. pestis product to the competitor product was plotted against the log of the known number of Y. pestis cells present. The standard curve was then determined by linear regression analysis and the resulting equation used to calculate the number of Y. pestis cells present in the unknown rat flea samples.

pestis in 21 replicate samples of three different cell densities by both Q-PCR and colony count. Seven of the 21 samples were assayed on a different day using a separate standard curve to assess run-to-run as well as sample-to-sample variation. Comparison of the mean and SD values of replicate samples (Figure 2A) indicated that the Q-PCR method was as reliable as the reference colony count method. Systematic error (accuracy) was evaluated by plotting the Q-PCR results against the plate count results for a series of 24 samples that incrementally encompassed the assay range. A bias plot showing the relationship between the Q-PCR and colony counts for these 32 samples indicated a high correlation between the two methods, with an R² value of 0.93 (Figure 2B).

Quantitation of Y. pestis in laboratory-infected fleas. The Q-PCR method was first used to quantitate Y. pestis in
Figure 2. Evaluation of accuracy and precision of the quantitative polymerase chain reaction (Q-PCR) method. A, mean and SD of Q-PCR and colony count quantitation results for sets of 21 replicate *Xenopsylla cheopis* rat flea samples containing $5 \times 10^3$, $5 \times 10^4$, or $8 \times 10^5$ *Yersinia pestis* cells as predetermined by direct count. B, scatterplot showing relationship between Q-PCR (ordinate) and colony count (abscissa) quantitation results for 24 different rat flea samples.

Quantitation of *Y. pestis* in laboratory-infected *X. cheopis* rat fleas. Seven days after infection, surviving fleas were stored at -70°C, and the number of bacteria per flea was later determined by Q-PCR and colony count. During the first week after infection, the bacteria are either cleared by the flea or else they successfully colonize the alimentary canal in the form of large bacterial masses, antecedent to the development of blockage. The results from 35 such infected but unblocked fleas (Figure 3A) show that most contained between $10^4$ and $10^5$ bacteria, as determined by either method. These results are in agreement with previous studies, and further indicate the efficacy of the Q-PCR method.

Because we were interested in using the Q-PCR to identify infective (blocked) fleas, a sample of blocked *X. cheopis* fleas was also examined. Blockage usually developed 2–4 weeks after infection. Blocked fleas died within one week of becoming blocked and were stored at -70°C within four days after death. Analysis of these fleas (Figure 3B) revealed a difference between the two quantitation methods. Thirty-four of 35 blocked fleas contained $10^5$–$10^7$ *Y. pestis* per flea as determined by the Q-PCR, whereas only 14 colony counts of the same 35 blocked fleas were that high, and 14 of the 35 colony counts were less than $10^3$ *Y. pestis* per flea. This discrepancy is probably due to loss of bacterial viability in dehydrated, dead fleas because no significant differences were evident between total count (Q-PCR) and viable (colony) count for five blocked fleas that had been frozen, while still alive, immediately after blockage was detected. These results indicate that the total count Q-PCR method is superior to viable count in quantifying bacteria in fleas of unknown condition, such as field-collected fleas.

Quantitation of *Y. pestis* in field-collected fleas. Forty-four *O. hirsuta* fleas (22 males and 22 females) that had been collected from the site of a prairie dog plague epizootic were prepared and analyzed by colony count and by a previously described PCR. This nonquantitative PCR was used to initially screen the fleas because it is more sensitive (able to detect as few as 10 *Y. pestis* per flea) and it is specific for *Y. pestis*. The Q-PCR primers also amplify *fur* gene DNA from *Y. pseudotuberculosis*, which can infect fleas rarely. The field-collected sample included many fleas that were markedly dehydrated, indicating that they were dead or dying when collected. Even though fleas were individually surface-sterilized, all colony count plates were overgrown with a variety of bacterial and fungal contaminants and were uninterpretable. This problem has been noted by others, and indicates that colony count quantitation requires special handling of flea samples, such as surface-sterilization and plating or frozen storage immediately after collection. However, 21 of the 44 fleas (48%; 14 males and seven females) were PCR positive, indicating that they were infected with *Y. pestis*. These positive fleas were further analyzed by Q-PCR (Figure 4). Seventeen of these 21 infected fleas contained less than $10^3$, three fleas contained $10^3$–$10^4$, and one flea contained $10^4$–$10^5$ *Y. pestis*.

Discussion

Competitive Q-PCR has been used to quantify viruses, protozoa, and bacteria in a variety of clinical and environmental samples, including bacterial endosymbionts within individual mosquitoes. In this study, we designed a standard-curve based, competitive Q-PCR method to quantify *Y. pestis* in fleas as a means to estimate the prevalence of infective (blocked) vectors in a population. The results of individual flea samples were interpolated from a calibration curve prepared from a single set of standard samples. The method proved to reliably indicate numbers of plague bacilli in individual fleas over three orders of magnitude. Standard curve-based Q-PCR methods similar to the one described here have been developed to quantitate bacteria in water, *Plasmodium falciparum* sporozoites in their mosquito vectors, and the expression of specific eukaryotic genes. Many plague workers have emphasized the complex ecology of plague, involving interrelationships between rodent
hosts, flea vectors, microorganism, and external environmental conditions.\textsuperscript{2-4} The factors that work together to produce a plague epizootic remain obscure, and epizootics recur at irregular, unpredictable intervals.\textsuperscript{28} An epidemic of a vector-borne disease is initiated by a sufficiently high vectorial capacity, the average daily number of potentially infectious bites experienced per individual in the host population.\textsuperscript{29, 30} A key component of vectorial capacity is the prevalence of infective vectors, but this parameter is often difficult to quantitate and must be estimated indirectly based on several factors and assumptions.\textsuperscript{29, 31}

Plague should provide an instructive system for modeling the dynamics of a vector-borne disease. Plague is common in the western United States, and the many populations of wild rodents in which it is enzootic provide ample opportunities for study. Fleas permanently reside on their hosts or in their hosts’ dwellings, so the relevant vector population can be directly sampled. The percentage of blocked fleas can be measured by using the Q-PCR because biological transmission requires a priori propagation of \textit{Y. pestis} to large numbers in the flea to produce the blockage. We found that 56 (96\%) of 58 blocked \textit{X. cheopis} contained greater than $10^5$, and 38 (65\%) of 58 contained greater than $10^6$ \textit{Y. pestis} (Figure 2B and unpublished data). Although this correlation

\begin{figure}[h]
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  \includegraphics[width=\textwidth]{figure3.png}
  \caption{Comparison of the quantitative polymerase chain reaction (Q-PCR) and colony count results for 35 individual laboratory-infected unblocked (A) and blocked (B) \textit{Xenopsylla cheopis} rat fleas. Unblocked fleas were collected, while still alive, one week after the infectious blood meal and blocked fleas were collected after they died, 2–4 weeks postinfection. The lines connecting the individual data points are to enhance visualization of the overall patterns; they do not indicate a functional relationship between the discrete flea samples.}
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\begin{figure}[h]
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  \caption{Agarose gel electrophoresis of the quantitative polymerase chain reaction (Q-PCR) of 22 individual field-collected \textit{Oropsylla hirsuta} prairie dog fleas. Samples used to prepare the standard curve are also shown. Arrows on the left indicate the 328-basepair (bp) and 263-bp bands that derived from the \textit{Yersinia pestis} and the competitor templates.}
\end{figure}
has not explicitly been made for other species, any blocked flea would be expected to contain large numbers of bacteria because blockage occurs by the same mechanism in all fleas for which this phenomenon has been described. Based on the X. cheopis data, a bacterial burden of $10^4$ or greater could be chosen to indicate potential infectivity, but this number should be verified whenever possible for a given flea species. This reckoning may overestimate the actual prevalence of blockage somewhat because large bacterial masses can develop in the flea that do not block the foregut (Figure 3A and unpublished data).

Using the $10^5$ criterion, none of the 44 prairie dog fleas examined was blocked, although one contained approximately $2 \times 10^4$ Y. pestis and may have been incipiently blocked. Further experience will be required to ascertain the bacterial levels achieved in this and other fleas. During a 1964–1966 survey of a Cynomys gunnisoni prairie dog epizootic, more than 12,000 fleas were collected from burrows and then pooled, triturated, and assayed by animal inoculation. Thirty percent of these pools were positive for Y. pestis during the first two summers of the epizootic. Both the number of fleas collected per burrow and the percentage of positive pools decreased during the third summer, by which time the colony was nearly exterminated. In this and other field studies, however, there are few data on individual flea infection rates and none on blockage rates.10, 15, 33

The relatively small bacterial load present in most of the positive prairie dog fleas supports the well-known distinction between infected and infective (blocked) fleas2 and the value of quantitation to distinguish them. The results may reflect the terminal stage of the epizootic during which these fleas were collected. Fewer bacteria present in the field-collected O. hirsuta than the laboratory-infected X. cheopis could result from lower bacteremia in the prairie dogs than the very high concentrations present in the laboratory mouse or artificial blood meal (laboratory-infected X. cheopis initially ingested $10^4$–$10^5$ bacteria),12 or greater excretion of ingested bacteria. A previous study showed that feeding on a bacteremic guinea pig infected 28% of O. hirsuta and 38% of X. cheopis, with the majority of fleas clearing themselves of infection. The average time between ingestion and development of blockage (the extrinsic incubation period) was only 21 days for X. cheopis, but 102 days for O. hirsuta.8 In general, different flea species vary in their susceptibility to stable colonization and blockage following an infectious blood meal.2, 8, 34 This is an important consideration because the flea index (the average number of each different flea species per host) of a rodent population fluctuates seasonally.1, 3, 15, 32, 35 A direct means to assess blockage, such as the Q-PCR, thus greatly simplifies estimation of the overall prevalence of infectivity among a heterogeneous vector population at a given time.

The Q-PCR to determine the prevalence of infective vectors, combined with data pertaining to the expected infective vector-host contact, would enable a more direct estimate of vectorial capacity. These other data include average number of attempted blood meals during the short life span of a blocked flea,2, 8 flea index, and the number of susceptible hosts in the population. Mathematical models to predict the future course of plague in the population could then be made.29 Furthermore, temporally monitoring the prevalence of blockage among fleas associated with a rodent population may in itself prove predictive of disease trends in an enzootic area, even in the absence of other epidemiologic data.31

The Q-PCR method described here provides a new tool for basic research into plague ecology and may help to answer some of the lingering questions concerning the enigmatic appearance and disappearance of plague. Although not primarily designed for routine plague surveillance, the ability to monitor flea blockage rates during the course of an identified epizootic would also provide a more reliable means to evaluate risks to human populations. The method should be applicable to other vector-borne diseases, particularly those for which the etiologic agent is difficult to quantitatively culture in vitro. It may also be useful in examining other epidemiologic issues, such as vertical or transovarial transmission of vector-borne disease agents.

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