Poor Vector Competence of Fleas and the Evolution of Hypervirulence in *Yersinia pestis*

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Population genetics and comparative genomics analyses of the pathogenic *Yersinia* species have indicated that arthropodborne transmission is an evolutionarily recent adaptation in *Yersinia pestis*, the agent of plague. We show that the infectivity of *Y. pestis* to its most proficient vector, the rat flea *Xenopsylla cheopis*, and subsequent transmission efficiency are both low. The poor vector competence of fleas likely imposed selective pressure that favored the emergence and continued maintenance of a hypervirulent *Y. pestis* clone. In particular, the rapidly fatal gram-negative sepsis that typifies plague is a consequence of the high threshold bacteremia level that must be attained to complete the transmission cycle. Epidemiological modeling predicts that, to compensate for a relatively short period of infectivity of the mammalian host for the arthropod vector, plague epizootics require a high flea burden per host, even when the susceptible host population density is high.

*Yersinia pestis*, the highly virulent agent of bubonic plague, is unique among the enteric group of gram-negative bacteria in having adopted an arthropodborne route of transmission. The results of population genetics analyses have indicated that *Y. pestis* strains worldwide constitute a highly uniform clone that diverged from the closely related *Yersinia pseudotuberculosis* within the last 1500–20,000 years, and comparative genomics analyses have confirmed a high degree of genetic identity [1–3]. Despite this recent common ancestry and genetic relatedness, the 2 sister species differ radically in natural history. *Y. pseudotuberculosis* is transmitted perorally in contaminated food and water and produces a relatively benign, self-limited enteric disease in humans and many animals. *Y. pestis*, by contrast, is transmitted primarily by fleas and is highly invasive and virulent. Presumably, these changes occurred during the evolutionarily short period since *Y. pestis* diverged from the *Y. pseudotuberculosis* progenitor.

To produce a transmissible infection, *Y. pestis* grows in the flea digestive tract as a biofilm that adheres to and fills the proventriculus, the valve that connects the esophagus to the midgut. The biofilm interferes with the normal valvular action and eventually blocks the flow of blood through the proventriculus. Transmission occurs when bacteria released from the biofilm are refluxed into the bite site when blocked fleas attempt to feed [4–6]. Within the first week after transmission from the flea, bacteria disseminate from the intradermal bite site to the regional lymph nodes to produce bubonic plague. From there, the bacteria rapidly invade the bloodstream and infect many internal organs. Infected tissues typically contain massive numbers of bacteria, and a high-density, usually fatal septicemia is a hallmark of plague [7]. Plague is characterized by an irregular pattern of periodic eruptive epizootics among susceptible rodents, which results in rapid amplification and geographic spread, followed by regression to enzootic focal areas [7].

Approximately 80 species of fleas, associated with some 200 species of wild rodents, have been found to be susceptible to *Y. pestis* infection [8]. Although the comparative vector efficiency of some of these fleas has been examined [8–14], their susceptibility to infection and transmission efficiency have not been directly mea-
sured. For the present study, we evaluated the vector competence of the rat flea *Xenopsylla cheopis*, the most efficient plague vector among ~40 species that have been examined [8–14]. The data indicate that the transmission of *Y. pestis* by fleas is inefficient, and they suggest that one consequence of the development of a vectorborne route of transmission was the emergence and continued maintenance of a highly virulent *Y. pestis* clone. These and other direct measurements of parameters of *X. cheopis* vectorial capacity presented here permit epidemiologic modeling of plague transmission dynamics and predictions about the outcome of vector control.

**MATERIALS AND METHODS**

**Infection of fleas with *Y. pestis***. *X. cheopis* fleas were infected with *Y. pestis* strain KIM6+ by use of an artificial feeding system and mouse blood that contained known bacterial concentrations, prepared as described elsewhere [5, 15, 16]. To determine the infectious dose of *Y. pestis* for fleas, blood meals that contained 4.0 × 10^6–4.2 × 10^8 *Y. pestis*/mL were used to infect *X. cheopis*. For each experiment, a sample of 20 female fleas was collected and stored at −80°C immediately after feeding; this sample was later used to determine the average dose of *Y. pestis* acquired in the blood meal. The remaining fleas were maintained and fed on healthy, uninfected mice on days 2 and 6 after infection, and a second sample of 20 female fleas was collected on day 7 to determine the infection rate and bacterial load. The volume of the blood meal was calculated in female fleas, collected immediately after their infectious blood meal, by dividing the average number of *Y. pestis* colony-forming units by the number of colony-forming units per milliliter of the blood they fed on. The results from 27 separate feeding experiments involving 469 fleas were used to determine the average and SD.

Other groups of fleas that ingested an infectious blood meal containing ~5 × 10^8 *Y. pestis*/mL were fed twice weekly for 4 weeks on uninfected mice. After each of these subsequent feedings, the fleas were examined under a dissecting microscope for evidence of proventricular blockage, which was diagnosed by the presence of fresh blood only in the esophagus [16].

**Flea survival and blockage rates**. Data were compiled from 930 *X. cheopis* infected with *Y. pestis* strains KIM6+ or 195/P in 10 separate experiments [16, 17]. All fleas fed on blood from either a membrane feeder or a mouse with a septicaemia level of ~5 × 10^8 *Y. pestis*/mL. Fleas were kept at 21°C and 75% relative humidity, fed twice weekly on uninfected mice, and monitored for mortality and proventricular blockage for 4 weeks after the infectious blood meal [16].

**Fleaborne transmission**. Individual blocked fleas were allowed to feed for 1 h on 0.3 mL of sterile heparinized mouse blood in a miniature artificial feeding device. Alternatively, blocked fleas were placed on shaved areas of uninfected mice. Immediately after feeding attempts, mice were killed; the resulting skin-biopsy samples of bite sites were stored at −80°C. For both the mouse and artificial feeding experiments, all fleas were examined microscopically, to verify blockage and attempted feeding.

**Quantitation of *Y. pestis* in fleas and tissues**. Individual fleas were surface sterilized and triturated, and dilutions of each triturate were plated on brain-heart infusion agar that contained 0.1% Irgasan (Ciba-Geigy), for colony-forming unit counts after a 72-h incubation at 28°C [5, 16]. To determine the number of *Y. pestis* colony-forming units transmitted by blocked fleas, blood was removed from the membrane feeder, the mouse skin membrane was washed 3 times with 0.1 mL of PBS, and blood and washings were spread on *Yersinia* selective agar (YSA) plates. The washed skin from the membrane feeder and the biopsies of bite sites from mice were digested with 5 μL of a 10% (wt/vol) solution of collagenase A (Roche Biochemicals) in 500 μL of 154 mmol/L NaCl, 5.6 mmol/L KCl, and 1.7 mmol/L CaCl_2 for 1 h at 37°C. The digests were then manually triturated and plated on YSA. Control experiments, in which colony-forming units from replicate aliquots of the buffer containing *Y. pestis* and uninfected skin with or without added collagenase, verified that the enzyme treatment did not affect bacterial viability or growth.

Quantitative TaqMan polymerase chain reaction (PCR) was performed on total DNA extracted from skin-biopsy samples of bite sites by use of a primer and probe set for the *Y. pestis* pla gene. Numbers of *Y. pestis* in the bite samples were determined by extrapolation from a standard curve generated from TaqMan results of uninfected skin-biopsy samples to which known numbers of *Y. pestis* were added.

**RESULTS AND DISCUSSION**

**Oral infectious dose of *Y. pestis* for *X. cheopis***. The ID<sub>50</sub> was determined by allowing groups of fleas to feed on blood that contained different concentrations of *Y. pestis*. The ID<sub>50</sub> for *X. cheopis* was 4.8 × 10<sup>7</sup> *Y. pestis* (95% confidence interval, 2.9 × 10<sup>7</sup>–1.4 × 10<sup>8</sup>), determined by linear-regression analysis of the data in table 1. In comparison, the ID<sub>50</sub> of *Y. pestis* for susceptible mammals is <10 bacteria [7]. The average volume of blood ingested by *X. cheopis* was calculated to be 0.12 ± 0.07 μL; estimates of 0.03–0.5 μL have been reported elsewhere [18]. Thus, the threshold septicemia level that would provide 1 ID<sub>50</sub> (4800 *Y. pestis*) in the 0.12 μL ingested by a feeding flea is ~4.0 × 10<sup>7</sup> bacteria/mL peripheral blood. The high ID<sub>50</sub> of *Y. pestis* for *X. cheopis* was not strain dependent or an artifact of the membrane feeder infection method; the rate and kinetics of infection of *X. cheopis* by the *Y. pestis* strain 195/P that fed on a mouse with a septicemia level of 4.3 × 10<sup>7</sup> *Y. pestis*/mL were the same as those observed for fleas artificially infected by use of blood with the same concentration of *Y. pestis* 195/P.
Y. pestis differs from a previous indirect estimate of 11,000–24,000 number of bacteria transmitted (data not shown). Our results quantitation of the colony count did not underestimate the actual measured the number of that break free from the biofilm [6]. Quantitative PCR (which also of various-sized clusters of bacteria through peristalsis and excretion in flea feces [4, 6].

Transmission efficiency of X. cheopis. Individual blocked fleas were allowed to feed on mice or on a membrane feeder, and the number of Y. pestis colony-forming units transmitted per flea was determined (figure 1). Less than one-half (14/31 [45%]) of the samples were positive; these contained 2–4970 Y. pestis (average ± SD, 636 ± 1324 Y. pestis; median, 82 Y. pestis). These results are consistent with the low LD₅₀ of Y. pestis in mammals [7] and with the results of previous reports that plague results from the bite of an individual blocked X. cheopis only 36%–47% of the time [9, 12–14]. Only 2 of 14 positive samples contained >1000 Y. pestis, even though the corresponding transmitting fleas contained an average of 4.8 × 10⁷ Y. pestis (median, 4.0 × 10⁷ Y. pestis; range, 2.1 × 10⁶–1.9 × 10⁸ Y. pestis)—a median transmission efficiency of <0.1%.

A likely explanation for the irregular distribution (figure 1) is that, because the transmitted bacteria derive from a biofilm in the flea proventriculus, the infectious units may consist not only of individual bacteria but also of various-sized clusters of bacteria that break free from the biofilm [6]. Quantitative PCR (which measured the number of Y. pestis genome equivalents transmitted) performed on an additional 25 skin biopsies verified that quantitation of the colony count did not underestimate the actual number of bacteria transmitted (data not shown). Our results differ from a previous indirect estimate of 11,000–24,000 Y. pestis transmitted per flea, which was based on a single blocked X. cheopis flea [9].

Evolution of increased virulence. The high infectious threshold for fleas and the low rate of transmission of small numbers of bacteria imply that only a strain able to produce high-density septicemia from a small intradermal infectious dose is able to complete the vector-host life cycle. Thus, the evolutionary change to fleaborne transmission would have imposed new selective pressures that strongly favored the coevolution of increased virulence. For the Y. pestis progenitor, the acquisition of 2 plasmids, one of which encodes a phospholipase D required for survival in the flea midgut and the other of which encodes a plasminogen activator that increases invasiveness in the mammalian host, were important steps in this evolutionary process [17, 19–21]. Epidemiological models have hypothesized that pathogens evolve a level of virulence that maximizes their basic reproduction ratio (R₀)—the number of new infections that a single infected host gives rise to during its infectious life span [22]. R₀ is directly proportional to the transmission rate and to the length of time during which a host remains infectious. A high transmission rate correlates with high parasite density in the host, but the associated virulence tends to shorten the host’s life span and to reduce the infectious period. A low level of pathogen reproduction in the host has little effect on host longevity (decreased virulence) but results in a lower transmission rate. Therefore, evolutionarily stable strains with an optimal R₀ are thought to have struck a balance between these 2 opposing tendencies that tempers virulence [22–24].

Does Y. pestis fit this model? The poor vector competence of fleas mandates that Y. pestis achieve high density in peripheral blood, and because gram-negative septicemias are rapidly fatal, the infectious period of the host to feeding fleas is brief. The transmission rate of an arthropodborne agent, however, depends only partly on vector competence or efficiency. Other factors, such as vector density, the number of bites experienced per day, the vector survival rate during the time it takes for a transmissible infection to develop, and the density of the sus-

<table>
<thead>
<tr>
<th>Y. pestis/mL in the blood meal</th>
<th>Average infectious dose/flea</th>
<th>Y. pestis/infected flea at 7 days, no. (%) of fleas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infection status at 7 days, no. (%) of fleas</td>
<td>Y. pestis/infected flea at 7 days, average no.</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>Not infected</td>
</tr>
<tr>
<td>4.0 × 10⁶</td>
<td>1100</td>
<td>1 (6)</td>
</tr>
<tr>
<td>1.9 × 10⁷</td>
<td>4900</td>
<td>3 (16)</td>
</tr>
<tr>
<td>2.0 × 10⁷</td>
<td>3800</td>
<td>6 (30)</td>
</tr>
<tr>
<td>5.0 × 10⁷</td>
<td>5600</td>
<td>11 (58)</td>
</tr>
<tr>
<td>1.8 × 10⁸</td>
<td>11,000</td>
<td>16 (89)</td>
</tr>
<tr>
<td>4.2 × 10⁸</td>
<td>20,000</td>
<td>15 (75)</td>
</tr>
</tbody>
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Table 1. Infectious dose threshold of Yersinia pestis for Xenopsylla cheopis.
ceptible host population also contribute to the overall vectorial capacity, which is a better measure of transmission rate [25]. Most rodents maintain a permanent flea population in their fur and in their nests or burrows, and fleas feed frequently. Thus, even though the time between threshold septicemia and death is short, if the flea burden is sufficiently high, there is good probability that >1 resident flea will ingest an infectious blood meal. Because fleas do not readily leave their host mammal, killing the host may actually be important to Y. pestis transmission. Death of the host compels the resident infected fleas to seek a new, healthy host—a necessary step to complete the transmission cycle [26]. The persistent feeding behavior of a blocked, starving flea also enhances vectorial capacity. These ecological factors weaken the linkage between extreme virulence and decreased opportunity for transmission, which is one of the key assumptions of most epidemiological models, and likely permitted the evolution of hypervirulence in Y. pestis.

For plague, models predicting a trade-off between virulence and transmission may better fit the insect host. Unlike most arthropodborne agents, Y. pestis is potentially fatal to its vector, because fleas with proventricular blockage rapidly starve to death [10, 11, 14]. However, only approximately one-half of chronically infected X. cheopis develop blockage, and this rate is lower for several other wild rodent flea species [5, 8, 9, 13, 16]. Thus, both the ID$_{50}$ and the LD$_{50}$ of Y. pestis are much higher for X. cheopis than for mammals, which may be important for the maintenance of transmission cycles. The evolution of virulence has been hypothesized to be more restricted in the arthropod than in the mammal, because an active vector is more important for transmission than a healthy, infected host [23].

**Vector density and transmission dynamics.** The X. cheopis density per primary host that is necessary to support an epizootic was calculated by use of a modification of Macdonald’s equation, a standard epidemiologic model of vectorborne disease dynamics [22, 25, 27]:

$$R_0 = \frac{ma(a')bp''}{r(\ln q)}$$,

where $m$ is the flea density per host; $a$ is the biting rate per day of an uninfected flea; $a'$ is the biting rate per day of a blocked flea; $b$ is the probability of blockage developing in a flea after it feeds on an infected host with septicemia at the threshold level or greater; $p$ and $q$ are the daily survival probability of unblocked and blocked fleas, respectively; $n$ is the number of days required for proventricular blockage to develop after the infectious blood meal (the extrinsic incubation period) and $p''$ is the probability of a flea surviving until blockage develops; and $1/r$ is the duration of infectivity (life expectancy) of the host after the threshold septicemia level is reached. In the modified equation, distinct terms were used for blocked (infective) fleas, because their daily biting rate and survival differ greatly from those of unblocked fleas. Experimental values for these parameters were determined from 930 infected fleas: 38% of the fleas developed proventricular blockage 7–28 days (median, 14 days) after the infectious blood meal ($b = 0.38$; $n = 14$), and mortality of unblocked fleas at 14 days was 15% ($p'' = 0.85$) for both artificially infected and naturally infected fleas [15, 16] (unpublished data). Unblocked fleas fed every ∼3 days ($a = 0.33$); blocked fleas attempted to feed every day ($a' = 1.0$), but they died from starvation within 2–5 days. In 2 separate infection kinetics studies, 14 Brown Norway rats survived only 2 days after their septicemia levels reached 1 × 10$^{6}$ Y. pestis/mL peripheral blood ($r = 0.5$; unpublished data).

Because $d'/\ln q$ represents the number of times during its life span that an infective flea bites a susceptible host, if we assume that host density is sufficiently high that every blocked flea finds a new host after its original infected host dies and

Table 2. Experimentally derived values of the vectorial capacity of *Xenopsylla cheopis* fleas used for epizootic modeling of bubonic plague.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Definition</th>
<th>Value</th>
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<tbody>
<tr>
<td>a</td>
<td>Daily biting rate (uninfected fleas)</td>
<td>0.33</td>
</tr>
<tr>
<td>a'</td>
<td>Daily biting rate (blocked fleas)</td>
<td>1.0</td>
</tr>
<tr>
<td>b</td>
<td>Probability of flea blockage after feeding on a host with septicemia at the threshold level or greater</td>
<td>0.38</td>
</tr>
<tr>
<td>n</td>
<td>Days between infectious blood meal and blockage (extrinsic incubation period [EIP])</td>
<td>14</td>
</tr>
<tr>
<td>p''</td>
<td>Probability of a flea surviving the EIP</td>
<td>0.85</td>
</tr>
<tr>
<td>1/r</td>
<td>Life expectancy (in days) of host after reaching the threshold septicemia level</td>
<td>2</td>
</tr>
</tbody>
</table>

**Figure 2.** Estimated *Xenopsylla cheopis* density per host required to sustain plague enzootics ($R_0 = 1$) and epizootics ($R_0 > 2$) in a population in which the no. of susceptible hosts is not limiting.
that the flea attempts to feed on the new host at least twice (transmission rate \( \geq 0.5 \); figure 1), the equation simplifies to

\[
R_s = \frac{ma}{r} \quad \text{or} \quad m = R_s \left( \frac{r}{ma} \right).
\]

The *X. cheopis* densities required to sustain plague enzootics and epizootics in a susceptible population were calculated by use of the experimentally derived terms for this equation (table 2). The calculated flea density per host required to maintain *Y. pestis* infection in a population \( (R_s = 1) \) was 4.7, and the flea density required to support an epizootic \( (R_s = 2) \) was 9.4 (figure 2). These are likely to be minimum estimates, because flea survival data were from optimal environmental conditions and saturation host density was assumed. During the last pandemic in India, where *X. cheopis* is the principal vector, the incidence of rodent and human plague correlated directly with seasonal changes in flea abundance [28, 29]. An *X. cheopis* index (the average number of fleas in the fur of live-trapped hosts) of 1–5 was cited as being critical for spread of an epidemic [8, 30]. However, the flea index underestimates the total flea density per host \( (m) \), because a majority of fleas may remain in the rodent nest or burrow [8, 14, 29, 31]. Field counts of *X. cheopis* per rat usually range from 1 to 12, but numbers fluctuate with climactic conditions, and flea indices of 20–80 have been recorded for *X. cheopis* [30, 32]. Our results support models that have postulated that plague epizootics depend on a coincidence of high vector and host densities and that flea control is a sufficient preventative measure and is less likely to engender cases of human plague than is rodent control [32, 33].

Proventricular blockage occurs more frequently and rapidly after an infectious blood meal in *X. cheopis* than in other flea species, which accounts for the greater vector efficiency of *X. cheopis* that has been reported in previous comparative studies [8–14]. However, complete blockage of the proventriculus is not essential for transmission. Partial obstruction and interference with the valvular function of the proventriculus can also result in efficient transmission [34]. For vector species in which complete blockage is less common, such as the rock squirrel flea *Diamanus* (*Oropsylla*) *montanus* in the western United States [9, 13], transmission dynamics may differ in some respects from those for *X. cheopis*. For example, the life span of infected and infective (transmission-competent) fleas has been reported to be higher in other vector species than in *X. cheopis* [13, 14]. Because partially blocked fleas can ingest blood, their daily survival probability \( (q) \) would be expected to be higher than that of blocked fleas. Thus, the number of times that a partially blocked infective flea feeds during its life span \( (d = \ln a/q) \) would be correspondingly higher than that for a fully blocked infective flea. This could compensate, in part, for a lower proventricular infection rate \( (b) \) and a longer extrinsic incubation period \( (n) \), compared with those in *X. cheopis*. Although oral transmission due to cannibalizing of dead nest mates is thought to play a role in sustaining plague in some rodent populations [35], direct transmission of pneumonic plague does not, because infected rodents, even those with lung involvement, do not generate aerosolized respiratory droplets [36, 37].

The vector competence of fleas is low, compared with those characterized for mosquito, sandfly, and tick vectors of other pathogens [38–42], which may be a reflection of the recent transition of *Y. pestis* to arthropodborne transmission. With a longer coevolutionary history, *Y. pestis* strains better able to colonize fleas and with a lower threshold of infection may evolve. Such strains would be predicted to have a selective advantage, which would be augmented if this advantage was coupled with reduced virulence that prolonged the infectious period of an infected host. The interaction of relatively few flea species with highly clonal *Y. pestis* strains that spread worldwide during plague pandemics during the past 700 years have been examined, and vector-host cycles with different transmission and disease dynamics may exist in the more ancient plague foci in central Asia [43].

Another example of a correlation between low vector competence and increased virulence is provided by the St. Louis encephalitis (SLE) virus. In the western United States, the primary vector is the most competent one, *Culex tarsalis*. This mosquito does not occur in the eastern United States, where the primary transmitter is *Culex pipiens*, a less competent vector with a higher threshold of infection for the virus. Eastern SLE strains produce higher levels of viremia in infected birds and cause more-severe disease in humans than do western strains, which suggests that reliance on the poorer vector has been compensated for by increased virulence [44]. The examples of *X. cheopis* and *C. pipiens* indicate that an inefficient vector is not necessarily a more benign one, and this has implications for disease-control strategies that are based on increasing vector resistance to infection.

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**References**


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