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Infection Prevalence, Bacterial Loads, and Transmission Efficiency in *Oropsylla montana* (Siphonaptera: Ceratophyllidae) One Day After Exposure to Varying Concentrations of *Yersinia pestis* in Blood

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Abstract

Unblocked fleas can transmit *Yersinia pestis*, the bacterium that causes plague, shortly (≤4d) after taking an infectious bloodmeal. Investigators have measured so-called early-phase transmission (EPT) efficiency in various fleas following infection with highly bacteremic blood (≥10^8cfu/ml). To date, no one has determined the lower limit of bacteremia required for fleas to acquire and transmit infection by EPT, though knowing this threshold is central to determining the length of time a host may be infectious to feeding fleas. Here, we evaluate the ability of *Oropsylla montana* (Baker) to acquire and transmit *Y.* pestis after feeding on blood containing 10^3 to 10^9cfu/ ml. We evaluated the resulting infection prevalence, bacterial loads, and transmission efficiency within the early-phase time period at 1d postinfection. Fleas acquired infection from bacteremic blood across a wide range of concentrations, but transmission was observed only when fleas ingested highly bacteremic blood.

Keywords

plague; early-phase transmission; *Oropsylla montana*; *Yersinia pestis*; flea

Plague, a primarily flea-borne zoonosis, is caused by the gram-negative bacterium, *Yersinia pestis*, and has been responsible for millions of human deaths throughout history (Gage and Kosoy 2005). The classical, biofilm-dependent regurgitative model of *Y. pestis* transmission, or the blocked flea model, was first described over a century ago (Bacot and Martin 1914, Eskey and Haas 1940, Burroughs 1947, Hinnebusch et al. 1996, Gage and Kosoy 2005, Hinnebusch 2012). According to this model, fleas develop a proventricular blockage composed of multiplying bacteria and biofilm. The blockage prevents the flea from successfully taking a bloodmeal, and through repeated attempts to feed, the flea transmits *Y. pestis* to naïve hosts typically 1wk or more postinfection (Bacot and Martin 1914, Hinnebusch et al. 1996, Jarrett et al. 2004).

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Given the relatively long extrinsic incubation period associated with blockage formation and the short infectious window following complete blockage of the flea, mathematical models indicate that the blocked flea model is insufficient to explain the rapid rates of *Y. pestis* spread that are typically observed during plague epizootics (Lorange et al. 2005, Eisen et al. 2006, Webb et al. 2006). In contrast, transmission by unblocked fleas ≤4d following infection, termed early-phase transmission (EPT), has been proposed as a model to describe the rapid spread of *Y. pestis* observed during epizootics (Eisen et al. 2006, 2015; Eisen and Gage 2009; Buhnerkempe et al. 2011). The term EPT underscores the importance of the short extrinsic incubation period in the flea-borne spread of *Y. pestis* during epizootic periods. Although previous studies have demonstrated that the genes required for the blocked flea model are not required for EPT (Vetter et al. 2010, Johnson et al. 2014), the biological or biomechanical interactions that ultimately result in EPT remain poorly defined (for review of this subject, see Eisen et al. 2015). Under the EPT model, fleas become infected after feeding on highly bacteremic hosts, which quickly succumb to *Y. pestis* infection. Plague bacteria spread when infectious fleas leave a highly infectious dead or dying host to feed on new, susceptible, naïve hosts. In recent years, EPT has been evaluated using blood containing $\geq 10^8$ cfu/ml to infect fleas, a level of bacteremia thought to be typical of those seen in the final hours before rodent hosts succumb to plague (Eisen et al. 2006, 2007a, 2008a,b; Wilder et al. 2008a, b). This design aims to replicate epizootic conditions and, owing to the consistent study design, allows for comparisons of transmission efficiency between flea species. However, to date, the range in concentration of *Y. pestis* in blood required to infect fleas such that they will reliably transmit the infection during the early phase has not been evaluated.

The concentration of *Y. pestis* in host blood required to generate a transmissible infection is important because it controls a number of ecologically relevant parameters (Eisen et al. 2015). One study suggested that bacteremia must be extraordinarily high ($\geq 10^7$ cfu/ ml) for fleas to reliably acquire and transmit plague bacteria through the blocked flea model (Engelthaler et al. 2000). This is compatible with earlier observations that bacterial acquisition rates are greatest during the terminal stage of infection when bacterial concentrations commonly exceed 10^6 cfu/ml (Douglas and Wheeler 1943, Burroughs 1947, Holdenried 1952, Engelthaler et al. 2000, Sebbane et al. 2005). Therefore, the period of time during which an animal is highly bacteremic and infectious to feeding fleas is thought to be short. However, there is some evidence to suggest that susceptible animals may also have lower bacterial concentrations in the blood during the hours or days leading up to terminal septicemia (Sebbane et al. 2005, Weller et al. 2012), or that resistant animals may exhibit transient bacteremia (Rust et al. 1971, Nichols et al. 2014). The length of the infectious window is an important factor when simulating the dynamics of *Y. pestis* spread under epizootic conditions (Eisen et al. 2006, Webb et al. 2006, Buhnerkempe et al. 2011); the longer the host is infectious, the greater the force of infection (Lorange et al. 2005). Recognizing the implications for epizootic transmission of a prolonged infectious period in the bacteremic host coupled with high transmission efficiency following a short extrinsic incubation period, we aimed to determine the minimum concentration of *Y. pestis* in blood required to infect feeding fleas and result in transmission during the early phase (≤4d, here

measured at 1d postinfection [p.i.]) by *Oropsylla montana* (Baker), an important bridging vector of *Y. pestis* to humans in the United States.

Materials and Methods

All animal procedures were approved by the Centers for Disease Control and Prevention Division of Vector-Borne Diseases Animal Care and Use Committee, protocol number 12–023. Use of *Y. pestis* was approved by the Centers for Disease Control, Division of Select Agents and Toxins, under registration number C20130826–1489.

Flea Infection With *Y. pestis* CO96–3188

Colony-reared O. *montana* (Centers for Disease Control and Prevention, Fort Collins, CO) were starved for 5d, then infected using an artificial feeding system described in detail previously (Eisen et al. 2006, 2007a). To generate the spiked bloodmeals for each feeder, fully virulent *Y. pestis* CO96–3188 was grown overnight (16h) in Heart Infusion Broth (BD Biosciences, Franklin Lakes NJ; 28°C, 200 rpm) to a concentration of ~10^9 cfu/ml, then pelleted and resuspended in Sprague Dawley defibrinated rat blood (Bioreclamation, Jericho, NY). Tenfold serial dilutions of this initial suspension yielded concentrations of 10^3 to 10^9 cfu/ml blood. We prepared three feeders for each concentration, and groups of 50 fleas were placed in each feeder. Fleas were allowed to feed for 1h, and those that successfully fed were pooled by concentration group, then held for 24h at 32°C and 85% relative humidity. The bacterial concentration within each feeder was verified by spread plating on 6% Sheep Blood Agar (SBA) immediately following the flea feed and counting colony-forming units after an ~48-h incubation at 28C. To obtain enough fleas to evaluate transmission, artificial flea feeds were conducted on four separate dates.

Transmission of *Y. pestis* to SKH-1 Mice

Twenty-four hours after feeding on spiked blood, 10 surviving fleas from each concentration group were pooled and allowed to feed on individual anesthetized 6- to 8-wk-old naïve outbred SKH-1 mice (Centers for Disease Control and Prevention). The 24-h time point was selected as representative of transmission efficiency during the early phase because earlier work showed statistically similar transmission rates from 1–4d p.i. After one hour, fleas were removed from the mice and immediately stored at 80°C for later bacterial quantitation. Mice were allowed to recover from anesthesia and then housed individually in HEPA-filtered Isocages (Techniplast, Exton, PA) to prevent transmission between cagemates. Mice were monitored daily and euthanized if signs of infection (e.g., slow response to stimuli, weight loss, and hunched posture) became evident. Infection with *Y. pestis* was confirmed in liver and spleen tissues using a direct fluorescence assay with fluorescein-conjugated rabbit polyclonal antibodies targeting the F1 antigen (Centers for Disease Control and Prevention, Division of Vector-Borne Diseases) followed by culture and use of specific bacteriophage lysis on resulting isolates (Chu 2000). Mice that did not show signs of illness during 21d of observation were euthanized, and blood serum was evaluated for the presence of *Y. pestis*-specific antibodies using a passive hemagglutination and inhibition assay (Chu 2000). Specific titers greater than 1:16 were interpreted as evidence of flea-borne transmission. For each *Y. pestis* concentration group, transmission was evaluated in at least 10 mice.

Infection Status and Quantitation of Bacterial Loads in Infected Fleas

To determine if fleas were infected with *Y. pestis* at the time of placement on mice, each flea was triturated in 1.0 ml heart infusion broth containing 10% glycerol. Infection status was determined by spread plating 100μl of undiluted triturate to duplicate SBA plates; fleas were considered infected if at least one *Y. pestis* cfu was present on a single plate at this concentration. To determine the bacterial load within each infected flea, quantitative plating to duplicate SBA plates was performed, and plates were enumerated following 48-h incubation at 28°C. Fleas yielding ≥1 colony per plate (10cfu/flea) were included in quantitative bacterial estimates. Because the fleas used in transmission evaluations are not kept in a sterile environment, contaminant growth was present on a subset of the plates evaluated. When contaminant growth inhibited accurate plate counts but *Y. pestis* colonies were present, that flea was included in infection prevalence estimates but excluded from quantitative bacterial estimates.

Evaluation of Transmission Success and Statistical Analyses

The proportions of fleas that remained infected with *Y. pestis* 24h after taking a bloodmeal were compared across the seven target concentration groups (10^3–10^9 cfu/ml) using contingency table analysis with Bonferroni correction for multiple pairwise comparisons (JMP 11.2.1, SAS Institute 2014). All fleas that took a bloodmeal, survived 24h, and fed on a naïve mouse were included in the analysis.

The relationship between concentration of *Y. pestis* in the feeding reservoir and the resulting bacterial load in infected fleas was measured using Spearman's q correlation. Analysis included all fleas that fed on infectious blood, were alive at 24h, and fed on naïve mice. Fleas were excluded that were found to be uninfected, did not take a bloodmeal from a naïve mouse at 24h, or for which accurate *Y. pestis* counts could not be obtained owing to nonspecific growth of contaminant organisms. Counts of *Y. pestis* in the blood (cfu/ml, averaged within concentration group for each artificial feed date) and in each flea (cfu/flea) were $\log_{10}$-transformed before analysis (JMP 11.2.1, SAS Institute 2014).

Transmission efficiency was estimated for fleas fed at seven different concentrations of *Y. pestis* in blood. Because fleas within each concentration group were pooled before placement on naïve mice, it was impossible to determine which individual flea (or if multiple fleas) transmitted plague bacteria to an individual mouse. Therefore, transmission efficiency of individual fleas within each concentration group was estimated using likelihood methods for pooled samples (PooledInfRate Excel Add-In 4.0, Biggerstaff 2009). To reflect uncertainty in point estimates of transmission efficiency that are based on the flea pool size, 95% confidence intervals are reported for each point estimate. To estimate differences of transmission efficiency between concentration groups, confidence intervals for the differences of proportions for pooled samples (Biggerstaff 2008) were computed using the binGroup package in R, version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria). The Bonferroni correction was used to adjust for multiple comparisons. For both analyses, only fleas that both fed on transmission mice and were infected (as confirmed by plating) were included in the calculation. Likewise, only mice that were exposed to at least one infected flea were included in the analyses.

To determine if transmission to mice was dependent on infected flea bacterial loads, bacterial counts for each flea were $\log_{10}$- transformed, and the median, minimum, and maximum bacterial load calculated for the flea pool associated with each mouse. Fleas that were not infected, did not feed on the mouse, or for which accurate plate counts could not be obtained were excluded from the analysis. A logistic regression model was then fitted to the data to determine if the median, minimum, or maximum bacterial load per flea pool was predictive of transmission outcome (JMP 11.2.1, SAS Institute 2014). For all analyses, statistical significance was measured at the $\alpha = 0.05$ level.

Results

Fleas that fed on blood containing a wide range of *Y. pestis* concentrations (10^3–10^9 cfu/ml) acquired and maintained infection for at least 24h p.i. (Table 1). At the lowest concentration of *Y. pestis* evaluated (10^3 cfu/ml), infection prevalence in fed fleas (28.0%) was significantly lower than in fleas fed at all higher concentrations, 10^4– 10^9 cfu/ml (range: 85.2–100.0%; $\chi^2 \geq 73.5$; $P<0.0001$ for all pairwise comparisons). Additionally, fleas fed at 10^8 or 10^9 cfu/ml were significantly more likely to be infected than fleas fed at 10^5 cfu/ml ($\chi^2 \geq 11.9$; $P \leq 0.0006$). All other pairwise differences among concentration groups were not significant when measured against the Bonferroni-corrected *P*-value of 0.002 ($\chi^2 \leq 8.7$; $P \geq 0.003$). The concentration of *Y. pestis* in the blood contained in the feeder was positively correlated with the resultant bacterial loads observed in infected fleas ($\rho_s = 0.81$, $P<0.0001$).

Transmission to naïve mice at 24h was observed for groups of fleas fed on blood containing concentrations of *Y. pestis* at $\geq 10^7$ cfu/ml (Table 1). Ten, 50, and 90% of mice exposed to fleas fed at 10^7, 10^8, and 10^9 cfu/ml, respectively, became infected, while transmission was not observed in mice exposed to fleas fed on lower concentrations of *Y. pestis*-infected blood. For concentration groups from which transmission was observed, per-flea transmission efficiency estimates ranged from 1.1% at 10^7 cfu/ml (95% CI: 0.07– 5.4%), to 18.5% for those fed at 10^9 cfu/ml (95% CI: 9.6–41.1%). Though transmission was not observed from flea pools fed at concentrations below 10^7 cfu/ml, transmission efficiency could have been as high as 3.6–4.2% for fleas fed on 10^4–10^6 cfu/ml, and as high as 13.7% for fleas fed at 10^3 cfu/ml. Notably, the confidence interval is the largest for the lowest concentration group because fewer fleas were infected, resulting in a small sample size of infected fleas feeding on naïve mice.

Transmission efficiency estimates were compared between concentration groups, and results are summarized in Table 2. Individual infected fleas fed at 10^9 cfu/ml were significantly more likely to transmit *Y. pestis* than those fed at 10^4–10^7 cfu/ml, but were not significantly more likely to transmit than those fed at 10^8 or 10^3 cfu/ml. All other differences of transmission efficiency between concentration groups were not statistically significant. Low infection prevalence among fleas fed at 10^3 cfu/ml yielded a small pool size and correspondingly wide confidence intervals, likely explaining why a significant pairwise difference in transmission efficiency could not be detected when these fleas were compared against the fleas fed at 10^9 cfu/ml.

Transmission outcome could be predicted by the bacterial load present in the fleas that took a bloodmeal on sentinel mice (Table 4). When considering all concentration groups from 10^3–10^9 cfu/ml, the log odds of a mouse becoming infected was positively related to the bacterial loads of individual fleas within the pool, regardless of whether minimum, median, or maximum cfu per flea was used to predict transmission outcome (Tables 3 and 4). With each increase of $\log_{10}$ bacterial load in the flea, the odds of transmission by the pool increased by as much as 8.5 times. Overall, the models fit the data significantly better than the null models ($\chi^2 \geq 34.1$; $P < 0.0001$ for all models). Finally, each model performed well, with area under the receiver operator curve >0.92, indicating that at least 92% of values were correctly classified. When considering only the concentration groups tested in previous studies ($\geq 10^8$ cfu/ml; Eisen et al. 2007a, Wilder et al. 2008a, Schotthoefer et al. 2011), a significant relationship between bacterial loads in individual fleas and transmission outcome was not observed ($\chi^2 \leq 2.4$; $P > 0.1$ for all models).

Discussion

For *O. montana*, a competent vector of *Y. pestis* that rarely forms proventricular blockage (Eisen et al. 2009), transmission during the early phase of infection (≤4d, measured here at 1d p.i.) requires that the flea feed on a highly bacteremic host. In this study, despite fleas acquiring infection across the concentration gradient from 10^3– 10^9 cfu/ml of blood, transmission was only observed for flea pools that fed on blood containing at least 10^7 cfu/ml and was significantly more likely to occur at 10^9 cfu/ml, the highest concentration we evaluated. At lower concentrations of bacteria ($<10^7$ cfu/ml), transmission was not observed.

Fleas acquired infection across all tested bacterial concentrations, although at a significantly lower rate in the lowest concentration group (10^3 cfu/ml) compared with other concentration groups. Bacterial loads in the fleas were positively associated with the bacterial concentration in the feeders, and in general, were roughly 3–4 logs lower than the feeder concentration; however, variation within pools was considerable (Tables 1 and 3). Taking into account that bacterial loads can increase by up to a log during the first 24h after blood feeding (Eisen et al. 2006), this finding is consistent with the range in bloodmeal volumes reported for *O. montana* and *Xenopsylla cheopis* Rothschild (0.03–0.5μl; Ledingham 1907, Douglas and Wheeler 1943, Perry and Fetherston 1997, Hinnebusch 2005, Oyston and Isherwood 2005). Some of the lower bacterial loads observed among flea pools may be attributable to ingestion of smaller blood volumes or partial feeding. Notably, fewer fleas took bloodmeals in the 10^5 cfu/ml concentration group compared with those in the 10^4, and 10^6–10^9 cfu/ml concentration groups. It is possible that those in the 10^5 cfu/ml concentration group also fed less fully, resulting in a lower infection prevalence for that group compared with others.

Despite the high prevalence of *Y. pestis* infection among pools of fleas exposed to blood containing at least 10^4 cfu/ml of blood, transmission was observed only in fleas exposed to at least 10^7 cfu/ml. Furthermore, the likelihood of transmission in the lower concentration groups was significantly lower than those exposed to 10^9 cfu/ml. Based on the sample sizes used in this study, we cannot rule out the possibility that fleas exposed to bacteremia at levels $<10^7$ cfu/ml are capable of transmitting plague bacteria 1 d p.i. Nonetheless, we

similar to results observed in studies of transmission by blocked fleas (Engelthaler et al. 2000), a high bacteremia ($\geq 10^7$ cfu/ml) is required for efficient transmission within the early phase (≤4d). However, it is important to note that our findings were restricted to an extrinsic incubation period of 1d p.i. and evaluations at later time points up to 4d would be needed to extrapolate across the full EPT time period.

Previous studies evaluating transmission efficiency during the early phase did not show a significant association between bacterial load and transmission outcome (Eisen 2007a, Wilder et al. 2008a, Schotthoefer et al. 2011). The perceived discrepancy in these results is likely an artifact of study design, as these earlier studies used blood containing $\geq 10^8$ cfu/ml to infect feeding fleas. Within this narrow range of bacteremia, the resulting bacterial loads of infected fleas were likely too similar to produce significant differences in transmission efficiency. Indeed, when we reexamined this relationship with our data and restricted analysis to fleas fed at $\geq 10^8$ cfu/ml, we did not identify a significant association between bacterial loads and transmission outcome. This suggests that beyond a critical threshold, increases in bacterial concentration in the blood (and thus bacterial loads in fleas) may not translate to increases in transmission efficiency.

Findings from a number of studies indicate that this rough threshold for reliable flea-borne transmission (between 10^7–10^8 cfu/ ml) is met or exceeded only during the terminal stage of *Y. pestis* infection (Eskey and Haas 1940, Douglas and Wheeler 1943, Holdenried 1952, Engelthaler et al. 2000). While few studies have explored how long a transmissible bacteremia can be sustained in a susceptible host, terminal end point studies show that the life expectancy after reaching this threshold is short. For example, Lorange et al. (2005) reported that once Brown Norway rats achieved bloodborne bacterial loads of $\geq 10^6$ cfu/ml, they survived only 2d. Similarly, Engelthaler et al. (2000) observed that mice with $\geq 10^7$ cfu of *Y. pestis*/ml of blood appeared “severely ill” and often died during the time it took for fleas to take a bloodmeal. Therefore, given that reliable flea-borne transmission of *Y. pestis* at 1d p.i. depends on a high host bacteremia (e.g., $\geq 10^7$ cfu/ml), it may also ultimately depend upon terminal illness of the host. Our findings support the concept that low transmission efficiency by fleas, particularly those fed on low concentrations of *Y. pestis* in the blood, exerts selection pressure for the bacterium to maintain a high level of virulence (Hinnebusch 2005, Lorange et al. 2005, Oyston and Isherwood 2005).

Although we did not observe transmission at concentrations $<10^7$ cfu/ml, it is interesting to note that when fleas were fed blood containing 10^4–10^6 cfu/ml, between 85.2 and 93.7% became infected and maintained infection for at least 24h. Further, for fleas fed on these lower concentrations of *Y. pestis* in blood, we estimated by maximum likelihood that transmission efficiency (while not observed) could have been as high as 4.2%. Given these findings, we cannot rule out the possibility that animals with mild bacteremia may contribute to infectious spread, either by extending the length of time the host is infectious (before reaching fulminant bacteremia), or by providing an infectious bloodmeal during a transient, lowlevel bacteremia, as in the case of animals that ultimately recover from infection.

There is some evidence to suggest that low-level bacteremia may occur in animals that are considered “resistant” to *Y. pestis* infection. For example, SEG strain mice, when compared

with susceptible B6 strain mice, are much more likely to survive *Y. pestis* challenge (10 and 95% mortality, respectively; Blanchet et al. 2011), and in at least one study, Demeure et al. (2012) demonstrated low-level bacteremias (mean levels roughly 10^1–10^3 cfu/ml) on days 2 and 3p.i. However, as groups of mice in the study were euthanized at 24-h intervals, neither recovery from infection nor duration of low-level bacteremia could be determined for individual mice. Similarly, Goldenberg et al. (1964) reported a bacteremia of 10^4 cfu/ ml in an otherwise asymptomatic wild-captured California vole (Microtus californicus), though it is unclear how long this bacteremia could have been sustained, as the culture was obtained at necropsy. Finally, both Rust et al. (1971) and Nichols et al. (2014) described cases of domestic dogs having quantifiable *Y. pestis* bacteremia before recovery from infection, though neither reported the bacterial concentrations they measured. While these studies point to the possibility of fleas acquiring infection from hosts that recover from plague infections, it is difficult to conceive a situation under which they could contribute meaningfully to epizootic transmission of *Y. pestis* in a natural system. Even if fleas were able to acquire infection from animals during a phase of transient, low-level bacteremia, subsequent bloodmeals would be noninfectious once the host animal recovered. Previous work has suggested that for *O. montana*, taking a noninfectious secondary bloodmeal may reduce EPT efficiency (Eisen et al. 2007b). Further, fleas would likely remain associated with hosts that survived infection, reducing the likelihood of transmission to new individuals. Indeed, to impact the spread of *Y. pestis*, fleas must not only become infected but also transmit infection to new hosts.

If *O. montana* could become infectious after feeding at very low bacterial concentrations, the infectious window provided by the host to feeding fleas would probably only be extended minimally. For example, in a rat model, blood-borne concentrations of *Y. pestis* were below the limit of detection ($<10^{1.3}$ cfu/ml) 24h postneedle inoculation. By 48 and 72h postinfection, bacteremia was as high as $10^{6.8}$ and 10^9 cfu/ml, respectively (Sebbane et al. 2005). In a mouse model, bacteremia was undetectable 24h postneedle inoculation, but reached levels up to 10^6 cfu/ml by 48–72h p.i. (Weller et al. 2012). In both studies, animals were humanely euthanized before the terminal stage of infection, so the duration of time that lapsed between achieving an infectious bacteremia and host death could not be determined. However, it seems that low-level bacteremia before fulminant sepsis is short-lived and likely contributes little to the total length of time a host might be infectious to feeding fleas.

Although fleas fed on weakly bacteremic hosts are unlikely to contribute significantly to EPT and epizootic spread of *Y. pestis*, it could be argued that they could occasionally acquire and maintain infection for sufficient lengths of time to become partially or completely blocked and thereby contribute later to enzootic maintenance of infection. Indeed, authors have discussed the potential for animals with low-level or transient bacteremia to contribute to plague maintenance between epizootics (Pollitzer 1954, Goldenberg et al. 1964). Although we did not observe transmission from fleas fed at the lowest concentrations of *Y. pestis* during the early phase (1d p.i.), we did not measure transmission at later time points; therefore, we cannot speculate as to whether or not they could contribute to enzootic maintenance via a blocked-flea or late-phase mechanism. Therefore, the contribution of weakly infected hosts to enzootic maintenance of *Y. pestis* by

O. montana, while an interesting speculation, remains outside the scope of our current study and has not yet been demonstrated.

In conclusion, transmission of *Y. pestis* following a short extrinsic incubation time (1d p.i.) likely depends on the host achieving a high bacteremia ($\geq 10^7$ cfu/ml) within the short period of time before death. Unblocked *O. montana* were able to acquire and maintain *Y. pestis* for 24h postinfection at rates exceeding 96% when feeding on $\geq 10^7$ cfu/ml of blood and transmit the plague bacterium efficiently following an extrinsic incubation period of only 1d. These findings support claims that EPT, defined as transmission by unblocked fleas within 4d of infection, could be an important driver of epizootic spread (Eisen et al. 2006, Eisen and Gage 2009, Buhnerkempe et al. 2011) and support theories that low vector efficiency, particularly among fleas exposed to low bacteremia, imposes selective pressure that favors the maintenance of virulence in *Y. pestis* (Hinnebusch 2005, Lorange et al. 2005, Oyston and Isherwood 2005).

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Table 1.

Flea infection status, observed transmission events, and estimated transmission efficiency of individual fleas 24 h after feeding on blood containing a target concentration of 10^3–10^9 cfu/ml *Y. pestis*

Target concentration of *Y. pestis*	Concentration of *Y. pestis* (cfu/ml) used in flea feed, range	Median bacterial load (range) of infected fleas that fed on naïve mice (cfu/flea)[a]	% of infected fleas that fed (no. of infected/no. of fed)	No. of mice infected (total no. of exposed)	Estimated transmission efficiency (%) (95% CI)[b]
1×10^3	1.6×10^3–3.9×10^3	28 (10–2.5×10^2), n=20	28.0 (23/82)	0 (9)	0.0 (0.0, 13.7)
1×10^4	2.0×10^4–3.3×10^4	2.0×10^2 (10–3.5×10^3), n=82	91.7 (88/96)	0 (10)	0.0 (0.0, 3.6)
1×10^5	2.3×10^5–3.0×10^5	9.2×10^2 (15–2.1×10^4), n=71	85.2 (75/88)	0 (10)	0.0 (0.0, 4.2)
1×10^6	2.2×10^6–2.7×10^6	2.0×10^3 (10–1.8×10^5), n=84	93.7 (89/95)	0 (10)	0.0 (0.0, 3.6)
1×10^7	2.0×10^7–4.1×10^7	1.1×10^4 (20–1.4×10^6), n=89	96.8 (90/93)	1 (10)	1.1 (0.1, 5.4)
1×10^8	2.3×10^8–3.4×10^8	2.9×10^5 (40–3.1×10^6), n=99	100.0 (100/100)	5 (10)	6.3 (2.4, 14.2)
1×10^9	1.7×10^9–3.4×10^9	1.0×10^6 (1.9×10^3–1.0×10^7), n=92	98.9 (92/93)	9 (10)	18.5 (9.6, 41.1)

[a] Calculated from fleas with≥10 cfu. Individual fleas were excluded if nonspecific growth impeded enumeration of plates.

[b] Calculated using maximum likelihood estimation (MLE) method. Results expressed as maximum likelihood of transmission per infected flea.

Table 2.

Pairwise comparisons of transmission efficiency estimates by concentration group

Concentration groups compared	Difference (95% CI) between transmission efficiency estimates
10^9 vs. 10^8	12.2 (−7.1, 83.9)
10^9 vs. 10^7 [a]	17.4 (2.7, 86.6)
10^9 vs. 10^6 [a]	18.5 (5.0, 47.1)
10^9 vs. 10^5 [a]	18.5 (4.5, 47.1)
10^9 vs. 10^4 [a]	18.5 (4.9, 47.1)
10^8 vs. 10^7	5.2 (−4.9, 19.6)
10^8 vs. 10^6	6.3 (−2.2, 25.0)
10^8 vs. 10^5	6.3 (−3.2, 25.0)
10^8 vs. 10^4	6.3 (−2.3, 25.0)
10^8 vs. 10^3	6.3 (−17.8, 25.0)
10^7 vs. 10^6	1.1 (−6.1, 17.8)
10^7 vs. 10^5	1.1 (−7.2, 17.8)
10^7 vs. 10^4	1.1 (−6.1, 17.8)
10^7 vs. 10^3	1.1 (−22.7, 17.8)
10^6 vs. 10^5	0.0 (−11.0, 9.4)
10^6 vs. 10^4	0.0 (−9.5, 9.4)
10^6 vs. 10^3	0.0 (−28.6, 9.4)
10^5 vs. 10^4	0.0 (−9.5, 11.0)
10^5 vs. 10^3	0.0 (−28.6, 11.0)
10^4 vs. 10^3	0.0 (−28.6, 9.5)

[a] Transmission efficiency between groups is significantly different when CI does not include zero (α=0.05 with Bonferroni correction).

Table 3.

Bacterial loads of infected fleas by individual flea pool and concentration group where transmission was observed

Target concentration of *Y. pestis* in artificial feeder (cfu/ml)	Mouse no.	Median (range) bacterial load of infected fleas within each flea pool (cfu/flea)[a]	No. of fleas infected (total fed on naïve mouse)	Transmission from flea pool to mouse
1×10^{7}	1	5.5×10^{3} (3.9×10^{2}–1.2×10^{5}), *n*=10	10 (10)	N
	2	4.0×10^{3} (75–8.0×10^{4}), *n*=10	10 (10)	Y
	3	1.7×10^{5} (2.3×10^{3}–9.3×10^{5}), *n*=9	9 (9)	N
	4	2.1×10^{4} (6.5×10^{2}–1.4×10^{6}), *n*=10	10 (10)	N
	5	1.9×10^{4} (4.9×10^{2}–5.0×10^{5}), *n*=10	10 (10)	N
	6	2.6×10^{5} (35–3.9×10^{5}), *n*=7	8 (8)	N
	7	2.1×10^{4} (70–1.8×10^{5}), *n*=9	9 (9)	N
	8	3.4×10^{2} (20–3.2×10^{4}), *n*=8	9 (9)	N
	9	2.8×10^{3} (1.4×10^{2}–7.0×10^{4}), *n*=10	10 (10)	N
	10	3.5×10^{4} (6.2×10^{2}–1.1×10^{6}), *n*=6	6 (9)	N
1×10^{8}	1	1.2×10^{6} (1.1×10^{2}–1.9×10^{6}), *n*=10	10 (10)	N
	2	5.9×10^{5} (40–3.1×10^{6}), *n*=10	10 (10)	N
	3	3.8×10^{5} (2.0×10^{4}–1.7×10^{6}), *n*=10	10 (10)	N
	4	2.2×10^{5} (5.1×10^{4}–1.0×10^{6}), *n*=10	10 (10)	Y
	5	3.1×10^{5} (85–8.8×10^{5}), *n*=10	10 (10)	Y
	6	8.6×10^{5} (5.1×10^{4}–1.6×10^{6}), *n*=9	10 (10)	N
	7	2.6×10^{5} (7.8×10^{4}–9.8×10^{5}), *n*=10	10 (10)	Y
	8	1.6×10^{5} (4.7×10^{4}–4.4×10^{5}), *n*=10	10 (10)	Y
	9	1.4×10^{5} (1.9×10^{4}–6.6×10^{5}), *n*=10	10 (10)	Y
	10	2.7×10^{5} (8.7×10^{3}–6.0×10^{5}), *n*=10	10 (10)	N
1×10^{9}	1	3.5×10^{6} (6.1×10^{5}–9.8×10^{6}), *n*=9	9 (10)	Y
	2	2.5×10^{6} (9.5×10^{5}–5.7×10^{6}), *n*=7	7 (7)	Y
	3	1.3×10^{6} (2.2×10^{5}–3.6×10^{6}), *n*=10	10 (10)	N
	4	1.2×10^{6} (1.9×10^{3}–8.0×10^{6}), *n*=10	10 (10)	Y
	5	1.4×10^{6} (1.5×10^{5}–5.5×10^{6}), *n*=9	9 (9)	Y
	6	1.3×10^{6} (6.3×10^{5}–2.8×10^{6}), *n*=9	9 (9)	Y

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Target concentration of *Y. pestis* in artificial feeder (cfu/ml)	Mouse no.	Median (range) bacterial load of infected fleas within each flea pool (cfu/flea)[a]	No. of fleas infected (total fed on naïve mouse)	Transmission from flea pool to mouse
	7	5.7×10^5 (5.8×10^4–1.0×10^7), n=10	10 (10)	Y
	8	7.4×10^5 (4.8×10^3–3.6×10^6), n=10	10 (10)	Y
	9	8.6×10^5 (6.0×10^4–2.1×10^6), n=9	9 (9)	Y
	10	$4.1\times\times10^5$ (3.8×10^4–3.0×10^6), n= 9	9 (9)	Y

[a]Calculated from fleas with ≥10 cfu. Individual fleas were excluded if nonspecific growth impeded enumeration of plates.

Table 4.

Logistic regression analysis of transmission of *Y. pestis* by infected flea pools to 68 individual mice

Predictor estimates					Overall model evaluation				
Single predictor[a]	**β (SE)**	**Wald's χ^2**	***P***	**e^{β} (odds ratio)**	**χ^2**	**df**	***P***	**R^2**	**AUC**
Minimum cfu	1.74 (0.42)	17.04	<0.0001	5.69	42.43	1	<0.0001	0.60	0.95
Median cfu	1.68 (0.46)	13.08	0.0003	5.37	34.05	1	<0.0001	0.48	0.92
Maximum cfu	2.14 (0.66)	10.42	0.0012	8.52	34.88	1	<0.0001	0.49	0.93

[a]Minimum and maximum cfu—counts from single flea in a pool of 1–10 infected fleas per mouse. Median cfu—calculated from all fleas in a flea pool of 1–10 infected fleas per mouse. All values were $\log_{10}$-transformed before analysis.