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Yersinia murine toxin is not required for early-phase transmission of *Yersinia pestis* by *Oropsylla montana* (Siphonaptera: Ceratophyllidae) or *Xenopsylla cheopis* (Siphonaptera: Pulicidae)

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Plague, caused by *Yersinia pestis*, is characterized by quiescent periods punctuated by rapidly spreading epizootics. The classical 'blocked flea' paradigm, by which a blockage forms in the flea's proventriculus on average 1–2 weeks post-infection (p.i.), forces starving fleas to take multiple blood meals, thus increasing opportunities for transmission. Recently, the importance of early-phase transmission (EPT), which occurs prior to blockage formation, has been emphasized during epizootics. Whilst the physiological and molecular mechanisms of blocked flea transmission are well characterized, the pathogen–vector interactions have not been elucidated for EPT. Within the blocked flea model, *Yersinia* murine toxin (Ymt) has been shown to be important for facilitating colonization of the midgut within the flea. One proposed mechanism of EPT is the regurgitation of infectious material from the flea midgut during feeding. Such a mechanism would require bacteria to colonize and survive for at least brief periods in the midgut, a process that is mediated by Ymt. Two key bridging vectors of *Y. pestis* to humans, *Oropsylla montana* (Siphonaptera: Ceratophyllidae) or *Xenopsylla cheopis* (Siphonaptera: Pulicidae), were used in our study to test this hypothesis. Fleas were infected with a mutant strain of *Y. pestis* containing a non-functional *ymt* that was shown previously to be incapable of colonizing the midgut and were then allowed to feed on SKH-1 mice 3 days p.i. Our results show that Ymt was not required for EPT by either flea species.

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INTRODUCTION

Plague is an often fatal, primarily flea-borne zoonotic disease caused by the bacterium *Yersinia pestis* and is characterized by quiescent periods punctuated by rapidly spreading epizootics (Barnes, 1982). Although maintenance of plague bacteria in nature is poorly understood, it is largely believed that *Y. pestis* circulates in enzootic cycles involving transmission between moderately resistant rodents and their fleas (Eisen & Gage, 2009; Gage & Kosoy, 2005; Politzer, 1954). The 'blocked flea' model, described by Bacot & Martin (1914), has been recognized as the primary mechanism of flea-borne transmission in the western literature for nearly a century (Eisen *et al.*, 2009). Under this scenario, *Y. pestis* adheres to and forms an obstruction in the flea proventriculus, the spine-filled valve connecting the oesophagus to the midgut; aggregation of bacteria in the proventriculus prevents its normal function and complete blockage prevents blood from reaching the midgut (Bacot & Martin 1914). More recently, the molecular mechanisms facilitating blocked flea transmission and survival of the plague bacterium in the flea midgut have been identified. Specifically, the gene encoding *Yersinia* murine toxin (*ymt*) and the genes in the haemin storage locus (*hms*) have been shown to be important in facilitating colonization (i.e. survival and reproduction) of the midgut and proventricular blockage, respectively, within the flea (Hinnebusch *et al.*, 1996, 1998, 2000, 2002; Jones *et al.*, 1999; Kirillina *et al.*, 2004; Perry *et al.*, 1990; Rudolph *et al.*, 1999).

Abbreviations: CDC, Centers for Disease Control and Prevention; d.f., degrees of freedom; EPT, early-phase transmission; p.i., post-infection; Ymt, *Yersinia* murine toxin.

The blocked flea model is likely to be important for the long-term maintenance of *Y. pestis*; however, mathematical modelling has suggested that it is unlikely that transmission by blocked fleas could completely explain the rapid rates of transmission typically observed during epidemics. Due to the long extrinsic incubation period, the low transmission efficiency, and the short infectious window from when a flea becomes blocked and infectious until it dies of starvation, blocked flea transmission could not drive epidemic or epizootic transmission unless the number of fleas per host is very high and the flea species involved frequently becomes blocked within relatively short periods of time (Drancourt *et al.*, 2006; Eisen *et al.*, 2006; Lorange *et al.*, 2005; Webb *et al.*, 2006). Recent work on what has been termed 'early-phase transmission' (EPT), has shown that transmission occurs efficiently during the first 1–4 days post-infection (p.i.) of the flea, a period prior to when block formation has been demonstrated to occur (Eisen *et al.*, 2006, 2007a, b, 2008a, b; Wilder *et al.*, 2008a).

Although EPT provides an ecological mechanism for the rapid spread of plague bacteria during epidemics and epizootics (Eisen *et al.*, 2006), the bacteria–vector interactions that result in EPT are not well understood. Potential mechanisms of EPT include: (i) regurgitation of *Y. pestis* from the flea's midgut through the proventriculus, (ii) reflux of *Y. pestis* from the foregut or internal mouthparts, (iii) mechanical transmission of *Y. pestis* from contaminated external mouthparts, or (iv) excretion of *Y. pestis* contaminated faeces (for a review, see Hinnebusch, 2012). Here, we evaluated whether Ymt, which promotes colonization of the midgut, a prerequisite for transmission via regurgitation from the midgut, was necessary for EPT by *Oropsylla montana* (Siphonaptera: Ceratophyllidae), an important bridging vector in North America, and *Xenopsylla cheopis* (Siphonaptera: Pulicidae), an important bridging vector worldwide. Both species are known to be efficient vectors during the EPT period (Eisen *et al.*, 2007b; Vetter *et al.*, 2010). Specifically, we infected *O. montana* and *X. cheopis* with a mutant strain of *Y. pestis* containing a non-functional *ymt*, which rendered this strain incapable of long-term colonization ($\geqslant$1 week p.i.) of the flea midgut (Hinnebusch *et al.*, 2002). We showed that EPT did not depend on the presence of Ymt, a protein that is important for colonization of the midgut, but not the proventriculus.

METHODS

Species and strains of bacteria, fleas and mice. Three strains of *Y. pestis* were used to infect colony-reared adult *O. montana* and *X. cheopis* (Table 1). Two fully virulent strains, CO96-3188 (Eisen *et al.*, 2006, 2007a, b, 2008a, b; Wilder *et al.*, 2008a) and KIM5(pCD1Ap)+ (Vetter *et al.*, 2010), were used previously to evaluate EPT of *Y. pestis*. The third strain, KIM6+*ymt*H188N, had a non-functional *ymt* in which a single amino acid change results in a >99 % decrease in phospholipase D activity and an inability to colonize the midgut of the flea (Hinnebusch *et al.*, 2002; Rudolph *et al.*, 1999). The KIM6+*ymt*H188N strain was electroporated with pCD1Ap to restore virulence factors including the type III secretion system and effector proteins to create the strain KIM5(*ymt*H188N, pCD1Ap)+ (Gong *et al.*, 2001). Transformation was verified by PCR amplification of a region of the pCD1 *repA* gene and plasmid profile analysis to confirm the presence of the plasmid (Chu, 2000; Kado & Liu, 1981).

Virulence of each strain in mice was confirmed by subcutaneously inoculating two mice per strain per flea transmission replicate with 10^4–10^5 c.f.u. *Y. pestis*. Mice were monitored daily for infection and euthanized when signs became apparent (e.g. hunched posture, slow response to stimulus). All mice used in the study were 14–16-week-old SKH-1 [Centers for Disease Control and Prevention (CDC), Division of Vector-Borne Diseases specific-pathogen-free colony] and were handled according to CDC Institutional Animal Care and Use Committee guidelines (Protocol 12-019). Presumptive positive infections were determined by direct fluorescence assays with fluorescein-conjugated rabbit polyclonal antibodies targeting the F1 antigen (CDC, Division of Vector-Borne Infectious Diseases) to stain slide preparations of liver and spleen smears. Infections were confirmed by specific bacteriophage lysis of *Y. pestis* bacilli isolated from the liver or spleen of infected mice (Chu, 2000).

Infection of fleas with *Y. pestis*. Fleas starved for 4–7 days were infected with *Y. pestis* using an artificial feeding system described in detail previously (Eisen *et al.*, 2006). Briefly, groups of colony-reared fleas were allowed to feed for 1 h in artificial feeders containing Sprague–Dawley strain defibrinated rat blood (Bioreclamation), and 1.3×10^8 and 1.6×10^9 c.f.u. *Y. pestis* ml^{-1}. Blood in the feeders was maintained at 37 °C whilst fleas fed. Only fed fleas (i.e. those with visible blood meals) were kept for use in transmission experiments. Fed, potentially infectious fleas were held at 23 °C and 85 % relative humidity for 72 h.

Transmission of *Y. pestis* to naïve mice. At 72 h p.i., pools of 10 potentially infectious fleas were placed in a feeding capsule and attached, using a beeswax/rosin mixture, to the dorsal side of an anaesthetized naïve SKH-1 mouse (50–75 mg ketamine kg^{-1}/0.5–1.0 mg dexmedetomidine kg^{-1}, intaperitoneally). After 1 h, fleas were removed from the capsule using a mechanical aspirator and examined using light microscopy to determine feeding success. Following exposure to potentially infectious fleas, anaesthetic was reversed using atipamezole (5 mg kg^{-1}, intaperitoneally) and mice were placed individually into HEPA-filtered isocages (Tecniplast ISOcage). Mice were monitored daily and euthanized when signs of infection with *Y. pestis* were evident (e.g. slow response to stimuli, hunched posture). Infection was confirmed as described above. Mice not exhibiting signs of infection were held for 22 days p.i., at which time mice were euthanized and blood was collected for serology. Serological evidence of exposure to *Y. pestis* was determined using passive haemagglutination and inhibition tests (Chu, 2000). Titres of $\geqslant$1 : 10 were considered positive and indicative of *Y. pestis* transmission from flea to mouse (Chu, 2000).

Quantification of bacterial loads in fleas and confirmation of *Y. pestis* strain infecting mice. Following the transmission feed, fed fleas were stored individually at −80 °C until being triturated in heart infusion broth with 10 % glycerol. Triturates were screened for the presence of *Y. pestis* on 6 % sheep blood agar. Bacterial loads were subsequently determined by plating serial dilutions of flea triturate in duplicate. All *O. montana* dilutions were plated on 6 % sheep blood agar. As screening revealed that 43 % of *X. cheopis* were infected with swarming *Proteus* sp., which overwhelmed *Y. pestis* growth, it was necessary to utilize a selective media (heart infusion agar supplemented with 0.25 µg Irgasan ml^{-1}) when plating triturates from *X. cheopis* (Graham *et al.*, 2014).

To confirm that the *ymt* mutation was maintained in the *ymt*H188N-infected mice, a portion of the *ymt* locus was sequenced in isolates

Table 1. Bacterial strains and plasmid

Strain or plasmid	Nomenclature used herein	Relevant characteristics	Reference or source
Strain			
CO96-3188	CO96-3188	Pgm$^+$, Ymt$^+$, pPCP$^+$, pMT$^+$, pCD1$^+$	Engelthaler *et al.* (2000)
KIM6+*	NA	Pgm$^+$, Ymt$^+$, pCD1$^-$, Pla$^+$, pPCP$^+$, pMT$^+$	Fetherston *et al.* (1992)
KIM5(pCD1Ap)+	KIM5+	Pgm$^+$, Ymt$^+$, pPCP$^+$, pMT$^+$, pCD1$^+$ (*'yadA::bla*); derived from KIM6+, Ap^r	Gong *et al.* (2001), Vetter *et al.* (2010)
KIM6(*ymt*H188N)+	NA	Pgm$^+$, pCD1$^-$, Pla$^+$, Ymt$^-$, pPCP$^+$, pMT$^+$; derived from KIM6+	Hinnebusch *et al.* (2002)
KIM5(*ymt*H188N, pCD1Ap)+	*ymt*H188N	Pgm$^+$, Ymt$^-$, pPCP$^+$, pMT$^+$, pCD1$^+$ (*'yadA::bla*); derived from KIM6+, Ap^r	This study
Plasmid			
pCD1Ap	NA	70.5 kb pCD1 with *bla* cassette inserted into *'yadA*; 71.7 kb, Ap^r	Gong *et al.* (2001)

NA, Not applicable; +, presence of a functional protein or plasmid, –, absence of a functional protein or plasmid, Ap^r, ampicillin/carbenicillin resistance.
*KIM6+ is the isogenic parent of KIM6(*ymt*H188N)+.

from all mice euthanized during the study using the primer pair YMT1.F (5′-GAGCGAGGACAATATTGGGA-3′) and YMT1.R (5′-CTGGGAGCCATAAGCAGAAG-3′). Primers were created using Primer3 (http://simgene.com/Primer3). Sequences were cleaned manually and aligned using Lasergene (DNASTAR).

Statistical analysis. Minimum transmission efficiency was calculated for each flea pool, as 1/number of infected fleas, presuming that at a minimum a single infected flea transmitted; minimum transmission efficiency was scored as 0 when no transmission was documented. Per mouse transmission efficiencies for each strain were estimated using maximum likelihood (Biggerstaff, 2009). These estimates were based on the number of infected fleas feeding on each mouse and accounting for whether or not transmission was observed. The bacterial concentrations contained in each feeder for all artificial infections, median bacterial loads, maximum bacterial loads (i.e. the highest bacterial load observed in an individual flea for each flea pool) and minimum transmission efficiencies were compared amongst or between strains of *Y. pestis* using non-parametric methods, including the Kruskal–Wallis and Wilcox rank-sum tests with χ^2 approximates. Spearman's correlations were used to test for correlations amongst median c.f.u. per flea or the number of infected fleas feeding per mouse and minimum transmission efficiency. Likelihood ratio tests were used to test for differences in flea feeding rates and infection prevalence amongst strains. We tested for an association between the numbers of infected fleas feeding or median and maximum bacterial load per flea pool and transmission success using logistic regression.

Bacterial loads within fleas were not normally distributed; therefore, we report median values instead of means. Evaluation of maximum bacterial loads, in addition to median bacterial loads, was used to account for heavily infected outliers in each group. This provided a greater sensitivity in identifying relationships between bacterial load and transmission outcome. *X. cheopis* used for all transmission studies were found to have high levels of *Proteus* sp. infections. To ensure that infection with *Proteus* sp. did not impact the capacity of *X. cheopis* to become infected with *Y. pestis* and transmit the infection, we compared c.f.u. per flea for all *Proteus*-infected and *Proteus*-uninfected fleas using Mann–Whitney *U* and Wilcox rank-sum tests with χ^2 approximates to test for differences within each of the three strains. Likelihood ratio tests were also used to determine if *Y. pestis* infection status differed based on *Proteus* infection status. All statistical tests were considered significant at $P<0.05$; all statistical tests were performed using JMP 10 statistical software (SAS Institute).

RESULTS

EPT was documented for all three *Y. pestis* strains and both flea species. The bacterial concentrations contained in the feeders for each artificial infection feed differed between flea species, but not amongst flea species (Fig. 1).

Transmission of *Y. pestis* strains by *O. montana*

Overall, transmission occurred in 80 % of mice fed upon by *O. montana* infected with CO96-3188 or *ymt*H188N and 60 % of mice exposed to KIM5+ (Table 2). Maximum-likelihood estimates of transmission efficiency were highest for *ymt*H188N and ranged from 8.80 to 16.08 % amongst all strains (Table 2); minimum transmission efficiency amongst strains was similar [Kruskal–Wallis test with χ^2 approximation: $\chi^2=3.74$, degrees of freedom (d.f.)=2, $P=0.15$]. Likewise, prevalence of infection was similar between *ymt*H188N and KIM5+, and together prevalence of infection with KIM strains was lower than that with CO96-3188 (likelihood ratio test: $\chi^2=6.90$, d.f.=1, $P=0.01$). Median bacterial loads in fleas infected with *ymt*H188N were significantly lower than those infected with KIM5+ (Kruskal–Wallis test with χ^2 approximation: $\chi^2=4.31$, d.f.=1, $P=0.04$) or CO96-3188 ($\chi^2=30.96$, d.f.=1, $P<0.0001$). Flea feeding rates were similar across strains and ranged from 80 to 100 % (likelihood ratio: $\chi^2=3.20$, d.f.=2, $P=0.20$; Table 2). There was no correlation between the number of infected fleas feeding per mouse or the median bacterial load and estimated minimum transmission efficiency for any strain ($P\geqslant0.09$). There was no association identified between the number of infected fleas feeding per mouse and transmission success for any strain (logistic regression: $\chi^2\leqslant0.22$, d.f.=1, $P\geqslant0.64$). Similarly, the maximum bacterial loads per flea were similar amongst strains (Kruskal–Wallis test with χ^2 approximation: $\chi^2=4.31$, d.f.=2, $P=0.12$), and there was no association detected between the maximum bacterial load, i.e. the maximum c.f.u. per flea in a pool of up to10

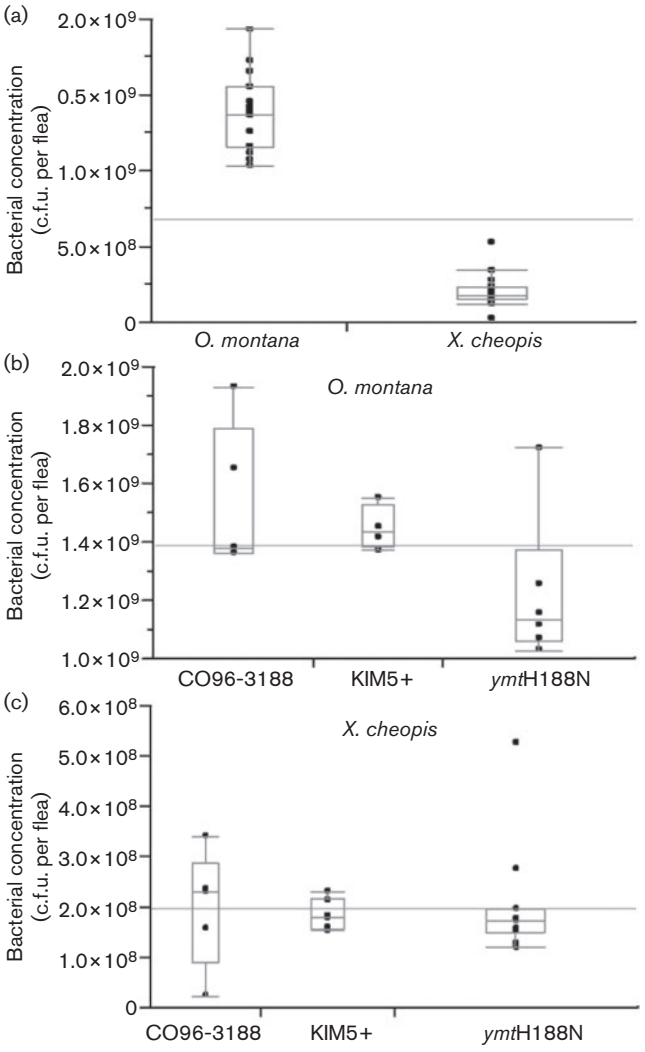

Fig. 1. (a) The bacterial concentrations contained in the feeders for each artificial infection feed differed between flea species (*O. montana* and *X. cheopis*; Kruskal–Wallis test with χ^2 approximation: $\chi^2=26.06$, d.f.=1, $P<0.0001$), but not amongst strains used to infect: (b) all *O. montana* ($\chi^2=5.03$, d.f.=2, $P=0.08$) or (c) all *X. cheopis* ($\chi^2=0.89$, d.f.=2, $P=0.64$). Although not statistically significant, the *Y. pestis* concentrations in the *ymt*-mutant-infected feeders were lower than those observed with the two WT strains for *O. montana*, but not for *X. cheopis*.

fleas, and transmission success (logistic regression: $\chi^2\leqslant 0.35$, d.f.=1, $P\geqslant 0.17$).

Transmission of *Y. pestis* strains by *X. cheopis*

Overall, transmission was observed in 30 % of mice fed upon by *X. cheopis* infected with *ymt*H188N, 14 % of CO96-3188-challenged mice and 17 % of mice fed upon by fleas infected with KIM5+ (Table 3). The point estimates of transmission efficiency of the three strains derived from maximum likelihood were again highest for *ymt*H188N-infected fleas and ranged from 1.82 (CO96-3188) to 4.59 % (*ymt*H188N) (Table 3). Minimum transmission efficiency amongst *Y. pestis* strains was similar (Kruskal–Wallis test with χ^2 approximation: $\chi^2=1.06$, d.f.=2, $P=0.58$). Prevalence of infection was similar between fleas infected with CO96-3188 and KIM5+, and was significantly lower in *ymt*H188N-infected fleas compared with either of the WT strains (likelihood ratio test: $\chi^2\geqslant 4.73$, d.f.=1, $P\leqslant 0.02$). There was no difference in median bacterial loads in fleas amongst strains (Kruskal–Wallis test with χ^2 approximation: $\chi^2=3.46$, d.f.=2, $P=0.177$). Flea feeding rates ranged from 70 to 100 % across strains and were similar across strains (likelihood ratio: $\chi^2=1.73$, d.f.=2, $P=0.42$; Table 3). Neither the number of infected fleas feeding per mouse nor the median bacterial load per flea group was correlated with estimated minimum transmission efficiency ($P>0.16$). There was no association identified between the number of infected fleas feeding per mouse and transmission success for any of the strains (logistic regression: $\chi^2\leqslant 0.32$, d.f.=1, $P\geqslant 0.21$). Similarly, the maximum bacterial loads per *X. cheopis* did not differ amongst strains ($\chi^2=2.94$, d.f.=2, $P=0.23$) nor was there any association detected between the median and maximum bacterial load, i.e. the maximum c.f.u. per flea in a pool of 10 fleas, and transmission success (logistic regression: $\chi^2\leqslant 0.29$, d.f.=1, $P\geqslant 0.59$).

Almost half (43 %) of *X. cheopis* transmission fleas were infected with *Proteus* sp. and infection with *Proteus* sp. did not differ amongst strains (Fig. 2). A slight trend appeared towards lower c.f.u. per flea in *Proteus*-infected fleas for KIM5+ and *ymt*H188N *Y. pestis* strains; the opposite trend was noted in CO96-3188 *Proteus*-infected *X. cheopis* (Fig. 2). Despite these apparent trends, there was no significant difference in median *Y. pestis* c.f.u. per flea between fleas infected or not infected with *Proteus* sp. for any strain (Mann–Whitney *U* test: $\chi^2<1.20$, $P>0.27$).

Confirmation that the *ymt* mutation was maintained in the *ymt*H188N-infected mice

Sequencing a 279 bp region targeting the single-base mutation from cytosine to adenine at nt 562 of the coding region of the *ymt* gene confirmed that all CO96-3188 and KIM5+ strains had cytosine at this position, whilst in all *ymt*H188N isolates the cytosine had been replaced by adenine (data not shown). All strains used here were confirmed to be virulent in needle-inoculated control mice, i.e. signs of infection and culture obtained from liver and spleen.

DISCUSSION

In contrast to the blocked flea model of transmission for which the physiological and molecular mechanisms are well understood, the interactions between the flea and *Y. pestis* that result in EPT remain elusive. However, the genetic mechanisms driving blocked flea transmission seem to be distinct from those involved in EPT. Under the blocked flea model, certain genes are required for blockage

Table 2. Bacterial loads and transmission efficiency for *O. montana* infected using an artificial feeder system containing defibrinated rat blood infected with *Y. pestis* at concentrations ranging from 1.03×10^9 to 1.93×10^9 c.f.u. ml^{-1}*

Strain/mouse	No. infected fleas fed on naïve mouse (total no. fed/total no. exposed)	Median (range) bacterial load per infected flea fed on naïve mouse	Transmission from flea to mouse (titre)†	Minimum observed transmission efficiency (%)
CO96-3188		7.80×10^4 (5.00 to 1.88×10^6)‡		14.56 (7.03–30.88)§
1	9 (10/10)	6.68×10^4 (2.25×10^3 to 8.45×10^5)	Yes (1 : 64)	11.1
2	8 (9/10)	6.83×10^4 (2.15×10^3 to 1.28×10^6)	No	0.0
3	10 (10/10)	2.30×10^4 (2.25×10^3 to 6.35×10^5)	Yes (1 : 128)	10.0
4	10 (10/10)	1.08×10^5 (2.10×10^3 to 7.10×10^5)	Yes	10.0
5	10 (10/10)	1.79×10^5 (9.40×10^3 to 1.24×10^6)	Yes	10.0
6	10 (10/10)	1.82×10^5 (4.25×10^2 to 1.09×10^6)	Yes	10.0
7	10 (10/10)	5.18×10^5 (7.50×10^1 to 1.88×10^6)	Yes (1 : 256)	10.0
8	9 (9/10)	1.43×10^5 (5.00 to 1.24×10^6)	Yes (1 : 256)	11.1
9	9 (9/10)	1.83×10^4 (6.90×10^2 to 1.59×10^6)	Yes	11.1
10	9 (9/10)	1.48×10^4 (3.00×10^1 to 3.65×10^5)	No	0.0
KIM5+		1.11×10^4 (5.00 to 1.42×10^7)‡		8.80 (3.77–18.73)§
1	9 (10/10)	3.75×10^3 (8.50×10^1 to 1.48×10^5)	Yes (1 : 256)	11.1
2	10 (10/10)	4.90×10^3 (5.00 to 1.07×10^5)	Yes (1 : 128)	10.0
3	9 (10/10)	9.00×10^2 (1.10×10^2 to 1.06×10^5)	Yes (1 : 256)	11.1
4	9 (9/10)	1.69×10^4 (3.15×10^3 to 1.42×10^7)	Yes (1 : 128)	11.1
5	8 (10/10)	1.07×10^4 (8.60×10^2 to 1.81×10^5)	Yes (1 : 256)	12.5
6	7 (10/10)	1.73×10^5 (4.05×10^2 to 3.75×10^6)	No	0.0
7	10 (10/10)	1.16×10^4 (2.50×10^1 to 9.70×10^5)	Yes (1 : 256)	10.0
8	10 (10/10)	2.65×10^4 (2.55×10^2 to 1.15×10^6)	No	0.0
9	10 (10/10)	1.97×10^5 (5.00 to 5.10×10^6)	No	0.0
10	10 (10/10)	1.39×10^4 (1.35×10^2 to 9.55×10^6)	No	0.0
***ymt*H188N**		4.10×10^3 (5.00 to 4.55×10^6)‡		16.08 (7.86–33.18)§
1	8 (9/10)	2.80×10^3 (1.00×10^1 to 1.67×10^4)	Yes	12.5
2	9 (10/10)	1.70×10^3 (2.00×10^2 to 3.35×10^4)	Yes	11.1
3	9 (9/10)	2.05×10^3 (2.50×10^2 to 8.00×10^4)	Yes	11.1
4	7 (8/10)	5.40×10^3 (5.00 to 1.53×10^5)	No	0.0
5	8 (9/10)	6.75×10^2 (3.00×10^1 to 3.45×10^4)	Yes	12.5
6	9 (10/10)	8.08×10^4 (5.00 to 7.30×10^5)	Yes	11.1
7	7 (10/10)	4.70×10^4 (3.00×10^1 to 4.55×10^6)	Yes	14.3
8	7 (10/10)	1.89×10^4 (3.50×10^1 to 1.87×10^6)	Yes	14.3
9	9 (9/10)	1.33×10^4 (2.20×10^3 to 2.58×10^5)	No	0.0
10	10 (10/10)	5.23×10^3 (1.30×10^2 to 1.22×10^6)	Yes (1 : 256)	10.0

*Range of bacterial concentrations in artificial feeders for each strain: CO96-3188 (1.36×10^9–1.93×10^9 c.f.u. ml^{-1}), KIM5+ (1.37×10^9–1.55×10^9 c.f.u. ml^{-1}) and *ymt*H188N (1.03×10^9–1.72×10^9 c.f.u. ml^{-1}).

†Day 22 p.i. titres by passive haemagglutination and inhibition tests.

‡Median and range of c.f.u. per flea for each strain.

§Estimated transmission efficiency (95 % confidence interval) per strain based on maximum likelihood (PooledInfRate, v. 4.0; Biggerstaff, 2009).

of the proventriculus (e.g. *hmsHFRS*). Both *ymt* and *hms* are known to be important in facilitating colonization (i.e. survival and reproduction) of the midgut and proventricular blockage, respectively, within the flea (Hinnebusch *et al.*, 1996, 1998, 2000, 2002; Jones *et al.*, 1999; Kirillina *et al.*, 2004; Perry *et al.*, 1990; Rudolph *et al.*, 1999). Our study, combined with a prior study that evaluated the role of biofilm formation in EPT (Vetter *et al.*, 2010), has shown that neither Ymt nor Δ*hmsR* or Δ*hmsT* are required for this mode of transmission.

Infection prevalence reported here for *X. cheopis* fleas infected with *ymt*H188N differs from that reported by Hinnebusch *et al.* (2002). Previous work has shown that at 24 h p.i., the *ymt*H188N mutant displayed an aberrant spheroplast form in the flea midgut that was assumed to have preceded elimination from the flea, as the mutant was not detected in the midgut at 7 days p.i. (Hinnebusch *et al.*, 2002). Although it is unlikely (Hinnebusch *et al.*, 2002) *ymt*H188N may survive long enough (>3 days p.i.) in the flea midgut to promote EPT, it is possible that the bacteria are occupying other regions of the flea that would leave *Y. pestis* more accessible for transmission (e.g. the proventriculus). We did not perform midgut dissections to determine the distribution of *Y. pestis* within each flea.

Table 3. Bacterial loads and transmission efficiency for *X. cheopis* infected using an artificial feeder system containing defibrinated rat blood infected with *Y. pestis* at concentrations ranging from 2.35×10^7 to 5.25×10^8 c.f.u. ml^{-1}*

Strain/mouse	No. infected fleas fed on naïve mouse (total no. fed/ total no. exposed to mouse)	Median (range) bacterial load per infected flea fed on naïve mouse	Transmission from flea to mouse	Minimum observed transmission efficiency (%)
CO96-3188		6.30×10^4 (5.00 to 2.27×10^6)†		1.82 (0.11–8.91)‡
1	5 (7/10)	3.20×10^4 (1.50×10^2 to 2.06×10^5)	No	0.0
2	9 (10/10)	8.15×10^4 (6.60×10^2 to 4.15×10^5)	Yes	11.1
3	10 (10/10)	2.58×10^5 (5.05×10^3 to 2.27×10^6)	No	0.0
4	5 (9/10)	3.80×10^4 (1.60×10^2 to 3.21×10^5)	No	0.0
5	9 (10/10)	3.17×10^4 (2.00×10^1 to 4.00×10^5)	No	0.0
6	10 (10/10)	3.60×10^4 (2.90×10^3 to 1.03×10^6)	No	0.0
7	9 (10/10)	1.90×10^4 (5.00 to 1.62×10^5)	No	0.0
KIM5+		1.83×10^5 (5.00 to 3.15×10^6)†		1.90 (0.11–9.41)‡
1	9 (10/10)	2.06×10^5 (6.00×10^1 to 3.15×10^6)	No	0.0
2	10 (10/10)	5.10×10^5 (3.20×10^3 to 1.95×10^6)	Yes	10.0
3	10 (10/10)	1.96×10^5 (5.00 to 1.61×10^6)	No	0.0
4	8 (10/10)	1.31×10^5 (1.50×10^1 to 8.20×10^5)	No	0.0
5	10 (10/10)	7.15×10^4 (3.00×10^1 to 2.95×10^5)	No	0.0
6	8 (9/10)	2.27×10^5 (1.00×10^1 to 1.10×10^6)	No	0.0
***ymt*H188N**		1.23×10^5 (5.00 to 3.45×10^6)†		4.59 (1.26–12.24)‡
1	8 (9/10)	2.45×10^4 (1.80×10^2 to 1.21×10^6)	No	0.0
2	9 (9/10)	6.70×10^2 (5.00 to 1.77×10^6)	No	0.0
3	5 (9/10)	6.25×10^5 (3.00×10^5 to 2.80×10^6)	No	0.0
4	5 (9/10)	1.94×10^5 (1.55×10^2 to 3.45×10^6)	Yes	20.0
5	8 (10/10)	3.80×10^5 (2.50×10^3 to 1.07×10^6)	No	0.0
6	6 (10/10)	1.90×10^5 (2.00×10^2 to 3.40×10^5)	No	0.0
7	9 (9/10)	6.35×10^4 (8.15×10^2 to 9.00×10^5)	Yes	11.1
8	6 (10/10)	2.60×10^4 (2.05×10^2 to 1.66×10^5)	Yes	16.7
9	7 (10/10)	1.58×10^5 (3.60×10^2 to 5.15×10^5)	No	0.0
10	4 (10/10)	5.98×10^4 (2.20×10^2 to 2.35×10^5)	No	0.0

*Range of bacterial concentrations in artificial feeders for each strain: CO96-3188 (2.35×10^7–3.40×10^8 c.f.u. ml^{-1}), KIM5+ (1.53×10^8–2.3×10^8 c.f.u. ml^{-1}) and *ymt*H188N (1.19×10^8–5.25×10^8 c.f.u. ml^{-1}).

†Median and range of c.f.u. per flea for each strain.

‡Estimated transmission efficiency (95 % confidence interval) per strain based on maximum likelihood (PooledInfRate, v. 4.0; Biggerstaff, 2009).

Consistent with previous early-phase studies, our results show no correlation between bacterial loads in the flea and transmission success (Ajl *et al.*, 1955; Eisen *et al.*, 2006, 2007a, b, 2008a, b Vetter *et al.*, 2010; Wilder *et al.*, 2008a, b). Our results support previous assertions that midgut colonization may not be necessary for EPT and that location of the bacteria within the flea is more important in predicting transmission outcomes than the flea's bacterial load (Eisen *et al.*, 2007a; Eisen & Gage, 2009). Specifically, the probability of transmission likely declines as bacteria move further posterior in the digestive tract during the early phase (Eisen *et al.*, 2007a).

One might ask why bacteria in the midgut would be less transmissible than those in the foregut or mouthparts. During the acquisition of a blood meal, blood is drawn up from the host by two pumps powered by muscles attached to the cibarial and pharyngeal regions of the foregut, respectively, in the head of the flea. The cibarial and pharyngeal pumps contract one after the other to force blood backwards through the oesophagus, into the proventriculus and finally into the midgut; at the same time, a salivary pump forces secretions from the salivary glands out through the hypopharynx (Furman & Catts, 1982). When the flea is feeding, the proventriculus opens and closes in concert with the cibarial and pharyngeal pumps in the head, maintaining the blood flow generally from anterior to posterior within the flea. Between blood meals, the proventriculus remains tightly closed, maintaining separation between the midgut and oesophagus. As a result, bacteria in the midgut might be less likely to be transmitted than bacteria found further anterior in the flea. However, because the proventriculus is constantly opening and closing during feeding, it is reasonable to presume that some midgut contents could escape and be regurgitated. If regurgitation from the midgut is a primary source of EPT, it would be expected that fleas infected with the *ymt* mutant strain would have exhibited lower transmission rates than the two WT strains tested here, which is contrary to what we

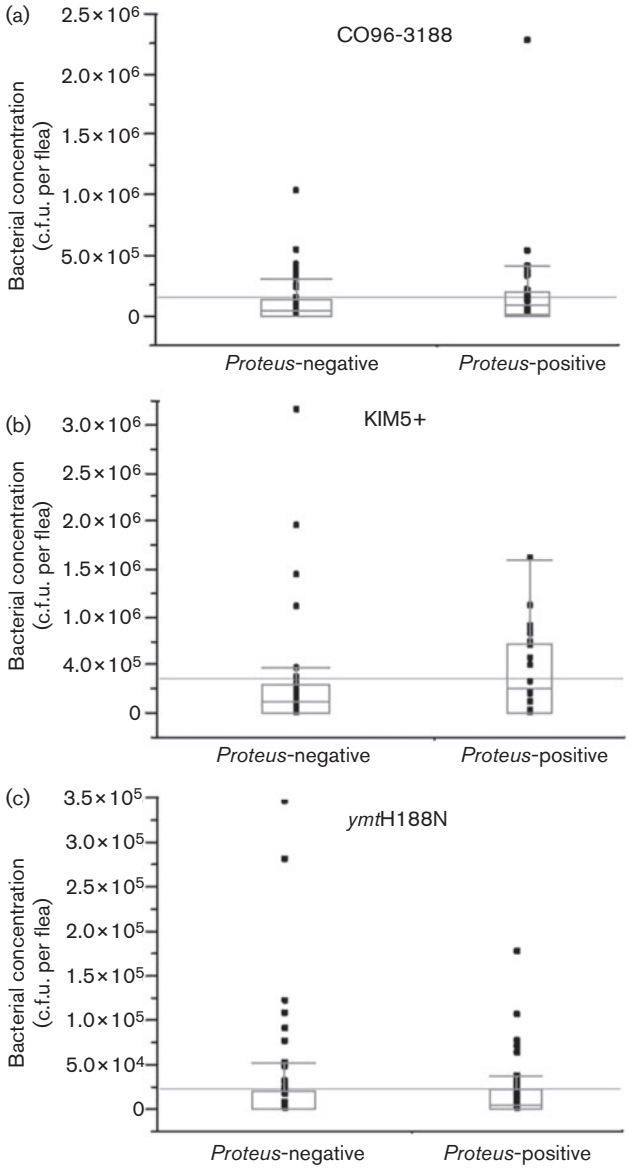

Fig. 2. Almost half (43 %) of *X. cheopis* were infected with *Proteus* sp. Infection with *Proteus* sp. did not differ amongst strains (likelihood ratio test: $\chi^2=0.83$, d.f.=2, $P=0.66$). (a) A slight but non-significant trend appeared towards higher *Y. pestis* c.f.u. per flea in *Proteus*-infected fleas for CO96-3188 (Mann–Whitney *U* test: $\chi^2=0.93$, d.f.=1, $P=0.33$); the opposite trend was noted in (b) KIM5+ ($\chi^2=0.57$, d.f.=1, $P=0.55$) and (c) *ymt*H188N ($\chi^2=0.29$, d.f.=1, $P=0.50$) *Y. pestis* strains.

observed in our study. If the proventriculus is being passively colonized during the initial ingestion of a blood meal and bacteria are being transmitted from the proventriculus during EPT, we would expect to observe no difference amongst strains regarding EPT efficiency. However, the likelihood of proventricular colonization differs between vector species and should be accounted for. *O. montana* are rarely observed to form a proventricular blockage and *Y. pestis* is rarely obvious in the proventriculus for the first few days following infection, whereas *X. cheopis* are often colonized by *Y. pestis* in both the proventriculus and midgut, with colonies of the bacterium being visible in the proventriculus soon after initial infection (Burroughs, 1947; Engelthaler *et al.*, 2000; Eskey, 1938). These well-known differences in blockage formation suggest for unknown reasons that the proventriculus of *O. montana* is less conducive to *Y. pestis* establishment than the proventriculus of *X. cheopis*, which is readily colonized and supports rapid growth of the plague bacterium in the days immediately following infection. This difference in colonization capacity, combined with the fact that *ymt*-mutant-infected *O. montana* received a lower concentration of *Y. pestis* than WT-infected fleas in the infectious meal, could explain why *ymt*H188N-infected *O. montana* had significantly fewer *Y. pestis* per flea compared with WT-infected *O. montana*, whilst we observed no difference in bacterial loads amongst the same strains within infected *X. cheopis*.

Compared with the need for midgut colonization, the extent to which the proventriculus may play a role in EPT is less clear; although it is apparent that the capacity for aggregation and biofilm formation is not required for EPT to occur (Vetter *et al.*, 2010). *Y. pestis* strains incapable of or with diminished abilities for making biofilm were transmitted as efficiently as WT strains during the early-phase period (Vetter *et al.*, 2010). However, we cannot rule out the possibility that, during the early phase, plague bacteria may become temporarily lodged within the spines of the proventriculus, and are then sloughed off and regurgitated during subsequent feeds. Hinnebusch *et al.* (2002) showed that a few *X. cheopis* challenged with *ymt*H188N had chronic infections at day 28 in the proventriculus and that a few (0.5 %) of these fleas became blocked.

Although the principal mechanism of EPT remains undefined, several additional potential mechanisms can and have been ruled out, whilst others await further investigation. Areas in the anterior portion of the flea, including the anterior pharynx and the posterior mouthparts, which are protected within the head capsule of the flea, may harbour viable *Y. pestis* for longer periods of time (Plague Commission, 1907; Hinnebusch, 2012). Residual blood has been observed within the pharynx and between the flea mouthparts after feeding. When a flea feeds, the mouthparts come together to form a feeding canal through which blood passes into the flea, as well as a salivary canal, which carries saliva out of the flea; transmission could occur if infected residual blood contaminates the grooves of the salivary canal and is transmitted to the bite site (Hinnebusch, 2012). Further, if midgut colonization is not essential for EPT, it is unlikely that faecal contamination is a primary mechanism of EPT. It should be noted, however, that we cannot rule out faecal contamination as a mode of transmission in our work or in the study by Vetter *et al.* (2010). Although *Y. pestis* KIM5+ strains *ymt*H188N and Δ*hmsR* (Vetter *et al.*, 2010) are unable to become established within the flea midgut and proventriculus,

respectively, some small possibility remains that viable *Y. pestis* might have been excreted in the faeces of potentially infected fleas resulting in EPT. The use of SKH-1 mice in this study, which are hairless, would have optimized the likelihood of this form of transmission compared with similar experiments done with a host covered by a pelt that might have reduced contact between the skin and flea faeces. Such results could also argue against the suggestion that EPT occurs as a result of contamination of blood feeding sites with infectious flea faeces.

We conclude that the pathogen–vector interactions that result in EPT are distinctly different from those described for the blocked flea transmission model and do not require the presence of Ymt. Although the mechanism of EPT remains undefined, our study suggests that future efforts should focus on early colonization events of the proventriculus or perhaps other portions of the flea foregut.

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