Biovar-related differences apparent in the flea foregut colonization phenotype of distinct *Yersinia pestis* strains do not impact transmission efficiency

Athena Lemon, Janelle Sagawa, Kameron Gravelle and Viveka Vadyvaloo*

Abstract

**Background:** *Yersinia pestis* is the flea-transmitted etiological agent of bubonic plague. Sylvatic plague consists of complex tripartite interactions between diverse flea and wild rodent species, and pathogen strains. Transmission by flea bite occurs primarily by the *Y. pestis* biofilm-mediated foregut blockage and regurgitation mechanism, which has been largely detailed by studies in the model interaction between *Y. pestis* KIM6+ and *Xenopsylla cheopis*. Here, we test if pathogen-specific traits influence this interaction by determining the dynamics of foregut blockage development in *X. cheopis* fleas among extant avirulent pCD1-*Y. pestis* strains, KIM6+ and CO92, belonging to distinct biovars, and a non-circulating mutant CO92 strain (CO92gly), restored for glycerol fermentation; a key biochemical difference between the two biovars.

**Methods:** Separate flea cohorts infected with distinct strains were evaluated for (i) blockage development, bacterial burdens and flea foregut blockage pathology, and (ii) for the number of bacteria transmitted by regurgitation during membrane feeding. Strain burdens per flea was determined for fleas co-infected with CO92 and KIM6+ strains at a ratio of 1:1.

**Results:** Strains KIM6+ and CO92 developed foregut blockage at similar rates and peak temporal incidences, but the CO92gly strain showed significantly greater blockage rates that peak earlier post-infection. The KIM6+ strain, however, exhibited a distinctive foregut pathology wherein bacterial colonization extended the length of the esophagus up to the feeding mouthparts in ~65% of blocked fleas; in contrast to 32% and 26%, respectively, in fleas blocked with CO92 and CO92gly. The proximity of KIM6+ to the flea mouthparts in blocked fleas did not result in higher regurgitative transmission efficiencies as all strains transmitted variable numbers of *Y. pestis*, albeit slightly lower for CO92gly. During competitive co-infection, strains KIM6+ and CO92 were equally fit maintaining equivalent infection proportions in fleas over time.

**Conclusions:** We demonstrate that disparate foregut blockage pathologies exhibited by distinct extant *Y. pestis* strain genotypes do not influence transmission efficiency from *X. cheopis* fleas. In fact, distinct extant *Y. pestis* genotypes maintain equivalently effective blockage and transmission efficiencies which is likely advantageous to maintaining continued successful plague spread and establishment of new plague foci.

**Keywords:** *Yersinia pestis*, Biovar, *Xenopsylla cheopis*, Glycerol, Transmission efficiency
Background

_Yersinia pestis_, the etiologic agent of bubonic plague, can be transmitted by a flea bite. In this model, the flea siphons blood into the midgut using muscles in its head that act as a peristaltic pump during feeding. The blood is drawn through the foregut which comprises the esophagus and proventriculus (PV), a valvular organ that ensures forward blood passage into the midgut for digestion while simultaneously preventing backflow [1]. However, when the flea acquires an infectious _Y. pestis_ blood meal, the bacteria forms a biofilm in the PV that partially or completely obstructs function of this organ and blood passage to the midgut. This is referred to as biofilm-mediated blockage of the flea foregut [2]. During blockage, active ingestion of a subsequent blood meal causes distention of the esophagus and a localized buildup of hydrodynamic pressure in the proximal region of the esophagus immediately preceding the blocked PV [3]. An elastic recoil of the blood-filled esophagus follows, resulting in regurgitation of some _Y. pestis_ biofilm back into the bite site thereby facilitating transmission. High blockage rates are thus synonymous with effective transmission, and both partial and complete blockage can cause blockage-mediated regurgitative transmission of _Y. pestis_ [2, 4].

The biofilm-mediated blockage localized to the PV is comprised of dense multicellular aggregates of _Y. pestis_ that are characterized by a self-produced extracellular polymeric substance (EPS). The EPS is primarily composed of poly-N-acetyl-d-glucosamine (PNAG) units [5]. The biosynthetic pathway of PNAG is encoded by the _hmsHFRS_ locus which is positively post-transcriptionally regulated by the second messenger molecule, c-di-GMP. The two functional diguanylate cyclase (DGC) enzymes HmsD and HmsT synthesize c-di-GMP. HmsD predominantly affects biofilm production during flea colonization, whereas HmsT plays a lesser role in biofilm formation in the flea environment [6–8].

Historically, _Y. pestis_ has been categorized into three clinically relevant biovars (bvs) differentiated by the biochemical abilities to ferment glycerol and arabinose, and reduce nitrate [9]: Antiqua (glycerol positive, arabinose positive and nitrate positive); Medievalis (glycerol positive, arabinose positive and nitrate negative); and Orientalis (glycerol negative, arabinose positive and nitrate positive). Recent phylogenetic analyses provide enhanced resolution of _Y. pestis_ strain characteristics, evolutionary history of biovars and associated lineages, strain geographical pattern distinctiveness, and plague spread [10, 11]. Additionally, mathematical modelling reveals that branch 1 Orientalis and branch 2 Medievalis lineage strains spread significantly faster than others [12–15].

In laboratory based experimental studies, the prototype model of the interaction between _Y. pestis_ strain KIM6+ and _Xenopsylla cheopis_ has forged an understanding of the details regarding _Y. pestis_ gut colonization, blockage development, transmission, and the bacterial genetic determinants for this process, including roles of _hmsH-FRS, hmsT, hmsD_ and _hmsP_ genes [6, 7, 16]. The KIM6+ strain is a Medievalis bv branch 2 strain that was isolated from a Kurdish Iranian man [17, 18]. The oriental rat flea, _X. cheopis_, is implicated in past plague pandemics and current plague outbreaks in Madagascar [19] and is reputed to be an efficient vector of _Y. pestis_ [20–22].

There remains minimal information regarding how different _Y. pestis_ strains influence vector transmission efficiency. Genetic differences in pathogen strains and pathogen fitness are demonstrated determinants for vector transmission efficiency in animal and plant diseases as exemplified in the interactions between Zika virus and _Aedes aegypti_, bluetongue virus and _Culicoides sonorensis_ midges, and _Xylella fastidiosa_ and sharpshooter leafhoppers [23–25].

Similarly, to understand if strain-specific _Y. pestis_ characteristics influence flea transmission efficiency, we comparatively characterized flea infection dynamics using two different _Y. pestis_ strains in _X. cheopis_ fleas, KIM6+ for which flea infection dynamics are well characterized and CO92. Use of the CO92 strain was motivated by several factors. First, CO92 belongs to the Orientalis branch 1 lineage comprising globally circulating strains, some directly implicated in causing the highest contemporary plague global case burden [12, 26, 27]. In contrast, Antiqua and Medievalis bvs are typically associated with enzootic rodent species in long term plague foci in Africa and Asia [28]. Secondly, CO92 was originally isolated from a fatal human case of pneumonic plague [29] and is primarily used for experimental infections in a murine plague model [30–32]. Thirdly, the most discerning genetic difference between CO92 and KIM6+ is that CO92 is incapable of metabolizing glycerol, allowing testing of a distinguishing metabolic trait in vector competency. Finally, CO92 produces significantly more biofilm than KIM6+ _in vitro_ suggesting that it may have higher blockage rates in fleas [6]. Given these factors, we posited that CO92 may have enhanced abilities to colonize fleas and be transmitted from these insects.

Methods

Bacterial culture conditions

_Yersinia pestis_ strains were cultured in heart infusion broth (HIB; BD Difco, New Jersey, USA) at 26 °C with aeration or grown on heart infusion agar (HIA). When appropriate, media were supplemented with carbenicillin (100 μg/ml), kanamycin (50 μg/ml) or trimethoprim [57x71][12–15].
GFP insertion into *Y. pestis*

*Yersinia pestis* strains were chromosomally tagged at the *attTn7* site with *gfpmut3* using a Tn7-based system [33]. To do this, *gfpmut3* under the control of the *P_{uc}* promoter was restricted from pGP-Tn7-*P_{uc} gfpmut3* [34] and cloned into matching restriction sites of pUC18R6KT-mini-Tn7T-Km [33] to create pUC18R6KT-mini-Tn7T-*P_{uc} gfpmut3* and electroporated into electrocompetent strains of *Y. pestis*. These plasmid constructs were verified by sequencing. Alternatively, *Y. pestis* electrocompetent strains were transformed with pAcGFP1 and selected on HIA containing carbenicillin.

Deletion of *hmsD* and *hmsT*

Deletion of *hmsD* and *hmsT* was achieved by homologous recombination using the lambda red recombinase system [35]. Flanking regions of approximately 500 bp upstream and downstream of the *hmsD* and *hmsT* genes were first amplified by PCR. The resulting PCR products were gel-purified and combined using splice-overlap extension (SOE) PCR with a kanamycin resistance cassette flanked by *frt* sites that were previously amplified from the plasmid pKD13 [35]. Electrocompetent *Y. pestis* CO92 carrying pKOBEG and expressing the recombinase was transformed with purified SOE PCR fragments [36]. Recombinant strains were picked on kanamycin and confirmed by PCR. The kanamycin resistance cassette introduced in the previous step was resolved by the introduction of pFLP3. Deletion mutants CO92 Δ*hmsD::frt* and CO92 Δ*hmsT::frt* were confirmed by sequencing.

Flea infections

*Yersinia pestis* strains confirmed as Pgm-positive were cultured overnight in heart infusion broth (HIB) as described previously [37]. Bacteria were added to 6 ml of sodium heparinized mouse blood (BioIVT, New York, USA) at a concentration of ~1 × 10^8 CFU/ml [38]. *Xenopsylla cheopis* fleas were then allowed to feed on the infected blood using a previously described artificial feeding apparatus [16, 39]. Only fleas that took full blood meals were selected. Fifty male and 50 female fleas were maintained at 21 °C and 75% relative humidity, fed twice weekly on uninfected mice and monitored for proven-tricular blockage over a period of 28 days as previously described [16]. To perform co-infections, the protocol was followed as previously described [39].

Determining *Y. pestis* CFU in the infectious blood meal and infected flea samples

After artificial infection, a sample of the infected blood meal was serially diluted and plated on Columbia blood agar (Hardy Diagnostics, California, USA) to determine *Y. pestis* CFU/ml in the blood. To quantify infection rate and bacterial load of the fleas, 20 infected fleas were collected immediately after the infectious blood meal, and at days 7, 14 and 28 post-infection. Fleas were subjected to individual plating on brain heart infusion (BHI) agar (BD Difco) supplemented with 1 μg/ml irgasan and 10 μg/ml hemin to determine CFU count. To determine CFU/flea from co-infected fleas, samples were treated as previously described, but plated on heart infusion agar with and without 50 μg/ml kanamycin to select for KIM6+:::kan^R on individual sets of plates respectively [39].

Imaging and esophageal scoring of fleas

Blocked fleas were dissected in PBS to isolate the flea gut. A 1.5 mm glass cover slip was then placed over the samples for imaging. Flea guts were imaged with a Leica DMi8 epifluorescence microscope (Leica Microsystems, Illinois, USA) with phase contrast and a GFP filter cube (Ex. 470/40nm, Em. 525/50nm). Esophageal images were first evaluated for valid scoring by determining if: (i) the esophagus was intact; and (ii) the entire esophagus was visible. If these criteria were met, the esophagus was scored for presence of GFP in which presence of GFP in < 1/3 the length of the esophagus was assigned a proximal colonization status, and presence of GFP in ≥ 1/3 the length of the esophagus was assigned a distal-medial colonization status.

Mass transmission experiment

For transmission experiments, fleas that took an infectious blood meal were housed as described above. On days 7 and 14, the fleas involved in the transmission experiment fed on 5 ml of sterile sodium heparinized CD-1 (ICR) mouse blood (BioIVT) using the artificial feeding system. After 60 min, fleas were examined microscopically to determine how many had taken a blood meal, and of those, how many were blocked. Transmission experiments were performed as previously described [40]. Immediately after each transmission feeding, all 5 ml of blood were removed and plated on Columbia blood agar plates. The interior of the feeder was rinsed 24 times with 1 ml of PBS and these washes were pooled. Pooled washes were centrifuged at ~12,000 × g for 10 min, supernatant removed, and the pellet was plated onto Columbia blood agar plates. The external surface of the mouse skin was disinfected with 70% ethanol twice to reduce surface contaminants and cut into small pieces that were homogenized in PBS in a FastPrep homogenizer (MP
| Yersinia pestis strains and plasmids used | Characteristics | Reference |
|------------------------------------------|-----------------|-----------|
| CO92                                     | Pgm + pCD1- pMT1- pPCP1 +, parental strain | [32]       |
| CO92 glmS-pstS::PlacGFP                  | CO92 with chromosomally inserted gfp for imaging | This study |
| CO92 glmD+                                | Defective glmD′ allele replaced with functional glmD gene from KIM6+ in the chromosome | [32]       |
| CO92 glmD+ glmS-pstS::PlacGFP             | Defective glmD′ allele replaced with functional glmD gene in the chromosome, with chromosomally inserted GFP for imaging | This study |
| CO92 gly                                 | Defective copy of the glmD′ allele replaced with functional glmD in the chromosome, and carries glmFX from KIM6+ on pT7pGLFFX6 | This study |
| CO92 gly glmS-pstS::PlacGFP               | CO92 gly with chromosomally inserted gfp for imaging | This study |
| CO92 ΔhmsD::frt                          | ΔhmsD           | This study |
| KIM6+                                    | Pgm + pCD1- pMT1- pPCP1 +, parental strain | [16]       |
| KIM6+ kanR                                | KIM6+ with chromosomally inserted kanamycin resistance for co-infections | [39]       |
| KIM6+ pAcGFP1                            | KIM6+ with GFP for imaging | This study |
| pAcGFP1                                   | Carries AcGFP1, Cbr | Clontech (Mountain View, CA, USA) |
| pTNS2                                    | TnsABC+D specific transposition pathway, Cbr | [33]       |
| pKD13                                    | Amplicon of kanamycin resistant cassette and flanking FRT sequence | [35]       |
| pFL3                                     | Recombinase, Cbr, Tet | [33]       |
| pUC18R6KT-mini-Tn7-Ts2                   | Cloning vector for Tn7 insertion, Cbr | [33]       |
| pUC18R6KT::PlacGFP                       | Source of PpT7-gfpmut3, Cbr | [34]       |
| pUC18R6KT-gfpmut3                        | Cloning vector for Tn7 insertion of PpT7-gfpmut3, Cbr | This study |

Abbreviations: Cbr, carbenicillin resistance; Kmr, kanamycin resistance; Tet, tetracycline

| Table 2 List of oligonucleotides used |
|--------------------------------------|
| Oligonucleotide  | Reference | Purpose | Sequence |
|------------------|-----------|---------|----------|
| p130             | [35]      | Amplify kanamycin cassette and check insertion | GTGAGCCTGGACGGGCTTTTTG |
| p131             | [35]      | Amplify kanamycin cassette and check insertion | ATCCGGAGGGATCAGGCTTTTTG |
| p407/pGPTn7pro   | This study| To clone gfpmut3 into pUC18R6KT-mini-Tn7-Km | ACGTGGTACATGGTGGTTG |
| p408/pGFP7Tn7R   | This study| To clone gfpmut3 into pUC18R6KT-mini-Tn7-Km | GAAGCAGCTGGAGGTGTTG |
| p544             | [51]      | To mutate hmsT | ACGTGGTACATGGTGGTTG |
| p545             | [51]      | To mutate hmsT | GAAGCAGCTGGAGGTGTTG |
| p546             | [51]      | To mutate hmsT | GAAGCAGCTGGAGGTGTTG |
| p547             | [51]      | To mutate hmsT | GAAGCAGCTGGAGGTGTTG |
| p548             | [51]      | To mutate hmsD | GAAGCAGCTGGAGGTGTTG |
| p549             | [51]      | To mutate hmsD | GAAGCAGCTGGAGGTGTTG |
| p550             | [51]      | To mutate hmsD | GAAGCAGCTGGAGGTGTTG |
| p551             | [51]      | To mutate hmsD | GAAGCAGCTGGAGGTGTTG |
| p552             | [51]      | To verify hmsD mutation | GAAGCAGCTGGAGGTGTTG |
| p553             | [51]      | To verify hmsD mutation | GAAGCAGCTGGAGGTGTTG |
| p554             | [51]      | To verify hmsD mutation | GAAGCAGCTGGAGGTGTTG |
| p555             | [51]      | To verify hmsD mutation | GAAGCAGCTGGAGGTGTTG |
| p556             | This study| To verify hmsD mutation | GAAGCAGCTGGAGGTGTTG |
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| p558             | This study| To verify hmsD mutation | GAAGCAGCTGGAGGTGTTG |
| p559             | This study| To verify hmsD mutation | GAAGCAGCTGGAGGTGTTG |
Biomedicals, California, USA) to release any bacteria deposited by flea bite into the mouse skin. Skin sample supernatants were pooled and treated in the same manner as the pooled washes. After incubation at 28 °C for 48 h, Y. pestis colonies were counted and confirmed by replicate patch plating on both Yersinia selective agar plates and Congo Red agar. At each time point post-infection 20 fleas were collected for enumeration of bacterial loads. To maintain the normal bi-weekly schedule of feeding, fleas were also fed on neonatal mice on days 4 and 11.

Statistical analysis
All analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, California, USA.). Fisher's exact test was performed with the fisher.multcomp package in R (3.6.1). The statistical tests that were used and relevant P-values are indicated in the figure legends and results.

Results
KIM6+ and CO92 infected X. cheopis fleas have similar blockage rates
To determine if Y. pestis strain specific distinctions can be made in the interactions of Y. pestis and its prototype flea vector, X. cheopis, blockage rates and flea colonization dynamics were directly compared between CO92 and KIM6+ strains. The distinctive phenotypic difference between KIM6+ and CO92 is the inability of CO92 to metabolize glycerol. Therefore, to simultaneously determine the role of glycerol utilization in potential differences that may be uncovered in flea infection dynamics between CO92 and KIM6+, we also tested a previously reported CO92 strain [32] that was restored in ability to metabolize glycerol (referred to as CO92gly). For this, X. cheopis fleas were artificially infected with CO92, KIM6+ or CO92gly strains to determine blockage rate, flea infection rate, and bacterial burden per flea over time. We found that the cumulative blockage rate of fleas infected with KIM6+ or CO92 over the period of a month averaged approximately 37% (SD ± 3.00%), and 40% (SD ± 5.78%) respectively (Fig. 1a). This was consistent with previous reports of blockage rates between 25–45% for the KIM6+ strain [40]. Strikingly, mean blockage rate of CO92gly infected fleas was 54% (SD ± 7.5%), significantly higher (t-test: \( t_{(4)} = 4.143, P = 0.0143 \)) than blockage rates for KIM6+ (Fig. 1a). However, no significant differences in bacterial burden per flea and rate of flea infection were noted for all three strains (Fig. 1b).

A detailed assessment of temporal incidence of blockage demonstrated that peak incidence of blockage occurred at 14.3 days post-infection for KIM6+ (Fig. 1c), but at 11 days post-infection for CO92 (Fig. 1d). In contrast, temporal incidence of blockage did not follow a normal distribution and was predicted to peak at a physiologically unrealistic 0.15 days (~3 h post-infection) for CO92gly (Fig. 1e).

KIM6+ biofilm blockage extends the full length of the X. cheopis esophagus
No quantitative differences in flea blockage rate, mortality, or infection were noted between the KIM6+ and CO92 strains. However, in preliminary studies we noted that KIM6+ colonized the entire length of the flea esophagus, as well as the PV, whereas CO92 primarily colonized the PV alone. Microscopic visualization prior to application of a coverslip eliminated any concerns that the phenotype observed resulted from the process of the coverslip laying down on the gut. To quantitatively assess this phenotype, we developed an esophageal pathology scoring rubric which enabled parsing of colonization status into major (distal-medial) and minor (proximal) with respect to Y. pestis esophagus colonization of the flea foregut. Intriguingly and consistent with preliminary observations, a significantly greater (Fisher’s exact test: OR: 1.339, 95% CI: 0.1164–0.5457, \( P < 0.0001 \)) 65% of KIM6+ blocked fleas compared to only 32% of fleas blocked with CO92 colonized most of the surface of the flea esophagus (Fig. 2a–e). The CO92gly strain maintained a similar foregut colonization phenotype to the parental strain (Fisher’s exact test: OR: 1.339, 95% CI: 0.6113–3.069, \( P = 0.4360 \)).

Transmission of KIM6+, CO92 and CO92gly is not significantly different
Full esophagus colonization by the sticky Y. pestis biofilm reflects that bacteria are in closer proximity to the feeding mouthparts and can likely be more efficiently regurgitated back into the flea bite site. Alternatively, the impediment caused by full Y. pestis esophageal colonization may reduce build-up of the hydrodynamic forces and pressure in the esophagus, resulting in fewer Y. pestis cells being dislodged and regurgitated from the

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biofilm blockage. To address this, we asked if the esophageal colonization differences observed among the CO92, CO92gly and KIM6+ strains might impact regurgitative transmission of bacteria into the flea bite site. Cohorts of *X. cheopis* fleas were infected with the *Y. pestis* strains of interest. Once a week for two weeks, mass-transmission experiments were conducted to determine the total CFU of each *Y. pestis* strain that is regurgitated by infected fleas into an artificial blood-feeding apparatus containing sterile blood. For all the strains tested, KIM6+, CO92 and CO92gly, a wide and variable range of *Y. pestis* CFU was transmitted per feeding (Fig. 3, Table 3). Although not statistically significant, fewer CO92gly CFUs were transmitted per blocked flea bite with similar numbers of blocked fleas occurring in all three strains (Fig. 3a, Table 3). Similarly, fewer CO92gly CFUs were transmitted per infected flea bite (Fig. 3b).

**KIM6+ and CO92 are equally fit during competitive flea co-infection**

The distinctive flea gut colonization phenotypes exhibited by the CO92 and KIM6+ strains may be important traits for flea infection that can only be discerned in more sensitive competitive fitness studies [37, 39]. To test this, we infected fleas with a 1:1 ratio of both strains. At 2 h post-infection, and by days 7 and 14 post-infection, there was no significant change in the ratio of each strain per flea. This data implies that KIM6+ and CO92 are equally fit during competitive co-infection in fleas (Fig. 4).
CO92 hmsT and hmsD diguanylate cyclase encoding genes have matching roles in blockage formation to KIM6+ hmsT and hmsD genes

We additionally considered that the phenotypic differences in foregut colonization noted between KIM6+ and CO92 was a consequence of disparate nutritional sensing due to the biochemical differences in the strains. Localized environmental signals including nutrient signals in the flea foregut may contribute to differential regulation of the diguanylate cyclase encoding genes [41–43] and alterations in c-di-GMP signaling and biofilm production. We know that in the KIM6+ strain, the hmsD gene is essential for blockage while the absence of hmsT reduced blockage rates by approximately 50% [7]. However, the individual contributions of HmsD and HmsT diguanylate cyclases to blockage development in CO92 strains is unknown [7]. We therefore compared blockage rates between CO92 mutants in hmsT and hmsD alongside the isogenic parental CO92 strain and found an 82% and 39% reduction in blockage for mutants relative to the
CO92 parental strain, respectively (Table 4). The roles of HmsT and HmsD in blockage formation by CO92, as such, recapitulated that occurring in the KIM6+ strain (Table 4).

Discussion
The present study aimed to understand the contribution of genetically and biochemically distinct Y. pestis strains to vector competence of the prototype flea vector species of plague, X. cheopis [37]. Our studies reveal that pathogen-specific differences in colonization of the flea foregut between the CO92 and KIM6+ strains do not result in differences in blockage rates, competitive fitness, in vitro transmission efficiency, or demonstrate different contributions of diguanylate cyclases to blockage during infection of X. cheopis fleas. The present blockage rate data of CO92 to KIM are consistent with previous results with two other Orientalis strains, 195/P (16) and GB [44]. In this study we additionally examined whether the ability to utilize glycerol, present in KIM6+ but absent in CO92, may contribute to vector competence. This was motivated by previous work demonstrating that genes encoding glycerol uptake are specifically induced in KIM6+ blocked fleas suggesting that active glycerol metabolism occurs in this strain during flea infection [37, 45]. Loss of glycerol uptake and utilization in CO92 is due to mutations in glpD and the glpFKX operon, but a previous study undertook restoration of this biochemical ability by complementing the CO92 strain with intact copies of glpD and the glpFKX genes [32]. Functionally restoring glycerol utilization to the CO92 wild-type strain did not impact virulence in a mouse model or biofilm production in vitro, suggesting that glycerol utilization is not essential for these functions [32].

Our study attributes re-establishment of glycerol utilization in the CO92gly strain to enhanced blockage rates but not changes in growth, flea bacterial burdens, or esophageal colonization in fleas. Principally, blockage is dependent on production of a biofilm EPS matrix that allows a robust adherence of bacterial aggregates to the PV a few days post-acquisition of the infected blood meal [46]. EPS production likely occurs at elevated levels in CO92gly leading to hyperbiofilm production in the flea gut. This may be counterproductive to transmission if the cohesive nature of the hyperbiofilm prevents easy dislodging of bacteria by hydrodynamic forces, resulting in lower numbers of regurgitated bacteria. Interestingly, CO92gly-infected fleas tended to transmit fewer bacteria despite a significantly higher blockage rate. The phosphodiesterase activity of Y. pestis HmsP is required
to degrade c-di-GMP and decrease EPS production [6, 47] and an HmsP mutant of Y. pestis is a hyperbi-
ofilm producer generating bacterial aggregates that are extraordinarily cohesive [47]. Alteration of c-di-GMP levels through regulation of its synthesis and degradation enzymes is suggested to occur through sensing of nutritional signals [37, 43], which, in this case could be conferred by the ability to ferment glycerol that is normally absent in CO92 strains. Glycerol utilization in the CO92 genetic background does not, however, appear to bode well for optimal transmission efficiency. First, the peak blockage incidence of the CO92gly strain three hours post-infection is physiologically improbable for EPS synthesis. Secondly, this improbable peak blockage incidence and blockage rate strays from the norm exhibited by the extant, circulating epidemic strains, CO92 and

| Table 3 Xenopsylla cheopis mass transmission summary |
|-----------------------------------------------|
| Days PI | No. of fleas fed | % infected | CFU/infected flea | No. of fleas blocked | CFU transmitted |
| X. cheopis infected with KIM6+ mass transmission summary |
| Experiment 1 (1.10 x 10^5 CFU/ml blood) |
| 0 | – | 80 | 1.20E+05 | – | – |
| 7 | 80 | 95 | 6.85E+05 | 8 | 607 |
| 14 | 88 | 100 | 7.81E+05 | 13 | 710 |
| Experiment 2 (2.46 x 10^5 CFU/ml blood) |
| 0 | – | 100 | 2.19E+05 | – | – |
| 7 | 206 | 95 | 6.35E+05 | 11 | 10,749 |
| 14 | 120 | 95 | 4.50E+05 | 7 | 1942 |
| Experiment 3 (6.70 x 10^5 CFU/ml blood) |
| 0 | – | 100 | 1.08E+05 | – | – |
| 7 | 206 | 100 | 2.62E+05 | 7 | 12,957 |
| 14 | 120 | 90 | 2.69E+05 | 7 | 620 |
| X. cheopis infected with CO92 mass transmission summary |
| Experiment 1 (1.03 x 10^6 CFU/ml blood) |
| 0 | – | 100 | 2.76E+03 | – | – |
| 7 | 132 | 90 | 1.11E+05 | 27 | 2583 |
| 14 | 65 | 95 | 3.46E+05 | 27 | 2353 |
| Experiment 2 (2.27 x 10^6 CFU/ml blood) |
| 0 | – | 100 | 8.87E+04 | – | – |
| 7 | 183 | 95 | 2.62E+05 | 13 | 1714 |
| 14 | 81 | 90 | 3.37E+05 | 15 | 405 |
| X. cheopis infected with CO92gly mass transmission summary |
| Experiment 1 (2.06 x 10^6 CFU/ml blood) |
| 0 | – | 95 | 2.51E+04 | – | – |
| 7 | 103 | 85 | 4.04E+05 | 12 | 143 |
| 14 | 52 | 85 | 1.81E+05 | 9 | 177 |
| Experiment 2 (1.45 x 10^7 CFU/ml blood) |
| 0 | – | 65 | 1.19E+04 | – | – |
| 7 | 92 | 90 | 7.14E+05 | 5 | 2233 |
| 14 | 54 | 75 | 8.94E+04 | 7 | 7 |
| Experiment 3 (6.70 x 10^7 CFU/ml blood) |
| 0 | – | 95 | 7.00E+04 | – | – |
| 7 | 88 | 75 | 7.32E+05 | 4 | 5 |
| 14 | 55 | 85 | 9.45E+05 | 5 | 961 |

Abbreviation: PI, post-infection
KIM6+. Thirdly, this strain shows a tendency to transmit fewer bacteria reflecting reduced transmission efficiency. Marked genome rearrangements also distinguish CO92 from KIM strains and may contribute to gene expression alterations relevant to metabolic processes in these strains [17]. Incorporation of an ability to metabolize glycerol within the existing CO92 metabolic networks may thus be deleterious to a metabolic physiology that supports optimal flea transmission competency. In KIM6+ glycerol utilization is likely metabolically innocuous. This may explain the loss of the pathway in Orientalis strains which have achieved global distribution.

One significant observation in this study is the predominant occurrence of extended colonization of the esophagus in KIM6+ blocked fleas. The physical anatomy of the flea foregut with regards to musculature surrounding the PV and the width of both the PV and esophagus have proven to be important determinants of flea vector transmission efficiency [40, 48]. The observation that the KIM6+ biofilm blockage ‘infiltrates’ the esophagus was made previously and described to be responsible for distending the esophagi width of X. cheopis and Oropsylla montana fleas [40]. The wider O. montana esophagus facilitates regurgitative transmission of greater numbers of bacteria in keeping with its greater width in these reports. However, our results did not reveal any difference in the CFU transmitted by KIM6+ and CO92 blocked X. cheopis fleas. In fact, this finding is consistent with previous studies that show similar variable ranges of bacteria being transmitted from X. cheopis fleas infected with KIM6+, and that transmission by flea bite is a relatively inefficient process requiring bolstering by high flea indices [38, 49]. We speculate that the relevance of esophageal colonization differences regarding regurgitative transmission may differ in other flea species with anatomical foregut characteristics that are distinct from that of X. cheopis. Certainly, the proposition that natural endemic foci harbor discrete co-evolved Y. pestis strain-flea vector species associations is a provocative one that may give credence to this notion [10, 50].

Table 4 CO92 c-di-GMP diguanylate cyclases have similar contributing roles to blockage formation as their homologs in KIM6+

| Y. pestis strain | CO92 | CO92 ΔhmsD::frt | CO92 ΔhmsT::frt | KIM6+ΔhmsD | KIM6+ΔhmsDΔhmsT | KIM6+ΔhmsTΔhmsD |
|------------------|------|-----------------|-----------------|------------|-----------------|-----------------|
| Mean % reduction in mutant blockage vs parental strain | na   | 82              | 39              | na         | 92              | 57              |
| Mean CFU/flea (0 dpi) | $3.1 \times 10^4$ | $9.4 \times 10^3$ | $2.3 \times 10^4$ | $1.3 \times 10^5$ | $5.1 \times 10^3$ | $5.2 \times 10^4$ |
| Mean CFU/flea (28 dpi) | $1.3 \times 10^5$ | $7.9 \times 10^5$ | $3.7 \times 10^5$ | $5.4 \times 10^5$ | $3.0 \times 10^5$ | $4.7 \times 10^5$ |

* All KIM6+ data derived from [7]

Notes: Data is reflective of one experiment, 20 fleas plated per time point

Abbreviations: na, not applicable; dpi, days post-infection
Abbreviations
PV: proventriculus; EPS: extracellular polymeric substance; SD: standard deviation; PNAG: poly-N-acetyl-glucosamine; DGC: diguanylate cyclase; bvs: biovars; HIB: heart infusion broth; HIA: heart infusion agar; SOE: splice-overlap extension; CFTL: colony forming units; GFP: green fluorescent protein; Ex: excitation, Em: emission.

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Authors’ contributions
VV and AL wrote the manuscript. AL, JS and VV analyzed and interpreted data. AL, JS and KG conducted experiments. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate
Use of mice in this study was performed in strict accordance with the recommendations by the National Research Council’s Guide for the Care and Use of Laboratory Animals (8th edition) and approved by the Washington State University Institutional Animal Care and Use Committee.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Snodgrass RE. The feeding apparatus of biting and sucking insects affecting man and animals. Washington: Smithsonian Miscellaneous Collections. 1944. p. 1–107.
2. Bacot AW, Martin CJLX. Observations on the mechanism of the transmission of plague by fleas. J Hyg. 1914;14(Suppl).423–39.
3. Hinnebusch BJ, Jarrett CO, Bland DM. “Fleaining” the plague: adaptations of Yersinia pestis to its insect vector that lead to transmission. Annu Rev Microbiol. 2017;71:215–32.
4. Bacot AW. Further notes on the mechanism of the transmission of plague by fleas. J Hyg. 1915;14(Suppl). 774–6.3.
5. Bobrov AG, Kirillina O, Forman S, Mack D, Perry RD. Insights into Yersinia pestis biofilm development: topology and co-interaction of Hms inner membrane proteins involved in exopolysaccharide production. Environ Microbiol. 2008;10:1419–32.
6. Bobrov AG, Kirillina O, Vadyvaloo V, Kostler BJ, Hinz AK, Mack D, et al. The Yersinia pestis HmsCDE regulatory system is essential for blockage of the oriental rat flea Xenopsylla cheopis, a classic plague vector. Environ Microbiol. 2015;17:947–59.
7. Sun YC, Koumouris A, Jarrett C, Lawrence K, Gherardini FC, Darby C, et al. Differential control of Yersinia pestis biofilm formation in vitro and in the flea vector by two ci-dGMP diguanylate cyclase. PLoS ONE. 2011;6:e19267.
8. Bobrov AG, Kirillina O, Ryjenkov DA, Waters CM, Price PA, Fetherston JD, et al. Systematic analysis of cyclic di-GMP signalling enzymes and their role in biofilm formation and virulence in Yersinia pestis. Mol Microbiol. 2011;79:533–51.
9. Zhou DS, Tong ZZ, Song YJ, Han YP, Pei DC, Peng X, et al. Genetics of metabolic variations between Yersinia pestis biovars and the proposal of a new biovar, microtus. J Bacteriol. 2004;186:5147–52.
10. Hinnebusch BJ, Chouikha I, Sun YC. Ecological opportunity, evolution, and the emergence of flea-borne plague. Infect Immun. 2016;84:1932–40.
11. Morelli G, Song Y, Mazzoni CJ, Eppinger M, Romagnac P, Wagner DM, et al. Yersinia pestis genome sequencing identifies patterns of global phylogenetic diversity. Nat Genet. 2010;42:1140–3.
12. Xu L, Sigle LC, Leirs H, Neerinckx S, Gage KL, Yang R, et al. Historical and genomic data reveal the influencing factors on global transmission velocity of plague during the third pandemic. Proc Natl Acad Sci USA. 2019;116:11833–8.
13. Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, Kusecek B, et al. Microevolution and history of the plague bacillus, Yersinia pestis. Proc Natl Acad Sci USA. 2004;101:17837–42.
14. Drancourt M, Roux V, Dang LV, Tran-Hung L, Castex D, Chenal-Francisque V, et al. Genotyping, Orientalis-like Yersinia pestis, and plague pandemics. Emerg Infect Dis. 2004;10:1585–92.
15. Drancourt M, Signoli M, Dang LV, Baozit R, Roux V, Tzortzis S, et al. Yersinia pestis Orientalis in remains of ancient plague patients. Emerg Infect Dis. 2007;13:332–3.
16. Hinnebusch BJ, Perry RD, Schwam TG. Role of the Yersinia pestis hms storage (hms) locus in the transmission of plague by fleas. Science. 1996;273:367–70.
17. Deng W, Burland V, Plunkett G 3rd, Boutin A, Mayhew GE, Liss P, et al. Genome sequence of Yersinia pestis KIM. J Bacteriol. 2002;184:4601–11.
18. Vetter SM, Eisen RJ, Schotthoefer AM, Montenieri JA, Holmes JL, Bobrov AG, et al. Biofilm formation is not required for early-phase transmission of Yersinia pestis. Microbiology. 2010;156:2216–25.
19. Harimalala M, Telfer S, Delatte H, Watts PC, Miarinjara A, Ramihangi-hajason TR, et al. Genetic structure and gene flow of the flea Xenopsylla cheopis in Madagascar and Mayotte. Parasite Vectors. 2017;10:347.
20. Eskey CR, Haas VH, Good NE. Plague in the western part of the United States. Washington: U.S. Government Printing Office; 1940.
21. Burroughs AL. Sylvatic plague studies: the vector efficiency of nine species of fleas compared with Xenopsylla cheopis. J Hyg. 1947;45:371–96.
22. Engelthaler DM, Hinnebusch BJ, Rittner CM, Gage KL. Quantitative competitive PCR as a technique for exploring flea-Yersinia pestis dynamics. Am J Trop Med Hyg. 2000;62:552–60.
23. Weger-Lucarelli J, Ruckert C, Chotiwan N, Nguyen C, Garcia Luna SM, Fauver JR, et al. Vector competence of American mosquitoes for three strains of Zika virus. PLoS Negl Trop Dis. 2016;10:e0005101.
24. Flannery J, Sanz-Bernardo B, Ashby M, Brown H, Carpenter S, Cooke L, et al. Evidence of reduced viremia, pathogenicity and vector competence in a re-emerging European strain of bluetongue virus serotype 8 in sheep. Transbound Emerg Dis. 2019;66:1177–85.
25. Estevés MB, Kleina HT, Sales TM, Oliveira TP, de Lara IAR, Almeida RPP, et al. Transmission efficiency of Xyelidae fastidioidae subsp. pauca sequence types by sharpshooter vectors after in vitro acquisition. Phytopathology. 2019;109:286–93.
26. Link VB. A history of plague in United States of America. Public Health Monogr. 1955;26:1–120.
27. Politzer R. Plague. Geneva: World Health Organization;1954. https://apps.who.int/iris/handle/10665/41628
28. Chain PS, Hu P, Mafatti SA, Radnedge L, Larimer F, Vergez LV, et al. Complete genome sequence of Yersinia pestis strains Antique and Nepal516: evidence of gene reduction in an emerging pathogen. J Bacteriol. 2006;188:4453–63.
29. Doll JM, Zeitz PS, Ettedat P, Bucholtz AL, Davis T, Gage K. Cat-transmitted fatal pneumonic plague in a person who traveled from Colorado to Arizona. Am J Trop Med Hyg. 1994;51:109–14.
30. Sha J, Rosenzweig JA, Kirtley ML, van Lier CJ, Fitts EC, Kozlova EV, et al. An non-invasive in vivo imaging system to study dissemination of bioluminescent Yersinia pestis CO92 in a mouse model of pneumonic plague. Microb Pathog. 2013;55:39–50.
31. Latham WW, Crosby SD, Miller VL, Goldman WE. Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity. Proc Natl Acad Sci USA. 2005;102:17786–91.
32. Williams SP, Chauhan S, Motin VL. Functional characterization of Yersinia pestis aerobic glycolytic metabolism. Microb Pathog. 2014;76:33–43.
33. Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, et al. A Tn7-based broad-range bacterial cloning and expression system. Nature Methods. 2005;2:443–8.
34. Knodler LA, Crowley SM, Sham HP, Yang H, Wrande M, Ma C, et al. Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. Cell Host Microbe. 2014;16:249–56.

35. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA. 2000;97:6640–5.

36. Derbise A, Lesic B, Dacheux D, Ghigo JM, Carniel E. A rapid and simple method for inactivating chromosomal genes in *Yersinia*. FEMS Immunol Med Microbiol. 2003;38:113–6.

37. Vadyvaloo V, Hinz AK. A LysR-Type Transcriptional regulator, RovM, senses nutritional cues suggesting that it is involved in metabolic adaptation of *Yersinia pestis* to the flea gut. PLoS ONE. 2015;10:e0137508.

38. Lorange EA, Race BL, Sebbane F, Hinnebusch BJ. Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis* J Infect Dis. 2005;191:1907–12.

39. Lemon A, Silva-Rohwer A, Sagawa J, Vadyvaloo V. Co-infection assay to determine *Yersinia pestis* competitive fitness in fleas. Methods Mol Biol. 2019;2010:153–66.

40. Jarrett CO, Deak E, Isherwood KE, Oyston PC, Fischer ER, Whitney AR, et al. Transmission of *Yersinia pestis* from an infectious biofilm in the flea vector. J Infect Dis. 2004;190:783–92.

41. Lorange EA, Race BL, Sebbane F, Hinnebusch BJ. Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis*. J Infect Dis. 2005;191:1907–12.

42. Petrova OE, Sauer K. Sticky situations: key components that control bacterial surface attachment. J Bacteriol. 2012;194:2413–25.

43. Rebeil R, Jarrett CO, Driver JD, Ernst RK, Oyston PC, Hinnebusch BJ. Induction of the *Yersinia pestis* PhoP-PhoQ regulatory system in the flea and its role in producing a transmissible infection. J Bacteriol. 2013;195:1920–30.

44. Carniel E, Hinnebusch BJ. *Yersinia* systems biology and control. Norfolk: Caister Academic Press; 2012.

45. Jarrett CO, Deak E, Isherwood KE, Oyston PC, Fischer ER, Whitney AR, et al. Transmission of *Yersinia pestis* from an infectious biofilm in the flea vector. J Infect Dis. 2004;190:783–92.

46. Bobrov AG, Krillina O, Perry RD. The phosphodiesterase activity of the HmsP EAL domain is required for negative regulation of biofilm formation in *Yersinia pestis*. FEMS Microbiol Lett. 2005;247:123–30.

47. Bland DM, Hinnebusch BJ. Feeding behavior modulates biofilm-mediated transmission of *Yersinia pestis* by the cat flea, Ctenocephalides felis. PLoS Negl Trop Dis. 2016;10:e0004413.

48. Bland DM, Jarrett CO, Bosio CF, Hinnebusch BJ. Infectious blood source alters early foregut infection and regurgitative transmission of *Yersinia pestis* by rodent fleas. PLoS Pathog. 2018;14:e1006859.

49. Bland DM, Jarrett CO, Bosio CF, Hinnebusch BJ. Infectious blood source alters early foregut infection and regurgitative transmission of *Yersinia pestis* by rodent fleas. PLoS Pathog. 2018;14:e1006859.

50. Anisimov AP, Linder LE, Per GB. Intraspecific diversity of *Yersinia pestis*. Clin Microbiol Rev. 2004;17:434–64.

51. Bellsow LE, Koestler BJ, Karaba SM, Waters CM, Latham WW. Hfq-dependent, co-ordinate control of cyclic diguanylate synthesis and catabolism in the plague pathogen *Yersinia pestis*. Mol Microbiol. 2012;86:661–74.

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