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The Role of Early-Phase Transmission in the Spread of *Yersinia pestis*

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Abstract

Early-phase transmission (EPT) of *Yersinia pestis* by unblocked fleas is a well-documented, replicable phenomenon with poorly defined mechanisms. We review evidence demonstrating EPT and current knowledge on its biological and biomechanical processes. We discuss the importance of EPT in the epizootic spread of *Y. pestis* and its role in the maintenance of plague bacteria in nature. We further address the role of EPT in the epidemiology of plague.

Keywords

early-phase transmission; *Yersinia pestis*; flea; course of infection; plague

Plague is a flea-borne zoonosis. The causative agent, *Yersinia pestis*, is extremely virulent in humans, eliciting a broad range of severe illness (Dennis and Gage 2003). Untreated, mortality for bubonic plague is usually cited as 40–60 percent and approaches 100 percent in septicemic and pneumonic plague, with death often occurring within 3–5 d of illness onset. In addition to its virulence, plague is known for its ability to spread explosively under certain conditions, including those seen during the three historical pandemics, less well-known regional epidemics, and epizootics (Pollitzer 1954). Humans are not the only victims of plague, as the disease is frequently fatal to rodent species that are essential for maintenance of *Y. pestis* in nature, rodent-consuming carnivores, and various other animals that are bitten by infective rodent fleas (Gage and Kosoy 2005). In some instances plague poses serious wildlife conservation issues, such as endangerment to prairie dog and black-footed ferret populations in North America (Antolin et al. 2002). Most cases of human and animal plague occur during sporadic epizootics when *Y. pestis* spreads rapidly between reservoir and incidental hosts through the bites of infectious fleas. Because of plague's threat to humans and wildlife, it is important to better understand factors contributing to its transmission.

The "blocked-flea" model of the transmission of plague bacteria (Bacot and Martin 1914) remained for decades the dominant paradigm for rat flea-borne transmission of *Y. pestis*. Under this scenario, rat fleas (*Xenopsylla cheopis* Rothschild) ingest a bloodmeal from a

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highly bacteremic host, allowing *Y. pestis* to colonize the flea's gut. Then, following an extrinsic incubation period typically of 7–31 d, but occasionally as short as 5 d postinfection (dpi), a blockage of blood and multiplying *Y. pestis* is formed in the flea's proventriculus (Burroughs 1947, Kartman and Prince 1956, Kartman et al. 1956, Engelthaler et al. 2000, Lorange et al. 2005, Eisen et al. 2009). This obstruction prevents subsequent bloodmeals from reaching the midgut, and as the flea begins to starve it increases its feeding attempts; this voracious feeding, coupled with regurgitation into a bite wound of fresh blood and pieces of infective blockage material, results in high vectorial capacity. Despite recognition of proventricular blockage and its importance a century ago, pathogen–host interactions underlying this phenomenon remained unknown until Hinnebusch and others demonstrated that it is induced by a *Y. pestis*-produced biofilm (Hinnebusch et al. 1996, Jarrett et al. 2004).

Although the blocked flea model is the classical mode of transmission of plague bacteria by *X. cheopis*, the occurrence of blockage in only a proportion of infected fleas of this species and rarely, if at all, in many other flea species that appear to be significant vectors based on ecological or epidemiological evidence (see review by Eisen et al. 2009), suggests that one or more alternative transmission mechanisms are important. Based on laboratory and field observations, others proposed that blocked, or partially blocked, fleas are principally responsible for interepizootic transmission, but that mechanical transmission is important during rapidly spreading epizootics (Burroughs 1947; Quan et al. 1953; Kartman et al. 1958a,b). More recently, mathematical modeling of sylvatic plague cycles provided support to the thesis that in the absence of extraordinarily high flea loads, the blocked flea model by itself is insufficient to explain the rates of spread typically observed during plague epizootics (Lorange et al. 2005, Eisen et al. 2006, Webb et al. 2006). This insufficiency is largely attributable to the long extrinsic incubation period required for blockage to occur, compounded by a short infective window that is often followed a few days later by death of the flea by starvation.

Recently, Eisen et al. (2006) revisited and highlighted an alternative form of transmission, long neglected, which they referred to as early-phase transmission (EPT), a term that emphasizes the phenomenon of a short time that elapses from when the flea becomes infected until it is infectious (extrinsic incubation period) in rapid flea-borne spread of plague. For the purposes of this paper, and based on experimental results obtained with a variety of flea species described in detail below, EPT can be defined as transmission of *Y. pestis* by unblocked fleas (biofilm-independent transmission; Hinnebusch 2012) following a short extrinsic incubation period (≤4 d). Because EPT can occur as soon as the flea vector takes its next bloodmeal after feeding on an infected host, an event that often occurs in a matter of only 1–2 d and sometimes within a few hours, the extrinsic incubation period is much shorter than for transmission that requires proventricular blockage in the flea. Here, the term extrinsic incubation period is neutral in relation to the mechanisms of transmission (e.g., biological vs. biomechanical); a discussion of evidence for the mechanism of EPT is presented later in this article. Because of this extremely short extrinsic incubation period and the high likelihood that fleas will survive long enough to feed again while still infectious,

EPT provides a contributory explanation for the rapid spread of plague observed during many epizootics and epidemics.

To date, EPT has been observed as early as 3 h postinfection and as late as 4 d postinfection (Eisen et al. 2006). EPT efficiency appears to be highest at the time of the first bloodmeal postinfection; despite fleas remaining infected, transmission efficiency wanes with subsequent bloodmeals (Eisen et al. 2007a; Wilder et al. 2008a,b). Biofilm-independent transmission may occur later than the 4 dpi that have been evaluated in recent studies (reviewed below) if the first bloodmeal following infection is delayed. However, to date, EPT studies spanning longer than 4 d have not been conducted, nor has a time course study been performed to determine if, and at what efficiency, transmission capability is regained subsequent to the second bloodmeal following infection.

When the early (biofilm-independent) phase of transmission ends and the later (biofilm-dependent) phase begins remains poorly defined. Here, conservatively, we define the early phase as up through 4 dpi. This time frame, which has been evaluated in recent EPT studies, is sooner than the earliest reported observation of blockage (5 dpi; Burroughs 1947), and earlier than when wild-type strains of *Y. pestis* were observed to colonize the proventriculus (Hinnebusch et al. 1996). After 4–7 dpi, it is reasonable to assume that bio-film-mediated partial or full blockage becomes important in the transmission of plague bacteria, a process referred to by Hinnebusch (2012) as biofilm-dependent regurgitative transmission in recognition of the role played by biofilm in proventricular blockage and increased transmission efficiency. By comparison, the mechanisms by which transmission occurs during the early phase period has been relatively unexplored and remains poorly defined, largely because the potential importance of EPT in epizootic and perhaps epidemic transmission has been seriously revisited and reported upon only recently (Eisen et al. 2006). In this forum article we contrast EPT, a phenomenon characterized by a short extrinsic incubation period, with biofilm-dependent regurgitative transmission (also referred to as blocked flea transmission) that occurs after a longer extrinsic incubation period. We also will discuss recent efforts to identify the mechanisms underlying EPT, expand on its role in the epizootic spread of plague, and explore its potential importance in the transmission of plague bacteria to and between humans.

Experimental Evidence Demonstrating Early-Phase Transmission

Following the initial description (Bacot and Martin 1914) of the formation of blockages in the proventriculus of *Y. pestis*-infected fleas and how these blockages increased the efficiency of *Y. pestis* transmission, experimental studies of flea-borne infection increasingly focused on this phenomenon. The belief that blocked fleas are the principal driving force behind epizootics and epidemics of flea-borne plague became so engrained that transmission occurring in the first few dpi was largely ignored or discounted. This situation persisted for many decades despite numerous experiments showing transmission by unblocked fleas prior to 5 dpi using rat fleas (Ledingham 1907, Verjbitski 1908, Quan et al. 1953) and diverse other flea species, including *Malaraeus telchinus* Rothschild (Burroughs 1947), *Oropsylla montana* Baker (McCoy 1910, Douglas and Wheeler 1943, Wheeler and Douglas 1945, Burroughs 1947, Engelthaler et al. 2000), *Polygenis gwyni* C. Fox (Holdenried 1952), and

Ctenocephalides felis Bouché, *C. canis* Curtis, and *Leptopsylla segnis* Schönherr (Verjbitski 1908). Although most of these studies used fleas that feed primarily on mammals other than humans, it is notable for our later discussions of interhuman transmission of plague bacteria that EPT of *Y. pestis* by *Pulex irritans*, the so-called human flea, has also been observed experimentally between animals (Ledingham 1907, Verjbitski 1908) and even between humans and animals (Blanc and Baltazard 1941).

Flea-borne infection of animals during the early phase in some older experiments was attributed to mass action in recognition of the large numbers of fleas (typically ≥ 50 fleas per host) placed on individual susceptible animals during transmission trials. Clearly, mass action (i.e., the exposure of animals to large numbers of infective fleas) is not an absolute precondition for EPT, as relatively high rates of transmission have been observed when hosts were exposed to the bites of individual *O. montana* infected less than 5 dpi (Wheeler and Douglas 1945).

EPT was demonstrated using a diversity of *Y. pestis* strains and flea species in a number of studies conducted 50 or more years ago (Verjbitski 1908, McCoy 1910, Douglas and Wheeler 1943, Wheeler and Douglas 1945, Burroughs 1947, Holdenried and Morlan 1955, Blanc 1956). This diversity speaks to the generality of EPT, at least under the conditions of the experiments. Fundamentals of early phase vector competence were also shown: 1) fleas were capable of acquiring *Y. pestis* while feeding on bacteremic hosts, 2) they maintained the infection until a subsequent bloodmeal was taken within 4 dpi, and 3) they transmitted plague bacteria within this 4-d period to a naïve host from which *Y. pestis* was later recovered. However, vector efficiency (i.e., the proportion of infected fleas that transmit the bacteria to susceptible hosts) is difficult to assess from these studies, either as a result of small sample sizes, or insufficient data reported. Comparing vector efficiency among the flea species tested across these historical transmission studies was confounded by unstandardized experimental designs (Eisen et al. 2009).

EPT and its potential ecological and epidemiological significance, neglected for decades, has recently been revisited in a series of experiments using standardized research designs and methods beginning with the description of this mode of transmission as EPT by Eisen et al. (2006). Several subsequent experiments conducted under controlled and comparable conditions consistently demonstrated EPT in flea species associated with rats (*Xenopsylla cheopis* (Eisen et al. 2007b, Vetter et al. 2010, Schotthoefer et al. 2011, Williams et al. 2013, Johnson et al. 2014)), ground squirrels (*Oropsylla montana* (Eisen et al. 2006, 2007a, 2008a,b; Johnson et al. 2014)), prairie dogs (*Oropsylla hirsuta* (Baker) (Wilder et al. 2008a,b) and *Oropsylla tuberculata* Baker (Wilder et al. 2008b)), mice (*Aetheca wagneri* Baker (Eisen et al. 2008a)), and cats and many other medium-sized mammals (*Ctenocephalides felis* (Eisen et al. 2008b)). EPT by *O. montana* also was observed at temperatures ranging from 6 to 23°C (Williams et al. 2013). In short, EPT was observed in all flea species evaluated and at varying temperatures. Transmission occurred occasionally as early as 3 h postinfection but usually was observed over 1–4 dpi. Although all flea species tested were capable of EPT, efficiency in these studies varied among species, suggesting that some fleas are likely to be more important than others in the rapid spread of plague in

nature, especially those that are both efficient transmitters and abundant on susceptible hosts.

Y. pestis–Vector Interactions Resulting in EPT

Despite the evidence that EPT occurs in multiple flea species and with different strains of *Y. pestis* under variable temperature conditions, the biological or biomechanical mechanisms behind this phenomenon have yet to be elucidated. In the following paragraphs we summarize what has been learned about mechanisms of EPT of *Y. pestis* and discuss several pathogen–vector interactions and biomechanical factors that might enable EPT.

In his discussion of the evolution of flea-borne transmission in *Y. pestis*, Hinnebusch (2005) emphasized the importance of two *Y. pestis* transmission factors, a biofilm that is produced by genes in the *hms* (hemin storage) locus and *Yersinia* murine toxin (Ymt), a phospholipase D encoded by the *ymt* gene. As noted previously, biofilm production by *Y. pestis* is responsible for blockage of the proventriculus and, therefore, essential for the biofilm-dependent regurgitative model of transmission (Hinnebusch et al. 1996, Jarrett et al. 2004). Ymt is required for survival of *Y. pestis* in the flea's midgut and strains of plague bacteria deficient in this transmission factor cannot survive there, although they can colonize its proventriculus (Hinnebusch et al. 2002). Because of the known importance of these two transmission factors for block formation in fleas or enabling survival of the plague bacterium in the flea's midgut, respectively, it is of interest to investigate their potential role in early-phase transmission.

Regurgitation or reflux of bacteria from a blocked or partially blocked proventriculus is fundamental to the biofilm-dependent regurgitative transmission model. Regurgitation of bacteria from the midgut or from sites anterior to the proventriculus-midgut junction also could explain EPT (Eisen et al. 2006, 2009; Eisen and Gage 2009; Hinnebusch 2012; Johnson et al. 2014). In support of a role for regurgitation in EPT, it was reported that when infected but unblocked fleas were fed either a subsequent infected or uninfected bloodmeal 5 dpi and then allowed to feed again on susceptible hosts on days 6–9 postinfection, despite similar bacterial loads in fleas from the two groups, transmission rates decreased significantly among those fleas exposed to uninfected blood. Presumably, the uninfected secondary bloodmeal forced plague bacteria further posterior in the flea's digestive tract, making them less readily transmissible (Eisen et al. 2007a). Normally, the proventriculus acts to impede retrograde flow of blood and reduces the likelihood of *Y. pestis* transmission from the midgut, but it has been observed that an "uncoordinated rhythm of contractions of the proventriculus and stomach" can lead to regurgitation of midgut contents to the esophagus in unblocked fleas (Bibikova 1965). Others have reported that fleas will regurgitate when disturbed (Cox et al. 1999). The frequency at which fleas regurgitate while feeding normally remains unknown but clearly could be a critical factor for EPT to reliably occur and should be further investigated.

Considering the essential role played by regurgitation of *Y. pestis*-laden biofilm in the biofilm-dependent regurgitative transmission model during late phase transmission, it would be reasonable to suspect that regurgitation by unblocked fleas of biofilm containing plague

bacteria from sites anterior to the proventriculus, the proventriculus itself, or the midgut might also play a role in EPT. If biofilm is required for EPT, it could be hypothesized that the mechanism behind transmission during early phase is not completely distinct from late phase biofilm-dependent regurgitative transmission model but rather represents the regurgitation of small pieces of *Y. pestis*-bearing biofilm that begin to accumulate in the flea's gut during the initial stages of *Y. pestis* colonization and continue to accumulate as growth of plague bacteria progresses and block formation begins.

Vetter et al. (2010) addressed whether biofilm is essential for EPT by comparing transmission rates for fleas infected 1–4 d earlier with *Y. pestis* wild-type strains that exhibited normal biofilm production with rates observed for fleas similarly infected with *Y. pestis* strains that completely lacked biofilm production, exhibited much reduced biofilm production, or over-produced biofilm. Results indicated that the two biofilm-deficient strains transmitted as efficiently as the wild-type parent strain. By contrast, only one transmission event was noted within 4 dpi among the fleas infected with the biofilm overproducing strain, resulting in an estimated early-phase transmission efficiency 7 to 23 times lower than for the biofilm-deficient or parent strains. These results indicate that biofilm is not required for EPT and, unexpectedly, overproduction of biofilm by *Y. pestis* is associated with greatly reduced EPT.

Although biofilm does not appear to be required for early-phase transmission, the work of Vetter et al. (2010) did not address regurgitation from the midgut. If regurgitation of midgut contents is essential for EPT to occur, strains deficient in *Yersinia* murine toxin (Ymt) should be incapable of supporting EPT because of the previously noted inability of such strains to survive in the flea's midgut. Recently, the role of this transmission factor in EPT was investigated by Johnson et al. (2014) who compared transmission of a *Y. pestis* mutant containing a nonfunctional *ymt* (Rudolph et al. 1999, Hinnebusch et al. 2002) during early-phase transmission by *X. cheopis* and *O. montana* with transmission of two wild-type *Y. pestis* strains by the same fleas over the same time period. Their results demonstrated that the Ymt-deficient strain of *Y. pestis* was transmitted during the early phase at rates similar to those observed for wild-type strains, indicating that Ymt and regurgitation of viable bacteria from a flea's midgut is not required for EPT. Notably, these experiments with Ymt-deficient *Y. pestis* do not eliminate the possibility that EPT occurs as the result of regurgitation of plague bacteria from sites in the foregut rather than the midgut.

The observations that the most commonly recognized *Y. pestis* transmission factors are not essential for EPT and that the extrinsic incubation period is very short could suggest that multiplication of *Y. pestis* within the vector is not essential and the plague bacterium plays a basically passive role in EPT because the underlying mechanism is biomechanical rather than biological. Host infections occurring in the mass action experiments described earlier were attributed later to mechanical transmission. This means of transmission, which has been compared to infection spread through inoculation by a pathogen-contaminated "dirty needle," requires no modification or multiplication of the pathogen in the vector for transmission to occur. In general, transmission by mechanical means is considered to be relatively inefficient and one assumption underlying the mass action experimental design is that the fleas being tested are inefficient vectors and the likelihood that any individual flea

will transmit *Y. pestis* is extremely low, hence the need to use large numbers of fleas to demonstrate transmission. If EPT could be explained by mechanical transmission, one might assume that transmission efficiency would be similar across flea species. However, under comparable experimental conditions EPT efficiency is quite variable among flea species (Eisen et al. 2009). Another requirement for mechanical transmission from contaminated external mouthparts to explain EPT, is that plague bacteria would have to remain viable on contaminated mouthparts for at least 4 d. Unfortunately, very little research has been done to investigate *Y. pestis* survivability on flea mouthparts. In one study plague bacteria reportedly remained viable for less than 3 h on flea mouthparts as determined by culturing material rinsed from the mouthparts of fleas previously fed on infected animals (Bibikova 1977), a period of time too short to account for the 4 dpi over which EPT has been observed. The survival time reported in Bibikova's study is similar to the reported survival times of *Y. pestis* that has been suspended in Butterfield buffer, a type of phosphate buffer, and then applied in small drops (20 μl) and allowed to dry on stainless steel, polyethylene or glass (Rose et al. 2003). However, *Y. pestis* suspended in this same buffer and applied to paper survived for more than 48 h, and when a BHI broth much richer in proteins and other potentially protective ingredients was substituted for the Butterfield buffer, plague bacteria survived for 4 d on stainless steel, polyethylene and glass, and for at least 5 d when the same BHI suspensions were applied to paper (Rose et al. 2003). The degree to which these last experiments inform our understanding of EPT and mechanical transmission is debatable, but it is clear that survival of *Y. pestis* on exposed surfaces depends on many factors and more research is needed to verify Bibikova's findings and determine to what extent mechanical transmission contributes to EPT compared to other possible mechanisms, including those discussed in the following paragraphs.

Blockage in fleas, associated with high infectivity by biofilm-dependent regurgitative transmission, is correlated with the numbers of plague bacteria present, requiring a threshold of at least 10^5 *Y. pestis* per flea (Hinnebusch et al. 1998). A similar relationship between infectivity during EPT and bacterial loads of fleas has not been observed. In several recent EPT studies, bacterial loads were quantified in *Y. pestis*-infected fleas that were frozen and stored at −80°C immediately after taking a bloodmeal on a naïve mouse (Eisen et al. 2006, 2007b, 2008b; Wilder et al. 2008a,b;Vetter et al. 2010; Schotthoefer et al. 2011; Williams et al. 2013). Unexpectedly, a positive correlation between bacterial loads in these fleas and EPT was not observed. Although a minimum threshold concentration of bacteria might be required for EPT, the lack of correlation between bacterial loads and transmission in these early phase experiments suggests that when fleas are infected initially by feeding on highly bacteremic blood the absolute number of bacilli present in a flea is a less important predictor of transmission outcome than their location within the flea. This finding is perhaps not so surprising when one considers the relatively low numbers of bacteria required to infect mice and other rodents (LD_{50} and ID_{50} <10 cfu; Perry and Fetherston 1997). It also could be consistent with a variant of mechanical transmission as an explanation for EPT because proliferation of *Y. pestis* in the flea vector would not be required.

A biomechanical pathway for EPT consistent with the above observations (Eisen et al. 2007a, Vetter et al. 2010, Johnson et al. 2014) was recently proposed by Hinnebusch (2012).

Under this scenario, as a flea feeds on *Y. pestis*-infected blood, plague bacteria lodge in and contaminate the grooved surfaces of the feeding and salivary canals (channels). These canals are in very close contact with each other and their hydrodynamic forces act in opposite directions (toward the mouthparts within the salivary canal and toward the midgut in the food canal). The individual bacteria most likely to be transmitted would be those that prior to feeding lie in the salivary grooves distal to where they join the salivary pump, or in other words in the direction of the maxilla through which the salivary canal passes as it carries saliva to the blood-feeding site. Regurgitation of blood containing *Y. pestis* from the esophagus, proventriculus, or midgut was considered less likely. Although it might seem that relatively few plague bacteria are likely to lodge in these salivary grooves and be transmitted in this manner, the high infectivity of the plague bacterium could allow infection of the vertebrate host to occur even when few bacteria are actually transmitted during flea feeding.

The Importance of EPT in Epizootic Spread

Vectorial capacity (a measure of the number of secondary infections that arise from a focal infection) is paramount to understanding epizootic spread of plague bacteria by fleas. Compared with vector competence or vector efficiency, vectorial capacity is more difficult to assess, as it is dependent not only on pathogen acquisition rates, vector efficiency, and the extrinsic incubation period, but also includes variables that are less easily quantified under field conditions. These variables include the daily biting rates of fleas, flea contact rates with susceptible and resistant hosts, daily survivorship of the vector, and the duration of an infectious bacteremia in the vertebrate host. Despite these difficulties, several recent studies have sought to determine how many fleas would be required per host for epizootic transmission employing a modified vectorial capacity model originally developed for malaria transmission (Macdonald 1961; Lorange et al. 2005; Eisen et al. 2006, 2007b, 2008b; Wilder et al. 2008a,b). Although these models greatly oversimplify natural transmission dynamics, they show that infestation rates commonly observed for some flea species would be sufficient for epizootic transmission to occur when vector efficiencies and extrinsic incubation periods associated with EPT are assumed in the models. More complex models that consider host population density, changing contact rates between fleas and hosts, and heterogeneity in host immunity also show that EPT is theoretically capable of driving plague epizootics (Buhnerkempe et al. 2011).

Experimental plague epizootics provide compelling evidence for the ability of EPT to enable natural epizootics. One study (Quan et al. 1953) used laboratory arenas to demonstrate epizootic transmission with rates of spread that were consistent with EPT. *Xenopsylla cheopis* that acquired *Y. pestis* by feeding on bacteremic rats were introduced into an arena containing naïve mouse populations that ranged in size from 200 to 400 mice. The study demonstrated a rapid spread of *Y. pestis* in the mouse populations. Over half of the mice died within 5 d of fleas being introduced and postmortem examination revealed over 95% of them had buboes or enlarged lymph nodes. *Y. pestis* was isolated from nearly all blood or spleen samples cultured. The reported observations are consistent with flea-borne rather than direct contact transmission and within a time course consistent with EPT. That is, transmission could not have occurred via the blockage mechanism given that block

formation is almost never observed prior to 5 d postinfection (Eskey and Haas 1940, Burroughs 1947, Kartman and Prince 1956, Kartman et al. 1956, Engelthaler et al. 2000), and at least a 2-d incubation period is expected in a mouse model (Douglas and Wheeler 1943). The study by Quan et al. (1953) demonstrated epizootic spread under optimal conditions that included high vector–host contact rates, a highly efficient vector (*X. cheopis*) for EPT, and highly susceptible hosts capable of developing high levels of bacteremia.

Determining epizootic potential of EPT under natural conditions is more challenging. One critical piece of information that is lacking for use in mathematical models is the duration that reservoir hosts are infective, a factor related to the level of bacteremia over the course of infection. The threshold bacteremia level required for fleas to become infected with and later transmit *Y. pestis* by EPT has yet to be precisely determined, although we believe it to be similar to that required to produce blocked fleas considering the following: 1) Flea bloodmeal volumes, primarily for *X. cheopis* or *O. montana*, were initially estimated to be as high as 0.5 μl (Ledingham 1907) or as low as 0.01–0.03 μl (Douglas and Wheeler 1943), and are now commonly estimated to be in the range of 0.1–0.3 μl (Hinnebusch 2005, Oyston and Isherwood 2005); 2) accepting the latter estimates for bloodmeal volume, evidence from transmission experiments suggests that bacteremia densities of at least 10^6 cfu/ml are needed to reliably infect feeding fleas (Burroughs 1947, Engelthaler et al. 2000, Lorange et al. 2005, Oyston and Isherwood 2005); 3) in laboratory animals, terminal *Y. pestis* concentrations usually exceed 10^5 or 10^6 cfu/ml of blood (Douglas and Wheeler 1943, Burroughs 1947, Sebbane et al. 2005). Eskey and Haas (1940) found infection rates of 32% in wild rodent fleas fed on guinea pigs with overwhelming bacteremia of late-stage illness (10 or more *Y. pestis* per microscopic field of peripheral blood), 25% in wild rodent fleas fed on animals with 1 or <1 *Y. pestis* organisms per field, and 10% in fleas fed on animals shown to be smear negative but weakly culture positive.

Studies by Verbitski (1908) showed that *X. cheopis* became infected only when fed on experimentally infected rats within 12–26 h of their death, when bacterial concentrations in the blood were at their highest. Sebbane et al. (2005) found that mean bacterial concentrations in the blood of *Rattus norvegicus* (BN strain) were roughly 10^5 cfu/ml, when the rats were euthanized at first signs of clinical illness; it is expected that bacterial concentrations would have been higher immediately preceding the natural death of the host. Although mice typically produce a higher level of bacteremia than *R. norvegicus*, the progression of growth in the blood is similar with greatest bacterial density just before death. Based on Douglas and Wheeler (1943), terminal bacterial densities in mice increase rapidly during the terminal four hours of infection, reaching as high as 10^7 bacilli per cmm of blood (10^{10} bacilli/ml). Indeed, Burroughs (1947) noted “it was found that a flea could not feed on mice with a terminal bacteremia without ingesting organisms.” Numerous other studies also noted that laboratory animals were highly infectious for fleas only in the terminal stage of the animal’s illness (Eskey and Haas 1940, Wheeler and Douglas 1945, Burroughs 1947, Engelthaler et al. 2000), at which times the numbers of plague bacteria in the host’s blood can reach or exceed 10^8 bacteria/ml or higher (Douglas and Wheeler 1943, Engelthaler et al. 2000). Natural selection favors *Y. pestis* strains capable of producing overwhelming bacteremia and death of reservoir hosts by increasing the likelihood that fleas feeding on

hosts infected with such strains will become infected and forced to seek new hosts, thus facilitating continued transmission and bacterial replication (Gage and Kosoy 2005, Hinnebusch 2005, Eisen and Gage 2009).

Potential Infectivity of Human Plague Cases to Fleas

A determining factor in whether flea-borne transmission, by EPT or other means, could be important in interhuman transmission of *Y. pestis* is the infectivity of humans to fleas. Flea infestation rates and the duration of time that persons remain bacteremic above a transmissible threshold are required to estimate vectorial capacity. Improved sanitation including reduced contacts between humans and fleas and availability of antibiotics which reduce the proportion of infected persons who become infectious to fleas have been associated with a marked reduction in plague incidence and epidemicity in modern compared with historical times (Tikhomirov 1999).

Data on the proportion of persons who develop bacteremia likely to be infectious to fleas are very limited. A majority of persons with untreated bubonic plague progress to sepsis, and septicemic and pneumonic cases typically die from overwhelming gram-negative bacteremia with its endotoxemic consequences (Butler 1972, 1983;Butler et al. 1976; Dennis and Meier 1997). In severe, untreated illness, *Y. pestis* organisms can typically be seen on stained peripheral blood films (Cantey 1974, Butler et al. 1976, Mann et al. 1984), which is an ominous prognostic sign (Cantey 1974, Butler et al. 1976, Dennis and Mead 2009). Although comparisons may be unreliable, the finding of a positive smear is generally considered equal to a bacteremia of 10^6 cfu/ml or greater (Douglas and Wheeler 1943, Torres and Bisno 1973).

The proportion of plague patients that progress to the stages of infection in which bacteremia is sufficient to infect feeding fleas was undoubtedly much higher in the pre-antibiotic era than subsequently. In early studies summarized by Pollitzer (1954) and others (Choksy 1903, Greig 1906, Wu Lien-Teh et al. 1936), plague bacilli were isolated from between 45% and nearly 100% of plague patients. Success in isolating bacteria from blood depends on timing and techniques; under current best practices, 2–4cc of blood from febrile patients is cultured in several types of media and with up to three samples collected from different venous sites, procedures much advanced beyond those applied in early studies. Early attempts at quantitating degrees of bacteremia in fatal cases gave varying results with concentrations ranging from < 1 bacillus to > 10^6 bacilli per ml of blood (Indian Plague Research Commission [IPRC] 1906). During the 1960s and 1970s in wartime Vietnam, determinations were made of prevalence and density of bacteremia in several case series composed mostly of Vietnamese civilians with bubonic illness (Marshall et al. 1967; Legters et al. 1970; Butler 1972; Butler et al. 1974, 1976; Cantey 1974). In the series reported by Cantey et al. (1974), *Y. pestis* was isolated from 5 of 13 (38.5%) bubonic cases; of these, plague bacilli were microscopically observed on stained peripheral blood smears in 1 of 11 cases. All 10 septicemic or pneumonic cases were culture positive and each had positive peripheral blood smears containing 3 to 15 or more bacilli per single oil immersion field. Combining results of the three Butler et al. series, *Y. pestis* was isolated from the blood in 66.7% of 104 mostly bubonic cases. In their largest single study, *Y. pestis* was isolated from

venous blood in 17 of 42 (40.5%) patients (Butler et al. 1976) with densities ranging from < 10 to 4×10^7 cfu/ml; four patients with $>10^6$ cfu/ml each had positive peripheral blood smears. These and other studies consistently support a positive correlation between density of bacteremia, severity of illness, and poor prognosis. Nearly all patients in the Vietnamese studies presented with bubonic plague and received treatment with appropriate antimicrobials without delay after the diagnosis was made; clearly, a much higher proportion of patients would have been culture and smear positive later in the course of untreated illness.

A series of 490 sporadic, sylvatic plague-associated human cases was recorded by the US Public Health Service during the antibiotic period 1956–2013, and 71 (14.5%) of these cases were fatal (CDC, unpublished). The presenting clinical diagnoses in 68 fatal patients with available information were: bubonic 40 (58.8%); septicemic 21 (38.9%); pulmonic 5 (13.6%); pharyngeal 2 (2.9%). The total number of septicemic cases (either primary or secondary) accounted for 59 (86.8%) of the 68 fatal cases for which information was available. Among the 57 fatal cases who had both blood smears and blood cultures taken with known result, 55 (96.5%) were culture positive and 25 (43.9%) were smear positive. Although lacking quantitative information on levels of bacteremia, these data indicate that, even in the era of advanced hospital care, rapid diagnosis, and early treatment with appropriate antibiotics, a substantial percentage of US plague cases experience the most severe forms of illness, associated with a high prevalence and intensity of bacteremia.

Epidemiologic Implications of EPT in Sylvatic Foci

A recently published evaluation of primary diagnoses in all 468 US human plague cases recorded in the period 1965–2012 identified bubonic cases as 80% of the total, septicemic 17%, pneumonic 2%, other 2% (Kugeler et al. 2015), which is similar to the profile reported in rat-borne plague epidemics at the turn of the 20th century. Assigned exposures in these nearly 500 recent US cases were: flea bite (22%), direct animal contact (7%), and unknown (51%), the latter presumably almost entirely due to flea bite because of the relative difficulty in making that assignment relative to direct animal exposures and to the high proportion of cases classified as bubonic. Infective exposures in the United States after 1925, other than direct animal contact, can be attributed almost entirely to bites by wild rodent fleas, such as *Oropsylla*, *Thrassis* and perhaps *Aetheca* species, that bite humans only occasionally, in small numbers, and that, based on a limited number of studies, appear to block rarely if at all (Gage and Kosoy 2005, Eisen et al. 2009, Eisen and Gage 2009). These epidemiological findings suggest that the majority of US human plague cases arise from bites by one or a few infective fleas. Similar conclusions are plausible in other important sylvatic plague foci around the world involving wild rodent reservoirs and flea species that don't typically block, as with suslik (*Citellus* sp.) associated plague in the steppes of southeastern Russia and Central Asia, and gerbil (*Meriones* sp.) associated plague on the Kurdistan plateau and in the south African grassland ecotypes (Pollitzer 1954, Gage and Kosoy 2005).

Implications of EPT for a Role in Human Plague Outbreaks

Evidence provided supports an important role of EPT in driving sylvatic epizootics and a potential role in infective human exposures in those circumstances. More difficult to assess is the role of EPT in epidemic plague. Unquestionably, *X. cheopis* and closely related species that readily block were the principal transmitters of plague bacteria to humans during the third pandemic (Pollitzer 1954) and in subsequent residual plague outbreaks in domestic rat-infested urban and rural settings. As shown in recent controlled laboratory experiments, and by earlier investigations, *X. cheopis* is capable of transmitting *Y. pestis* to mammalian hosts in the early, unblocked, phase of infectivity (Ledingham 1907, Verjbitski 1908, Burroughs 1947, Eisen et al. 2009). The epidemiologic evidence for rat fleas as the principal route of infection in the third pandemic was based on time and space observations of epizootic rat deaths, or “rat falls,” a variable period to allow for decimation of the rat population, and then a period of delay averaging 3 d for potentially infectious fleas leaving dead rats to seek human blood and transmit infection, and a 3- to 4-d incubation period in humans prior to onset of symptoms (Wu Lien-Teh et al. 1936, Pollitzer 1954). This time course implies that either fleas seeking human bloodmeals are already blocked, that EPT is occurring, or both; otherwise, the expected period from rat deaths to human cases might be extended as much as 8–16 d or more to account for the extrinsic period required for *Y. pestis* colonization, multiplication, and blocking. If principal transmission to humans was by already blocked fleas leaving their rat hosts, the period between rat deaths and human cases could be reduced by several days as blocked fleas are starved, dehydrated, and seek immediate and repeated meals. Although the classic time-line neither supports nor weakens an auxiliary role for EPT, there is reason to consider this possibility, especially in circumstances of higher than expected rates of spread of disease.

More controversial is the question of flea-borne interhuman transmission of *Y. pestis*, discussed in older reviews (Wu Lien-Teh et al. 1936, Pollitzer 1954, Blanc 1956, Baltazard et al. 1960, Hopla 1980), and more recently by Drancourt et al. (2006). The flea most commonly mentioned in this regard is the human flea, *Pulex irritans*. *Y. pestis*-infected *P. irritans* have been collected on a number of instances in association with modern plague outbreaks (Laudisoit et al. 2007, Ratovonjato et al. 2014), and it has been suggested that the human flea was an important adjunct in the spread of plague during the medieval pandemic, helping to explain its occurrence in portions of Europe where black rats and *X. cheopis* were supposedly absent or very rare (Drancourt et al. 2006). Although clinical and epidemiologic descriptions of acutely ill persons coughing up blood and exhibiting other signs of fulminant respiratory disease leave little doubt that pneumonic plague occurred during the Black Death and was responsible for some direct spread of plague, including local outbreaks, narratives from this pandemic far more frequently describe illness with the swollen lymph glands characteristic of flea-borne bubonic plague and frequently associate its introduction and spread with the presence of rats and rat deaths (Pollitzer 1954). However plausible, interhuman transmission by *P. irritans* under natural conditions has not been proven. Evidence in its favor is based on epidemiological observations, with two exceptions. Blanc and Baltazard (1941) produced fatal plague in guinea pigs by inoculating them with *P. irritans* fed in separate experiments on two patients in their terminal phase of illness,

providing the best available evidence that bacteremia in humans can be sufficient to infect fleas with *Y. pestis* that then maintain virulence. These same authors also demonstrated that *P. irritans* that acquired plague bacteria through feeding on moribund human plague patients were capable of transmitting *Y. pestis* to a guinea pig via flea bite.

Epidemiological support in favor of interhuman flea-borne transmission comes from records of limited bubonic plague outbreaks in isolated rural communities under exceptional circumstances of heavy human flea infestations, high familial attack rates, and a lack of evidence for concurrent rat-flea borne plague. These include early studies of nomadic populations in North Africa (Blanc 1956) and the Middle East (Blanc and Balthazar 1942), and of remote communities in the Andean region (Ramos Diaz 1938). In a more recent investigation of a limited outbreak of bubonic plague in an isolated community in the Bolivian Andes, a joint epidemiologic and entomologic study by Pan American Health Organization (PAHO) and the US Centers for Disease Control (CDC) was made of one community of 245 persons in which 95 (39%) cases occurred over a 5–6-week period (Beasley 1965). Based on an epidemiologic pattern of person to person spread, especially the high attack rates among contacts of the sick, an absence of domestic rats, and an unusual abundance of *P. irritans* infesting villagers and their homes, investigators concluded that the outbreak resulted from infective bites by *P. irritans*. No mention was made of the presence or absence of guinea pigs, which are a common domestic food source in Andean villages and a favorable host of both *P. irritans* and *Y. pestis*. Recent reports from Tanzania (Laudisoit et al. 2007) and Madagascar (Ratovonjato et al. 2014) circumstantially implicate *P. irritans* as a possible vector in human plague at heavily infested rural sites in these countries.

In conclusion, EPT is a well-documented phenomenon that appears to be important in plague epizootics and possibly in transmitting plague bacteria to humans. Recent studies have provided insights into the pathogen–vector interactions involved in flea borne transmission of *Y. pestis*, but further research is needed, especially to define the molecular-genetic, anatomical, and biomechanical determinants of EPT. To better understand the ecological and epidemiological importance of EPT, we need to know the level of *Y. pestis* bacteremia required for fleas to reliably acquire infection and transmit it within 4 dpi. Determination of fluctuations in the levels of bacteremia in natural hosts over time would complement these studies and provide better parameters in models of epizootic spread of plague bacteria. Finally, studies aimed at determining EPT efficiency of *P. irritans*, a flea that has proven difficult to rear in the laboratory, would be of use in evaluating its role in modern and historical human plague outbreaks.

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