Natural History of *Yersinia pestis* Pneumonia in Aerosol-Challenged BALB/c Mice

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Abstract

After a relatively short untreated interval, pneumonic plague has a mortality approaching 100%. We employed a murine model of aerosol challenge with *Y. pestis* to investigate the early course of pneumonic plague in lung, blood and spleen. We fit a mathematical model to all data simultaneously. The model fit to the data was acceptable. The number of organisms in the lung at baseline was estimated at 135 (median) - 1184 (mean) CFU/g. Doubling time was estimated at 1.5-1.7 hr. Between hrs 1 and 12 post-exposure, counts declined, but then increased by hr 24, a finding hypothesized to be due to innate immunity. The model predicted that innate immunity declined with a half-time of 3-3.8 hr. The threshold for bacteremia was $6.4 \times 10^4$ to $1.52 \times 10^6$ CFU/g. By 42-48 hr, stationary phase was obtained. Lung bacterial burdens exceeded 10 Logs/g. Obviating early defenses allows rapid amplification of *Y. pestis* with bacteremia, making the rapid course with high mortality understandable.

Keywords: Plague pneumonia  Septicemia  Mathematical Modeling
Plague is one of the oldest bioterror weapons known to man. In 1346, the Mongols catapulted plague-infected corpses into the Crimean City of Caffa (14). Infection with \textit{Yersinia pestis}, the causative organism of plague, results in three major syndromes (15). The most familiar syndrome is bubonic plague. Here organisms arrive in lymph nodes and multiply there, generally after being introduced by the bite of infected fleas. In untreated cases of bubonic plague mortality is approximately 50-70%. With treatment, mortality decreases to 15% or less.

In some cases, bubonic plague progresses to septicemic plague which increases mortality and may result in pneumonic plague. In contrast to other agents of bioterror like \textit{Bacillus anthracis}, pneumonic plague can result in person-to-person transmission once infection is established. Untreated pneumonic plague has a mortality approaching 100%. Intentional release scenarios will cause great confusion in the therapeutic situation, as there will initially be no diagnosis due to lack of familiarity in the medical community and an unknown drug susceptibility profile. It will certainly take a considerable number of hours to days until the exposed populations become symptomatic and individuals entered into the health care system. Pneumonic plague, unlike many other pneumonias, has a very foreshortened course, so that after approximately 2-3 days without appropriate treatment, mortality becomes very high and response to therapy after this time is poor (15).

We employed a whole body murine aerosol challenge model to explore the rapid course of this disease and to better understand why pneumonic plague becomes poorly treatable after a relatively short interval following the onset of symptoms, with
consequent very high mortality. Because this murine infection model is used as a proof of principle for antibiotic therapies before advancement to the non-human primate models, a better understanding of the treatment trigger and justification of that timing has been needed. The treatment point of 42h post challenge was based on previous studies of effective survival by initiating therapy at different times between 24 and 60 hours post challenge (2). While the 42h treatment initiation time point has proven valid in other studies (8), the disease history and distribution of *Y. pestis* in the murine model before, around and after this time point has not been fully defined.

**Materials and Methods**

*Mice.* Female BALB/c mice, 7-9 weeks old, (20 g) were obtained from the National Cancer Institute/Charles River Laboratories and were used for all experiments. The mice had free access to food and water throughout the course of the study. For all experimental procedures described here, we adhered to the guidelines promulgated in the *Guide for the Care and Use of Laboratory Animals* (3). Research was conducted in the BSL3 laboratory of the Univ. of Florida in Albany, New York and were in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. The facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

*Preparation of the Y. pestis challenge strain for aerosolization.* *Y. pestis* CO92 (obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH) was originally isolated in 1992 from a person with a fatal case of pneumatic plague (5). The LD$_{50}$ in mice for this strain is $6.8 \times 10^4$ cfu inhaled when
administered as an aerosol (whole body) (8). The inoculum for aerosol challenge was prepared as described elsewhere (2), and the suspension of Y. pestis was diluted to the appropriate aerosol challenge dose. Colonies were counted after serial dilution and plating on sheep blood agar plates (SBAPs). These plates were incubated for 2 days at 28°C.

Aerosol infection. Inhaled doses of 20 LD$_{50}$ (LD$_{50}$ equals $6.8 \times 10^4$ cfu) of Y. pestis were administered to mice by whole-body aerosol. Aerosol was generated using a 3-jet Collison nebulizer (12). All aerosol procedures were controlled and monitored using the Automated Bioaerosol Exposure system (7) operating with a whole-body rodent-exposure chamber. Integrated air samples were obtained from the chamber during each exposure using an all-glass impinger. Aerosol bacterial concentrations were serially diluted and plated on SBAPs, as described above. The inhaled dose (in colony-forming units per mouse) of Y. pestis was estimated using Guyton’s formula (6).

Assessment of animals. Cohort size for statistical evaluation was 10-15 mice. At 1, 12 24, 30, 36, 42, 45, 48 and 51 hr post challenge a group of 15 (10 at 1, and 12 hrs) animals were anesthetized by injecting a mixture containing ketamine/acepromazine/xylazine (6/0.30/0.66 mg/ml), in a 0.1ml volume, intramuscularly in the caudal thigh. Whole blood was collected by cardiac puncture and serially diluted in sterile saline. After bleeds animals were sacrificed and lungs and spleens removed, weighed, homogenized in 1 ml sterile saline and serially diluted in sterile saline. Serial dilutions of blood, lung homogenates and spleen homogenates were plated within 30 minutes after harvest onto SBAPs and incubated at 28°C for 2 days to determine bacterial load.
Mathematical model of *Yersinia pestis* growth in the lung, blood and spleen of the mouse. A series of inhomogeneous differential equations described the growth of *Y. pestis* after inhalational challenge.

\[
dX(1)/dt = IC(1) + K_{\text{growth-L}} \cdot X(1) \cdot (1-(X(1)/\text{POPMAX})) \cdot K_{\text{BL-L}} \cdot X(1) - K_{\text{INNATE}} \cdot X(1) \cdot e^{(-\text{INNATE}_\text{DECL} \cdot t)} 
\]

\[dX(2)/dt = K_{\text{TR-BL}} \cdot X(1) - K_{\text{TR-BL}} \cdot SPLIT \cdot X(2) \]

\[\text{IF (}X(1) \leq \text{THRESHOLD}) \text{ THEN} \]
\[XP(2) = 0 \]
\[\text{ELSE} \]
\[XP(2) = K_{\text{TR-BL}} \cdot X(1) \]
\[\text{END IF} \]

\[dX(3)/dt = K_{\text{TR-BL}} \cdot SPLIT \cdot X(2) \]

In Equation 1, the growth of the organism in lung is described. IC(1) is the Initial Condition of the number of CFU/ml in the lung at time = 0 after challenge spray cessation. \(K_{\text{growth-L}}\) is the first order growth rate constant for organisms in the Lung. POPMAX is the maximal population density and the term \((1-(X(1)/\text{POPMAX}))\) is a logistic carrying function designed to prevent unconstrained bacterial growth and for the organism population to achieve stationary phase. \(K_{\text{BL-L}}\) is a first order rate constant for transfer from Lung to Blood. \(K_{\text{INNATE}}\) is a first order kill rate constant generated by Innate immunity. Since this was rapidly overwhelmed as evidenced by the raw data after hour 12, a term decrementing the size of the kill rate constant over time was introduced by multiplying \(K_{\text{INNATE}}\) by \(e^{(-\text{INNATE}_\text{DECL} \cdot t)}\). Equation 2 describes the time course of organisms in blood. Again, by examining the raw data it became clear that no organisms were
detected by blood culture at hours 1 and 12 and were first detected in low numbers at hour 24. Consequently, we coded an “If/Then/Else statement that allowed organisms from the lung to spill over into the blood only after a THRESHOLD value had been achieved. Equation 3 describes the time course of organisms in the Spleen. From the Blood, organisms were filtered by the Spleen and $K_{BL-SPL}$ is the first order rate constant for transfer of organisms from the Blood compartment to the Spleen.

All animal data from all three system outputs (Lung, Blood and Spleen) were simultaneously modeled with Big NPAG (NON-PARAMETRIC ADAPTIVE GRID) a fully mathematically consistent non-parametric population modeling program described by Leary, Jelliffe, VanGuilde and Schumitzky (10). As the model was a destructive one and we never had more than one datum point per animal for each output we did not use the “Adaptive $\gamma$” feature of BigNPAG. Weighting was as the inverse of the observation variance for each output for all the animals within a time cohort. Post-Bayesian estimates were obtained for each animal employing the “Population of One” utility within the program.

“Goodness of fit” was examined by pre- and post-Bayesian observed-predicted plots and their regressions for each output. The Mean Weighted Squared Error served as the measure of Bias and the Bias-Adjusted Mean Weighted Squared Error served as the measure of precision.

Results

The CFU time-curves for Lung (per g), Blood (per ml) and Spleen (per g) are presented in Figure 1. As can be seen, there is a decline in the number of CFUs/g in the lung between hour 1 and hour 12 ($p < 0.001$ by t-test). This decrease resulted in the
inclusion of an innate immunity term in our model. By hour 24 post-exposure, there is again a significant change (increase) in the number of CFU/g of lung tissue and at this point we first see organisms measured in the cultures of blood and spleen. Of note, there were more organisms measured in spleen relative to blood, most likely because of the filtering action of the spleen and whereas the whole of the spleen is cultured, only a sample of limited volume is cultured for blood and at low density there is a Poisson distribution of organisms in the blood and the probability of a sample being positive is governed by the sample size. In the lung, at the time of first positive blood culture there were circa $10^5$ CFU/g of tissue. The rise in counts in the lung between 12 and 24h and the continuing increase in colony counts past hour 24 resulted in a model term wherein the Innate Immunity term is decremented in an exponential fashion. After hour 24, we see exponential growth in all three compartments until approximately hour 48 for lung and hour 42 for blood and spleen. The timing of achieving stationary phase counts in all three compartments may have an impact on the clinical course of pneumonic plague in the untreated state.

The fit of the model to the data prior to the Bayesian step was quite acceptable. It is displayed in Figure 2, panels A-C. In Panel A (Lung), the $r^2$ was 0.911 ($p << 0.001$), the Bias was 1.277 and the Precision was 16.601, which are quite reasonable. For Panel B (Blood), these values were $r^2 = 0.379$ ($p << 0.001$), 2.523 and 43.65. The latter value is likely due to three outlier points in the pre-Bayesian estimation. In Panel C (Spleen) these values were $r^2 = 0.627$, 0.881 and 1.965.

The Observed-Predicted regressions after the Bayesian step are shown in Figure 3, Panels A-C. For lung, the $r^2 = 0.964$ ($p << 0.001$) with a Bias of 0.228 and a Precision
of 0.869. For Blood, these values were $r^2 = 0.811$, 1.703 and 37.292. For Spleen, the values were $r^2 = 0.832$, 0.348 and 0.485. Consequently, the fit of the model to the data for all three outputs explained a substantial portion of the variance.

In Table 1, the mean, median and standard deviation of the population parameter estimates are displayed. $K_{\text{growth-L}}$ in the lung was 0.409 h$^{-1}$ (mean) or 0.461 h$^{-1}$ (median), indicating a doubling time of *Y. pestis* in the lung of 1.5 – 1.7 hours. This is quite consistent with the observed increase in lung colony counts seen between hours 24 and 48 (14 – 16 doubling times).

The initial condition (number of lung organisms at the end of spray challenge) was between 135 and 1184 CFU/g (median and mean, respectively). Because of the population nature of the estimation, it is likely that the mean values are somewhat influenced by outliers and it is likely that the median is the better measure of central tendency for the number of organisms in the lung at the end of aerosol challenge. The whole body spray in the mouse produces a baseline lung load of 2-3 Logs of organisms.

Again, by examining Figure 1, very low counts are seen in the blood first at hour 24. All earlier blood cultures were negative. Therefore the Threshold at which blood spillover occurs should be around the lung counts observed at this time. Indeed, the THRESHOLD parameter identified $6.4 \times 10^4$ CFU/g (median) to $1.52 \times 10^6$ CFU/g (mean) as the value. The value of $K_{\text{sPL-BL}}$ is quite small, but consistent with the rather large organism burden in the lung at the time of spill over into the blood. It should be noted that the increase in bacterial burden in the three sites occurs in parallel, suggesting that the lung is the driver for all three (spill over into the blood and then filtered from blood to spleen).
The transfer rate constant from blood to spleen is large, when either mean or median is examined and is testimony to the efficiency of the spleen’s ability to filter the blood.

The decrement of CFU/g between 1 and 12 hours we hypothesize to be attributable to innate immunity (rate constant of kill is substantial at 3.37 to 4.93 h\(^{-1}\)), but is overwhelmed by 24h. The decline in kill rate due to innate immunity has a half time of about 3.0-3.8 h, indicating that between hours 12h and 24h there has been a decline of about 88.5% to about 93.75%. Given the growth thereafter, this is a physiologically plausible result.

**Discussion**

Pneumonic plague is a horrific disease. Unless treatment is started in short order after onset of symptoms, mortality is 80-100% (15). In contradistinction to Anthrax, Plague pneumonia is highly transmissible from person-to-person (9). Consequently, early in the time sequence after an intentional release, there may be a number of patients in the first cohort who present in the later stages of the disease and, consequently, will have a low likelihood of survivorship, even with the most aggressive antimicrobial therapy. As it is likely to take a considerable period of time to confirm the identity of the causative pathogen as *Yersinia pestis* and even longer to obtain a sensitivity profile in the index case, it is highly likely that secondary spread will occur. Obviously early aggressive chemotherapy is critical to patient survivorship.

Perhaps as importantly, such early aggressive therapy may play a role in blocking person-to-person transmission. If true, this leaves us with the question of why the therapeutic window for plague pneumonia is so foreshortened and why there is such a
propensity for person-to-person transmission. In order to obtain some insight into these questions, we employed a mouse model of whole-body aerosol challenge with Yersinia pestis CO92 and then employed a mathematical model to help understand the time course of the disease. As seen in figures 2-3, the fit of this model to the data was quite acceptable and allows insight into the pathophysiological process.

The raw colony counts in lung, blood and spleen shed considerable light on the issue and are displayed in Figure 1. The one hour time point after aerosol challenge shows an average CFU/g of lung tissue colony count of 3.64\pm0.3 \log_{10} (CFU/g) . Application of the mathematical model (see Table 1) indicates that the Initial Condition of the lung after the cessation of the aerosol challenge was (median estimate) 2.13 \log_{10} (CFU/g) to (mean estimate) 3.07 \log_{10} (CFU/g). This number increases quickly to the 1 hr point but then, surprisingly, decreases to 2.92\pm0.22 \log_{10} (CFU/g) at hour 12 (p < 0.01). At hour 24, counts increase to 5.07\pm0.28 \log_{10} (CFU/g). We hypothesized that the decrement initially was at least partially attributable to innate immunity. Since there was a rapid increase in counts, we also hypothesized that Yersinia pestis overcame innate immunity, perhaps attributable to Yersinia Outer membrane ProteinS (YOPS: 12, 13). The ability of the mice to suppress Y. pestis amplification was estimated to decline with a half time of about 3-4 hours.

At and after 24 hours, organisms are first found spilling over into the blood and filtering into the spleen. The model demonstrates that counts ranging from 4.81 to 6.18 \log_{10} (CFU/g) in the lung (Threshold value) that we first see bacteremia. Exponential increase is seen thereafter until stationary phase is obtained at around 48 hours for all three compartments. Now the foreshortened course of pneumonic plague becomes
understandable. Once the YOPS (Yersinia Outer-membrane ProteinS) allow innate immunity to be bypassed, the relatively low challenge in lung can rapidly amplify, so that by 24 hours sepsis is seen and, in the absence of intervention, colony counts at 48 hours are 6-7 Log₁₀ (CFU/g) in the blood, even with an intact spleen. The numbers in lung are in excess of 10 Log₁₀ (CFU/g). These numbers are larger than would normally be seen in VABP patients and occur rapidly. There seems to be a breakpoint of around 42-48 hours in the course of untreated pneumonic plague where the ability of antibiotics alone to improve survivorship becomes limited (2,8). It is clear that the ability of plague to circumvent innate immunity so quickly allows organisms to reach densities so great that sepsis becomes overwhelming. One may also speculate that because organisms appear near or at stationary phase in the window of 42-48h that many of them may be in Non-Replicative Persister (NRP) phenotype and, therefore, less sensitive to the lethal effects of antibiotics (14, 15). These results lend greater support to the 42h post exposure time point for evaluating therapies in the murine model. Successful evaluation in the murine model under these conditions, allows easier decisions to move to NHP studies where body temperature, telemetry and other triggers can be used to define when treatment should begin. As NHPs are an expensive and limited resource, in addition to the cost of the GLP studies with telemetry the importance of an accurate and robust defined treatment time in the murine model has been needed.

In the case of an intentional release the key to an effective response will be early diagnosis and the key to that is a high index of suspicion with regard to the causative pathogen on the part of “first responders” in the healthcare community. Early intervention with an appropriate antibiotic should provide near optimal therapy.
Interestingly, as shown by Byrne et al (2), β-lactam antibiotics often accelerated death (hypothesized to be due to endotoxin release) and, therefore, initial treatment with β-lactam antibiotics alone is likely contraindicated in the case of pneumonic plague. The ability to initiate treatment when the burden of organisms in the lung is at or below THRESHOLD should not only improve individual patient survivorship but also minimize the number of secondary cases. It is likely that increasing the numbers of patients who survive after the therapeutic window of opportunity has passed may be a function of development of adjunctive therapeutics aimed at countering the sepsis syndrome. Treatment of pneumonic plague remains a great challenge, even with the availability of a modern therapeutic armamentarium.

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**Conflict of Interest:** The authors have no conflict of interest to declare.
References


Figure 1: Counts of *Yersinia pestis* in lung, blood and spleen (CFU/g – Lung or Spleen; or CFU/ml - Blood) after an aerosol challenge to cohorts of mice (n = 15)

![Graph showing CFU counts over time for Spleen, Blood, and Lung.](http://aac.asm.org/)
Figure 2A: Observed-predicted plot prior to the Bayesian step from the model for lung counts of *Yersinia pestis*
Figure 2B: Observed-predicted plot prior to the Bayesian step from the model for blood counts of *Yersinia pestis*
Figure 2C: Observed-predicted plot prior to the Bayesian step from the model for spleen counts of *Yersinia pestis*
Figure 3A: Observed-predicted plot after the Bayesian step from the model for lung counts of *Yersinia pestis*
Figure 3B: Observed-predicted plot after the Bayesian step from the model for blood counts of *Yersinia pestis*.
Figure 3C: Observed-predicted plot after the Bayesian step from the model for spleen counts of *Yersinia pestis*
**Model Parameters**

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