Natural History of *Francisella tularensis* in Aerosol-Challenged BALB/c Mice

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Abstract

The objective of this study was to evaluate the natural history and pathogenesis of \textit{Francisella tularensis} in a murine model of inhalational tularemia with the SchuS4 strain. Before the efficacy of antimicrobials can be assessed in this model, further model development was required to determine the optimal time to start therapy. This study helped define the time course of infection after aerosol challenge by quantifying the presence of bacteria in the lung, blood, and spleen at multiple harvest points.

In this study, mice were infected via a targeted inhaled dose of 100 LD50 (LD50 = 300 CFU) of \textit{F. tularensis} by whole-body aerosol. At 1, 24, 36, 48, 60, 72, 75, 78, 81, 84, 87 and 90 h post challenge groups of 15 animals were sacrificed with blood, lung, and splenic tissue harvested, homogenized, plated, and incubated to evaluate the bacterial load in those tissues.

It was determined that of the 3 tissues harvested, splenic tissue provided the most consistent bacterial counts which steadily increased with the progressing infection. Further, it was determined that at 75 hours post aerosolization all animals’ (15/15) lungs were positive for infection and 14/15 animals had positive splenic tissue counts. Bacterial levels in blood were not predictive of treatment initiation. For future therapeutic evaluation studies in this model using \textit{F. tularensis} (SchuS4), it was determined that therapy should be initiated at 75 hours post challenge and validated by spleen involvement.

**Keywords:** Tularemia  Septicemia  Mathematical Modeling
Introduction

*Francisella tularensis* is the causative agent of tularemia, normally a zoonosis producing flu-like symptoms in humans. The low numbers of organisms required to cause infection makes *F. tularensis* a potentially effective agent for a biowarfare or terrorist attack. Given the added possibility of resistance to current treatments through genetic engineering or natural emergence, identifying effective antibiotics with novel mechanisms of action is critical. Before the efficacy of antimicrobials can be assessed in a “treatment” model, further model development was required to determine the optimal time to start therapy versus “post-exposure prophylaxis”. This study helped define the time course of infection after aerosol challenge and quantify the presence of bacteria in the lung, blood, and spleen at the harvest points with the goal of identifying a time post infection associated with consistent systemic disseminated disease.

We employed a whole body murine aerosol challenge model to explore the course of the disease and to better understand why pneumonic tularemia becomes poorly treatable after a relatively short interval following the onset of symptoms, with consequent very high morbidity. As this murine infection model is used as a proof of concept for antibiotic therapies before advancement to the non-human primate models, a better understanding of how to define the treatment trigger and justification of that timing is needed. Differentiation between post-exposure prophylaxis and a true treatment model must be based upon a disease history and tissue distribution of *F. tularensis* in the murine model and in turn can identify acceptance criteria for validation of therapeutic results in the infection model.
The data was also applied to a mathematical model to describe the growth of *F. tularensis* in blood, lung and spleen tissues after inhalation.

**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* (1). 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 was fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

**Preparation of the *F. tularensis* challenge strain for aerosolization.**

*F. tularensis* strain SchuS4 (obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH) was used for this study. The 50% lethal dose (LD$_{50}$) in mice for this strain is 300 colony-forming units (CFU) inhaled, based on respiratory rates derived from Guyton (2) when administered as a whole body aerosol. The inoculum for aerosol challenge was prepared as follows, *F. tularensis* SchuS4 was grown overnight in 2% isovitalex-(Becton Dickinson, Sparks Md.) supplemented Brain Heart Infusion broth. For the aerosol challenge the overnight
culture was adjusted to the challenge dose of approximately $1 \times 10^9$ CFU/ml. To verify final bacterial concentrations and exposure doses, colonies were enumerated after serial dilution and plating on chocolate agar plates. The plates were incubated at 35°C and colonies enumerated after 48 hours.

**Aerosol infection.**

A target inhaled dose of 100 LD$_{50}$ (LD$_{50} = 300$ CFU) of *F. tularensis* was administered to 7-9 week old female BALB/c mice (Charles River) by whole-body aerosol. The aerosol was generated using a three-jet Collison nebulizer (3). All aerosol procedures were controlled and monitored using the Automated Bioaerosol Exposure system (Biaera Technologies, Md) (4) operating with a whole-body rodent exposure chamber. The aerosol was driven by compressed air at 26 lb/in$^2$ and a flow rate of 7.5 liter/min. Animals were exposed for 10 min followed by a 10 minute clean air exposure. Integrated air samples are obtained from the chamber during each exposure in 10 ml of BHI using an all-glass impinger (AGI). Integrated air samples are serially diluted, plated on chocolate agar, and incubated for 2 days at 35°C to quantify the samples. The inhaled doses (CFU/mouse) of *F. tularensis* were estimated using the quantification of the integrated air samples and mouse respiratory rates per Guyton (2). Sixty mice were challenged at a time. Mice from each aerosol challenge run were divided evenly into each experimental time-point group to balance the impact of potential exposure differences in each run. Actual challenge doses for the three aerosol runs were 2, 4 and $4 \times 10^4$ CFU/mouse respectively, representing, 61,124 and 125 LD$_{50}$s, average 104 LD$_{50}$s,

**Assessment of animals.** Cohort size for statistical evaluation was 15 mice.
At 1, 24, 36, 48, 60, 72, 75, 78, 81, 84, 87 and 90 h post challenge groups of 15 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 with EDTA by cardiac puncture and serially diluted in sterile saline. After bleeds animals were euthanized and lungs and spleens removed, weighed, homogenized in 1 ml sterile saline and serially diluted in sterile saline. Serial dilutions of blood, lung and spleen homogenates were plated within 30 minutes after harvest onto Chocolate agar and incubated at 35°C for 2 days to determine bacterial load. Limits of detection were 5 CFUs/ml for blood samples, 5 CFUs/gm for lung and 10 CFUs/gm for spleen.

An additional set of animals were observed and temperatures recorded 2× per day by applying an infrared probe (infrared body surface BIOSEB IR Rodent Thermometer model 153IRB) against the chest between the front legs.

**Mathematical model of *F. tularensis* growth in the lung, blood and spleen of the mouse.** A series of inhomogeneous differential equations described the growth of *F. tularensis* after inhalational challenge.

\[
\frac{dX(1)}{dt} = IC(1) + K_{\text{growth-L}} \left(1-(X(1)/\text{POPMAX_L})\right)X(1)
\]

**IF** \(X(1) \leq \text{THRESHOLD}\) **THEN**

\[
\frac{dX(2)}{dt} = 0
\]

**ELSE**

\[
\frac{dX(2)}{dt} = K_{\text{TR_L_BL}}X(1) - K_{\text{TR_BL_SPL}}X(2)
\]

**END IF**

\[
\frac{dX(2)}{dt} = K_{\text{TR_L_BL}}X(1) - K_{\text{TR_BL_SPL}}X(2)
\]
\[
\frac{dX(3)}{dt} = (K_{TR\_BL\_SPL} \times X(2) + K_{growth\_SPL} \times X(3))^\ast(1-(X(3)/POPMAX\_SPL)) \quad (3)
\]

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. \(K_{growth\_L}\) is the first order growth rate constant for organisms in the Lung. \(POPMAX\_L\) is the maximal population density and the term \((1-(X(1)/POPMAX\_L))\) is a logistic carrying function designed to prevent unconstrained bacterial growth and for the organism population to achieve stationary phase. \(K_{tr\_L\_BL}\) is a first order rate constant for transfer from Lung to Blood. Equation 2 describes the time course of organisms in blood. The raw data demonstrated that no organisms were detected by blood culture by hour 24 and were first detected in low numbers at hour 36. 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. The spleen serves as a natural filter and in Equation 2, there is loss of organisms from the blood into the Spleen. \(K_{TR\_BL\_SPL}\) is a first order transfer rate constant describing this process. Equation 3 describes the time course of organisms in the Spleen. \(POPMAX\_SPL\) serves an analogous function to that noted above in the lung.

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, VanGuilder and Schumitzky (5). 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. 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 1A. Of note is the small numbers and late detection of organisms in 
the blood. While the mean counts (n=15) in the blood did increase with time, the number 
of animals with bacteremia did not increase with duration of infection, but was variable 
across the time points from 13% to 87% (mean 49%); see Table 1. In the lung, counts 
increase to about 5 x 10^6 CFU/g by hour 36 and remained plateaued at this level. As 
expected due to the point of infection, all lungs at all time points were positive. After 
hour 24, we see exponential growth in the spleen and less variability in terms of the 
number of animals with positive results, 80 to 100% from hour 75 on. The spleen data 
are provided in Table 1.

Because it is easily measured and non-invasive, body temperature is often used 
as a marker for the onset of disease and a trigger for therapy initiation in animal models 
where therapies are being evaluated. Examination of Figure 1B demonstrates that 
temperature is relatively constant out to hour 96. Thereafter, temperature markedly 
decreases when the mice are in the terminal stages of the infection.
We next sought to describe the progression of disease by modeling the outgrowth of bacteria in each tissue compartment. The fit of the mathematical 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 0.726 (p << 0.001), the Bias was -0.761 and the Precision was 3.427. For Panel B (Blood), these values were \( r^2 = 0.631 \) (p << 0.001), -6.068 and 312.1. The latter value is due to the weights (unweighted Precision was 0.484). In Panel C (Spleen) these values were \( r^2 = 0.769 \), -1.06 and 2.562.

The fit of the model to the data after the Bayesian step was quite acceptable. It is displayed in Figure 3, panels A-C. In Panel A (Lung), the \( r^2 \) was 0.956 (p << 0.001), the Bias was 0.024 and the Precision was 0.519, which are quite reasonable. For Panel B (Blood), these values were \( r^2 = 0.920 \) (p << 0.001), -0.0636 and 58.2. The precision was due to the relative weights (the unweighted Bias-adjusted Precision was 0.106). In Panel C (Spleen) these values were \( r^2 = 0.918 \), 0.145 and 0.864. The model described the data well.

In Table 2, the mean, median and standard deviation of the population parameter estimates are displayed. \( K_{g-L} \) (growth rate in the lung) was 0.113 h\(^{-1}\) (mean) or 0.118 h\(^{-1}\) (median), indicating a doubling time of \( F. tularensis \) in the lung of 5.9-6.1 hours. This is quite consistent with the observed increase in lung colony counts seen between hours 1 and 24 (about 8.5 doubling times).

The initial condition (number of lung organisms at the end of spray challenge) was between 136 and 1467 CFU/g (median and mean, respectively). The colony count observed in the mouse at 1 h was 127 CFU/g.
Again, by examining Figure 1, very low counts are seen in the spleen first at hour 24. Earlier spleen cultures were negative and only 47% of the animals were positive at hour 24. While spleen loads steadily increased with time, the percent positive did not peak/plateau until 72-75 hours where for the remaining time points it fluctuated between 72 and 100% for an average of 84.8% between hours 72 and 90 hours.

Discussion

The objective of this study was to evaluate the natural history and pathogenesis of \textit{F. tularensis} strain SchuS4 in a murine model of inhalational tularemia. The intent was to identify a time point or signal (e.g. temperature change) that would inform when systemic dissemination of disease had occurred. The rationale for this is that more challenging “treatment” models share this trait in common, in contrast to a “post exposure prophylaxis” models where therapy is initiated at a fixed time point after aerosol challenge, regardless of progression of disease (6-8). Therefore this study informs the appropriate time to test a therapeutic agent for efficacy in a murine “treatment” model of inhalational tularemia.

In this study, mice were infected via a targeted inhaled dose of 100 LD$_{50}$ of \textit{F. tularensis} by whole-body aerosol. Between 1 and 90 hours post challenge groups of 15 animals were euthanized with blood, lung, and splenic tissue collected to evaluate the presence of bacterial load in those tissues. Body temperature was also evaluated as a potential marker for therapeutic intervention. It was determined that of the 3 tissues harvested, splenic tissue provided the most consistent bacterial counts which steadily increased with the progressing infection (Table 1). While the lung tissue was
consistently culture positive, this was the source of infection and was near the tissue max at 24 hrs (post-exposure) making it less useful for a treatment trigger criteria. The lack of temperature change out to hour 96 indicates that temperature should not be used as a trigger for therapeutic intervention as it occurs in the terminal stages of the infection but could be used as a criteria for therapeutic failure and euthanasia (9). This presents a unique challenge compared to primate models where fever is a reliable measure for systemic disease (10) because the justification for treatment initiation in the mouse model requires sacrificing the animals.

The natural history of *F. tularensis* differs substantially from the model for *Yersinia pestis* that we have describer earlier (11). The first and most straightforward difference is in replication rate in the lung, where the doubling time was approximately four fold longer with *F. tularensis*, relative to *Y. pestis*. The longer doubling time is well described for *F. tularensis*. The model here did not require a term for innate immunity that declined with time, as it did with *Y. pestis*. However, as the experimental designs were not identical, this observation needs further study with an identical early study design. Another major difference was the dominance of the splenic burden. In *Y. pestis*, the lung dominates the disease after aerosol exposure, whereas in *F. tularensis*, the spleen continues to have greater and greater bacterial burden, even after 72 h, when the lung burden is at stationary phase.

Again in contrast to *Y. pestis*, *F. tularensis* has an early and prominent phase of macrophage interaction. After phagocytosis, there is transient interaction with early and late endosomes and phagosome acidification. Proteins from the organism’s pathogenicity island are then expressed, mediating phagosomal escape and replication.
in the cytoplasm (12). We speculate that the dominance of the spleen in the natural history of tularemia is due to its ability to rapidly escape from the phagosome after ingestion and reproduce in the cytoplasm. This locale may shield it to some degree from drugs like β-lactams which do not efficiently penetrate cells (13). It may also explain the utility of fluoroquinolones like ciprofloxacin which are able to efficiently achieve therapeutic concentrations in these cells.

Finally, *F. tularensis* is not known to be transmitted person-to-person, again in contrast to pneumonic *Y. pestis*. This has major implications for treatment of patients. It is unlikely that patients need to be isolated, even in the case of a deliberate release. Clean uninfected mice added in with previously tularemia exposed mice did not become infected (H. Heine, unpublished observation).

As important are the factors that were quite similar between the two investigations. In both, the threshold value for colony counts in the lung resulting in breakthrough bacteremia were quite similar. The values were (*F. tularensis* vs. *Y. pestis* (11) median and mean values) 5.9 and 6.1 Log$_{10}$(CFU/g) vs. 4.81 and 6.18 Log$_{10}$(CFU/g). This may have implications for the immune mediated clearance of these pathogens being saturable (as we have described previously for other pathogens (14-16)).

The transfer rate constants from blood to spleen are also similar and are large (*F. tularensis* vs. *Y. pestis* median and mean values) at 8.06, 11.2 h$^{-1}$ vs. 12.5, 9.4 h$^{-1}$. This is not surprising, as the spleen, as part of the reticuloendothelial system serves as a natural filter for bacteremic pathogens, as seen in pneumococcal pneumonia, with
patients without a spleen having higher bacteremic burdens and consequent poorer outcomes (1, 18).

The difference in pathogenesis of these infections is also important. In contrast to Y. pestis, F. tularensis has initial involvement in macrophage penetration as part of the process (11, 19). This likely explains the difference in infection course, with breakthrough bacteremia occurring at approximately the same point for both pathogens, but with overwhelming burdens of bacteria in the blood leading to very early death in Y. pestis, but with much lower burdens of bacteremia probably resulting from a greater affinity for macrophage infection for F. tularensis resulting in a more tissue-focused profile of infection. These observations may help explain the difference in the types of antimicrobials effective for these two pathogens.

In the case of an aerosol intentional release, it is clear that the earlier therapy is initiated, the better the outcome will be. Examination of Figure 1A shows that near maximal bacterial burdens are developed after 72 h, including bacteremia. Overall, the model appears robust and suitable for use in examining the efficacy of antibiotics against tularemia in an active infection situation. At 75 hours, 14 of 15 animals had positive bacterial cultures from splenic tissue suggesting that this is the appropriate time to begin therapy with an antibiotic agent in mice under a treatment mode. Furthermore, it was observed that neither temperature change nor bacterial counts in the bloodstream (Table 1) accurately reflected the extent of infection thus we recommend euthanizing and culturing a small subset of animals to establish >90% splenic involvement at 75 hours post challenge, as the inclusion criteria for mouse “treatment” experiments.
Unfortunately the actual therapy would be well underway before that validation result becomes available.

The model should allow testing of new antimicrobials alone and in combination and provide insight into therapeutic utility in man. Of equal importance, understanding the central role of macrophages and the reticuloendothelial system as exemplified here by the spleen, the model provides a venue for testing host-directed therapeutics.

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


Figure 1: A. Progression of *F. tularensis* bacterial burden in blood, lung, and splenic tissue over the course of the study. (n=15, error bars are SD)
B. Temperature course of mice challenged with *F. tularensis* (n=14 to 108h, n=10 at 132h, error bars are SD)
Figure 2A: Observed-predicted plot from the pre-Bayesian (population) step from the model for lung counts of *Francisella tularensis*. 

Pre-Bayesian (Population) Predictions

Observed: 0.866 x Predicted + 1.030

$R^2 = 0.726; P << 0.001$
Figure 2B: Observed-predicted plot from the pre-Bayesian (population) step from the model for blood counts of *Francisella tularensis*.
Figure 2C: Observed-predicted plot from the pre-Bayesian (population) step from the model for spleen counts of *Francisella tularensis*.
Figure 3A: Observed-predicted plot after the Bayesian step from the model for lung counts of *Francisella tularensis*
Figure 3B: Observed-predicted plot after the Bayesian step from the model for blood counts of *Francisella tularensis*
Figure 3C: Observed-predicted plot after the Bayesian step from the model for spleen counts of *Francisella tularensis*.
Table 1: Mean bacterial burden in splenic tissue and number of culture-positive animals spleen and blood by group.

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<th>Log_{10} CFU/g mean (group)</th>
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<th># positive bloods</th>
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*Animals sacrificed at moribund or found dead, time point noted
Table 2: Estimates of the Parameter Values for the Model

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Definitions are provided in Methods