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

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The objective of this study was to evaluate the natural history and pathogenesis of Francisella tularensis in a murine model of inhalational tularemia with the SchuS4 strain. Before the efficacy of antimicrobials could 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 lung, blood, and spleen at multiple harvest points. In this study, mice were infected via a targeted inhaled dose of 100 50% lethal doses (LD50s) (LD50 = 300 CFU) of F. tularensis by whole-body aerosol. At 1, 24, 36, 48, 60, 72, 75, 78, 81, 84, 87, and 90 h postchallenge, groups of 15 animals were sacrificed and blood, lung, and splenic tissue samples were harvested, homogenized, plated, and incubated to evaluate the bacterial load in those tissues. It was determined that of the 3 sample types harvested, splenic tissue provided the most consistent bacterial counts, which steadily increased with the progressing infection. Further, it was determined that lung samples from all (15/15) animals were positive for infection at 75 h postaerosolization and that 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 F. tularensis (SchuS4), it was determined that therapy should be initiated at 75 h postchallenge and validated by spleen involvement.

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 “postexposure prophylaxis.” This study helped define the time course of infection after aerosol challenge and quantify the presence of bacteria in lung, blood, and spleen at the harvest points with the goal of identifying a time postinfection 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 with novel mechanisms of action, a better understanding of how to define the treatment trigger and justification of that timing are needed. Differentiation between postexposure 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 were 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 to 9 weeks old and 20 g in body weight) 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 biosafety level 3 (BSL3) laboratory of the University of Florida in Albany, NY, 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 inhaled 50% lethal dose (LD50) in mice for this strain is 300 CFU, based on respiratory rates derived from Guyton (2) for administration of the strain 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 (BHI) broth. For the aerosol challenge, the overnight culture was adjusted to the challenge dose of approximately 1 X 10^6 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 h.

Aerosol infection. A target inhaled dose of 100 LD50s (LD50 = 300 CFU) of F. tularensis was administered to 7-to-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 an automated bioaerosol exposure system.
(Biaera Technologies, MD) (4) operating with a whole-body rodent exposure chamber. The aerosol was driven by compressed air at a pressure of 26 lb/in² and a flow rate of 7.5 liter/min. Animals were exposed for 10 min followed by 10 min of clean air exposure. Integrated air samples are obtained from the chamber during each exposure in 10 ml of BHI broth 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 (quantified as CFU counts/mouse) of F. tularensis were estimated using the quantification of the integrated air samples and mouse respiratory rates per the method of Guyton (2). Sixty mice were challenged at a time. Groups of mice from each aerosol challenge run were divided evenly into experimental time point groups to balance the impact of potential exposure differences in each run. Actual challenge doses for the three aerosol runs were 2 × 10⁴, 4 × 10⁴, and 4 × 10⁵ CFU/mouse, respectively, representing, 61, 124, and 125 LD₅₀ s (average, 104 LD₅₀ s).

**Assessment of animals.** The cohort size for statistical evaluation was 15 mice. At 1, 24, 36, 48, 60, 72, 75, 78, 81, 84, 87, and 90 h postchallenge, groups of 15 animals were anesthetized by intramuscular injection of a mixture containing ketamine/acepromazine/xylazine (6/0.30/0.66 mg/ml) in a 0.1-ml volume in the caudal thigh. Whole blood was collected using EDTA by cardiac puncture and serially diluted in sterile saline solution. After the bleed, animals were euthanized and lungs and spleens removed, weighed, homogenized in 1 ml sterile saline solution, and serially diluted in sterile saline solution. Serial dilutions of blood, lung, and spleen homogenates were plated within 30 min after harvest onto chocolate agar and incubated at 35°C for 2 days to determine bacterial loads. Limits of detection were 5 CFU/ml for blood samples, 5 CFU/gm for lung samples, and 10 CFU/gm for spleen samples.

An additional set of animals were observed and temperatures recorded twice a day by applying an infrared probe (infrared body-surface Bioset 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.

\[
\frac{dX(1)}{dt} = IC(1) + K_{\text{growth-L}} \times \left[1 - \frac{X(3)}{\text{POPMAX-L}}\right] \times X(1) \quad (1)
\]

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

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

ELSE

\[
\frac{dX(2)}{dt} = K_{\text{TR-L-BL}} \times X(1) - K_{\text{TR-L-SBL}} \times X(1)
\]

END IF

\[
\frac{dX(3)}{dt} = K_{\text{TR-BL-SPL}} \times X(2) + K_{\text{growth-SPL}} \times X(3) \times \left\{1 - \frac{X(3)}{\text{POPMAX-SPL}}\right\} \quad (3)
\]

In equation 1, the growth of the organism in lung is described. IC(1) is the initial condition of the number of CFU per milliliter 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 (L). POPMAX-L is the maximal population density, and the term \(\left[1 - \frac{X(3)}{\text{POPMAX-L}}\right]\) is a logistic carrying function designed to prevent unconstrained bacterial growth and to cause the organism population to achieve stationary phase. \(K_{\text{TR-L-BL}}\) is a first-order rate constant for transfer from lung to blood (BL). Equation 2 describes the time course for organisms in blood. The raw data demonstrated that no organisms were detected by blood culture by h 24 and that they were first detected in low numbers at h 36. Consequently, we cored 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 (SPL). \(K_{\text{TR-BL-SPL}}\) is a first-order transfer rate constant describing this process. Equation 3 describes the time course for organisms in the spleen. POPMAX_SPL serves a function analogous to that noted above for 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 nonparametric population modeling program described by Leary et al. (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 γ” feature of BigNPAG. Weighting was determined 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 by 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 gram), blood (per milliliter), and spleen (per gram) are presented in Fig. 1A. The small numbers and late detection of organisms in the blood are of note. While the mean counts (\(n = 15\)) in the blood did increase with time, the numbers of animals with bacteremia did not increase with duration of infection but were variable (13% to 87%; mean, 49%) across the time points (see Table 1). In the lung, counts increased to about \(5 \times 10^5\) CFU/g by h 36 and remained plateaued at this level. As expected due to the point of infection, all lung results were positive at all of the time points. After h 24, we saw exponential growth in the spleen and less variability in terms of the numbers of animals with positive results (80% to 100% from h 75 on). The spleen data are provided in Table 1.

Because measurements are easy to perform and noninvasive, 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 Fig. 1B demonstrates that temperature was relatively constant to h 96. Thereafter, temperature markedly declined when the mice were 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. The corresponding data are displayed in Fig. 2. In panel A (lung), the $r^2$ value was 0.726 ($P < 0.001$), the bias value was $-0.761$, and the precision value was 3.427. For panel B (blood), these values were $0.631$ ($P < 0.001$), $-6.068$, and $312.1$. The latter value was a consequence of the weights (the unweighted precision value was 0.484). In panel C (spleen), these values were $0.769$, $-1.06$, and $2.562$.

The fit of the model to the data after the Bayesian step was quite acceptable. It is displayed in Fig. 3. In panel A (lung), the $r^2$ value was 0.956 ($P < 0.001$), the bias value was 0.024, and the precision value was 0.519, which are quite reasonable values. For panel B (blood), these values were $0.920$ ($P < 0.001$), $-0.063$, and $58.2$. The precision value was a consequence of the relative weights (the unweighted bias-adjusted precision was 0.106). In panel C (spleen), these values were $0.918$, $0.145$, 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. $K_{p+1}$ (the 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 to 6.1 h. This is quite consistent with the observed increase in lung colony counts seen between h 1 and h 24 (about 8.5 doubling times).

The values corresponding to the initial condition (numbers of lung organisms at the end of spray challenge) were between 136 and 1,467 CFU/g (median and mean, respectively). The colony count observed in the lung at h 1 was 127 CFU/g.

Again, examining Fig. 1, very low counts are first seen in the spleen at h 24. Earlier spleen culture results were negative, and only 47% of the animals gave positive results at h 24. While spleen loads steadily increased with time, the percentage of positive results did not peak or plateau until 72 to 75 h, whereupon, for the remaining time points, it fluctuated between 72% and 100% (average, 84.8%) between h 72 and h 90.

### DISCUSSION

The objective of this study was to evaluate the natural history and pathogenesis of $F. tularensis$ 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 indicate when systemic dissemination of disease had occurred. The rationale for this is that the more challenging “treatment” models share this trait, in contrast to a “postexposure prophylaxis” model(s), where therapy is initiated at a fixed time point after aerosol challenge, regardless of progression of disease (6–8). Therefore, this report gives 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 $F. tularensis$ by whole-body aerosol. Between h 1 and h 90 postchallenge, groups of 15 animals were euthanized and 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 maximum at 24 h (postexposure), making it less useful for a treatment trigger criteria. The lack of temperature change to h 96 indicates that temperature should not be used as a trigger for therapeutic intervention, as temperature change occurs only 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 described earlier (11). The first and most straightforward difference is in replication rate in the lung, where the doubling time was approximately 4-fold higher with *F. tularensis* than with *Y. pestis*. The longer doubling time is well described for *F. tularensis*. The model used here did not require a term for innate immunity that declined with time such as was the case with *Y. pestis*. However, as the experimental designs were not identical, this observation needs further study.

### TABLE 1 Mean bacterial burden in splenic tissue and number of animals with spleen culture- and blood culture-positive results by group

<table>
<thead>
<tr>
<th>Experiment group</th>
<th>Sacrifice time point (h)</th>
<th>$n$</th>
<th>CFU/g mean (group)</th>
<th>Log$_{10}$ CFU/g mean (group)</th>
<th>No. of spleen culture-positive animals/total no. of animals</th>
<th>No. of blood culture-positive animals/total no. of animals</th>
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<tr>
<td>1</td>
<td>1</td>
<td>15</td>
<td>0.00E + 00</td>
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<td>11/15</td>
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<td>5.00</td>
<td>8/15</td>
<td>3/15</td>
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<td>72</td>
<td>15</td>
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<td>7</td>
<td>75</td>
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<td>8.11</td>
<td>13/15</td>
<td>5/15</td>
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$^a$ Animals sacrificed at moribund stage or found dead; time point noted.

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FIG 2 (A) Observed-predicted plot from the pre-Bayesian (population) step from the model for lung counts of *Francisella tularensis*. Observed = 0.866 × predicted + 1.030, $r^2 = 0.726$; $P \ll 0.001$. (B) Observed-predicted plot from the pre-Bayesian (population) step from the model for blood counts of *Francisella tularensis*. Observed = 0.982 × predicted + 0.688, $r^2 = 0.631$; $P \ll 0.001$. (C) Observed-predicted plot from the pre-Bayesian (population) step from the model for spleen counts of *Francisella tularensis*. Observed = 1.139 × predicted − 0.290, $r^2 = 0.769$; $P \ll 0.001$. 
FIG 3 (A) Observed-predicted plot after the Bayesian step from the model for lung counts of *Francisella tularensis*. Observed = 0.975 × predicted + 0.129, $r^2 = 0.956$; $P < 0.001$. (B) Observed-predicted plot after the Bayesian step from the model for blood counts of *Francisella tularensis*. Observed = 1.026 × predicted − 0.124, $r^2 = 0.920$; $P < 0.001$. (C) Observed-predicted plot after the Bayesian step from the model for spleen counts of *Francisella tularensis*. Observed = 1.007 × predicted − 0.124, $r^2 = 0.918$; $P < 0.001$. 
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 a 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 the ability of the pathogen to rapidly escape from the phagosome after ingestion and to reproduce in the cytoplasm. This locale may shield it to some degree from drugs such as β-lactams which do not efficiently penetrate cells (13). It may also explain the utility of fluoroquinolones such as ciprofloxacin which are able to efficiently achieve therapeutic concentrations in these cells.

Finally, and again in contrast to pneumonic *Y. pestis*, *F. tularensis* is not known to be transmitted from person to person. 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 introduced into the presence of previously tularemia-exposed mice did not become infected (H. Heine, unpublished observation).

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

The rate constants of transfer from blood to spleen are also similar and are large (with respect to *F. tularensis* versus *Y. pestis* median and mean values) at 8.06 and 11.2 h$^{-1}$ versus 12.5 and 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 (17, 19).

The differences in the pathogenesis of these infections are also important. In contrast to *Y. pestis*, *F. tularensis* has initial involvement in macrophage penetration as part of the process (11, 18). This likely explains the differences in the infection courses, with breakthrough bacteremia occurring at approximately the same time point for the two pathogens; however, overwhelming burdens of bacteria in the blood lead to very early death in cases of *Y. pestis* infection, whereas lower burdens of bacteremia, probably resulting from a greater affinity for macrophage infection for *F. tularensis*, result 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 intentional aerosol release, it is clear that the earlier therapy is initiated, the better the outcome is. Examination of Fig. 1A shows that near-maximal bacterial burdens, including bacteremia, are developed after 72 h. Overall, the model appears robust and suitable for use in examining the efficacy of antibiotics against tularemia in an active infection situation. At 75 h, 14 of 15 animals had positive splenic tissue bacterial cultures, 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 specimens from a small subset of animals to establish >90% splenic involvement at 75 h postchallenge as the inclusion criterion for mouse “treatment” experiments. Unfortunately, the actual therapy would be well under way before the corresponding validation result would become available.

The model should allow testing of new antimicrobials alone and in combination and should provide insight into therapeutic utility in humans. Of equal importance for 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.

**FUNDING INFORMATION**

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**REFERENCES**


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**TABLE 2** Estimates of the parameter values for the model

<table>
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<th>Parameter</th>
<th>Unit or value</th>
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*Parameter definitions are provided in Materials and Methods.*


