Abstract #185

# Onset of Bacteremia in Mice Following Nose-Only Inhalation of *Bacillus anthracis* Ames Spores

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## Abstract

**Background**
*Bacillus anthracis* is the etiological agent of the disease anthrax in humans and animals. The 50% lethal dose ($LD_{50}$) has been well described in several animal species. However, the onset of bacteremia following nose-only inhalation exposure has not been well described. The accurate timing of onset of bacteremia is critical for experimental design when testing anti-microbial and therapeutic agents for post-exposure prophylactic treatment to prevent anthrax disease.
**Methods**
Six groups of BALB/c mice, consisting of five males and five females, were exposed by nose-only inhalation to an average inhaled dose of 2.6+E06 CFU of *B. anthracis* Ames spores. Mice were euthanized at T=0 (immediately following exposure), and 6, 12, 24, 36, and 48 hours post-exposure. Blood samples were serially diluted, plated, incubated, and enumerated.
**Results**
Bacteremia was observed in 100% of mice euthanized immediately after exposure. Subsequently, bacteremia rapidly dropped to 0% by 12 hours and increased to 70% at 24 hours. By 48 hours, 100% of mice were positive for *B. anthracis* bacteremia. Bacterial load analysis of blood demonstrated high bacterial loads (i.e. >3.0+E05 CFU/mL) as early as 24 hours post-exposure.
**Conclusions**
These results suggest that initiating anti-microbial treatment may be necessary earlier than 24 hours post-exposure for successful prevention of inhalational anthrax disease.

## Introduction

*Bacillus anthracis*, the etiological agent of anthrax, is a Gram-positive, aerobic, rod-shaped, spore-forming bacterium. Spores are produced under nutrient starvation, and are highly resistant to a variety of harsh environmental and chemical conditions. Spores are capable of long-term survival in the environment and may germinate and outgrow into vegetative bacteria upon encountering favorable conditions such as a mammalian host. The bacteria may cause cutaneous, gastrointestinal, and inhalational anthrax disease, with the inhalational form being the greatest bioterrorism threat. Following deposition in the lung, spores are phagocytized by alveolar macrophages and rapidly begin producing the lethal and edema toxins. The spores may cause fatal anthrax disease regardless of antibiotic therapy due to the prolonged presence of the toxins in the host[1]. This aspect of anthrax disease makes the timing of antibiotic therapy critical for successful treatment. This study focuses on the timing of onset of bacteremia in a murine model of nose-only inhalation exposure to *B. anthracis* Ames spores.

## Methods

**Spore Preparation**
*B. anthracis* Ames (BAA) spores were prepared by growing cultures in Difco Sporulation Media until nutrient exhaustion. Sporulated cultures were harvested by centrifugation and washed with USP-WFI. Washed spores were then purified over Renografin-60 gradients (20% : 50% in USP-WFI (v/v)) to remove vegetative cells, debris, and phase dark spores. Purified spores were washed again with USP-WFI and stored at 4°C in USP-WFI. Spore preparations were observed using phase contrast microscopy to quantify the percent of phase bright spores, phase dark spores, vegetative cells, and debris. Endotoxin levels were tested using the limulus amebocyte lysate assay (Charles River Labs EndoSafe PTS System). Spore number was quantified by serial dilution in USP-WFI and plating on TSA with 5% sheep's blood plates to determine the number of CFU/mL. Prior to exposure, the spore suspension was diluted in USP-WFI to achieve a concentration of 5.0E+09 CFU/mL.

**Exposure System**
The exposure system consisted of a Collison 3-jet nebulizer, passive air diluter, radial mixer, stainless steel delivery line, and radial nose-only exposure plenum. The exposure plenum used in this study was an In-Tox Products radial nose-only 36-port chamber fitted with Positive Flow-By™ nose cones. Aerosol monitoring devices consisted of a DustTrak ™ Aerosol Monitor for real-time aerosol concentration, stainless steel impingers and an Aerosol Particle Sizer (TSI, Inc.) to determine aerosol particle sixe distribution reported as mass median aerodynamic diameter, µm and geometric standard deviation.

**Inhalation Exposure**
Mice (BALB/c from Charles River Labs) were loaded into restraint tubes and placed on the exposure chamber. Animals were exposed for 60 minutes and oxygen concentration was monitored constantly with a Model 5800 Intelligent Oxygen Monitor (Hudson RCI). Actual aerosol concentration was determined by impinging into USP-WFI and subsequent plate count analysis on TSAB plates.

**Animal Monitoring and Microbiological Analysis**
Following exposure, animals were observed three times daily for signs of mortality and moribundity. Body temperature was recorded three times each day. Clinical observations to observe sings of illness such as ruffled coat, hunched back, dyspnea, and lethargy were performed once daily. Animals were bled at time of scheduled euthanasia or upon being found dead or moribund. Blood was collected into tubes containing sodium polyethanesulfonate, serially diluted in USP-WFI, and plated on TSAB plates to determine the number of CFU/mL. Lungs were manually homogenized, serially diluted in USP-WFI, and plated on TSAB plates to determine the mean deposited dose.

**Table 1. Exposure Schedule and Design**

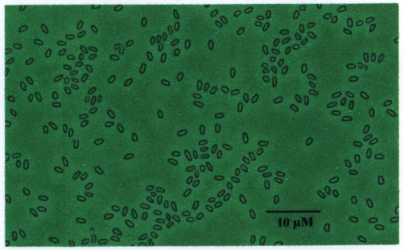
| Exposure Group(s) | Nebulizer Concentration (CFU/mL) | Target Inhaled Dose (CFU) | Target $LD_{50}$ Equivalents | Target Aerosol Concentration (CFU/L) | Number of Mice per Group |
|---|---|---|---|---|---|
| 1 | 0.0E+00 | 1.5E+07 | 0 | 0.0E+00 | 5M/5F |
| 3,5 | 5.0E+09 | 1.5E+07 | 100 | 6.3E+06 | 5M/5F |
| 2,4 | 5.0E+09 | 1.5E+07 | 100 | 6.3E+06 | 5M/5F |
| 6,7 | 5.0E+09 | 1.5E+07 | 100 | 6.3E+06 | 5M/5F |

## Results

### Spore Preparation

*B. anthracis* Ames spores were grown in DSM and purified over Renografin gradients. Microscopic analysis determined that the spore preparation was 99% phase bright spores. Additionally, the spore preparation contained < 0.7 endotoxin units / mL. A phase contrast micrograph of the purified preparation is shown in Figure 1.

**Figure 1. Renografin purified *B. anthracis* Ames spores.**




### Particle Size Distribution

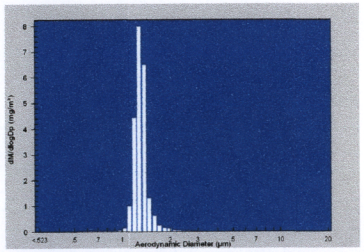
Particle size distribution was determined from Aerodynamic Particle Sizer (APS 3321) samples collected from the inhalation exposure plenum. All APS samples were 20 seconds in duration and collected at approximately t = 30 minutes. The mean count median aerodynamic diameter (CMAD) for all spore exposed groups was 1.3 ± 0.0 µm. The mean geometric standard deviation for all spore exposed groups was 1.2 ± 0.0. The mean mass median aerodynamic diameter (MMAD) for all spore exposed groups was 1.3 ± 0.0 µm. The mean geometric standard deviation for all spore exposed groups was 1.2 ± 0.0. BAA particle size distribution data is shown in Table 2. A representative graph of the mass distribution is shown in Figure 2.

## Results

**Table 2. *B. anthracis* Ames Particle Size Distribution Data**

| Group Number | MMAD (µm) | GSD | CMAD (µm) | GSD |
|---|---|---|---|---|
| 3,5 | 1.3 | 1.2 | 1.3 | 1.2 |
| 2,4 | 1.3 | 1.2 | 1.3 | 1.2 |
| 6,7 | 1.3 | 1.2 | 1.3 | 1.2 |
| Mean | 1.3 | 1.2 | 1.3 | 1.2 |
| Stdev | 0.0 | 0.0 | 0.0 | 0.0 |
| %CV | 0.5 | 0.5 | 0.4 | 0.5 |

**Figure 2. *B. anthracis* Ames Spore Mass Distribution**




### Inhaled Dose

Inhaled dose (CFU) was defined as the product of aerosol concentration, murine minute volume, and exposure time. Murine minute volume was dependent on the weight of the animals on the day of challenge and calculated using Guyton's formula[2]. Group mean body weight, minute ventilation and inhaled dose data are shown in Tables 3a&b.

**Table 3a. Inhaled Dose for Males**

| Group Number | Mean Body Weight (g) | Mean Aerosol Concentration (CFU/L) | Mean Minute Volume ($cm^3$/min) | Mean Inhaled Dose (CFU) |
|---|---|---|---|---|
| 1 | 23.64 | 0.0E+00 | 22.51 | 0.0E+00 |
| 2 | 25.28 | 2.1E+06 | 23.68 | 3.0E+06 |
| 3 | 25.26 | 2.1E+06 | 23.66 | 3.0E+06 |
| 4 | 24.20 | 2.1E+06 | 22.91 | 2.9E+06 |
| 5 | 24.94 | 2.1E+06 | 23.44 | 3.0E+06 |
| 6 | 25.48 | 1.9E+06 | 23.82 | 2.7E+06 |
| 7 | 24.84 | 1.9E+06 | 23.37 | 2.7E+06 |
| | | MEAN | 23.34 | 2.9E+06 |
| | | STDEV | 0.47 | 1.4E+05 |
| | | % CV | 2.0 | 4.9 |

**Table 3b. Inhaled Dose for Females**

| Group Number | Mean Body Weight (g) | Mean Aerosol Concentration (CFU/L) | Mean Minute Volume ($cm^3$/min) | Mean Inhaled Dose (CFU) |
|---|---|---|---|---|
| 1 | 18.62 | 0.0E+00 | 18.82 | 0.0E+00 |
| 2 | 18.42 | 2.1E+06 | 18.67 | 2.4E+06 |
| 3 | 18.90 | 2.1E+06 | 19.04 | 2.4E+06 |
| 4 | 19.48 | 2.1E+06 | 19.47 | 2.5E+06 |
| 5 | 19.02 | 2.1E+06 | 19.13 | 2.4E+06 |
| 6 | 19.22 | 1.9E+06 | 19.28 | 2.2E+06 |
| 7 | 19.10 | 1.9E+06 | 19.19 | 2.2E+06 |
| | | MEAN | 19.08 | 2.3E+06 |
| | | STDEV | 0.27 | 1.1E+05 |
| | | % CV | 1.4 | 4.9 |

### Deposited Dose

Deposited dose was calculated by euthanizing mice immediately following exposure and analyzing the lung homogenates by plating on TSAB. For male mice the mean deposited dose (MDD) was 8.2E+05 CFU with as standard deviation of 6.7E+04. For female mice the MDD was 6.4E+05 CFU with a standard deviation of 1.4E+05.

## Results

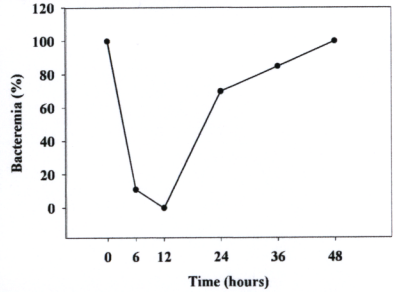
### Bacteremia

Bacteremia was analyzed at time of scheduled euthanasia, time of death, or time of moribundity. Bacterial load levels were up to >3.0E+05 CFU/mL in bacteremic animals. Initial group size was 10 animals per group. However, results of bacteremia analyses were interpreted based on actual time of blood collection. The final group sizes and percent bacteremia are listed in Table 4. Onset of bacteremia is presented in Figure 3.

**Table 4. Final Group Sizes**

| Group Number | Time (h) | Bacteremia (%) | Number of Animals |
|---|---|---|---|
| 7 | 0 | 100 | 10 |
| 2 | 6 | 11 | 9 |
| 3 | 12 | 0 | 10 |
| 4 | 24 | 70 | 10 |
| 5 | 36 | 85 | 13 |
| 6 | 48 | 100 | 5 |

**Figure 3. Onset of Bacteremia in BALB/c Mice**




## Conclusions

The MDD for male and female mice was 8.2E+05 CFU and 6.4 E+05 CFU, respectively. Bacteremia was observed immediately following inhalation exposure. It is likely that the observation of bacteremia at T=0 and T=6 was the result of residual BAA spores in the blood. The presence of vegetative cells was likely first observed at T=24[3]. Because administration of antibiotic treatment after the onset of bacteremia may not effectively resolve anthrax[1], this study suggests that it may be necessary to begin antibiotic therapy earlier than 24 hours post exposure.

## References

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3. **Sanz, P., L.D Teel, F. Alem, H. M. Carvalho, S. C. Darnell, and A. D. O'Brien.** 2008. Detection of *Bacillus anthracis* spore germination in vivo by bioluminescence imaging. Infect. Immun. 3:1036-47.

## Acknowledgements

**This contract was funded by the National Institutes of Health, National Institute of Allergy and Infectious Disease, Contract N01-AI-30063.**