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Comparison of Blood Collection Methods in the Early Stages of Anthrax Bacteremia in Mice

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Abstract

Background
Bacillus anthracis is the causative agent of the disease anthrax in humans and animals. The route of blood collection could affect bacterial counts from potential ocular and dermal contamination during a nose-only inhalation exposure. The comparison of different blood collection routes to determine bacterial load during the early stages of bacteremia has not been well described.

Methods
In this study we compared the retro-orbital sinus and intra-cardiac puncture blood collection routes during the early stages of the onset of bacteremia. Seven groups of BALB/c mice, consisting of five males and five females, were exposed by nose-only inhalation to an average aerosol concentration of 3.2E+06 CFU of *B. anthracis* Ames spores. Mice were euthanized at T=0 (immediately following exposure), 6, 12, 18, and 24 hours post-exposure. Blood samples were collected via retro-orbital sinus or intra-cardiac puncture at 0 and 6 hours and by retro-orbital bleeding at subsequent time collections. Blood was serially diluted, plated, and incubated. The plates were counted to determine the number of CFU/mL.

Results
For retro-orbital blood collection, bacteremia was observed in 100% of mice at T=0 and 50% at 6 hours. The average bacterial counts were 1.1E+03 CFU/mL and 77 CFU/mL, respectively. For cardiac puncture blood collection, bacteremia was observed in 90% of mice at T=0 and 40% at 6 hours. The average bacterial counts were 5.9E+03 CFU/mL and 323 CFU/mL, respectively. By 12 hours, 100% of mice were negative for *B. anthracis* in the blood followed by 100% and 50% at 18 and 24 hours, respectively.

Conclusions
These results suggest there was there was a difference in the percent of bacteremia at T=0 and 6 hours post exposure between collection methods, but the differences were insignificant. Initial bacterial concentrations ranged between 7.7E+01 to 5.9E+03 at 0 to 6 hours. The bacterial load from different routes of collections was insignificant.

Introduction

Blood can be collected from animals using different techniques with differences in handling, restraining, anesthesia, invasiveness and the volume taken. Blood collection via the retro-orbital sinus or intra-cardiac puncture are standard techniques to obtain blood samples from mice. Comparisons of blood collection techniques and their effects on blood analyses have been documented.[1-3] However, there is a lack of information on collection methods for bacterial samples from mice in an aerosol challenge model.

The onset of bacteremia of *Bacillus anthracis* Ames (BAA) during the first 24 hours in mice is crucial to determining the optimal time for antibiotic or post therapeutic treatment. Accurately determining the presence of bacteremia is critical to eliminate the possibility of ocular and dermal contamination.

In development of the inhalational anthrax murine model, an initial study on the onset to bacteremia resulted in mice challenged with an aerosol concentration of 2.0E+06.[4] Blood samples were collected at 0, 6, 12, and 24 hours post challenge for bacterial load. All mice euthanized immediately after aerosol challenge had bacteremia. Colony counts ranged from 1.3E+02 CFU/mL to greater than 3.0E+05 CFU/mL. The onset to bacteremia started 12 hours earlier and the bacterial counts were higher than published data.[5] It was not clear if the retro-orbital blood samples were contaminated from the aerosol challenge route at 0 and 6 hours. Therefore, we proposed to compare blood collection via intra-cardiac puncture and retro orbital sinus immediately after exposure and six hours after aerosol challenge.

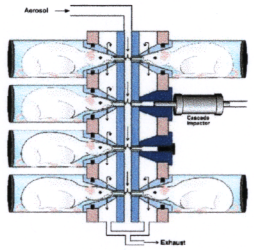
Methods

Challenge Material Preparation:
BAA spores were prepared by growing cultures in Difco Sporulation Media to nutrient exhaustion. The sporulated culture was harvested by centrifugation. Spores were then purified over Renografin-60 density gradients to remove vegetative cells and debris. The purified spores were resuspended in USP-Water For Injection (WFI). The spores were observed using phase contrast microscopy to quantify the number of phase bright spores, phase dark spores, vegetative cells, and other debris. Endotoxin levels were tested using the limulus amebocyte lysate assay to ensure that the spore preparation contained less than 1 endotoxin unit per milliliter. Spore number was quantified by serial dilution in USP-WFI and plating on TSA with 5% sheep's blood plates and determining the number of CFU/mL. Prior to exposure, the spore suspension was serially diluted in USP-WFI to achieve a concentration of 5.0E+09.

Methods

Aerosol Challenge of BALB/c Mice:
Seven groups consisting of 5 male and 5 female BALB/c mice were placed in nose-only restraint tubes and connected to the exposure chamber using Positive Flow-By™ nose cones (In-Tox Products, LLC; Albuquerque, NM) as shown in Figure 1. The Collison nebulizer was filled with a pre-mixed nebulizer stock suspension of 5.0E+09 BAA and operated at a constant pressure. The start of the exposure period (T = 0) began once the nebulizer was activated. The actual concentration of challenge material in the exposure atmosphere was determined by analysis of stainless steel impinger samples collected from the breathing zone of the animals during the exposure. Impinger samples were analyzed on the day of collection. Aerosol concentration were reported as colony forming units per liter of air (CFU/L). Real-time aerosol concentration was monitored using a laser-based aerosol photometer. Each group was exposed for approximately 60 minutes.

Figure 1. Positive Flow-By™ Nose Cones



Blood Collection Methods:
Blood from the retro-orbital sinus was collected at 0, 6, 12, 18, and 24 hours. Two additional groups were bled via the intra-cardiac puncture at 0 and 6 hours. All blood collections were terminal bleeds. Blood was serially diluted, plated, and incubated. The plates were counted to determine the number of CFU/mL.

Intra Cardiac Puncture Blood Collection Method:
The mice were anesthetized prior to euthanasia and the thoracic cavity was wiped with 70% ethanol. The thoracic cavity was palpated to identify the bottom of the rib cage, the needle inserted at the base and under the rib cage to the heart. Blood was collected into the syringe and placed into the collection tube.

Retro-orbital Sinus Blood Collection Method:
The mice were anesthetized prior to euthanasia and the head immobilized. The heparinized microhematocrit capillary tube was inserted into the eye socket. The first drop of blood was discarded to flush out any heparin and then held so that the blood flows from the capillary tube into the collection tube.

Microbiological Analyses:
Blood samples were serially diluted in USP-WFI, plated in triplicate on blood agar and incubated for colony formation. The plates were then counted to determine the number of CFU/mL.

Results

Inhalation:
BALB/c mice were exposed by nose-only inhalation to a mean aerosol concentration of 3.2E+06 CFU/L of BAA spores. Individual aerosol concentrations with for each group are shown in Table 1. Using Guyton's formula[6] the calculated mean inhaled dose for male and female mice combined was 4.1E+06 CFU and corresponded to an inhaled dose of 44 LD_{50} (1 LD_{50} = 9.4E+04 CFU based on prior data).[7]

Table 1. Summary of Aerosol Concentration Data

Time of Blood Collection (Hour)	Impinger Concentration (CFU/mL)	Aerosol Concentration (CFU/L)	Mean Inhaled Dose (CFU)
0	3.20E+07	2.60E+06	3.30E+06
6	5.70E+07	4.50E+06	5.90E+06
12	3.20E+07	2.60E+06	3.25E+06
18	5.70E+07	4.50E+06	5.85E+06
24	3.20E+07	2.40E+06	3.10E+06

Results

Retro-orbital Sinus vs. Intra-cardiac Puncture Blood Collection:

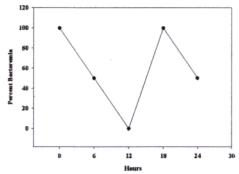
Percent Bacteremia
The percent of bacteremia is shown in Table 2. The percent of bacteremia in mice was 100% and 90% at 0 hours in retro-orbital sinus and intra-cardiac puncture blood collections, respectively. At 6 hours 50% and 40% of mice had bacteremia in retro-orbital sinus and intra-cardiac puncture blood collections, respectively. Subsequent blood collections were only taken via the retro-orbital sinus. Figure 2 illustrates the percent of bacteremia from retro-orbital samples collected at all time points within 24 hours post exposure.

Table 2. Percentage of Mice with *Bacillus anthracis* Ames Bacteremia

Route of Blood Collection*	Time of Blood Plating (Hour)	Percent Bacteremia (%)
RO	0	100
IC	0	90
RO	6	50
IC	6	40
RO	12	0
RO	18	100
RO	24	50

*RO – retro-orbital sinus
*IC- intra-cardiac puncture

Figure 2. Percentage of Mice with *Bacillus anthracis* Ames Bacteremia From Blood Collected Via the Retro-orbital Sinus



Bacterial Concentration
Table 3 summarizes the bacterial loads of BAA in the blood taken at T=0, 6, 12, 18, and 24 hours post exposure. Initial concentrations were 1.1E+03 CFU/mL and 5.9E+03 for retro-orbital sinus and intra-cardiac puncture blood collections, respectively at T=0. At 6 hours the concentrations decreased to 77 CFU/mL and 323 CFU/mL for retro-orbital sinus and intra-cardiac puncture blood collections, respectively. Bacteremia was characterized by an initial spike followed by a rapid decline with no detectable bacteria at 12 hours as shown in Figure 3. A rapid increase was identified with concentrations of 3.4E+02 at 18 hours and 3.4E+06 at 24 hours.

Subsequent analysis was performed using the criteria listed by the Food and Drug Administration.[8] Bacterial counts less than 25 CFU per plate were excluded from the additional bacterial count analyses as they were below the detectable limit of quantification (LOQ) for aerobic bacteria. One sample at 6 hours post challenge had a detectable colony count, all other samples were below the level of quantification.

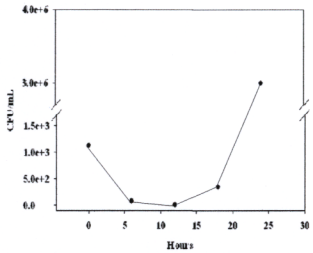
Table 3. Average Concentration of *Bacillus anthracis* Ames in Blood

Time Collection (Hours)	Initial Data Analysis RO[1] (CFU/mL)	Initial Data Analysis IC[2] (CFU/mL)	Subsequent Data Analysis RO[1] (CFU/mL)	Subsequent Data Analysis IC[2] (CFU/mL)
0	1.12E+03	5.91E+03	2.87E+03	1.40E+04
6	7.67E+01	3.23E+02	BLOQ[3]	2.97E+03
12	0.00E+00		BLOQ[3]	
18	3.43E+02		BLOQ[3]	
24	3.39E+06		BLOQ[3]/TNTC[4]	

RO[1] – retro-orbital sinus
IC[2]- intra-cardiac puncture
BLOQ[3] - Below the level of quantification
TNTC[4] - Too numerous to count

Results

Figure 3. Concentration of *Bacillus anthracis* Ames From Blood Collected via the Retro-Orbital Sinus



Conclusions

Bacteremia was characterized by an initial spike followed by a rapid decline and then rapid increase in mice euthanized from 0 hours to 24 hours. These results suggest that blood collection from the retro-orbital sinus is an acceptable method following an inhalation exposure.

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Acknowledgements

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