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Development of a Murine *Bacillus anthracis* Treatment Model Using a 21-Day Ciprofloxacin and Doxycycline-Dosing Schedule

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ABSTRACT

Objective: An aerosol model of infection for *Bacillus anthracis* was developed to evaluate therapeutic delayed treatment strategies with ciprofloxacin and doxycycline. Beginning at 42 and 48 hours post-challenge, mice were dosed with ciprofloxacin and doxycycline for 21 days. The relapse potential after antibiotic treatment was also evaluated during a post-dosing recovery period.

Methods: *B. anthracis* Ames spores were prepared using the medium and growth conditions of Leighton and Doi (1971). The spore titer was determined using serial dilution plating on Trypticase Soy Agar. For the determination of the LD_{50}, challenge concentrations of 0, $1.19x10^2$, $1.70x10^4$, $1.85x10^5$, and $2.91x10^6$ CFU/mL were used.

An inhaled dose of $1.30x10^6$ *B. anthracis* spores was administered to BALB/c mice via nose-only exposure. Ciprofloxacin (twice/day at 30 mg/kg/dose) and doxycycline (twice/day at 40mg/kg/dose), were initially administered at either 42 or 48 hours after aerosol challenge via the intraperitoneal (IP) route for 21 days. Control animals were administered sterile saline IP twice/day. Animals were monitored for mortality twice daily after challenge through day 60.

Results: The LD_{50} was determined to be $3.16x10^4$ CFU/mouse. The administered inhaled dose was $1.30x10^6$ CFU/mouse. Survival values for mice treated with doxycycline at either 42 or 48 hours after challenge were 90% and 70%, respectively, following the 21 day dosing period. Survival values of mice treated with ciprofloxacin at either 42 or 48 hours after challenge were 100% for both groups. At the conclusion of the 60 day study, survival was 100% for ciprofloxacin groups dosed at either 42 or 48 hours post-challenge, while the doxycycline groups dosed at either 42 or 48 hours post-challenge showed decreased survivals of 70% and 60%, respectively. Control mice had a 20% survival rate.

Conclusions: Ciprofloxacin demonstrated complete protection against aerosolized *B. anthracis* throughout the recovery phase for both 42 and 48 hours delayed treatment groups, while doxycycline demonstrated partial protection during the dosing and recovery phases in both of the delayed treatment groups.

INTRODUCTION

Bacillus anthracis, a Gram-positive bacterium with the capability to form endospores, is the causative agent for inhalational anthrax. Additionally, *B. anthracis* is a potential agent to be used for bioterrorism purposes especially by release as an aerosol. Inhalational anthrax symptoms in mice are generally observed within a few days and may approach 100% lethality (Twenhafel, 2010). IITRI's historical data indicate that, depending on the dose, untreated mice generally succumb to inhalational anthrax as soon as 24 hours.

Our goals for this study were three-fold:

1) to develop and characterize a BALB/c murine model for inhalation anthrax using an IITRI-developed nose-only bioaerosolization system;
2) to determine the aerosol LD_{50} of a *B. anthracis* Ames in BALB/c mice; and
3) to evaluate and compare the efficacy of a delayed treatment schedule with either the bactericidal ciprofloxacin or the bacteriostatic doxycycline in BALB/c mice exposed to an aerosol challenge of *B. anthracis.*

MATERIALS AND METHODS

***B. anthracis* Challenge Material:** *B. anthracis* Ames stocks, previously prepared using medium and growth condition of Leighton and Doi (1971) and stored at approximately 2-8°C, were heat shocked for approximately 45 minutes at 60−65°C. The titer of the *B. anthracis* spores was subsequently determined by serial dilution with sterile ASTM 1 water, and the stocks were plated on Trypticase Soy Agar (TSA) and incubated at 37°C±2°C for approximately 18−22 hours. The colonies were approximately 2-4 mm in diameter with a cream, flat, matte appearance with irregular edges.

***B. anthracis* Aerosol Generation:** The culture was serially diluted to the desired concentrations in sterile water for injection for inhalation exposure to minimize foaming during the aerosolization process. *B. anthracis* aerosols were generated using the IITRI bioaerosol system that utilizes a 64-port, flow-past, nose-only inhalation exposure chamber (Lab Products Inc., Seaford, DE) and six Pari LC Plus jet nebulizers (Pari, Germany). With 20 PSI of pressurized air, each nebulizer has a flow rate of approximately 5 LPM each with a cumulative total of approximately 30 LPM flow rate with all six nebulizers operating concurrently. All exhaust air was passed through a series of HEPA filters prior to exiting the facility. Viable aerosol sampling from the breathing zone of the exposure chamber was determined by two all glass impingers (AGI) connected in series with each containing 10 mL of sterile water for injection. Additionally, 3 mm glass beads were included in the upstream impinger to improve collection efficiency. Aerosol particle size distribution was measured by a viable 6-stage Andersen cascade impactor (Tisch Environmental Inc., OH). TSA plates were used as the collection medium in the Andersen cascade impactor. Time of exposure for a typical run was approximately 10 minutes. All titers of *B. anthracis* were determined on TSA and incubated at 37°C±2°C for approximately 48 hours.

LD_{50} Determination: Animals were 9-week-old female BALB/c mice weighing on average 18 g to 20 g. Each experimental group had 10 mice.

Antibiotic Administration: Based upon methods developed by Heine *et al.* (2007), ciprofloxacin (30 mg/kg) or doxycycline (40 mg/kg) was administered twice/day (q12h) via the intraperitoneal dosing route. The dosing schedule was initiated 42 or 48 hours after exposure for the delayed treatment assessment. After completion of the dosing phase, animals were monitored for an additional 39 days each (relapse observation phase).

RESULTS

Table 1. Summary of Aerosol Exposure Parameters for LD_{50} Determination in BALB/c Mice

		Group 1	Group 2	Group 3	Group 4	Group 5
B. anthracis Concentration	Post-Challenge Titer (CFU/mL)	0	$1.90x10^5$	$1.79x10^7$	$3.30x10^8$	$2.73x10^9$
	Presented Dose (CFU/animal)	0	$1.19x10^2$	$1.70x10^4$	$1.85x10^5$	$2.91x10^6$
Average Particle Size	Particle diameter (µm)	NA	~1			
Spray Factor (Average: $3.0x10^6$)			$1.63x10^{-6}$	$2.74x10^{-6}$	$3.06x10^{-6}$	$4.59x10^{-6}$

Table 2. Summary of Mean Time to Death and Survival for LD_{50} Determination in BALB/c Mice

		Number of Animals*		
	Actual Challenge	Females (Total)		Survival
Group	(CFU/animal)	Dead	Alive	(%)
1	0	0	10	100%
2	$1.19x10^2$	0	10	100%
3	$1.70x10^4$	3	6	67%
4	$1.85x10^5$	6	3	33%
5	$2.91x10^6$	10	0	0%

The calculated LD_{50} (Reed-Munch) was determined to be $3.16x10^4$ CFU/Animal.

*Excludes animals that died prior to exposure that were found dead immediately after exposure or had an unexplained death.

Figure 1. Survival of BALB/c Mice at Different Exposure Concentrations of *B. anthracis*

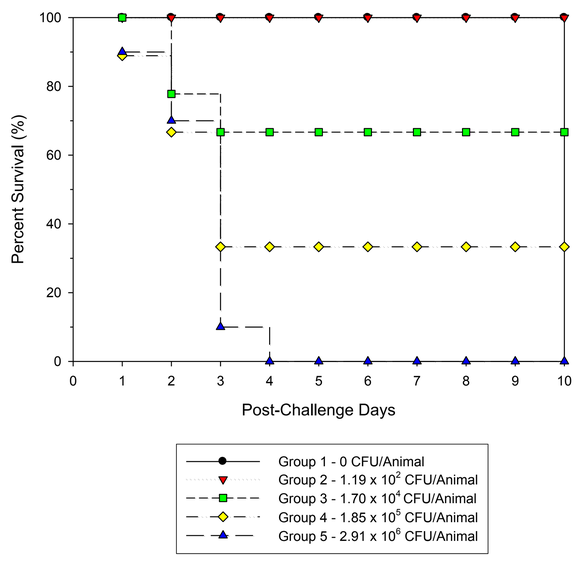
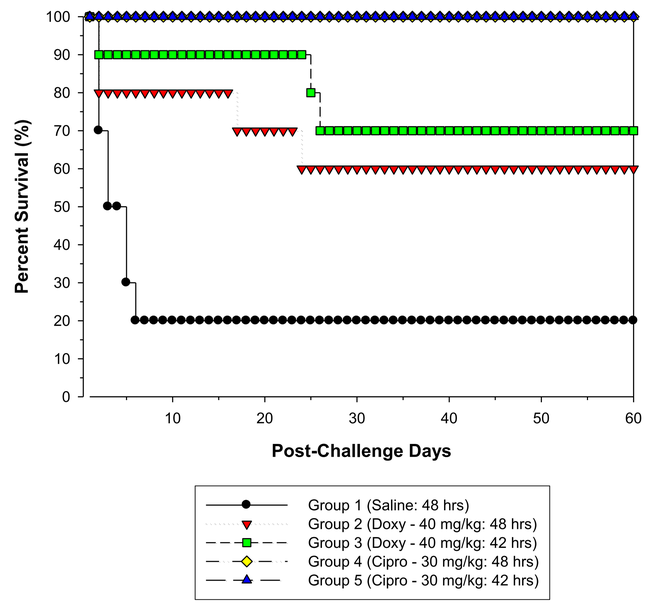


Table 3. Survival of Ciprofloxacin and Doxycycline Dose Results

Group	Test/Control Material	Time of Initial Dose (Post-Challenge)	Dose Concentration (q12h schedule) (mg/kg)	Challenge Dose CFU/mouse	Percent Survival after 21 days of dosing	Percent Survival after 60 days	Mean Time to Death (MTD-Days)
1	Saline	48	--	$1.30x10^6$	20%	20%	3.5
2	Doxycycline (40 mg/kg)	48	40 mg/kg		70%	60%	11.3
3	Doxycycline (40 mg/kg)	42	40 mg/kg		90%	70%	17.7
4	Ciprofloxacin (30 mg/kg)	48	30 mg/kg		100%	100%	0.0
5	Ciprofloxacin (30 mg/kg)	42	30 mg/kg		100%	100%	0.0

Figure 2. Survival of BALB/c Mice after Initiation of Ciprofloxacin or Doxycycline Dosing at 42 or 48 Hours Post-challenge



CONCLUSIONS

- LD_{50} was determined to be $3.16x10^4$ CFU/mouse.
- When challenged with *B. anthracis* at $1.30x10^6$ CFU/animal, all BALB/c mice survived with 30 mg/kg ciprofloxacin (q12h) when the first dose was administered within 42 or 48 hours post-exposure. Dosing continued for 21 days thereafter. No relapse was observed during the relapse observation period (days 22-60).
- When challenged with *B. anthracis* at $1.30x10^6$ CFU/animal, there was 90% and 70% survival after 21 days of dosing with 40 mg/kg doxycycline (q12h) with the first dose administered 42 and 48 hours after challenge, respectively. During the relapse observation period (days 22-60), 70 and 60% of the animals survived in the doxycycline 42 and 48 hour groups, respectively.
- A 20% survival was observed in mice that received saline. Mice died within 2-6 days post-exposure.
- While protection from aerosolized *B. anthracis* was evident from either the bactericidal or the bacteriostatic antibiotics (ciprofloxacin and doxycycline, respectively), this study demonstrates that bactericidal treatment for 21 days is effective in preventing relapse of the disease. However, treatment of a bacteriostatic antibiotic such as doxycycline may require an extended period of treatment to prevent a relapse of the disease.

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