The *in vitro* Evaluation of the Effect of Linezolid and Levofloxacin on *Bacillus anthracis* Toxin Production, Spore Formation and Cell Growth

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Abstract:

Bacillus anthracis, the etiological agent of anthrax, is a spore- forming, toxin- producing bacterium. Currently, treatment of *B. anthracis* infections requires a 60- day antibiotic regimen. However, better therapeutics are required. Therefore, this study looked at the effect of levofloxacin and linezolid on *B. anthracis* cell viability, toxin production and spore formation using *in vitro* static models and a pharmacodynamic model. It was hypothesized that the combination would be the most effective at preventing toxin and spore production resulting in greater bacterial killing. However, these studies suggest otherwise. Nevertheless, clinically, the combination therapy may be more effective in rapid killing of vegetative *B. anthracis* and may be able to reduce the duration of therapy (by reducing the likelihood of spore survival). Therefore, the clinical benefit of combined therapy on long-term recurrence cannot be determined from these *in vitro* models. Further investigation with combination therapy is warranted.

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List of Copyrighted Material for Which Permission was Obtained:

List of Abbreviations:

AbrB - transition state regulator

ANOVA- Analysis of Variance

ATP- adenosine triphosphate

ATR- anthrax toxin receptor

AtxA- anthrax toxin activator

AVA- Anthrax Vaccine Absorbed

AVP- Anthrax Vaccine Precipitated

BA- Blood Agar

BID- twice a day

Blast- basic local alignment search tool

Blood agar- Tryptic soy agar containing 5% Sheep blood

Bp- basepairs

BWC- Biological Weapons Convention

CA- Cutaneous anthrax

CAD- Canadian dollars

cAMP- 3, 5'-cyclic adenosine monophosphate

CB-M- Columbia broth with $MnSO_4$

CFU- colony forming units

CMG-2- capillary morphogenesis protein-2

CO_2- carbon dioxide

DNA- deoxyribonucleic acid

EDTA- ethylene diamine tetra acetic acid

EF- edema factor

ELISA- enzyme-linked immunosorbant assay

ET- edema toxin

FDA- Food and Drug Administration

FICI- fractional inhibitory concentration index

g- gravity

GA- Gastrointestinal anthrax

IA- Inhalational anthrax

IBM- International Business Machines Corporation

IPDM- *in vitro* pharmacodynamic model

Kb- kilobase

kDa- kilodalton

LB- Luria- Bertani

LD_{50}- lethal dosage able to kill 50 percent of the exposed individuals

LF- lethal factor

LT- Lethal toxin

MAPKK- mitogen-activated protein kinase kinase

MBC- Minimum bactericidal concentration

MHB- Cation- adjusted Mueller Hinton Broth

MIC- Minimum inhibitory concentration

mM- millimolar

$MnSO_4$- manganese sulfate

MRSA- methicillin- resistant *Staphylococcus aureus*

NCBI- National Center for Biotechnology Information

NHPs- non-human primates

NML- National Microbiology Laboratory

PA- protective antigen

PA83- 83 kDa PA protein

PAI- pathogenicity island

PCR- polymerase chain reaction

QD- once a day

SC- subcutaneous

SDS- sodium dodecyl sulfate

SEM- standard error of the mean

SigH- sigma factor H

SPSS- Statistical Package for the Social Sciences

TBE- Tris-Borate EDTA

TEM-8- tumor endothelial marker 8

UK- United Kingdom

US- United States

UV- ultraviolet

WHO- World Health Organization

Zn^{2+}- zinc

CHAPTER 1.0: INTRODUCTION

1.1 Introduction to *Bacillus anthracis*

1.1.1 Bacteriology and Epidemiology

Bacillus anthracis, the causative agent of anthrax, is an aerobic, toxin-producing, Gram-positive, rod shaped bacterium that belongs to the *Bacillus cereus* subgroup in the *Bacillaceae* family [1,2]. This subgroup is comprised of both pathogenic and non-pathogenic bacilli including *Bacillus weihenstephanensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, *B. anthracis*, and *B*. *cereus* (the parent strain) that are recognized for having complex genomes (containing multiple plasmids of various sizes, prophages or unique repeats) and their ability to form resistant endospores [3-7]. Although the majority of the members share several phenotypic properties, *B. anthracis* is distinguishable since it is non-motile and non-hemolytic on sheep blood agar [1]. These differences are due to the fact that *B. anthracis* has a mutation in the global transcriptional regulator gene, PlcR, which is responsible for activating the transcription of numerous *B. cereus* genes encoding virulence factors (including enterotoxins and degradative enzymes), transporters and environmental sensors [8,9].

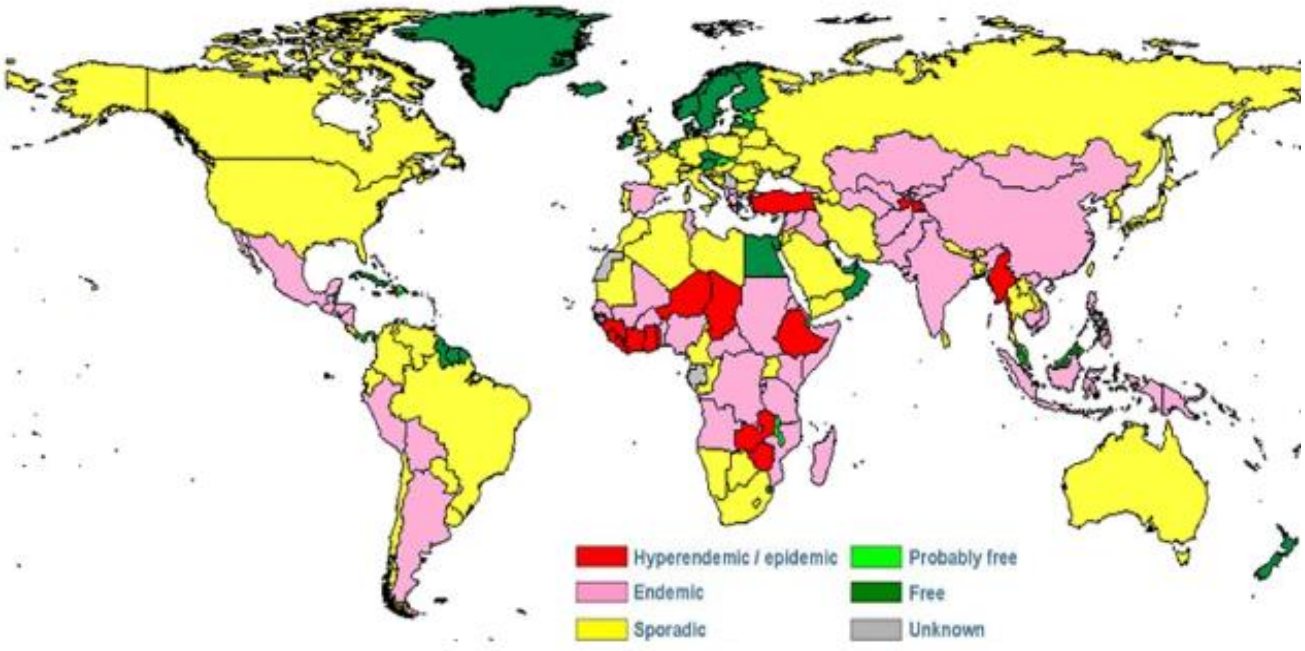
Like other bacilli, *B. anthracis* is readily found in the soil where it primarily exists as a dormant spore. It can infect most mammals (including humans) and is responsible for causing disease in grazing animals which include livestock (cows, sheep, and goats) and wild animals (bison, buffalo) [10]. Previous animal outbreaks have been recorded, with the largest epidemic occurring in 1945 in Iran where more than 1 million sheep died due to anthrax [11-14]. Once

the animal is dead, carcasses containing vegetative bacteria in the tissues, fluids and hides can lead to further contamination of the soil.

Human *B. anthracis* infections, although rare in the west, have been reported all over the world and is a particular problem in endemic countries in Africa, Latin America, Eastern Europe and Asia (Figure 1.1.1), having infected 20 000 to 100 000 people in the first half of the 20th century [10,15-19]. Turkey, Greece and Bangladesh are particularly affected due to common practices of animal husbandry, lack of protective measures (such as animal vaccinations) and lack of knowledge about *B. anthracis* [20-22]. Between August 2009 and October 2010, 14 outbreaks of anthrax occurred in Bangladesh resulting in 140 animal infections and 273 cases of cutaneous anthrax [22]. In all of the human cases, infection occurred after butchering anthrax-infected animals, or handling contaminated meat or animal hides. In addition, since the habitants were unaware of the proper way to dispose of the dead animals, the anthrax-diseased animals were discarded in fields or rivers which may have led to further contamination of the environment.

In Europe and the United Kingdom (UK), contaminated heroin originating in Afghanistan likely contributed to the 2009 outbreak due to casing the drug in skins of goats that died from anthrax [23,24]. Similarly, in Andhra Pradesh, India in 2011, 9 villagers were infected with cutaneous anthrax (CA) following exposure to anthrax- ridden dead livestock (i.e. handling, burying, skinning, and slaughtering) [25].

Figure 1.1.1. World distribution map of anthrax as determined by the World Health Organization (WHO) (updated in 2003)



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1.1.2 Clinical Presentations

B. anthracis can be transmitted to humans when the endospores enter the body through one of four routes which include direct contact, ingestion, aerosolization or injection resulting in cutaneous, gastrointestinal, inhalational and injectional anthrax, respectively [26].

1.1.2.1 Cutaneous Anthrax

CA, the least severe, albeit the most common form of anthrax, represents approximately 95% of all reported cases with an annual estimate of 2 000 cases worldwide [15,27,28]. To date, the largest CA epidemic was reported in Zimbabwe (1979-1985) and affected almost 10 000 people [14]. Symptoms of CA usually manifest 1- 8 days after infection and presents as an isolated infection on the exposed area (most often the face, neck, hands or arms) following abrasion [15,27]. Initially, a small, painless papule (less than 4 cm in diameter) develops at the site of infection along with a low-grade fever [29]. Within a week a black necrotic skin eschar with extensive edema and erythema develops [15,27]. Although CA is often self- limiting and is rarely fatal, it can lead to extensive edema and shock [17,30-32]. Therefore, prompt treatment is critical. The CA lesions can be easily treated with antibiotic therapy [27,29]. In 90% of cases, within 1- 2 weeks the anthrax eschar dries and sloughs off without scaring or complications [27].

1.1.2.2 Gastrointestinal Anthrax

Gastrointestinal anthrax (GA) is more severe although rare, with no cases having been previously reported in the United States (US) [16]. GA occurs following the consumption of

poorly cooked *B. anthracis*- contaminated meat and has been responsible for outbreaks in Asia and Africa [16,33-35]. GA can present as either an intestinal or oropharyngeal infection (less common) and typically has an incubation period of 1 to 6 days [35]. Symptoms of intestinal GA are considered non-specific (nausea, vomiting, fever, bloody diarrhea and malaise) and are often misdiagnosed, leading to treatment delays and high mortality rates of over 50% [10,16,36]. In fact, this was the case in the 1960's GA outbreak in Lebanon [16]. Patients admitted to initially having low-grade fever, headaches, asthenia, nausea and fainting spells although many of them did not seek medical attention since they assumed they had a viral infection. Mild to severe symptoms persisted for 24 hours and was followed by constant abdominal pain. Next, ascites developed, along with some gastrointestinal bleeding, red conjunctivae and shock which was quickly followed by death. Upon autopsy, extreme edema of the bowel walls was noted in addition to a central necrotic lesion approximately 2- 3 cm in diameter [16].

1.1.2.3 Inhalational Anthrax

Inhalational anthrax (IA) is the most severe form of anthrax and has a mortality rate of up to 90% if left untreated or treated improperly [37-39]. IA is biphasic in that it has two distinct stages during disease progression. The first stage can last from 1 to 7 days and is characterized by non-specific symptoms (fever, cough, chills, sweating, fatigue, dyspnea and chest or abdominal pain) which are often are misdiagnosed [37,38]. The initial phase of IA lasts for approximately 2- 4 days. Often prior to the second stage, the patient's status will slightly improve before a sudden and rapid progression into the fulminant stage [40]. Symptoms in this

phase include fever, and cardiac and pulmonary shock. The infection also commonly spreads to the brain resulting in meningitis. Usually 24 hours following respiratory distress, the patient dies [37,38].

IA infections require prompt and aggressive antibiotic therapy in order to increase the patient's chance of survival. Indeed, this was the case in 2001 when *B. anthracis* spores were sent through the US post to 7 US states including Florida, Connecticut, New Jersey, Pennsylvania, Virginia, New York and Maryland. 6 of the 6 patients that were admitted to hospital and treated with antibiotics with activity against *B. anthracis* on the same day of admittance all survived. In contrast, patients that were only administered antibiotics during the fulminant stage all perished [39].

1.1.2.4 Injectional Anthrax

Injectional anthrax is the most recently identified clinical form of anthrax and has primarily been associated with heroin drug users in the UK and Europe [10]. Since 2009, over 50 systemic cases have been reported with a mortality rate of approximately 33% [10,41-44].

Clinical symptoms usually take 1 to 10 days to manifest and often appear at the site of injection [10]. Symptoms present initially as a soft tissue infection with erythema and edema with no pus or necrosis [10,41]. In addition, unlike CA, there is no type of eschar present. Injectional anthrax is often associated with severe pain and can rapidly progress to a systemic infection leading to cardiovascular shock and death [10,41,45].

Similar to IA, if the patient is treated improperly, their chances of survival decrease drastically. This was seen in 2013 when two male patients were admitted to the hospital with injectional anthrax in Denmark [45]. The first patient, a 55- year old male, had severe pain in his thigh and abdomen, while the second patient, a 39 year old male, was admitted with a fever and a swollen arm. Although both patients were admitted with similar symptoms, the first patient was initially treated for a soft tissue infection and was not administered proper antibiotics until 24 hours after clinical symptoms had manifested. At this time he had already progressed to systemic shock and ultimately died on day 4. The second patient, on the other hand, was treated rapidly and was released after 29 days in the hospital. These cases demonstrate how crucial it is for prompt identification and treatment of *B. anthracis* and any delays in either could lead to increased fatalities.

1.1.3 *B. anthracis* Pathogenesis and Virulence

1.1.3.1 The Anthrax Toxins

B. anthracis possess two plasmids, pXO1 and pXO2, which are required for full virulence (Figure 1.1.2). pX01, the larger of the two plasmids, is approximately 182 kilobases (kb) and encodes a total of 140 genes which includes a pathogenicity island [46]. The pathogenicity island (PAI) is 44.8 kb long and contains the anthrax toxin genes pagA, lef and cya that make up the protective antigen (PA), lethal factor (LF), and the edema factor (EF), respectively as well as the toxin regulatory genes, atxA and pagR [47-51].

The atxA gene is 1.5 kb long and is located between the cya and the pag genes [52]. It is controlled by the transcription factors AbrB (the transition state regulator) and SigH (a sigma

factor), which are encoded on the *B. anthracis* chromosome [53,54]. Once atxA is activated, it produces a 56 kilodalton (kDa) cytoplasmic protein, called AtxA (anthrax toxin activator), that directly regulates toxin gene expression [52]. Toxin production has been shown to be at its highest when *B. anthracis* is grown at 37°C with 5% carbon dioxide (CO_2) in a chemically defined growth medium [50,55]. Under these conditions, toxin gene expression is at its peak at the end of the exponential growth phase [50,55-57].

Following the activation of the toxin genes, the 3 toxin components, PA, LF, and EF are produced. PA, the non-enzymatic portion of the toxin, is an 83 kDa protein (PA83) that binds to both EF and LF and facilitates toxin entry into the host cell by binding multiple cell surface receptors including the anthrax toxin receptor (ATR), tumor endothelial marker 8 (TEM-8) and capillary morphogenesis protein-2 (CMG-2) which are found on endothelial cells throughout the body [58-62]. Once PA83 is bound to host receptors it is cleaved by host proteases (furin) into two fragments referred to as PA20 and PA63 [61]. Following the disassociation of PA20, the remaining cell-bound PA63 heptamerizes and binds up to 3 molecules of LF and/or EF to form the lethal toxin (LT) or edema toxin (ET), respectively [63,64]. The toxin complexes are subsequently translocated into the host cell via receptor-mediated endocytosis and delivered to the endosome. Here, the acidic pH induces a conformational change resulting in the release of LF and EF into the cell cytosol where they can exert their enzymatic properties (Figure 1.1.3) [65,66].

EF is an 89 KDa calcium and calmodulin-dependant adenylate cyclase that catalyzes the reaction of adenosine triphosphate (ATP) to 3, 5'-cyclic adenosine monophosphate (cAMP) [67].

Following calmodulin binding, EF undergoes a conformational change resulting in a sudden increase in cAMP. This increase in cAMP prevents apoptosis, leads to impaired phagocytic, macrophage and neutrophil abilities and impairs the release of several inflammatory cytokines [68-70].

LF, a zinc (Zn^{2+}) -dependant metalloproteinase, cleaves members of the mitogen-activated protein kinase kinase (MAPKK) family leading to the disruption of several pathways including macrophage activation, maturation and chemotaxis, as well as disrupts the release of pro-inflammatory cytokines which leads to cell death [70- 72]. Together with EF, these toxin components compromise both the host innate and adaptive defenses and enable bacterial growth resulting in systemic shock, meningitis, and eventually death [37,38].

Figure 1.1.2. The two plasmids, pXO1 (181.6kb) and pXO2 (96.2kb), required for a fully pathogenic *B. anthracis* strain.

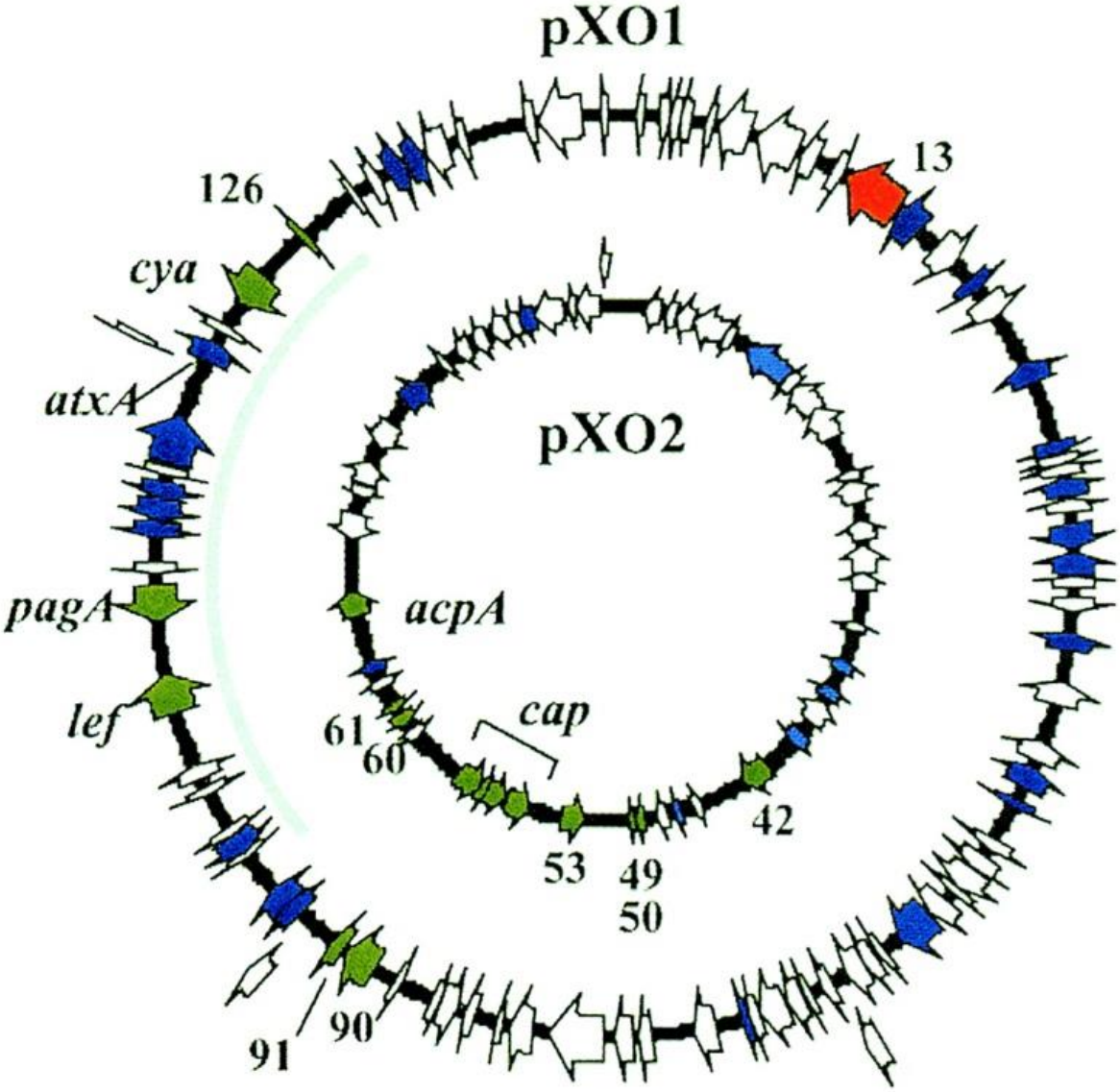

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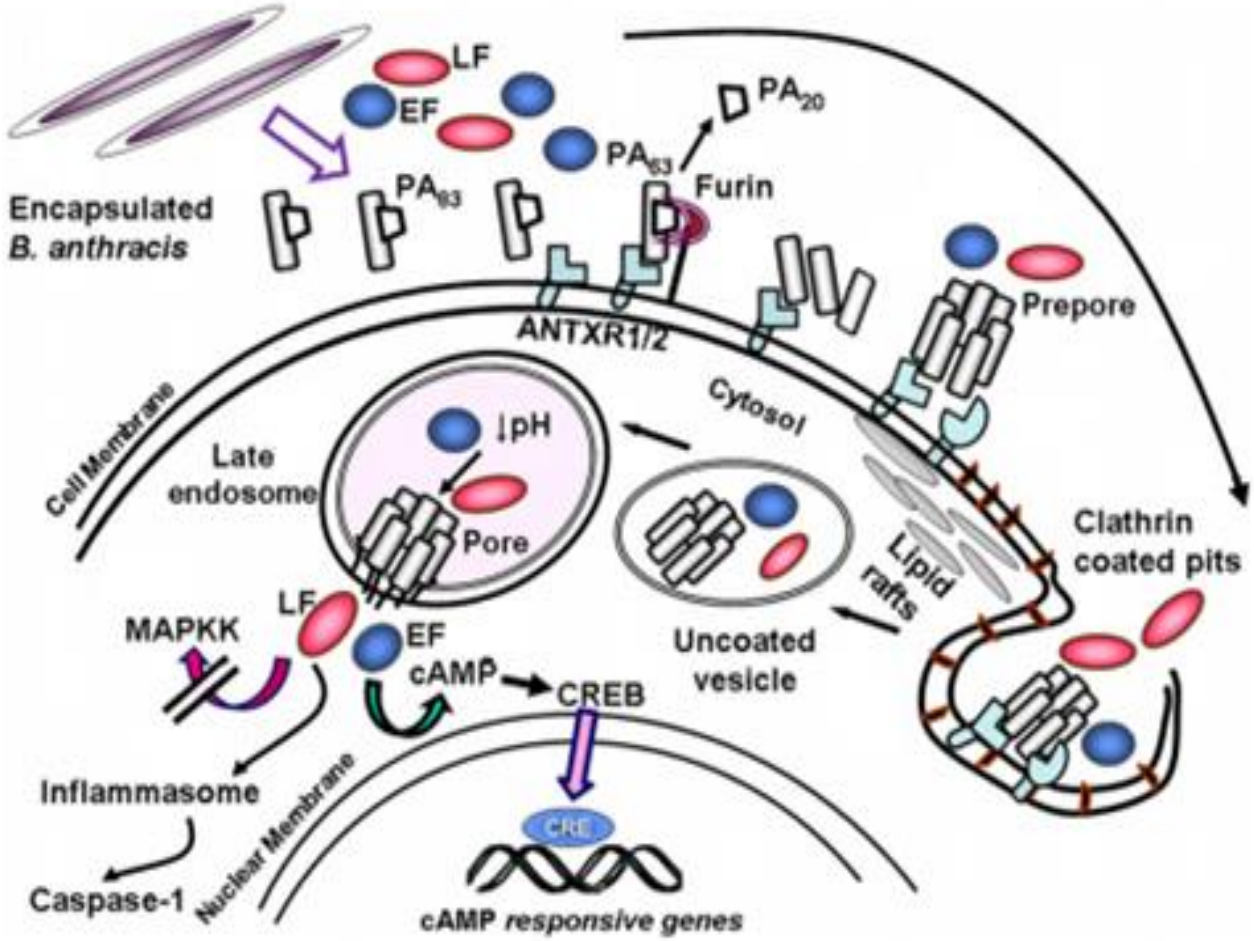
Figure 1.1.3. Schematic representation of the *B. anthracis* toxin entry process



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1.1.3.2 The Anti- Phagocytic Capsule

The capsule genes, although regulated by the atxA gene on pXO1, are encoded on pXO2, the other plasmid produced by *B. anthracis*. This plasmid is the smaller of the two and is approximately 95 kb in size [46]. It encodes the capBCAE operon which contains the capB, capC, and capA genes that are responsible for synthesizing the unique poly-γ-D-glutamate capsule that *B. anthracis* possesses. In addition, pXO2 also encodes the capsule degradation gene (dep) and the regulatory genes (acpA and acpB) [75-78].

Capsule gene expression, similar to toxin gene expression, is controlled by atxA, however, in contrast to toxin production, the capsule genes are activated indirectly by atxA through the use of two transcriptional intermediates, AcpA and AcpB [49,73,79]. Once these intermediates are synthesized, they positively regulate capB which in turn upregulates capsule synthesis [78].

The unique capsule of *B. anthracis* has been shown to impart several advantages to this pathogen [1,46,47,72,75,80]. It provides the bacterium with a negative charge that aids in both host immune evasion and macrophage intracellular survival allowing *B. anthracis* to survive and thrive within the host [1,46,47,72].

1.1.3.3 The Endospore

1.1.3.3.1 Sporulation

In unfavorable conditions (such as starvation, desiccation or extreme heat), *B. anthracis* can undergo a process called sporulation in order to ensure its future survival and dissemination. This metabolically inactive endospore can survive a wide range of environments including soil, water and various hosts that the vegetative cell otherwise cannot [81,82]. Consequently, the

spore of *B. anthracis* is the infectious particle that enters the host and leads to disease. Based on previous studies in non-human primates (NHPs), the LD_{50} for humans (i.e. the lethal dosage able to kill 50 percent of the exposed individuals) is between 8 $x10^3$ and 5 $x10^4$ spores depending on strain pathogenicity, delivery system and route of entry [40,83].

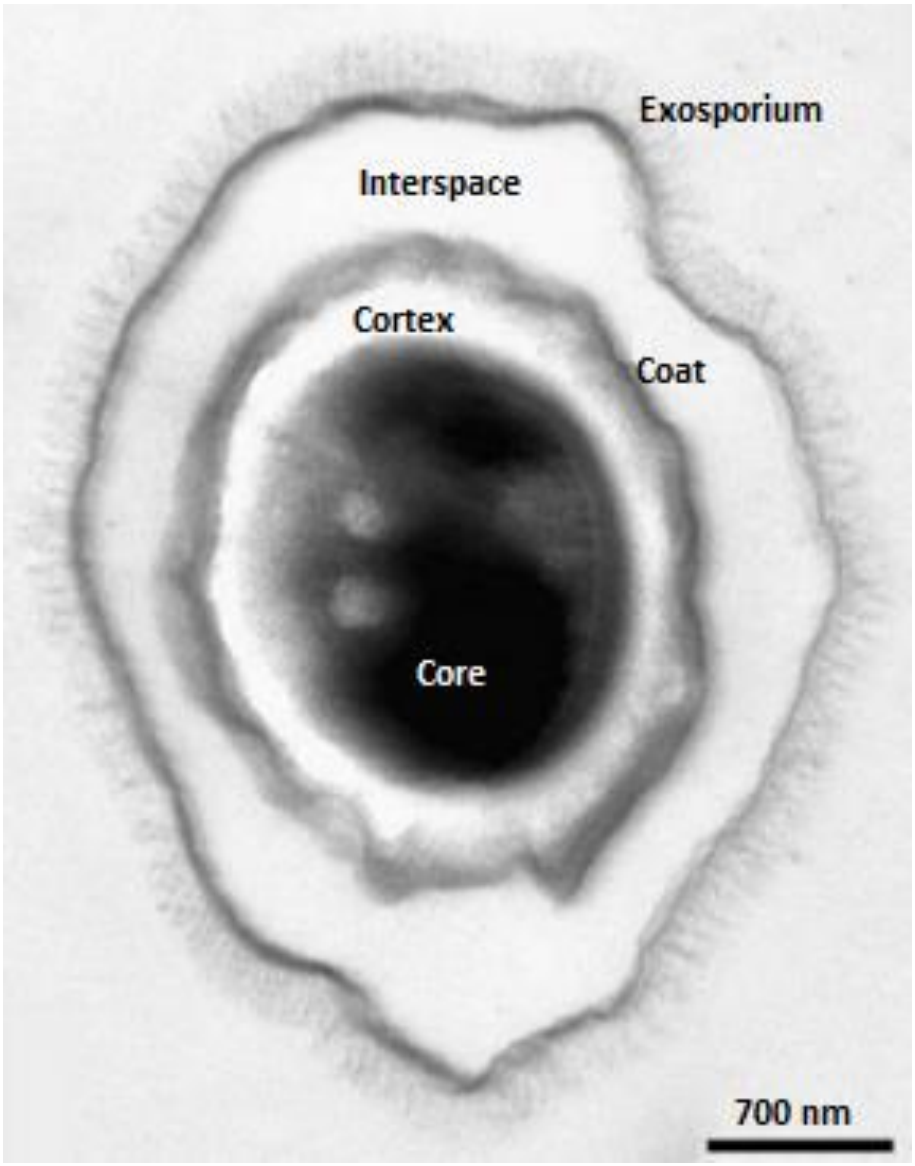
Sporulation is a carefully controlled process that takes approximately 8 hours to complete and is initiated by several factors including lack of nutrients, the state of the cell cycle, and secreted factors [84,85]. However, the exact chemical signals that trigger sporulation have yet to be elucidated [86].

Once, sporulation has commenced, the forespore forms at the center or sub-terminal end of the mother cell and is approximately 1- 1.5 µm [86]. Once forespore maturation is complete, the spore will have several layers (i.e. the outer exosporium, an interspace, a coat, a cortex and a core) (Figure 1.1.4) [86]. The core is the innermost structure of the spore and it houses the chromosome which is tightly complexed to acid-soluble proteins [87,88]. These interactions combined with the high presence of calcium dipicolinic acid and other ions, protect the bacterial chromosome from heat and ultraviolet (UV) radiation. The surrounding peptidoglycan layer (the cortex) increases spore resistance as well as keeps the inner core dry [89]. The cortex is covered by a protein shell coat which aids in spore resistance by preventing the passage of large molecules such as degradative enzymes or reactive molecules [90-93]. The final and outermost layer of the *B. anthracis* endospore consists of an exosporium that surrounds the interface. The function of this layer is currently unknown. This exosporium is predominantly

made up of two collagen-like glycoproteins (BclA and BclB) that form protruding hair-like filaments [94-96].

Although the role of the exosporium is still not well defined, studies have found that BclA reduces the interactions between the spore and the host matrix as well as targets the spores to macrophages and dendritic cells [97-99].

Figure 1.1.4. Transmission electron micrograph of the *B. anthracis* Sterne strain spore.



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1.1.3.3.2 Germination

Once the spore enters the macrophage (the primary site of germination) or if more favorable conditions are met (i.e. an increase in the abundance of nutrients), the *B. anthracis* spore will germinate and return to its vegetative form [100]. Germination is triggered by the binding of various germinants (amino acids such as L-alanine, ribonucleotides and peptidoglycans) to the spore receptors located in the spore inner membrane [89,101-103]. Binding of these germinant receptors leads to an influx of water, which results in spore swelling. This swelling is followed by a period of vegetative outgrowth where the cortex and coat are dismantled and the spore resumes metabolic activity [104-106].

1.1.4 *B. anthracis* and Biological Warfare

B. anthracis has been used numerous times throughout history as a bioterrorism agent since it can cause a highly contagious and fatal disease [12,13,107-112]. In World War I, offensive biological warfare programs were underway in various countries including England, France and Germany [13,107,108]. Allegedly, the Germans attempted to ship disease-ridden horses, cattle and sheep (inoculated with *B. anthracis* or *Pseudomonas pseudomallei*) to the US, Russia and other countries [107,108].

During World War II, biological weapon research was continued in Germany, England and France and was commenced in several other countries including Russia and Japan [12]. From 1932 until the end of the war, Japan conducted extensive research for the Japanese Biological Warfare Program (also known as “Unit 731”) under the direction of Shiro Ishii and Kitano Misaji [109,113,114]. This program, located at Pingfan, Manchuria, consisted of over 150 buildings and

3000 scientists studying various microorganisms (i.e. *B. anthracis*, *Neisseria meningitidis*, *Vibrio cholerae*, and others) [113,115]. By the same token, the British Biological Warfare Program was ongoing to determine the most effective means to disseminate lethal bioaerosol particles in order to ensure the highest number of casualties in the target area [116,117]. Weaponized *B. anthracis* bomb experiments were carried out extensively on Gruinard Island, Scotland which led to the contamination of the entire island [118]. In addition, the War Reserve Service in the US produced 5000 bombs filled with *B. anthracis* spores at its research facility in Camp Detrick, Maryland (known today as the US Army Medical Research Institute of Infectious Diseases) as well as extensively studied *B. anthracis* and *Brucella suis* at other testing facilities in Mississippi, Utah and Indiana [113,114,119].

It wasn't until 1972 that the Biological Weapons Convention (BWC) was developed in the hopes of terminating the offensive bioweapons programs of many countries [13]. This treaty prohibits the development, production, and stockpiling of pathogens for malicious use and required the 103 members (nations) to destroy their stockpiles and any bioterror delivery systems [108,113]. Despite the treaty agreement, offensive biological research has continued globally due to both the ambiguous definition of prohibited research and the lack of BWC reinforcement [113,120].

Over the last 30 years, numerous anthrax outbreaks have occurred due to the study and malicious use of *B. anthacis* [2,39,111,112,121,122]. In 1979 in Ekaterinburg, Russia (formerly known as Sverdlosk), the accidental release of *B. anthracis* spores from a military microbiology laboratory (Compound 19) lead to the death of numerous livestock as well as over 60 cases of IA with 42 deaths due to an air filtration malfunction [10,121,122]. In 1993, the Aum Shinrikyo

cult intentionally released aerosolized *B. anthracis* spores over Kameido, Japan. However, since they used the commercially available attenuated *B. anthracis* Sterne 34F2 vaccine strain, no infections were recorded [112]. Most recently, in 2001, *B. anthracis* Ames strain spores were intentionally released when they were sent through the US post office to various news and government offices [121,122]. Out of the thousands of individuals thought to have been exposed, only 22 cases were reported including 5 deaths [2,39,121,122].

Studies have estimated that in the event of a *B. anthracis* bioterrorism attack in an urban city of approximately 5 million people, there would be over 250 000 anthrax cases with approximately 100 000 deaths [123,124]. In addition, the economic burden of a large-scale anthrax attack would cost billions of dollars due to direct costs (hospitalization and medical costs, post-exposure prophylaxis), loss of productivity, disruption in commerce, animal illnesses and deaths, and decontamination and disposal [125].

1.2 Pre- Exposure Prophylaxis

Vaccination is recommended to aid in the development of an active immune response and to prevent *B. anthracis* infections. Currently, there are two anthrax vaccines, Anthrax Vaccine Precipitated (AVP) and Anthrax Vaccine Absorbed (AVA), that are licensed and available in the UK and the US, respectively, for pre-exposure prophylaxis. However, these vaccines are only recommended for high-risk populations such as military personnel, researchers or veterinarians [126]. AVP, first licensed in 1979, is a PA-based vaccine made from an avirulent *B. anthracis* Sterne 34F2 strain and requires 4 intramuscular doses over a period of 32 weeks [127]. AVA or Biothrax (Emergent BioDefense Operations Lansing LLC), a cell-free PA-based vaccine made

from the V770-NP1-R *B. anthracis* strain, contains an aluminum adjuvant and is part of the US strategic national stockpile [16,128]. A subcutaneous (SC) dose of 0.5 mL of AVA must be administered as five doses at 0 and 4 weeks and 6, 8 and 12 months to be the most effective as pre- exposure prophylaxis [129,130]. In some studies, AVA has also been shown to be effective in post-exposure situations [128,131,132].

Aside from requiring multiple doses, there are many other points to be considered with the current anthrax vaccine. Specifically, AVA has varying amounts of PA per batch, and a limited shelf life (stocks must be replaced every 4 years) [133-135]. Although it is considered safe, adult patients have reported some side effects (lymphadenopathy, immune system disorders, tremors, ulnar nerve neuropathy, as well as musculoskeletal, connective tissue and bone disorders) [130]. Also, the safety of AVA in children less than 18 years of age is currently unknown. In addition, in a 2008 observational study it was found that pregnant US military women that had been vaccinated with AVA during their first trimester had slightly elevated rates of birth defects compared to non-vaccinated pregnant women. Consequently, AVA is not recommended for pregnant women or people under 18 years of age unless all other options have been exhausted [136]. Furthermore, although PA can elicit a humoral immune response, it is limited in its ability to promote long-lasting immunity (due to a declining anti-PA response over time) [137,138]. Although the exact duration of efficacy of AVA is currently unknown, data from animal studies have suggested that two inoculations of AVA are only efficacious for 1 to 2 years [139-141].

1.3 Current Post- Exposure Prophylaxis

In order to effectively treat anthrax disease, prompt recognition and therapeutics are essential. Once the secreted toxins accumulate in the body, antibiotics are rendered ineffective and the patient's chances of survival are decreased drastically [2]. As an example, in the 2001 attacks, 5 patients perished due to pulmonary anthrax despite the administration of antibiotic therapy [39,122]. Once *B. anthracis* is suspected, antibiotic administration should commence immediately and be administered for 60 days [2]. If there are no clinical symptoms, doxycycline or penicillin G should be administered at a dosage of 100 mg orally or intravenously twice a day (BID) and 1,200,000 units every 12 hours, respectively [142]. Conversely, if clinical symptoms do manifest, an oral or intravenous formulation of 400 mg of ciprofloxacin BID is recommended [143]. Despite this lengthy administration period, in NHPs spores have been shown to germinate up to 100 days post infection resulting in death rates of up to 30% illustrating that even a 60 day course of antibiotics may not be sufficient [144-146]. Furthermore, this lengthy period tends to result in a decrease in patient compliance, as seen in the 2001 attacks which had a compliance rate of only 40 percent [147,148]. Moreover, the possibility of selection of antibiotic resistant mutants due to high antibiotic usage is a risk [139]. Although resistance is considered to be low in *B. anthracis,* natural strains resistant to penicillin and tetracycline have been documented [149,150]. Additionally, reports have been published showing that penicillin, ciprofloxacin and doxycycline strains can be easily bioengineered [149,150]. Therefore, in the event of a bioterrorist attack it should be assumed that the microorganism being used might be resistant to first line antibiotic therapy (penicillin, doxycycline, or ciprofloxacin) until confirmed otherwise.

Storage of both the 5 dose vaccine along with the necessary 60 day course of antibiotics in sufficient quantities to treat the quarter of a million people estimated to be affected in a large scale bioterrorism attack in addition to the required constant patient monitoring makes the aforementioned treatment strategies far from ideal [123-125]. Consequently, treatment following a scenario of a biological anthrax attack where a large number of individuals are exposed would not be feasible using the current treatment regimens.

1.4 Alternative Antibiotic Treatment for *B. anthracis* infections

1.4.1 Levofloxacin

Levofloxacin, like ciprofloxacin, is a broad-spectrum fluoroquinolone that functions by inhibiting the bacterial deoxyribonucleic acid (DNA) gyrase and topoisomerase IV [151-153]. In 2008, the US Food and Drug Administration (FDA) approved this third generation quinolone, under the name Levaquin (Janssen Incorporated, Toronto, Ontario), as a suitable alternative for the treatment of IA [151]. Although in the event of a bioterrorism attack it is recommended to assume that the agent in question is resistant to first line antibiotics until confirmed otherwise, a study by Athamna *et al* has demonstrated that being resistant to one fluoroquinolone (i.e. ciprofloxacin) does not necessarily indicate resistance to all others [149]. Moreover, spontaneous resistance to this class of antibiotic has been assessed *in vitro* and appears to be quite rare ($10^{-9} – 10^{-10}$) [151].

Currently, a daily dose of 500 mg of levofloxacin is recommended for children and adults weighing more than 50 kg [151]. In contrast, a dosage of 8 mg/kg BID is recommended for

children weighing less than 50 kg due to their underdeveloped renal function and limited clearance ability [154]. Side effects have only been assessed in adult populations and are similar to those of other fluoroquinolones (tendon rupture and tendinopathy, peripheral neuropathy, arthralgia, myalgia, dermatologic reactions, thrombocytopenia, and interstitial nephritis) [151]. In addition, in some juvenile animal studies quinolones have been associated with osteochondrosis [155,156]. Although the safety profile of using levofloxacin long-term (for example in a 60 day regimen) is currently unknown, due to the gravity of IA and to the current lack of approved alternative antibiotics, the benefits of using a fluoroquinolone such as levofloxacin greatly out-weigh the risks.

1.4.2 Linezolid

To date linezolid has not been approved by the FDA for the treatment of *B. anthracis* infections, although it has been approved for the treatment of a variety of other Gram-positive microorganisms including methicillin - resistant *Staphylococcus aureus* (MRSA) and multidrug resistant *Streptococcus pneumonia* under the name Zyvox (Pfizer Canada Incorporated, Kirkland, Quebec) [157]. Linezolid is a bacteriostatic oxazolidinone that inhibits bacterial protein synthesis by preventing the formation of the 70S ribosomal complex [158]. Since linezolid has such a unique mode of action, cross resistance with other antibiotic classes is unlikely and has not yet been observed making it an attractive therapeutic for penicillin or fluoroquinolone resistant bioterrorism agents. *In vitro* linezolid resistance occurs at a frequency of approximately 10^{-10} and has been associated with point mutations in the bacterial 23S rRNA gene [157]. Studies have shown that linezolid can reduce the production of the *S. aureus toxin,*

toxic shock syndrome toxin 1 [158]. Since linezolid is a protein synthesis inhibitor that can decrease toxin production in other Gram-positive organisms it is reasonable to consider that it may also prevent the toxin production of *B. anthracis.* A study by Louie A. *et al* looked at the effect of ciprofloxacin and linezolid on *B. anthracis* PA production at various times throughout a 10 day experiment [160]. They found that although PA was present in the control after 3 hours and in the levofloxacin group from 3 to 8 hours, no PA was observed at any of the time points in the linezolid group [160]. Their study supports the hypothesis that using a protein synthesis inhibitor, such as linezolid, may be a practical means at preventing the deleterious effects of the anthrax toxins. In a later study, Louie A. *et al* also looked at resistance to, and the pharmacodynamics of, linezolid using dose-ranging and dose fractionation studies [161]. They found that their pharmacodynamically optimized regimen of linezolid (700 mg once a day [QD]) did not amplify for resistance and was just as effective at killing as the clinically prescribed linezolid (600 mg BID) and the gold standard ciprofloxacin (500 mg BID). Not only does this dosing regimen decrease the total dosage patients would be exposed to but it would also aid in patient compliance by decreasing the dose frequency from BID to QD making the pharmacodynamic linezolid regimen more feasible and more cost effective.

Aside from being a protein synthesis inhibitor, linezolid also has excellent bioavailability and is available in both oral and intravenous formulations [157,160-162]. One drawback to this antibiotic is that it is quite costly. The average price of medication for one patient for one day is approximately $140.00 Canadian dollars (CAD) which is much higher than levofloxacin ($2.00 CAD), ciprofloxacin ($2.50 CAD), and doxycycline ($3.00 CAD) combined making linezolid undesirable if a 60 day regimen is required [163-166]. In addition, although linezolid is

considered relatively safe when used for a short term (less than 2 weeks), side effects such as peripheral neuropathy, thrombocytopenia, and neutropenia have been associated with long-term use (28 days) [162,167-170]. This toxicity may, however, be reduced by modifying the currently acceptable linezolid regimen to the dosage schedule suggested by Louie *et al* [161]. By the same token, linezolid could be used initially in the event of a bioterror attack and once susceptibilities of the agent have been determined patients could switch to alternative antibiotics such as ciprofloxacin, penicillin or doxycycline.

1.5 Research Goals

To date the effect of linezolid on *B. anthracis* toxin production has not been quantified. Furthermore, the combined effect of a bactericidal antibiotic (such as levofloxacin) with a protein synthesis inhibitor (like linezolid), on *B. anthracis* total growth, spore production and toxin formation has not yet been examined. Our hypothesis is that a bactericidal antibiotic in conjunction with an antibiotic that is a protein synthesis inhibitor will be the most effective therapy for *B. anthracis* killing and for preventing toxin and spore production. To evaluate this hypothesis, an *in vitro* flow-rate dependant pharmacodynamic model was used to compare the impact of levofloxacin and linezolid, alone and in combination, on *B. anthracis* killing. Then, using two static models, the effect of these antibiotics (alone and in combination) on *B. anthracis* spore formation and toxin production was determined. Ideally, the combination therapy would cause rapid killing of *B. anthracis* while preventing further spore formation and toxin buildup thereby potentially shortening the course of infection and by extension the duration of prophylaxis.

CHAPTER 2.0: MATERIALS AND METHODS

2.1 Microorganisms

Two *B. anthracis* isolates, the clinical pathogenic 03-0191 strain and the Sterne (34F2) strain, were acquired from the National Microbiology Laboratory (NML) stocks for this study. The 03-0191 strain is a fully pathogenic strain, possessing both the pXO1 and pXO2 plasmids (Figure 1.1.2), and is therefore able to produce infectious toxins and a working capsule, respectively [73]. The Sterne strain lacks the pXO2 plasmid and is avirulent since it cannot produce a capsule. Although Sterne is classified as a level 2 microorganism, since 03-0191 is a level 3 microbe, all studies were conducted in a level 3 biocontainment laboratory at the NML. Stock cultures (1 mL containing culture medium with 20% glycerol) with approximately 10^9 spores/mL of each individual strain were stored in a freezer at -80^{o}C. Once a strain was required, a sample from the frozen stock was removed from the freezer and streaked across a Luria- Bertani (LB) - Miller plate. The plate was incubated at 37^{o}C with 5% CO_2 for 24 hours [171]. If a bacterial suspension was required, it was made by taking a few colonies off the surface of the plate and directly suspending them in the medium.

2.2 Strain Confirmation

To confirm that the provided isolates were the *B. anthracis* Sterne and 03-0191 strains, multiple biochemical and biological analyses were conducted including colony morphology, hemolysis, ability to form spores, 16s rRNA sequencing and polymerase chain reaction (PCR) and agarose

gel electrophoresis of genes unique to *B. anthracis*. The *Bacillus cereus* ATCC 14579 strain was provided from the NML stocks and served as a control.

2.2.1 Colony Characteristics and Hemolysis

Colonies were grown on Tryptic soy agar containing 5% Sheep blood (Blood agar) and their characteristics were compared to those described by the New York State Department of Health [170]. Subsequently, hemolysis was determined by inspecting the plates for any discoloration or clearing in the medium.

2.2.2 Spore Formation

To determine if the strains were spore formers, colonies were grown up on LB- Miller plates at 37^{o}C with 5% CO_2 for 24 hours. Bacterial suspensions were made from the fresh plates by placing one isolated colony into 1 mL of cation-adjusted Mueller Hinton Broth (MHB) [161]. Suspensions were mixed thoroughly and then heat shocked (65^{o}C for 30 minutes) [172,173]. Following heating, the samples were serially diluted 1:10 in 900 µl of MHB up to 10^{-5}. The undiluted and diluted samples were spread over the surface of Blood Agar (BA) plates. The plates were then incubated aerobically at 37^{o}C with 5% CO_2 overnight at which time the colonies were counted.

2.2.3 16s rRNA Analysis

The 16s rRNA amplification and sequencing was carried out on both *B. anthracis* isolates as well as on the *B. cereus* control strain by using primers described by Sacchi *et al* (Table 2.2.1) [174].

2.2.3.1 Polymerase Chain Reaction

The first PCR product was amplified using the primers 67F and 1671R (Table 2.2.1) resulting in a fragment of 1686 bp while the second amplification for the nested PCR resulted in a 1656 bp fragment. A single colony from a BA plate was resuspended in 5 µl of double distilled nuclease-free water by pipetting then used for PCR in a Mastercycler Personal Thermal Cycler (Eppendorf, Mississauga, Ontario). Each 100 µl PCR reaction contained 50 µL of 1 X GoTaq® Green Master Mix (Promega Corporation, Madison, Wisconsin), 5 µl of whole cell suspensions, 10 µl of the upstream primer (1 µM), 10 µl of the downstream primer (1 µM) and nuclease- free water. The initial denaturation reaction was incubated at 95^{o}C for 5 minutes. PCR cycles (35 cycles in total) were performed as follows: 30 seconds at 94^{o}C (denaturation), 15 seconds at 52^{o}C (annealing), followed by 90 seconds at 72^{o}C (extension). The final extension step consisted of an incubation step of 72^{o}C for 5 minutes. The annealing temperature for the nested PCR was 50^{o}C.

Table 2.2.1. Primers used for the amplification and sequencing of the 16s rRNA *B. anthracis* and *B. cereus* gene. Adapted from Sacchi *et al* [174].

Primer	Nucleotide Sequence (5' to 3')	Purpose
67F	TGA AAA CTG AAC GAA ACA AAC	Nested PCR (outside)
1671 R	CTC TCA AAA CTG AAC AAA ACG AAA	
23 F	ACA AAC AAC GTG AAA CGT CAA	Nested PCR (inside)
136 R	AAA CGA AAC ACG GAA ACT T	
104 R	CAC GTG TTA CTC ACC CGT CC	Sequencing
357 F	TAC GGG AGG CAG CAG	
530 R	GTA TTA CCG CGG CTG CTG	
790 F	ATT AGA TAC CCT GGT AG	
981 R	GGG TTG CGC TCG TTG CGG G	
1230 F	TAC ACA CGT GCT ACA ATG	
1390 R	CGG TGT GTA CAA GGC CC	

2.2.3.2 Gel Electrophoresis

PCR products were then purified by agarose gel electrophoresis. A 1.0 % agarose gel was made using 1 gram of agarose powder in 100 mL of 1 X Tris-Borate EDTA (TBE) buffer. The PCR amplicons were mixed with 5 µl of 1x loading dye (0.017% sodium dodecyl sulfate [SDS], 2.5% ficoll, 11 millimolar (mM) ethylene diamine tetra acetic acid [EDTA], 3.3 mM Tris-HCl and 0.015% bromophenol blue) (New England BioLabs Limited, Whitby, Ontario) and run in parallel to a 1 Kb Plus DNA ladder (New England BioLabs Limited, Whitby, Ontario) on the agarose gel in 1X TBE buffer at 120 volts for 30 minutes.

Once the bands were confirmed using an ultraviolet illuminator, they were excised from the gel using a clean razor blade and were placed into the filter cup of a Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Column (Bio- Rad Laboratories Incorporated, Mississauga Ontario). The spin column was placed in to a -20^{o}C freezer for 5 minutes then spun at 13 000 x gravity (g) for 3 minutes at room temperature according to the manufacturer's instructions. The collected DNA diluent was placed in a fresh microcentrifuge tube which was surface decontaminated before being removed from the biocontainment level 3 laboratory space.

2.2.3.3 Sequencing

Once in level 2, the DNA amplicons were sent to the NML DNA Sequencing Core Facility for sequencing using the primers from Table 2.2.3.1.1. The nucleotide sequences generated were entered in to the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the Basic local alignment search tool (blast). A nucleotide blast was performed for the NML- acquired *B. cereus* and *B. anthracis* isolates

against the *B. cereus* ATCC 14579 (accession number NC_004722) and *B. anthracis* Sterne (accession number NC_005945) strains, respectively.

2.2.4 Agarose Gel Electrophoresis of *B. anthracis* PCR Products

To further confirm that one of the *B. anthracis* strains was pathogenic and the other was avirulent, *B. anthracis* specific genes were amplified and visualized using PCR and agarose gel electrophoresis. Six primers that amplified 330 base pairs (bp) of the pXO1 plasmid, 290 bp of the pXO2 plasmid and 150 bp (corresponding to the Ba813 sequence) unique to the *B. anthracis* chromosome were designed according to Vahendi *et al* [175,176]. The primers were purchased from Eurofins MWG Operon Incorporated (Huntsville, Alabama) and their corresponding sequences are shown in Table 2.2.2. These primers have previously been shown to be very specific for *B. anthracis* [175,176]. Each strain was grown on BA and a well-isolated colony was resuspended by pipetting in 5 µl of double distilled nuclease- free water to serve as template. If needed, 2 or more colonies were selected. Each 100 µL PCR reaction contained 50 µL of 1 X GoTaq® Green Master Mix (Promega Corporation, Madison, Wisconsin), 5 µl of whole cell suspension, 10 µl of the upstream primer (1 µM), 10 µl of the downstream primer (1 µM) and nuclease- free water. PCR was performed in a Mastercycler Personal Thermal Cycler (Eppendorf, Mississauga, Ontario) and cycling consisted of the following: 1 x (95^{o}C for 5 minutes), 35 x (94^{o}C for 50 seconds, 58^{o}C for 50 seconds and 72^{o}C for 50 seconds), and 1 x (72^{o}C for 5 minutes). PCR products were then subjected to agarose gel electrophoresis. A 2 % agarose gel was prepared using 2 grams of agarose powder in 100 mL of 1 X TBE buffer. Each amplicon was mixed with 5 µl of 1 x loading dye (New England BioLabs Limited, Whitby,

Ontario) as described above and run with a 1 Kb Plus DNA ladder (New England BioLabs Limited, Whitby, Ontario) on the agarose gel in 1 x TBE buffer at 120 volts for 30 minutes. The bands were illuminated using ultraviolet light.

Table 2.2.2. Primers used for the PCR amplification of *B. anthracis* specific genes. Adapted from Vahedi *et al* [175].

Primer	Nucleotide Sequence (5' to 3')	Targeted Gene Location	Expected Amplicon Size (bp)
Bac F	AAT GAT AGC TCC TAC ATT TGG AG	Chromosome	150
Bac R	TTA ATT CAC TTG CAA CTG ATG GG		
PA F	CGA AAA GGT TAC AGG ACG G	pXO1	330
PA R	CAA GTT CTT TCC CCT GCT A		
Cap F	GTA CCT GGT TAT TTA GCA CTC	pXO2	290
Cap R	ATC TCA AAT GGC ATA ACA GG		

Note: Bac- Bacterial chromosome, PA- Protective antigen, Cap- Capsule, F- Forward primer, R- Reverse primer

2.3 Antibiotics

Levofloxacin (Hospira Incorporated, Atlanta, Georgia) and linezolid (Zyvoxam, Pfizer Canada Incorporated, Kirkland, Quebec) were both purchased from the pharmacy at St. Boniface General Hospital (Winnipeg, Manitoba). Levofloxacin was supplied in a 5% dextrose intravenous solution at a concentration of 5 mg/mL. Aliquots of the stock solutions were refrigerated at 4^{o}C. Linezolid was supplied in a dextrose intravenous formulation at a concentration of 2 mg/mL. Stock solutions were wrapped in foil in order to be protected from light and were stored at room temperature. All preparations were used prior to their stated expiry date.

2.4 Susceptibility Testing

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined in triplicate by the microdilution broth method for both *B. anthracis* strains for linezolid and levofloxacin according to the Clinical and Laboratory Standards Institute guidelines [177]. Freshly prepared bacterial suspensions (no older than 24 hours) equal to a 0.5 McFarland Standard (Thermo Fisher Scientific, Ottawa, Ontario) (approximately 10^5 colony forming units [CFU]/mL) were prepared in MHB and inoculated in triplicate into 96-well plates containing serial 2- fold dilutions of either linezolid or levofloxacin. Three wells containing no antibiotic were used as a positive growth control and 3 uninoculated wells with only MHB served as a negative control. The plates were incubated aerobically at 37^{o}C with 5% CO_2 for 20 hours and the MIC was determined. The MIC was defined as the lowest concentration of antibiotic that resulted in lack of visible growth compared to the positive growth control. The MBC was determined by plating 1 µl from each non-turbid well (in duplicate) on to LB-Miller

plates and incubating aerobically overnight at 37^{o}C with 5% CO_2. The MBC was defined as the concentration at which there was a 3 log reduction in CFU compared to the original inoculum. The *Staphylococcus aureus* ATCC 25923 strain was used for quality control.

2.5 Synergy Testing

Synergy in efficacy between linezolid and levofloxacin was tested on both *B. anthracis* strains in a microdilution assay in a 96-well microtiter plate in triplicate. Both drugs were dispensed in a two- dimensional checkerboard fashion in 2- fold serial dilutions in concentrations ranging from 0.031 µg/mL to 32 µg/mL [178,179]. Freshly prepared bacterial suspensions (no older than 24 hours) equal to a 0.5 McFarland Standard (Thermo Fisher Scientific, Ottawa, Ontario) (approximately 10^5 CFU/mL) were prepared in MHB and were inoculated into each well containing the appropriate combination of linezolid and levofloxacin. Once again, 3 wells containing no antibiotic were used as a positive growth control and 3 uninoculated wells with only MHB served as a negative control. Plates were incubated for 20 hours at 37^{o}C with 5% CO_2 then results were recorded. A positive result was defined as growth or visible turbidity and a negative result was recorded as lack of growth (no turbidity). Once growth was determined, the fractional inhibitory concentration index (FICI) was calculated for each antibiotic in each combination using the formula $FIC_A + FIC_B = FICI$ where FIC_A is the MIC of linezolid in the combination divided by the MIC of linezolid alone and FIC_B is the MIC of levofloxacin in the combination divided by the MIC of levofloxacin alone. FICI that were less than 0.5 were considered synergistic, FICI between 0.5 and 4 were considered additive or indifferent and FICI greater than 4 were considered antagonistic [178-180].

2.6 *In vitro* Pharmacodynamic Infection Model

An *in vitro* flow-rate dependant pharmacodynamic infection model was carried out to study the response of *B. anthracis* to antibiotic exposure under simulated *in vivo* conditions [181]. All individual parts of the *in vitro* pharmacodynamic model (IPDM) were prepared in advance and were individually wrapped or bagged and autoclaved at 132^{o}C for 30 minutes to ensure sterility. The fresh media was infused with the independent treatments (either no antibiotic, linezolid, levofloxacin or a combination of linezolid and levofloxacin). The concentrations of antibiotics which were used were determined by susceptibility and synergy testing.

At the onset of every experiment, the pump (Masterflex, Cole-Parmer, Chicago, Illinois) was set to a flow rate of 1 mL/minute and calibrated according to the manufacturer's instructions. Since this model is a closed system, while 1 mL of fresh, sterile medium was being pumped into the reaction flask, 1 mL from the reaction flask was also being eliminated into the waste flask at an identical rate. The reaction flasks were placed in a temperature controlled 37^{o}C water bath for optimal growth, and sterile magnetic stir bars were used in each flask to ensure adequate mixing of all contents. The schematic of the apparatus set up can be seen in Figure 2.6.1.

Bacterial suspensions equal to a 0.5 McFarland Standard (Thermo Fisher Scientific, Ottawa, Ontario) (approximately 10^5 CFU/mL) were prepared fresh (no older than 24 hours) by placing several colonies of each strain in MHB broth and diluting accordingly. 2.5 mL of the bacterial culture was inoculated into a 250 mL round bottom reaction flask. Bacteria were allowed to acclimate at 37^{o}C for 30 minutes at which time 1 mL of culture was removed from the reaction flask to determine the starting bacterial load and the initial spore counts. 2 mL samples were

taken from the IPDM at 2, 4, 6, 8, 24, and 48 hours post-inoculation and the samples were divided into two portions of 1 mL each for total growth quantification and spore quantification.

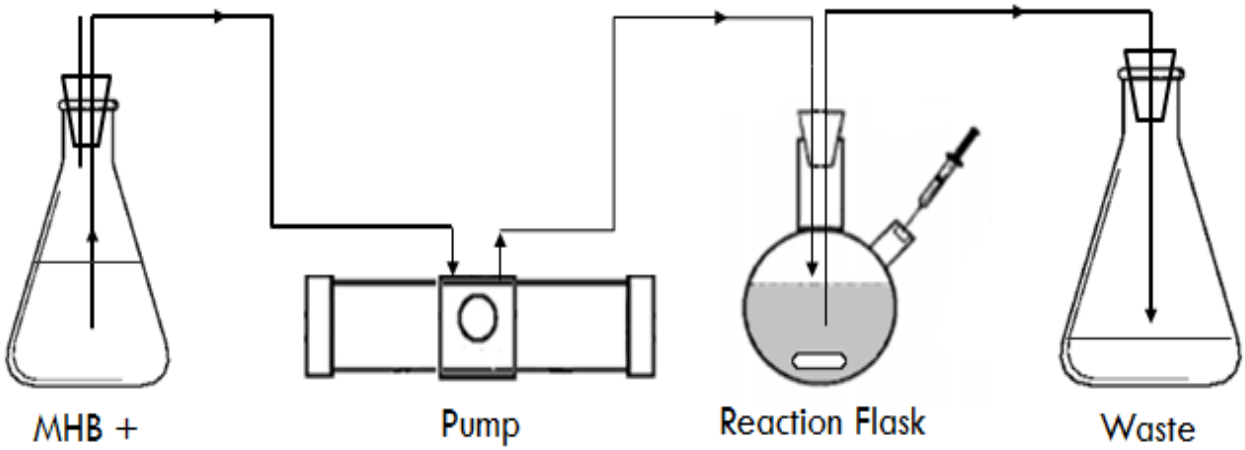
2.6.1 Bacterial Cell Growth

Bacterial colony counts were determined by serially diluting the samples in 900 µl of MHB to 10^{-6}. Serially diluted samples and the undiluted samples were spread plated on to LB-Miller plates and incubated aerobically at 37^{o}C with 5% CO_2 for 24 hours then counted.

2.6.2 Spore Count Determination

Spores were quantified by the Heat Shock Method i.e. the samples were heated at 65^{o}C for 30 minutes then serially diluted 1:10 (in 900 µl of MHB to 10^{-5})[182-184]. The samples were then spread plated onto BA and incubated at 37^{o}C with 5% CO_2 for 24 hours at which time they were enumerated.

Figure 2.6.1. Schematic of the *in vivo*-simulating *in vitro* flow-rate dependant pharmacodynamic infection model. MHB containing either no antibiotic, linezolid, levofloxacin or a combination of linezolid and levofloxacin is pumped from the medium reservoir to the reaction flask (which contains the active bacterial cultures). As 1 mL of fresh medium is pumped into the reaction flask, used medium (plus bacterial culture) is pumped out into the waste at a similar rate.



2.7 Static Spore Studies

The static spore model experiments were performed in triplicate on 3 different occasions. At the start of each trial, both *B. anthracis* strains were removed from the -80^{o}C freezer and sub-cultured 3 times on LB- Miller plates (24 hours at 37^{o}C with 5% CO_2) prior to commencing testing. Tubes containing 10% Columbia Broth containing 0.1 mM manganese sulfate ($MnSO_4$) were made up containing no antibiotic (positive control), linezolid, levofloxacin, or a combination of linezolid and levofloxacin. The antibiotic MICs, determined from susceptibility and synergy testing, concentrations were used for testing. Columbia broth with $MnSO_4$ (CB-M) was used to further stress the bacteria out and help promote spore formation. Aliquots of the CB-M media in tubes were stored in a 4^{o}C fridge until they were required.

The treatment CB-M tubes were removed from the fridge and allowed to reach room temperature prior to use. Bacterial suspensions equal to a 0.5 McFarland Standard (Thermo Fisher Scientific, Ottawa, Ontario) were prepared fresh (no older than 24 hours) in CB-M and 1 mL of the suspension was inoculated into each tube. 1 mL samples were taken at 0, 24, and 48 hours to determine the effect of each treatment on bacterial growth and spore formation.

2.7.1 Bacterial Cell Growth

Total bacterial growth was determined as discussed previously (Section 2.6.1).

2.7.2 Spore Count Determination

To determine spore counts, the samples were heat shocked at 65^{o}C for 30 minutes and then serially diluted 1:10 in 900 µl of 1/10 CB-M to 10^{-5}. The diluted and undiluted samples were

spread plated on to BA plates. All of the plates were incubated at 37^{o}C with 5% CO_2 for 24 hours and then enumerated.

2.8 Toxin Quantification

Toxin quantification experiments were performed in triplicate on 3 different occasions. At the start of each trial, both *B. anthracis* strains were removed from the -80^{o}C freezer and sub-cultured 3 times on LB- Miller plates (replating a single colony and incubating for 24 hours at 37^{o}C with 5% CO_2) prior to commencing testing. Tubes containing MHB and either no antibiotic (positive control), linezolid, levofloxacin, or a combination of linezolid and levofloxacin were prepared and stored in a 4^{o}C fridge until they were required. The concentrations of antibiotics that were used were the MICs determined by susceptibility and synergy testing.

The positive and negative controls were assayed first to ensure that both *B. anthracis* strains were producing quantifiable amounts of toxin prior to commencing studies with the experimental treatment groups.

Bacterial suspensions equal to a 0.5 McFarland Standard (Thermo Fisher Scientific, Ottawa, Ontario) were prepared fresh from cultures no older than 24 hours in MHB. The treatment tubes were removed from the fridge and allowed to reach room temperature at which time they were inoculated with 1 mL of the bacterial suspension. Initial baseline inoculum levels and baseline toxin levels were then determined. Samples were taken at 24 and 48 hours post-inoculation for cell and toxin quantification. The toxin samples were filtered through a 0.2 µm sterile cellulose acetate membrane filter (VWR International LLC. Mississauga, Ontario) in order to remove all bacteria and spores present. The flow-through was kept and placed in a 4^{o}C fridge

until further use. The cell samples were used immediately to determine the bacterial burden. The cells were serially diluted (100 µl of the sample placed in to 900 µl of MHB) and the diluted and undiluted samples were plated in duplicate on LB- Miller plates. The plates were incubated at 37^{o}C with 5% CO_2 for 24 hours and then enumerated.

2.8.1 PA83 Concentration

Due to the low concentration of PA83 in the experimental positive controls, the samples were concentrated at each time point in order to properly quantify the level of toxin. PA83 was concentrated using Amicon Ultra- 0.5 mL Centrifugal Filters (EMD Millipore, Billerica, Massachusetts). The cut- off size of the concentrator is 30 kDa therefore the 83 kDa PA protein was retained within the Amicon device during the filtration process. Samples (4 mL) from each time point for each treatment group were concentrated according to the manufacturer's instructions. Briefly, 0.5 mL of the sample was loaded per Amicon Centrifugal Filter and the filter was spun in an Eppendorf microcentrifuge model 5424 (Mississauga, Ontario) at 14 000 x g for 10 minutes. The concentrated samples were recovered by placing the inverted Amicon Filter in to a new microcentrifuge tube and spinning the tubes for 2 minutes at 1 000 x g in an Eppendorf microcentrifuge model 5424 (Mississauga, Ontario). The filtrate was collected and run in an enzyme-linked immunosorbant assay (ELISA) alongside the positive recombinant ELISA controls (provided by the kit).

2.8.2 Enzyme- Linked Immunosorbant Assay

For toxin quantification, PA83 was used since PA is the common component between both the ET and the LT [63,64]. PA83 was quantified using a commercial sandwich ELISA assay kit (Alpha

Diagnostic International Incorporated, San Antonio, Texas). A practice run was performed with the supplied positive recombinant PA83 controls to ensure the reproducibility of the ELISA kit. Subsequently, the treatment samples were run in parallel with the positive recombinant controls according to the manufacturer's instructions and the absorbance was read at 450 nm using a DTX 880 Multimode microplate reader and analyzer (Beckman Coulter Incorporated, Fullerton, California). Once the absorbance values were determined, the recombinant controls were used to create a standard curve. The standard curve was then used to determine the PA concentration (ng/mL) that corresponded to each absorbance value for each experimental treatment sample.

2.9 Statistical Analysis

The data is expressed as the mean plus or minus (±) the standard error of the mean (SEM). The error bars on the graphs represent the SEM. The relationship between time, treatment and strain on viable cell counts, spore production or toxin formation was assessed using a Three-Way Analysis of Variance (ANOVA) from the International Business Machines Corporation (IBM) Statistical Package for the Social Sciences (SPSS) software. If the ANOVA was significant, multiple comparisons tests or Tukey's Post Hoc HSD analyses were carried out to determine the significance of the differences between the experimental groups. If the Three- Way ANOVA was not statistically significant, a Two- Way ANOVA was carried out looking at only the effect of time and treatment on viable cell counts, spore production or toxin formation. If this ANOVA was significant, post hoc tests were carried out as mentioned above. A value was considered statistically significant if the P value was less than an alpha of 0.05.

CHAPTER 3.0: RESULTS

3.1 Strain Confirmation

B. anthracis colonies appeared white, flat and irregular with some protrusions (Table 3.1.1.). The average colony diameter was approximately 2 mm. *B. cereus* colonies were slightly larger with an approximate colony diameter of 4 mm. They were a cream color and flat and irregularly shaped. *B. cereus* was beta hemolytic while both of the *B. anthracis* isolates were non-hemolytic. All three strains were able to form spores.

The 1656 bp nucleotide sequences containing the entire 16s rRNA gene (1554 bp) from all three strains were aligned and compared to either the *B. cereus* ATCC 14579 strain or the *B. anthracis* Sterne strain in the NCBI database. The *B. cereus* and *B. anthracis* isolates in this study had 100% sequence similarity to the *B. cereus* ATCC 14579 strain and Sterne, respectively (Table 3.1.1.). These results indicate that the NML- acquired strains were in fact *B. cereus* and *B. anthracis.* However, these tests alone could not confirm *B. anthracis* virulence and therefore could not distinguish between a pathogenic and non-pathogenic strain.

In order to confirm strain virulence, primers previously described by Vahedi *et al* were used to amplify a portion of the PA gene (330 bp), the capsule gene (209 bp) and a chromosomal segment unique to *B. anthracis* (150 bp) (Figure 3.1.1.) [175]. The anthrax chromosome and the PA segments were successfully amplified in the provided NML Sterne strain (lanes 4- 6). No amplification of the capsule gene was documented confirming that this strain was attenuated since it lacked the pXO2 plasmid. In contrast, 03-0191 (lanes 7- 9) had a positive signal for all

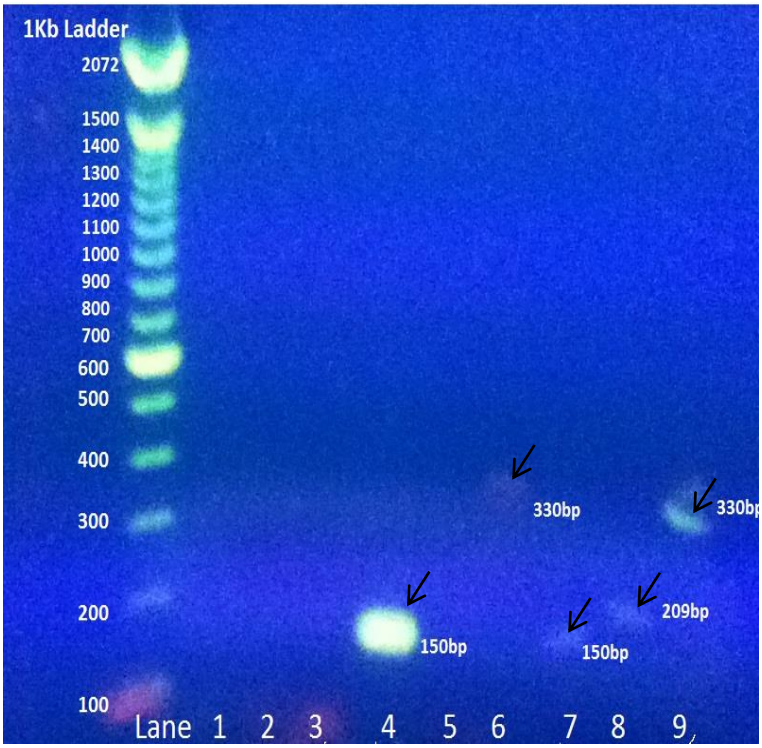
three of the amplified fragments confirming that it possessed both virulence plasmids. No signal was detected for the *B. cereus* strain (lanes 1- 3).

Table 3.1.1. The identification of *B. cereus* ATCC 14579, and *B. anthracis* Sterne and 03-0191 strains based on colony characteristics, 16s rRNA sequencing and hemolytic and spore-forming abilities

	B. cereus	**Sterne**	**03-0191**
Hemolytic	Yes	No	No
Colony Morphology	Flat, irregular, cream color, 4mm	Flat, irregular, white, 2mm	Flat, irregular, white, 2mm
Spore- Former	Yes	Yes	Yes
16s RNA Sequence Similarity	100% *	100%**	100%**

Note: **B. cereus* 16s rRNA sequence was compared to *B. cereus* ATCC 14579; ** *B. anthracis* 16s rRNA sequence was compared to the *B. anthracis* Sterne strain

Figure 3.1.1 Agarose gel electrophoresis of PCR products of *B. cereus* (lanes 1-3), Sterne (lanes 4-6) and 03-0191 strain (lanes 7-9).



3.2 Susceptibility and Synergy Studies

Using the broth microdilution method, the MIC and MBCs for the *B. anthracis* Sterne and 03-0191 strains were determined (Table 3.2.1). Both *B. anthracis* strains had similar MICs for linezolid having an MIC of 2.00± 0.22 μg/mL and 2.00± 0.11 μg/mL for Sterne and 03-0191, respectively. Both *B. anthracis* strains had MBCs greater than 32 μg/mL for linezolid. MICs of levofloxacin on Sterne and 03-0191 were 0.25± 0.03 μg/mL and 0.25± 0.03 μg/mL, respectively. Both strains exhibited MBCs that were similar to their MIC values (Table 3.2.1). The MIC values generated were used for all further testing in this project.

Synergy titrations were conducted on the *B. anthracis* strains by the checkerboard method to determine the optimal concentration required for the combination therapy. For the Sterne strain, linezolid (0.5 ± 0.17 μg/mL) and levofloxacin (0.13± 0.04 μg/mL) in combination exhibited a borderline synergistic effect with a FICI of 0.5. For the pathogenic strain, the combination of linezolid (0.13 ± 0.04 μg/mL) and levofloxacin (0.13± 0.05 μg/mL) exhibited indifference with a FICI value of 0.56 (data not shown).

Table 3.2.1 MIC and MBC of linezolid and levofloxacin on *S. aureus* ATCC 25923 and *B. anthracis* Sterne and 03-0191

	Linezolid		Levofloxacin	
Microorganism	**MIC**	**MBC**	**MIC**	**MBC**
S. aureus ATCC 25923	2.00(±0.22)	>16	0.25(±0.17)	0.50(±0.17)
B. anthracis Sterne	2.00(±0.22)	>32	0.25(±0.03)	0.25(±0.03)
B. anthracis 03-0191	2.00(±0.11)	>32	0.50(±0.04)	0.50(±0.06)

Note: Testing was conducted in MHB and streaked on LB-Miller plates. MIC and MBC are measured in μg/mL and are reported as the mean ± SEM. Results for 3 trials, conducted in duplicate, are reported.

3.3 *In vitro* Flow-Rate Dependant Pharmacodynamic Infection Model

Both *B. anthracis* strains grew well in the control arm. At time 0, the starting total populations (spore and vegetative cells) were 4.14± 0.03 $\log_{10}$ CFU/mL and 4.63± 0.04 $\log_{10}$ CFU/mL for Sterne and 03-0191, respectively (Figures 3.3.1. and 3.3.2). By 48 hours, both strains reached similar $\log_{10}$ CFU/mL of approximately 5.50.

All of the antibiotic therapies (linezolid, levofloxacin, and combination) were able to reduce the Sterne bacterial burden to undetectable levels (< 1 $\log_{10}$ CFU/mL) by 48 hours. In contrast, all of the treatments were able to reduce the 03-0191 bacterial burden to undetectable levels by 24 hours.

Heat shock studies revealed that the populations in the various treatment groups (including the control) were primarily in the vegetative form since spores were undetectable (<1 $\log_{10}$ CFU/mL) under these experimental conditions.

Figure 3.3.1. The effect of linezolid and levofloxacin on *B. anthracis* Sterne strain cell growth in a flow rate dependant *in vitro* pharmacodynamic model. The Y axis represents viable counts (measured in $\log_{10}$ CFU/mL) while the X axis represents time (in hours). Results are from three separate trials and are reported as the mean ± SEM (error bars). Asterisks (***) indicate that the values are significantly different (P< 0.05) from the control.

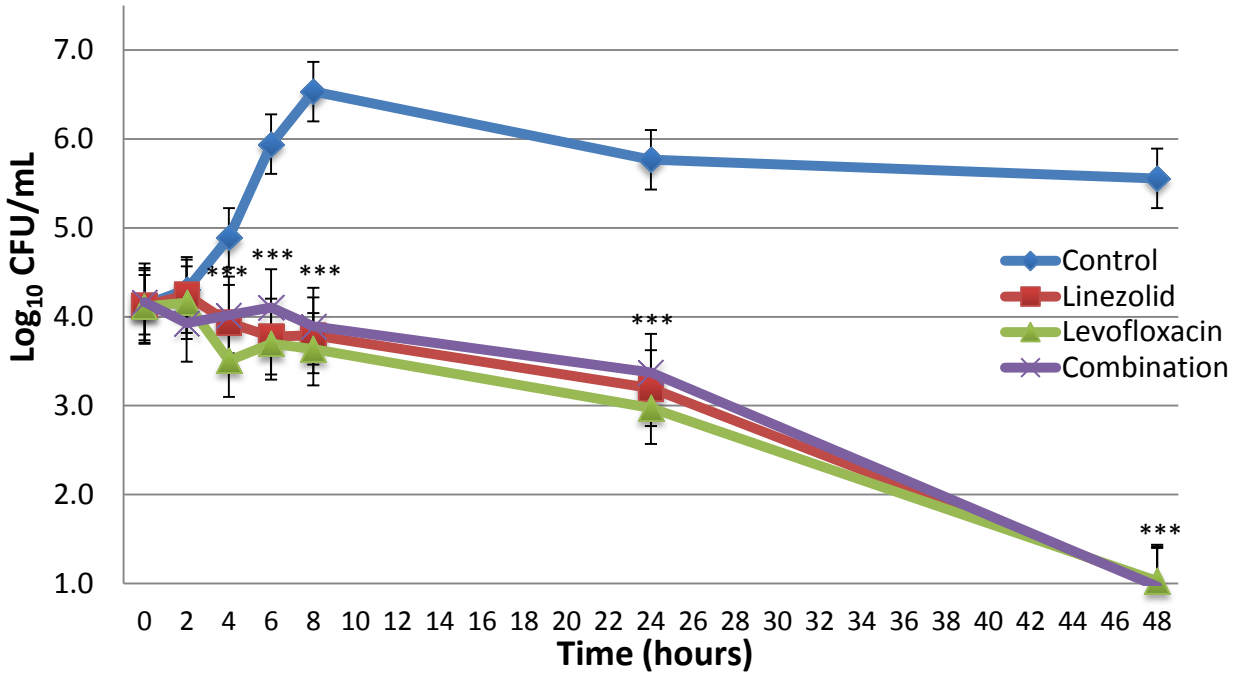
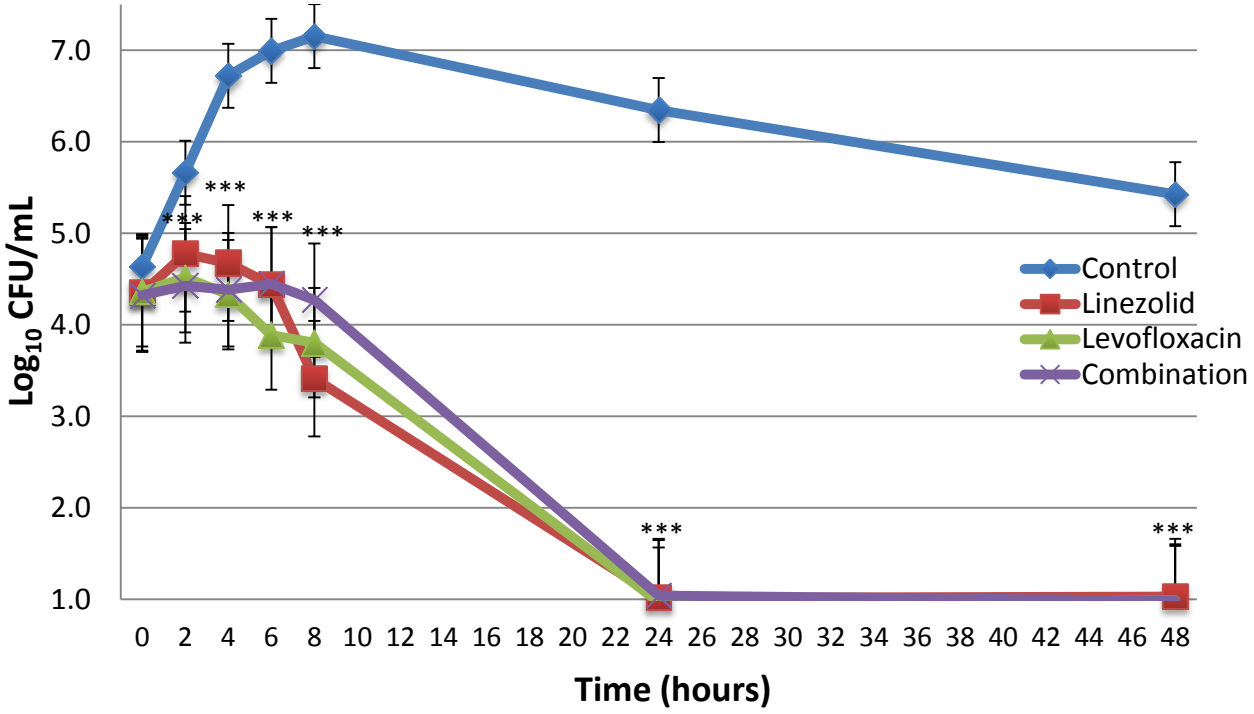


Figure 3.3.2. The effect of linezolid and levofloxacin on *B. anthracis* 03-0191 strain cell growth in a flow rate dependant *in vitro* pharmacodynamic model. The Y axis represents viable counts (measured in $\log_{10}$ CFU/mL) while the X axis represents time (in hours). Results are from three separate trials and are reported as the mean ± SEM (error bars). Asterisks (***) indicate that the values are significantly different ($P< 0.05$) from the control.

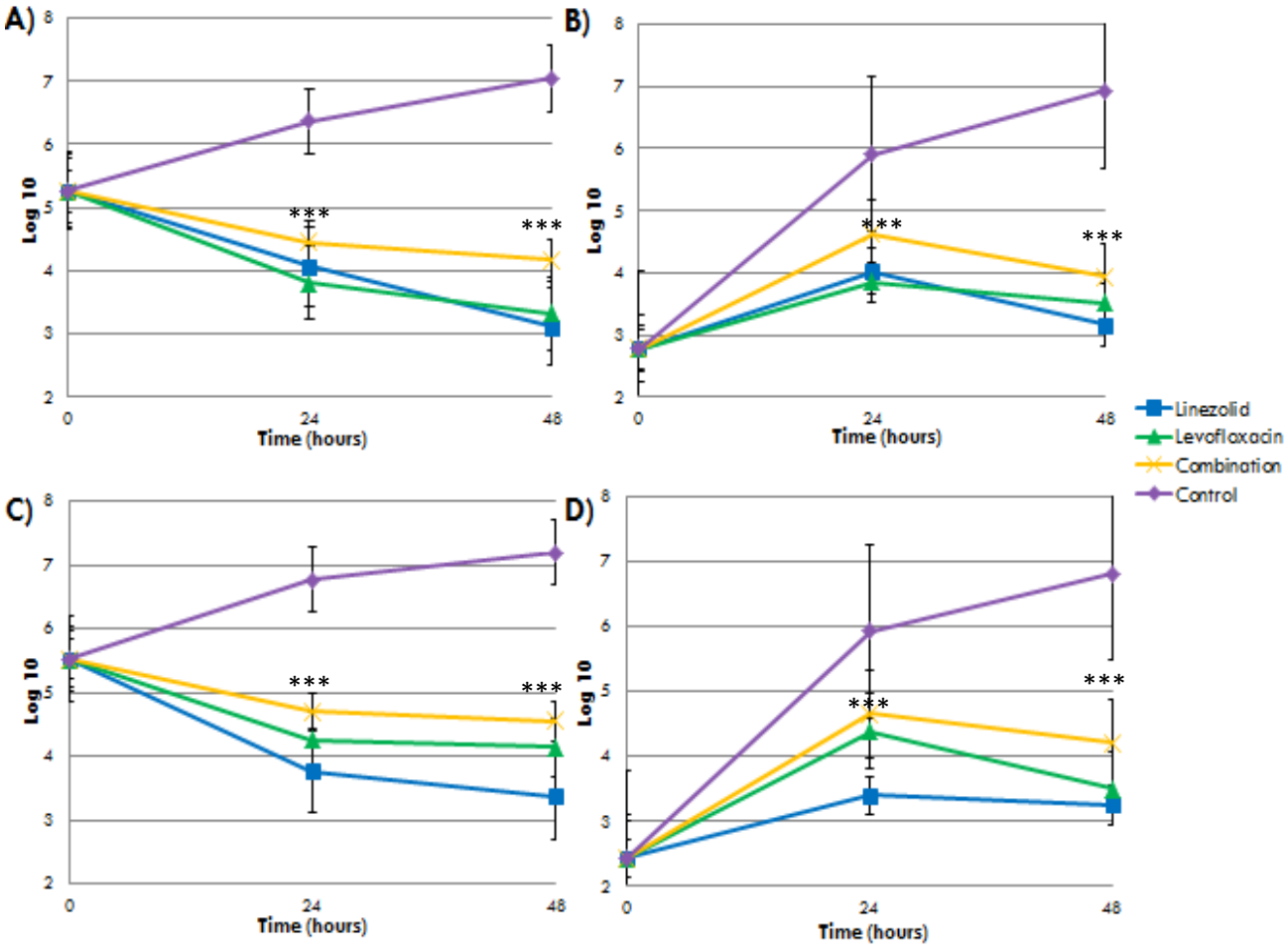


3.4 Static Spore Studies

In the static spore model, both *B. anthracis* strains started with a total (vegetative plus spore) population of approximately 5.40 $\log_{10}$ CFU/mL that contained a spore population of approximately 2.60 $\log_{10}$ CFU/mL for all test groups (Figure 3.4.1). The total control populations grew steadily to 7.00± 0.37 $\log_{10}$ CFU/mL and 7.20± 0.19 $\log_{10}$ CFU/mL for Sterne and 03-0191, respectively over the 48 hour trial period and consisted of a predominantly spore population (98.0 %).

For linezolid therapy, the Sterne and 03-0191 total populations decreased from 5.40 $\log_{10}$ CFU/mL to 3.10± 0.46 $\log_{10}$ CFU/mL and 3.40± 0.25 $\log_{10}$ CFU/mL, respectively. Although linezolid was able to reduce the bacterial burden by approximately 4 logs compared to the control, it did not appear to hinder spore formation since heat shock studies revealed that the total viable population was in the spore form at the end of 48-hour experiment. Similarly, the levofloxacin and combination therapies also resulted in a population that consisted predominantly of spores. Levofloxacin led to a decrease in the bacterial burden resulting in a final population of 3.30± 0.72 $\log_{10}$ CFU/mL (Sterne) and 4.10± 0.48 $\log_{10}$ CFU/mL (03-0191) and a spore population of 3.50 $\log_{10}$ CFU/mL for both strains. Although the combination treatment resulted in a population reduction of approximately 2.70 logs compared to the control for both strains (ending with a final total population of 4.20± 0.27 $\log_{10}$ CFU/mL and 4.60± 0.12 $\log_{10}$ CFU/mL for Sterne and 03-0191, respectively), it did not reduce the overall population more effectively than either of the antimicrobials alone. In addition, the final spore burden of Sterne and 03-0191 was 3.90± 0.43 $\log_{10}$ CFU/mL and 4.20± 0.27 $\log_{10}$ CFU/mL, respectively.

Figure 3.4.1. The effect of linezolid and levofloxacin, alone and in combination, on Sterne cell growth (A), Sterne spore formation (B), 03-0191 cell growth (C), and 03-0191 spore formation (D). The Y axis represents viable counts (measured in $\log_{10}$ CFU/mL) while the X axis represents time (in hours). Results are from three separate trials and are reported as the mean ± SEM (error bars). Asterisks (***) indicate that the values are significantly different ($P < 0.05$) from the control.

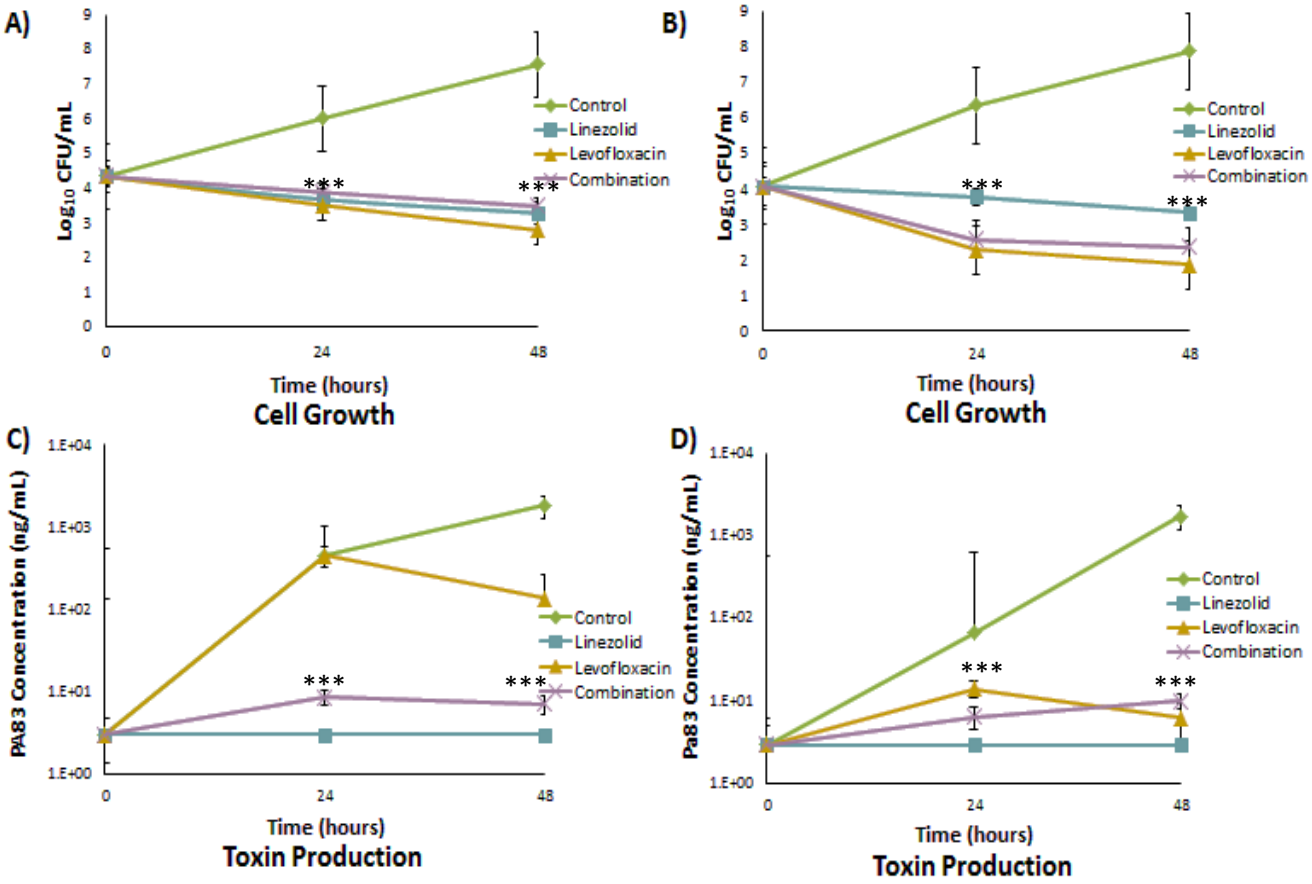


3.5 Toxin Quantification

3.5.1 Total Cell Growth

In the static growth model, total (vegetative and spore) cell growth as well as PA83 production were determined. Sterne had a starting population of 4.35± 0.03 $\log_{10}$ CFU/mL while 03-0191 had a starting population of 4.08± 0.04 $\log_{10}$ CFU/mL (Figure 3.5.1. panels A and B). By the end of the 48 hour experiment, without any antibiotic therapy, both strains reached a total population of 7.57± 0.06 $\log_{10}$ CFU/mL (Sterne) and 7.85± 0.08 $\log_{10}$ CFU/mL (03-0191). In contrast, the linezolid therapy was able to prevent *B. anthracis* growth and by 48 hours resulted in an approximate log reduction for both Sterne (3.30± 0.05 $\log_{10}$ CFU/mL) and 03-0191 (3.33± 0.03 $\log_{10}$ CFU/mL). Treatment with levofloxacin resulted in the greatest reduction in the bacterial burden of both isolates, with a final population of 2.81± 0.03 $\log_{10}$ CFU/mL and 1.85± 0.19 $\log_{10}$ CFU/mL for Sterne and 03-0191, respectively. The combination therapy of linezolid and levofloxacin lead to a reduction in the overall bacterial population resulting in 3.49± 0.02 $\log_{10}$ CFU/mL and 2.35± 0.03 $\log_{10}$ CFU/mL for Sterne and the clinical pathogenic strain, respectively.

Figure 3.5.1. The effect of linezolid and levofloxacin, alone and in combination, on Sterne total cell growth (A), 03- 0191 total cell growth (B), Sterne PA83 production (C), and 03-0191 PA83 production (D). The Y axis for panel A and B represents bacterial growth and is measured in $\log_{10}$ CFU/mL. The Y axis for panels C and D represents the concentration of PA83 (measured in ng/mL). The X axis represents time (measured in hours). Results are from three separate trials and are reported as the mean ± SEM (error bars). Asterisks (***) indicate that the values are significantly different (P< 0.05) from the control.

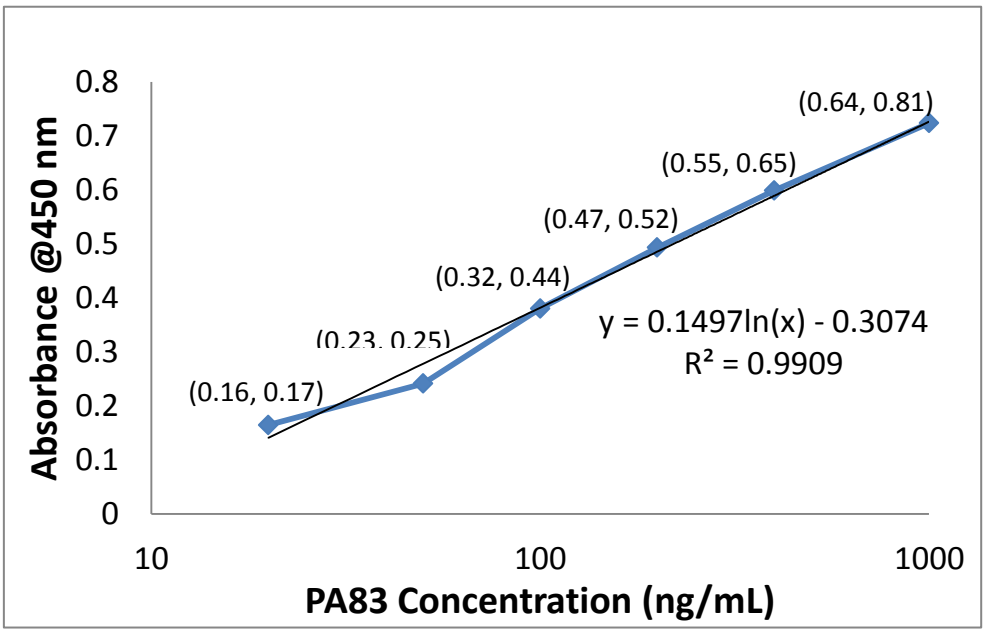


3.5.2 PA83 Standard Curve

A PA83 standard curve was generated using the positive control recombinant PA83 provided by the Alpha Diagnostic International ELISA kit. The recombinant controls contained 20, 50, 100, 200, 400 and 1000 ng/mL and were assayed in duplicate. Their absorbance ranged from 0.17± 0.01 ng/mL to 0.72± 0.04 ng/mL for the lowest and highest PA83 controls, respectively (Figure 3.5.2). The absorbance values were plotted against the various PA83 concentrations and a logarithmic best-fit line was overlaid. The best-fit line had an R^2 value of 0.99 and an equation of:

$$\text{Absorbance}_{450nm} = 0.1497 \ln (\text{PA83 concentration}) - 0.3074$$

Figure 3.5.2. Standard curve of anthrax protective antigen (PA83) measured at 450 nm. Brackets represent a 95% confidence interval with the first number representing the lower bound and the second representing the upper limit.



3.5.3 PA83 Production

Using the standard curve best-fit line equation, toxin concentrations were determined for each sample. In this static growth model, both *B. anthracis* strains started with an initial toxin measurement of 3 ng/mL, which was at the lower limit of detection. Within 48 hours, the Sterne strain produced a total of 1816± 797 ng/mL of PA83 while the pathogenic 03-0191 strain produced 1716± 466 ng/mL (Figure 3.5.1 panels C and D). No toxin was detected in the linezolid treatment group for either of the isolates throughout the 48 hours. Likewise, no toxin production was recorded for either strain when treated with the combination therapy (less than 10 ng/mL). Although basal levels of PA83 was detected in the levofloxacin treatment arm for the 03-0191 strain (15 ng/mL), the Sterne strain produced amounts similar to the untreated control at 24 hours (451± 70 ng/mL). By 48 hours, however, only 133± 38 ng/mL of PA83 was quantifiable in the levofloxacin group.

CHAPTER 4.0. DISCUSSION

Anthrax, although rare in the western world, still remains a global issue. In areas where *B. anthracis* is endemic (Africa, South America, and Asia), more than 4000 anthrax cases are reported annually [19]. Furthermore, due to its ability to form highly resistant spores and deadly toxins, this pathogenic microbe can cause high morbidity and mortality if used as an agent of bioterrorism [12,13,107-112]. Mortalities associated with anthrax may be prevented if antibiotic therapy is commenced immediately after exposure. However, any delay in treatment drastically reduces the outcome of the patient. While the current antimicrobials can be effective against the multiplying vegetative bacteria, treatment is lengthy and recovery is not guaranteed [39,122]. In addition, none of the "gold standard" antibiotics (i.e. ciprofloxacin, doxycycline or penicillin) nor their alternative (levofloxacin) currently interfere with the bacterium's ability to form hardy spores or harmful toxins. Although several studies have looked at alternative therapeutics for anthrax, few studies have looked at the combined effect of an antibiotic that is a protein synthesis inhibitor with a bactericidal antibiotic on *B. anthracis*.

In this study, we looked at the effect of linezolid and levofloxacin (alone and combined) on *B. anthracis* 03-0191 and Sterne strain with respect to spore production, toxin formation and cell viability. Several models were utilized in this research including two static models for the spore and toxin studies in addition to an *in vitro* flow rate- dependant IPDM to simulate *in vivo* conditions of *B. anthracis* infections. Initially, susceptibility testing was performed to determine the appropriate antibiotic dosages. In accordance with previous studies, these preliminary tests indicated that linezolid was bacteriostatic against *B. anthracis* while levofloxacin was

bactericidal [151,158]. Synergy titrations indicated that the combination of these two drugs would work in an indifferent or slightly synergistic manner. However, in all of the models tested here, the combination of linezolid and levofloxacin was indifferent at best and was not more effective than either of the antimicrobials alone.

When looking at the effect of the antibiotics on bacterial killing, the combination was indifferent in both the IPDM and in the static toxin model. In the IPDM, no significant difference was noted between the antibiotic therapies. All of the treatments were equally effective (P<0.001) compared to the control and were able to decrease the bacterial burden of both *B. anthracis* strains to undetectable levels (<1 $\log_{10}$ CFU/mL) by 48 hours. Similarly, when looking at bacterial growth in the static toxin studies, all antimicrobial therapies were able to significantly reduce the bacterial population compared to the untreated control (P< 0.001). In this static model, as expected, linezolid killed the slowest (due to its bacteriostatic nature) while the bactericidal antibiotic, levofloxacin, killed more rapidly. Post hoc analyses using Tukey's test for significance revealed that at 24 hours, there was a significant difference between levofloxacin and the combination on Sterne killing (P=0.016). However, no difference was noted between linezolid and levofloxacin. In contrast, at 48 hours levofloxacin therapy resulted in a significantly lower viable population compared to both the linezolid and the combined treatment (P< 0.001). Likewise, when treating the 03-0191 strain, levofloxacin reduced the bacterial burden the most (P< 0.001 compared to linezolid and the combination) while linezolid reduced the bacterial burden the least (P< 0.001 compared to levofloxacin and the combination). These results support previously published studies and extend them by showing that the combination of levofloxacin and linezolid was not synergistic.

Next, the effect of linezolid and levofloxacin on *B. anthracis* toxin production was quantified. Three- way ANOVA results indicated that there was a significant three- way relationship between strain, treatment and time $F(6,48)=53.744$, $P< 0.001$. Post hoc analyses were used to determine the differences between each treatment at all three time points (i.e. 0, 24 and 48 hours) on each strain. For the clinical 03-0191 strain, all treatments were able to significantly reduce toxin production compared to the control ($P <0.001$). When this strain was treated with any of the antibiotics (i.e. linezolid, levofloxacin or the combination) no toxin production was observed (<10 ng/mL). In contrast, for the Sterne strain, only linezolid and the combination were able to significantly reduce toxin production ($P=0.001$ compared to the control) to <10 ng/mL despite the fact that there was still approximately 3.80 and 3.40 $\log_{10}$CFU/mL of bacteria at 24 and 48 hours, respectively. Conversely, treatment with levofloxacin led to an increase in toxin production at 24 hours similar to those seen in the controls. At 24 hours, 451± 70 ng/mL of PA83 was observable in the levofloxacin populations, which was significantly different from the linezolid and combination populations ($P= 0.001$ and $P=0.010$, respectively). Likewise, at 48 hours toxin levels in the levofloxacin samples (133± 38 ng/mL of PA83) were approximately 10 fold greater than those observed for linezolid or the combination ($P< 0.030$). Since all of the antibiotic treatments had similar CFU/mL at 24 hours (approximately 3.5 $\log_{10}$CFU/mL) and since no PA83 was present in the linezolid (or the combination with linezolid) populations, we conclude that the protein synthesis inhibitor prevented the production of PA83 and by extension the anthrax toxins. These findings confirm the previous studies by Louie *et al* that looked at the presence or absence of PA in Sterne populations treated with ciprofloxacin or linezolid in a hollow fiber model [160]. They found that levofloxacin treatment was able to

decrease toxin production (due to rapid bacterial killing) compared to the control while linezolid was able to prevent toxin production altogether. Our data extend the findings of Louie *et al* as the clinical strain 03-0191 that we tested also showed the same effect on toxin production when exposed to levofloxacin and linezolid as seen for the Sterne strain.

Since toxin studies suggested that linezolid and the combination therapy prevented the formation of the bacterial toxins, it was logical to next look at their ability to prevent spore formation. Prior to looking at spore production in a static model, we had looked at the effect of each treatment on sporulation in the IPDM using two different mediums (i.e. MHB as well as CB-M). Interestingly, however, no spores were detectable in any of the treatment flasks (including the untreated control) with either type of media. It is hypothesized that using a rich medium such as MHB in this dynamic model led to idyllic conditions (i.e. constant influx of fresh media, constant removal of depleted media, and a consistent temperature) thereby encouraging the bacteria to remain in their vegetative state. Additionally, any spores that may have been formed in either media may have been removed due to the constant dilution effect of the growth system.

Consequently, the efficacy of the antimicrobials was assessed using a static *in vitro* spore model. A three- way ANOVA was carried out to examine the effect of strain type, treatment and time on spore formation and total viable cells. The results demonstrated that there was no significant three- way interaction between these factors. There was, however, a significant interaction between time and treatment $F(6,48)= 19.506$, $P<0.001$ on total cell viability as well as a significant interaction between time and treatment $F(6,48)= 4.334$, $P<0.001$ on spore

production. Post hoc comparisons using the Tukey's test indicated that all of the antibiotic treatments were significantly different from the untreated controls at 24 and 48 hours in the total population and the spore population experiments (P<0.001). However, very few differences were noted between the efficacies of the antibiotic treatments on either the total or spore populations. When looking at the effect these antimicrobials had on spore formation, no statistically significant difference was detected. In contrast, when looking at the total viable counts, a significant difference was seen between linezolid and the combination therapy at 48 hours (P=0.010). At this time, the bacteria treated with the combination had the highest overall counts whereas the linezolid treated populations had the lowest counts. Since linezolid treatment also led to the lowest increase in spore formation it is reasonable to conclude that, in this static model, linezolid is a more effective alternative for the treatment of *B. anthracis* compared to the combination therapy.

The effect of antibiotics on *B. anthracis* has been previously examined. In accordance with the present study, Louie *et al* found that the bactericidal (i.e. ciprofloxacin, moxifloxacin, and meropenem) and bacteriostatic antibiotics (i.e. linezolid and doxycycline) reduced the overall bacterial population at similar rates (within 1 $\log_{10}$ CFU/mL of each other) over a 10-day period [184]. When assessing the proportions of vegetative bacteria to spores, they found that when the *B. anthracis* Sterne strain was treated with ciprofloxacin, the total population consisted primarily of spores [160]. Conversely, when it was treated with linezolid more than 90 percent of the population was vegetative. Likewise, in a later study it was found that when treated with bactericidal antibiotics (moxifloxacin, ciprofloxacin, or meropenem) the *B. anthracis* population

was predominantly spores whereas when treated with protein synthesis inhibitors (doxycycline or linezolid) the population was predominantly vegetative [184].

In the current study, however, after 48 hours both the linezolid and the levofloxacin populations were present predominantly in the spore form (> 85 percent). This discrepancy compared to previous studies may be attributable to the model chosen since the studies here used a static spore model while the studies conducted by Louie A. *et al* used a dynamic, *in- vivo* simulating hollow fiber model [160,184]. Furthermore, in their studies, approximately 10- 15% of their starting inoculum consisted of spores whereas in this study, approximately 50% of our starting inoculum was already in the spore state. Spores have a profound impact on the extent of bacterial killing by antibiotics. Therefore, starting with such a high number of spores at time 0 may have influenced the results presented here. Performing this same experiment with a uniformly vegetative population prior to promoting sporulation may shed some light to see if the differences observed herein are due to the starting population proportions (i.e. approximately half spores and half vegetative) or if they are due to the model chosen.

Although studies have looked at the efficacy of single antimicrobials on *B. anthracis* spore formation *in vitro*, to date no studies have looked directly at their effect (alone or in a combination) *in vivo*. However, several studies looking at the spore- forming *Clostridum difficile* have found protein synthesis inhibitors to be more effective than other antimicrobials both *in vitro* and *in vivo* [185-189]. Mathur *et al* looked at the *in vitro* and *in vivo* efficacy of RBx 11760, a novel oxazolidinone, on *C. difficile* infections [187]. They found that this antibiotic was more effective than both metronidazole and vancomycin at preventing spore formation *in vitro*.

Furthermore, in an *in vivo* hamster gastrointestinal model, they found that treatment with RBx 11760 lead to increased survival rates compared to either of the other antibiotics. Likewise, cadazolid, an oxazolidinone derivative, demonstrated similar abilities to RBx 11760 [188]. This novel antibiotic was able to substantially inhibit spore formation *in vitro* and was equally as effective as vancomycin in conferring full protection in both the hamster and mouse *C. difficile* models. Moreover, cadazolid demonstrated good efficacy against *C. difficile* in a human gut model [189]. Similarly, in a study by Ochsner *et al,* the protein synthesis inhibitor REP3123 was determined to be superior compared to vancomycin and metronidazole in attenuating sporulation *in vitro* and was also more effective compared to vancomycin in an *in vivo* hamster infection model [186]. Collectively, these studies indicate that *in vitro* efficacy of a protein synthesis inhibitor has translated well into *in vivo* efficacy. Therefore, it is reasonable to assume that since linezolid was effective against *B. anthracis* spore production in the *in vitro* studies described here, it would also be effective *in vivo*. Additionally, since several studies indicate that the combined effect of a protein synthesis inhibitor and a bactericidal antibiotic works indifferently *in vitro,* it is hypothesized that these antibiotics will not be more effective *in vivo.* Since it is unethical to conduct *B. anthracis* studies in humans, animal models will be required to confirm whether this is actually the case *in vivo*.

Since linezolid is a protein synthesis inhibitor and levofloxacin is a bactericidal antibiotic, it was hypothesized that linezolid would inhibit the production of the molecules required for spore and toxin production while levofloxacin would work rapidly to kill the vegetative bacteria [148,158]. Consequently, this combination would result in a reduction in toxin formation and spore production resulting in a more rapid reduction in the *B. anthracis* total population

compared to treatment with either antimicrobial alone. However, the results of the present studies indicate otherwise. This discrepancy might be explainable by looking at the mode of actions of these two drugs. Linezolid functions by binding to the bacterial 23S ribosomal RNA of the 50S subunit preventing the formation of a functional 70S initiation complex, and ultimately protein production [157,158,190]. Levofloxacin, on the other hand, works by inhibiting the bacterial topoisomerases II and IV in addition to inhibiting DNA gyrase [151-153]. According to previous studies, bactericidal drugs, such as levofloxacin, are often most potent with actively growing cells [191-193]. Therefore, the inhibition of protein synthesis induced by a bacteriostatic such as linezolid (which prevents bacterial growth) could have resulted in a reduction of levofloxacin efficacy [192,193].

Although this is the first study looking at the combined effect of linezolid and levofloxacin on *B. anthracis* killing, previous studies looking at the efficacies of other antibiotic combinations on *B. anthracis* and various other bacterial species have come to similar conclusions [159,184,194-197]. Athamna *et al* found that the combination of ciprofloxacin and linezolid was indifferent against both the *B. anthracis* Sterne and ST-1 strains with a FICI index of 2.03 [194]. In addition, against the same strains, they found that the combination of ciprofloxacin and clindamycin (another protein synthesis inhibitor) was also indifferent compared to the effect of either antimicrobial alone. In accordance with the studies by Athamna *et al*, the results of the present studies found that levofloxacin and linezolid was also indifferent or had an additive effect when combined. Although their studies used ciprofloxacin, since both ciprofloxacin and levofloxacin have similar mechanisms of action and since they both worked indifferently with linezolid, it is reasonable to assume that fluoroquinolones in general may function indifferently when

combined with protein synthesis inhibitors. Further studies looking at other quinolone-protein synthesis inhibitor combinations may be able to confirm or refute this hypothesis.

Numerous antimicrobial studies have looked at the effect of various antibiotics on multiple *B. anthracis* strains (e.g. Sterne, ST-1, Ames). However, few differences have been documented between their sensitivities [184,194,198]. In these studies, however, although the Sterne and the 03-0191 strains shared many similarities, they exhibited some differences in their antibiotic sensitivity. A significant difference was noted between the susceptibilities of the two strains in the IPDM at 24 hours (P<0.001). At this time point, no growth was detected in the 03-0191 strain. In contrast, approximately 3 $\log_{10}$ CFU/mL were observed for the Sterne strain and it was not until 48 hours that the strain population was reduced to undetectable levels. A possible explanation for the difference between the sensitivities of the two strains in the IPDM could be attributed to the fact that the Sterne strain is a reference strain while 03-0191 is a clinical isolate. The Sterne strain has been around for several decades and has been used in multiple laboratories. Therefore, it is possible that through several passages Sterne may have developed some differences in antimicrobial resistance compared to the clinical strain. However, since the two strains exhibited comparable behaviors in many of the other experiments in this study it is unlikely that this difference is attributable to resistance.

An alternative explanation for the strain differences observed in the IPDM could be due to the sampling method. Although approximately 3 $\log_{10}$ CFU/mL were observed at 24 hours for the Sterne strain, since no data was collected at any time points between 24 and 48 hours we cannot deduce when, precisely, the bacterial burden reached <1 $\log_{10}$ CFU/mL. Therefore, it is

possible that the amount of time required to decrease the total population of Sterne might be closer to the 03-0191 results than the data might suggest. Studies looking at more time points (i.e. 26, 28, 30, 32 hours post- inoculation) would be able to determine if the latter is indeed the case.

A consideration of the present study is that the effect of the antibiotics on *B. anthracis* using MICs alone was examined. It is possible that higher antibiotic concentrations which may be clinically achievable in serum may have shown different (and more promising) effects on *B. anthracis*. In addition, these studies did not investigate whether either *B. anthracis* strain developed resistance during exposure to the single antimicrobials or the combination studied. Previous studies with the Sterne strain have demonstrated that resistance to fluoroquinolones requires 9 passages to develop and requires prolonged exposure to the antibiotic [149]. Moreover, *in vitro* oxazolidinone resistance occurs at a low frequency of approximately 10^{-10}, therefore bacterial resistance is unlikely [158].

Overall, this study indicates that the combination of linezolid and levofloxacin is not a better treatment option for *B. anthracis* infections. However, in a clinical setting, the combination of levofloxacin and linezolid may be more effective in rapid killing of vegetative form of *B. anthracis* and may be more effective at reducing the duration of therapy (as it would reduce the likelihood of spore survival). Therefore, the clinical benefit of the combined therapy on long-term recurrence cannot be determined from these *in vitro* models.

Although linezolid on its own was not able to rapidly kill *B. anthracis*, it was able to prevent toxin formation and resulted in a greater decrease in sporulation compared to the other

antibiotic therapies. The impact of linezolid on spore formation may reduce the risk of recurrence. Thus, linezolid may be a promising alternative for the treatment of anthrax. However, since linezolid would likely not be used on its own because of slow kill rate against vegetative *B anthracis* – it may be necessary to have combined therapy to see the most rapid kill of vegetative as well as the prevention of spore formation.

In vivo studies looking at the effect of linezolid and the combined therapy on spore production and toxin formation are warranted. Furthermore, since we only investigated the effect of linezolid combined with one other antibiotic, it is recommended that future research investigating the efficacy of other linezolid combinations be investigated both *in vitro* and *in vivo*.

An alternative approach for treating *B. anthracis* infections could be to use sequential antibiotic cycling rather than combination therapy. Although antibiotic cycling is a rather new approach, it has been shown to be effective in the treatment of *Helicobacter pylori*, *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumonia* [199-202]. Furthermore, sequential cycling has also been shown to be effective at slowing bacterial resistance [203,204]. An alternating regimen of linezolid and levofloxacin (i.e. one week of each with repeat cycles), for example, may be a possible substitute to the current regimen. Initial treatment with linezolid would theoretically be able to halt toxin production as well as prevent spore formation and vegetative growth while subsequent treatment with levofloxacin would lead to rapid bacterial killing. The benefit to cycling these two antibiotics rather than combining them would also bypass the issue of the protein synthesis inhibitor (i.e. linezolid) decreasing the efficacy of

levofloxacin as seen in these studies. Since antibiotic cycling is a relatively new avenue, studies are still required to determine how reliable this approach is. In order to implement an effective cycling regime, optimization of drug dosage and length of time is crucial [205-207]. Furthermore, determining the order in which the antibiotics should be administered is also important [205-207]. It is recommended that studies looking at the efficacy of drug cycling on *B. anthracis* be conducted both *in vitro* and *in vivo* to determine if this would be a feasible option for the treatment of anthrax.

CHAPTER 5.0. CONCLUSIONS

B. anthracis, the etiologic agent of anthrax, is a spore forming, toxin producing bacterium that is classified as a high-risk bioterrorism agent [1,2]. Currently, treatment of this microbe consists of a 60-day course of antibiotics (i.e. ciprofloxacin, doxycycline or penicillin). However, due to bacterial resistance, poor patient compliance and the ability for toxin accumulation the recommended antimicrobial regimen is currently far from ideal. Furthermore, antibiotics are known to be ineffective against the metabolically inactive bacterial spore [81,82]. Since sporulation occurs *in vivo* in the final stages of infection and is essential for future bacterial propagation, it is logical to look at ways to inhibit this pathway [199]. Therefore, therapeutics that can prevent spore formation (by directly inhibiting sporulation through protein synthesis inhibition or indirectly by rapidly killing the vegetative bacteria before they are able to sporulate) are necessary. Alternative regimens that target protein synthesis, thereby targeting both toxin production and spore formation, could prevent the build-up of harmful toxins and hardy spores resulting in the attenuation of two of the main virulence factors of *B. anthracis*. In addition, a regimen that could both target protein synthesis while rapidly killing the vegetative bacterial populations would increase our ability to combat *B. anthracis* in a more effective manner while potentially shortening the treatment period.

Although to date information on the efficacy of antimicrobials in combination on *B. anthracis* have been lacking, this study was able to quantify the effect of linezolid and levofloxacin on *B. anthracis* cell growth, spore formation and toxin production filling in some gaps. Although it was hypothesized that the combination would be the most effective antibiotic therapy, *in vitro*

studies indicate that this was not the case. In both the static models and the *in vivo*-simulating IPDM, the combination worked indifferently. Overall, these studies indicate that the combination of linezolid and levofloxacin is not more effective at killing the *B. anthracis* vegetative form than treatment with either of the antimicrobials alone. However, in the *in vivo* simulated model, the combination was able to reduce bacterial populations to undetectable levels by 48 hours. Furthermore, not only was the combination able to abolish toxin production but it was also able to significantly reduce sporulation. Targeting two main virulence factors such as sporulation and toxin production could lead to increased patient survival as well as reduce the length of treatment. In a clinical setting, the combination therapy may be more effective in killing the vegetative form of *B. anthracis* and may be more effective at reducing the duration of therapy. Consequently, the clinical benefit of the combined therapy on long-term recurrence cannot be determined from these studies and should be investigated *in vivo.* In addition, since antibiotic combination therapy remains an important option as a treatment strategy aimed at controlling the rise of resistance and for combating *B. anthracis* virulence, other combinations aside from linezolid and levofloxacin should be investigated.

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