Bacillus anthracis Spore Concentrations at Various Carcass Sites

Pamala Rose Coker
Louisiana State University and Agricultural and Mechanical College

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BACILLUS ANTHRACIS SPORE CONCENTRATIONS
AT VARIOUS CARCASS SITES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
The Veterinary Medical Sciences through the
Department of Pathobiological Sciences

by

Pamala R. Coker
B.S., Cameron University, 1986
D.V.M., Louisiana State University 1998
December 2002
DEDICATION

To my family for their support, faith and love.

To Kimothy, my mentor, my friend, my love.

Two wholes
when they coincide…

That is
beauty

that is
love.
ACKNOWLEDGEMENTS

I would like to thank my major professor Dr. Martin Hugh-Jones for his guidance and kindness. His confidence and support in my abilities encouraged me to push the envelope. He helped me to realize I can be so much more than I ever imagined.

This would not have been possible without the help of my examining committee: Drs. Gus Kousoulas, Wayne Taylor, Richard Corstvet, David Huxsoll, and Diana Williams. Their advice and guidance have been indispensable. This group kept me on firm ground and always gave me a push away from the quicksand.

My collaborators, Wood Buffalo National Park (WBNP), Northwest Territories, Canada; Washoe County District Health Department, Nevada; and Kim Conseco, Cuidad Acuna, Mexico made all of this possible. The people of these organizations were vital to the project from reporting cases, helping to collect samples and financial support. My appreciation and gratitude go beyond words. I am forever in their debt. I must specifically extend my gratitude to my fellow samplers at WBNP, Dan Dragon and Christy Wickenheiser; in Nevada, Scott Monsen, Mike Murray, Judith Saum, Gary Cole, Jerome Peterson, and Jack Spencer; and in Mexico, Kim Conseco. Thanks y’all! When can we do it again?

Preston Fulmer and Erin McCarty made processing a ton of soil possible and fun. Without their help I would still be extracting DNA. Thank you for your help and generosity.
I wish to thank Dr. Kimothy Smith for guidance and support with the statistical analyses utilized in this document. Dr. Smith was instrumental in the development of the project and was indispensable as a mentor and advocate.

Dr. Gus Kousoulas, Director of the Division of Biotechnology and Molecular Medicine, greatly supported this project by providing the technical assistance of Galena Rybachuk for development of the primers, probes, and clones used in the quantitative PCR assay. I am extremely grateful for his support and assistance.

Chapter three, *Bacillus anthracis* Virulence in AVA Vaccinated Guinea pigs is Linked to Plasmid Quantities and Clonality, would not be the powerful paper it is without the vaccinated guinea pig data provided by Patricia F. Fellows. Mrs. Fellows willingly provided her data after learning of my work and findings of differences in plasmid copy number associated with clonality after she noticed differences in survival between isolates used in the vaccine study. This chapter will be submitted to the Journal of Clinical Microbiology with Patricia Fellows and Kimothy Smith as co-authors.

My family’s emotional, spiritual, and financial support were always available and given freely. You have my heartfelt appreciation. Thank you for your guidance and unconditional love throughout my life.

Last, though far from least, I thank Kimothy for being my rock. You are always there, your faith in me is unwavering, and you are my best friend. You have helped make me a whole person. Thank you for everything.
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ABSTRACT

Bacillus anthracis is a bacterial pathogen of great importance, both historically and in the present. Despite this importance, many questions remain regarding defending against its use as a biological weapon, the bacteria’s variation in virulence, and its epidemiology in nature.

Using Etest strips (AB BIODISK, Solna, Sweden) to measure the minimum inhibition concentrations (MICs), 25 genetically diverse isolates of B. anthracis were tested to determine their susceptibility to seven clinically relevant antimicrobial agents. Using the National Committee for Clinical Laboratory Standards (NCCLS) MIC breakpoints for staphylococci, three isolates were found to be resistant to penicillin and negative for beta-lactamase production.

From a group of investigations, results indicated B. anthracis virulence is related to clonality and the copy numbers per cell of the virulence plasmids, pXO1 and pXO2. Isolates were characterized with respect to their plasmid copy number (pXO1/2) using a novel method of quantitative PCR and the numbers differ greatly from previous reports. Anthrax Vaccine with Adjuvant (AVA) vaccinated guinea pigs were challenged with 20 B. anthracis strains representative of worldwide genetic diversity. A virulence model was constructed by combining the survival, plasmid copy number, and genotyping (based on multilocus variable number tandem repeat analysis typing) data of each isolate. The model obtained was validated using a randomly chosen set of 12 B. anthracis isolates and verified model robustness.
Two carcass disposal methods, incineration and burial, are recommended to decrease or prevent environmental spore contamination. The extent of contamination from an anthrax carcass is almost totally unknown in either method of disposal. Studies of environmental contamination by spores of *B. anthracis* from infected carcasses have only recently been possible because of new technologies. A method utilizing real-time quantitative PCR was developed to quantitate *B. anthracis* in environmental samples. Absolute quantitation was made possible by the use of clones. This method has allowed the evaluation of the environmental contamination by the different carcass disposal methods and by scavenging of the carcass. The results support the complete burning of a carcass soon after death as the method choice to decrease environmental contamination for the disposal of anthrax affected carcasses.
CHAPTER ONE

GENERAL INTRODUCTION

1.1. Background

Anthrax is an acute bacterial disease of primarily herbivores, which is transmissible to humans. The etiologic agent, *Bacillus anthracis*, is a gram-positive spore forming rod shaped bacterium. Animals become infected by ingesting spores or possibly by being bitten by flies that have fed on an infected animal or carcass (7). Infected animals are usually found dead as death can occur within 24 hours. (27).

Anthrax can be found worldwide affecting wildlife, livestock, and humans. During epidemics in 1959/60 and 1970 in the Kruger National Park, South Africa, anthrax deaths numbered in the thousands (4). Livestock cases are known to contribute to human cases through the cutaneous gastrointestinal and inhalation route. In 2000, the first gastrointestinal cases were reported in the United States after the family ate beef from an infected carcass (5).

In North America in the year 2000, 413 livestock cases were confirmed and in the year 2001, 795 (Table 1.1). For every confirmed anthrax case, it is estimated that at least five cases go unreported. Though an effective vaccine for anthrax does exist and is obtainable through a veterinarian, it is often not used until an epidemic occurs. Vaccine complacency seems to arise after three to five years of vaccine use.

Although environmental contamination with *B. anthracis* spores occurs because of wildlife and domestic livestock cases, the degree or level of contamination from each case is unknown. Anthrax spores are known to persist in the environment for years and
are resistant to environmental factors (19). Spores may be found in soil contaminated by
diseased animals or in diseased animal products such as hair, wool, hides, and bones (2,
12). Very little research has been done on anthrax spore survival under natural conditions.

Table 1.1 Reported livestock deaths in North America for the years 2000 and 2001

<table>
<thead>
<tr>
<th>Location</th>
<th>Reported Anthrax Livestock Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Year 2000</td>
</tr>
<tr>
<td>WBNP, Canada</td>
<td>105</td>
</tr>
<tr>
<td>Alberta, Canada</td>
<td>0</td>
</tr>
<tr>
<td>Hook Lake, Canada</td>
<td>0</td>
</tr>
<tr>
<td>Manitoba, Canada</td>
<td>30</td>
</tr>
<tr>
<td>Nevada</td>
<td>83</td>
</tr>
<tr>
<td>N. Dakota</td>
<td>150</td>
</tr>
<tr>
<td>S. Dakota</td>
<td>15</td>
</tr>
<tr>
<td>Minnesota</td>
<td>25</td>
</tr>
<tr>
<td>Texas</td>
<td>0</td>
</tr>
<tr>
<td>California</td>
<td>0</td>
</tr>
<tr>
<td>Cuidad Acuna, Mexico</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>413</td>
</tr>
</tbody>
</table>

To prevent or decrease the number of spores contaminating an area, several
methods of carcass disposal have been suggested and utilized. These methods, including
incineration, burial, or rendering of the carcass, are recommended with no scientific data
to back them. Once a site becomes seeded with spores, the disease becomes enzootic or
at a constant risk of a persistent series of outbreaks, and sporadic occurrences of the
disease occur in susceptible animals (26).

A search of the literature shows little work has been done to ascertain the level of
contamination from a carcass regardless of the manner of carcass disposal. Spores are
known to survive in the environment for years (19). The worldwide distribution of
anthrax and the difficulty encountered in combating it is the result of the marvelous
tenacity of the anthrax spore (17). Spores prepared by Pasteur in 1888 were found to be
viable 68 years later. Bones recovered in the Kruger National Park and estimated by
carbon dating to be 200±50 years old are reported to have yielded *B. anthracis* on culture
(4). Recent studies on the ecology of anthrax have reported a correlation between the
disease and specific soil factors, such as alkaline pH, high moisture, and high organic
content (16, 23, 24). Review of the properties of spores of *B. anthracis* suggests that the
specific soil factors linked to epidemic areas reflect important environmental conditions
that aid the anthrax spores in causing epidemics (6). The Office International Des
Epizooties (OIE) and World Health Organization (WHO) Anthrax Guidelines
recommend incineration of infected carcasses as the most desirable method of disposal
followed by rendering and then by deep burial of the carcass after covering it with
quicklime (1, 15, 20). Nothing definitive exists in the literature quantifying or comparing
environmental contamination by carcass disposal methods.

1.2. Significance of the Work

The 2000 and 2001 North American epidemics raised many questions. Questions
about how to treat, vaccinate, quarantine, and dispose of the carcasses were asked by
everyone from the farm hand to the veterinarian and the department of public health.
Treatment, vaccination, and quarantine were handled by the attending veterinarian in
collaboration with the responsible public health departments. However, there was still
the question of carcass disposal, which is an interesting dilemma.
Incineration is thought to be the best way to dispose of the carcass and reduce or eliminate contamination by the anthrax spores. However, there exist many problems with the incineration of carcasses. Most cases occur in July and August in geographical areas that are very dry and have a high fire risk. The labor and materials to burn a 2000-pound carcass can be prohibitive, especially if there are several deaths.

Burial is another option that is suggested especially for high-risk fire areas. Also prior to backfilling, ‘experts’ suggests that quicklime be poured over the carcasses. Yet again burial raises many questions regarding how, where, and what to bury. No guidelines exist on how deep to bury a carcass and how much quicklime should be applied, though suggestions and opinions abound.

C.D. Stein first suggested quicklime for use in the 1950s though no reason was given (18). Recently, it has been proven that more cases occur in calcium rich soil, and as quicklime is a calcium based agent there seems to be no logic supporting this suggestion (16). High levels of calcium in the soil may buffer the spores’ supply of calcium and help maintain the spores viability in the environment for longer periods of time, thereby increasing their chances of coming into contact with and successfully infecting new hosts (14, 16).

Also questions regarding single or group burial are numerous. The burial of each carcass where it lay would be the logical choice, but, if there are several cases, group burial may be more appropriate. Group burial presents its own problems with transportation of carcasses and contamination.
In some areas where anthrax occurs, neither burning nor burial is a viable option. WBNP is a prime example of such an area as their cases are usually found by fire spotters flying over the park. The carcasses are usually found in remote areas inaccessible except by helicopter or all terrain vehicles. Getting the equipment necessary for the burial of a two-ton bull buffalo carcass into these areas is impossible.

Unless carcasses are found and disposed of quickly, scavenging occurs from buzzards, canines, felines, and other wildlife. Scavenging should be kept to a minimum as the scavengers help contaminate the environment. Scavengers open the carcass, which allows more sporulation to occur by releasing the vegetative organism from the acidic environment of the decaying carcass, thereby, enabling it to sporulate. Scavengers are not as susceptible to anthrax as herbivores; hence; after eating the diseased carcass spread spores in their feces.

Public health departments and liable parties are interested in environmental contamination not just for the possible re-infection of livestock but also for liability and safety reasons. Many acres of pastureland are fast becoming urban ranchettes, and some of this pastureland is known as an anthrax area. They need to know about contamination for risk assessment purposes. They want to know the risk of building homes and apartments in areas where anthrax infected carcasses have been disposed and the risk of human infection in these areas.

These arguments lead to the question of what is the best disposal method of anthrax carcasses and how much contamination can be expected utilizing each method. The answers will provide scientific data on which to base a decision.
The virulence of *B. anthracis* is associated with two megaplasmids, and isolates lacking either plasmid are either avirulent or significantly attenuated. Plasmid pXO2 (60 MDa) carries genes required for the synthesis of an antiphagocytic poly-D-glutamic acid capsule (8, 10, 13, 21, 22, 25). The 110-MDa plasmid pXO1 is required for synthesis of the anthrax toxin proteins, edema factor (EF), lethal factor (LF), and protective antigen (PA) (21). These proteins act in binary combinations to produce the two anthrax toxins: edema toxin (a protective antigen and edema factor) and lethal toxin (a protective antigen and lethal factor) (11). Despite the important roles of pXO1 and pXO2 in *B. anthracis* virulence, it has always been assumed that each plasmid is represented once in a bacterial cell.

The original scope of this thesis was the environmental contamination posed by anthrax carcass disposal. During the controlled studies and preparation of the 5' nuclease assay it was discovered that plasmid to chromosome ratio was 1:1:1. This discovery led to a study to quantitate the number of plasmids per bacteria.

The bioterrorism events in the Northeast United States in October 2001 also changed the scope of this work, as the event involved the mailing of anthrax spores. The first cases of inhalation and cutaneous anthrax resulting from the intentional release of *B. anthracis* were seen with this event (3, 9). This attack offered a tragic proof of principle of the danger of *B. anthracis* and has spurred numerous efforts by the biological community to improve forensics and medical countermeasures against the bacterium. My contribution to the immediate threat was an antimicrobial panel against 25 geographically and genetically diverse *B. anthracis* isolates to assess minimum inhibitory
concentrations. This panel consisted of penicillin, cephalaxin, cefaclor, tobramycin, doxycycline, ciprofloxacin, and cefuroxime.

1.3. Hypotheses

1. A difference in antimicrobial minimum inhibitory concentrations (MICs) exists based on the genotype of \textit{B. anthracis} isolates.

2. The chromosome and plasmid quantity of a vegetative \textit{B. anthracis} cell is dependent on genotype and affects an isolates’ virulence.

3. The burning of anthrax infected carcasses contributes the least to the environmental contamination by \textit{B. anthracis} spores.

1.4. References


CHAPTER TWO

ANTIMICROBIAL SUSCEPTIBILITIES OF DIVERSE

BACILLUS ANTHRACIS ISOLATES*

2.1. Introduction

The etiologic agent of anthrax, *Bacillus anthracis*, is an acute disease of primarily herbivores, which is transmissible to humans. Susceptible animals are primarily infected by the spores formed by the vegetative state of the bacteria. Spores may be found in soil contaminated by diseased animals or in diseased animal products such as hair, wool, hides, and bones. The importance of treatment of the disease in humans has been underscored by the bioterrorism events of October 2001 in the United States. Ciprofloxacin was the antimicrobial of choice for prophylactic treatment after exposure to the spores of *B. anthracis* that were used in the bioterrorism events. Penicillin, traditionally the drug of choice for treatment, is still recommended in other parts of the world despite reports of penicillin-resistance (3,4). The Centers for Disease Control has recommended ciprofloxacin, penicillin, and doxycycline for the treatment of human anthrax and for use as a prophylactic measure prior to and during the October event (2). In the past streptomycin, penicillin, gentamicin, and chloramphenicol have also been recommended (7).

Other articles have been written concerning *B. anthracis* and antimicrobial susceptibilities, two over ten years ago and one within the past year. The first paper in

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1990 by Lightfoot et al. determined the antimicrobial susceptibility of 70 isolates of *B. anthracis* against penicillin, amoxycillin, cefuroxime, gentamicin, streptomycin, erythromycin, tretracycline, chloramphenicol, and ciprofloxacin by agar dilution (6). Penicillin resistance and beta-lactamase production was noted in two isolates. This paper was quickly followed in 1991 by Doganay and Aydin (3). They tested 22 *B. anthracis* isolates against 27 antimicrobial agents by agar dilution and reported 19 isolates showed resistance to the five 3rd generation cephalosporins tested. The isolates were shown to be sensitive to the other antimicrobials tested. These two papers were very basic in their approach and reporting. The latest paper, Mohammad et al, tested 65 isolates with nine antimicrobial agents using the National Committee for Clinical Laboratory Standards (NCCLS) broth microdilution method (7). The results obtained from 50 of the isolates were compared to the Etest strips. One isolate was penicillin resistant and no statistically significant difference was found between the broth microdilution, and Etest results.

The two earlier papers distinguished between sensitive and resistant in regards to the MIC noted for each isolate(3,6). Unfortunately, no basis is given, such as what standard they used for these designations. The Mohammed et al. paper used the NCCLS breakpoints for *Staphylococcus*, which we have also chosen to use to discriminate between sensitive (S), intermediate (I), and resistant (R). According to the NCCLS literature, studies for *Bacillus* spp. are not yet adequate to develop reproducible, definitive standards to interpret results (8). The infections caused by *Staphylococcus* spp. are very similar to the infections caused by *B. anthracis*. In addition, the distributions of
the aforementioned antimicrobial data are in line with the *Staphylococcus* MIC breakpoints for antimicrobials used in this study.

In this study, we examined 25 genetically diverse isolates of *B. anthracis* to determine their susceptibility to seven clinically relevant antimicrobial agents. Etest strips (AB BIODISK, Solna, Sweden) were used to measure the MICs of the isolates.

2.2. Materials and Methods

2.2.1. Selection of Isolates

A total of 25 isolates diverse in time, space, and genotype were used in this study (Table 2.1). These 25 isolates are a representative subset of 89 distinct genotypes characterized by multiple locus variable number tandem repeat analysis (5).

2.2.2. Study Design

MICs of seven antimicrobial agents—penicillin, cephalexin, cefaclor, tobramycin, doxycycline, ciprofloxacin, and cefuroxime—were determined, using the Etest (AB BIODISK Solna, Sweden) methodology described elsewhere (1). Tryptic soy agar plates containing 5% sheep blood (Remel) were inoculated with a swab taken from a colony suspension equal to that of a 0.5 McFarland standard. One Etest strip was placed per plate after ten minutes. Quality control was assessed using *Staphylococcus aureus* ATCC 29213. The plates were incubated overnight at 37°C and the plates were read at 16 and 24 hours. This was repeated three times for each isolate.

2.2.3. Beta Lactamase Activity

Beta-lactamase activity of each isolate was determined with the use of nitrocefin disks (BBL), which utilizes a chromogenic cephalosporin. A drop of sterilized water was
applied to the disk, and then with a loop, a colony was removed from the agar plate and applied to the disk. The inoculum was taken from an overnight TSA plate for each isolate. A positive reaction was denoted by the formation of a pink or red color. The reactions were held for one hour to confirm negative results.

Table 2.1. *B. anthracis* isolates used by genotype and cluster

<table>
<thead>
<tr>
<th>Genotype #</th>
<th>Cluster</th>
<th>Country</th>
<th>State/Location</th>
<th>Source</th>
<th>Year isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>A1a</td>
<td>Canada</td>
<td>unknown</td>
<td>bovine</td>
<td>1974</td>
</tr>
<tr>
<td>10</td>
<td>A1a</td>
<td>USA</td>
<td>South Dakota</td>
<td>bovine</td>
<td>1996</td>
</tr>
<tr>
<td>15</td>
<td>A1a</td>
<td>Poland</td>
<td>Krakow</td>
<td>bovine</td>
<td>1962</td>
</tr>
<tr>
<td>20</td>
<td>A1a</td>
<td>Italy</td>
<td>Campania</td>
<td>ovine</td>
<td>1994</td>
</tr>
<tr>
<td>23</td>
<td>A1b</td>
<td>Turkey</td>
<td>Yozgat</td>
<td>human</td>
<td>1991</td>
</tr>
<tr>
<td>25</td>
<td>A1b</td>
<td>USA</td>
<td>Florida</td>
<td>bovine</td>
<td>1937</td>
</tr>
<tr>
<td>28</td>
<td>A1b</td>
<td>Turkey</td>
<td>Sivas</td>
<td>human</td>
<td>1984</td>
</tr>
<tr>
<td>29</td>
<td>A2</td>
<td>Pakistan</td>
<td>unknown</td>
<td>ovine</td>
<td>1978</td>
</tr>
<tr>
<td>30</td>
<td>A3a</td>
<td>Zambia</td>
<td>Mongu</td>
<td>bovine</td>
<td>1992</td>
</tr>
<tr>
<td>34</td>
<td>A3a</td>
<td>South Korea</td>
<td>Kyungjoo</td>
<td>human</td>
<td>1994</td>
</tr>
<tr>
<td>35</td>
<td>A3a</td>
<td>Namibia</td>
<td>Etosha Nat'l Park</td>
<td>zebra</td>
<td>1993</td>
</tr>
<tr>
<td>38</td>
<td>A3a</td>
<td>Germany</td>
<td>Giessen</td>
<td>porcine</td>
<td>1971</td>
</tr>
<tr>
<td>41</td>
<td>A3a</td>
<td>Turkey</td>
<td>Sivas</td>
<td>human</td>
<td>1985</td>
</tr>
<tr>
<td>45</td>
<td>A3a</td>
<td>Argentina</td>
<td>Buenos Aires</td>
<td>bovine</td>
<td>1980</td>
</tr>
<tr>
<td>51</td>
<td>A3a</td>
<td>USA</td>
<td>Maryland</td>
<td>bovine</td>
<td>1939</td>
</tr>
<tr>
<td>55</td>
<td>A3a</td>
<td>Australia</td>
<td>Queensland</td>
<td>bovine</td>
<td>1994</td>
</tr>
<tr>
<td>57</td>
<td>A3b</td>
<td>China</td>
<td>Changping</td>
<td>bovine</td>
<td>unknown</td>
</tr>
<tr>
<td>62</td>
<td>A3b</td>
<td>USA(Ames)</td>
<td>Texas</td>
<td>bovine</td>
<td>1981</td>
</tr>
<tr>
<td>67</td>
<td>A3a</td>
<td>South Africa</td>
<td>Kruger Nat’l Park</td>
<td>Kudu</td>
<td>1993</td>
</tr>
<tr>
<td>68</td>
<td>A3d</td>
<td>USA</td>
<td>Ohio</td>
<td>human</td>
<td>1968</td>
</tr>
<tr>
<td>69</td>
<td>A4</td>
<td>Pakistan</td>
<td>unknown</td>
<td>wool</td>
<td>1976</td>
</tr>
<tr>
<td>77</td>
<td>A4</td>
<td>UK (Vollum)</td>
<td>S. Oxfordshire</td>
<td>bovine</td>
<td>1937</td>
</tr>
<tr>
<td>80</td>
<td>B1</td>
<td>France</td>
<td>unknown</td>
<td>bovine</td>
<td>1997</td>
</tr>
<tr>
<td>85</td>
<td>B2</td>
<td>Mozambique</td>
<td>Maputo</td>
<td>porcine</td>
<td>1944</td>
</tr>
<tr>
<td>87</td>
<td>B2</td>
<td>South Africa</td>
<td>Kruger Nat'l Park</td>
<td>Kudu</td>
<td>1975</td>
</tr>
</tbody>
</table>
2.3. Results

The MIC results are summarized in Table 2.2. Using the *Staphylococcus* MIC breakpoints all isolates were susceptible to the antimicrobials except for cefuroxime and penicillin.

Only one isolate, GT 69, was sensitive, five isolates (GT’s 23, 28, 34, 55, 77) were resistant, and the remaining 19 were categorized as intermediate to cefuroxime. The MIC\(_{90}\) was 32 µg/ml, which is considered the breakpoint for resistance. The range for cefuroxime was wide, from 6 – 48 µg/ml.

Of the three recommended antimicrobials for the treatment of anthrax- ciprofloxacin and doxycycline exhibited potent activity against the *B. anthracis* isolates. Twenty-two of the isolates were susceptible to penicillin and three (GT’s 20, 68, 85) were classified as resistant. None of the isolates examined showed any beta-lactamase activity.

Ciprofloxacin, the drug-of-choice, had an MIC range of 0.032 to 0.38 µg/ml with a MIC at which 90% of the isolates tested are inhibited (MIC\(_{90}\)) of 0.094 µg/ml. This was the same as the MIC\(_{50}\) and was the most active agent tested. All of the isolates were susceptible to doxycycline and the other antimicrobials- cephalexin, cefaclor, and tobramycin.

2.4. Discussion

In this study, we have examined a spatially, temporally, and genetically diverse group of *B. anthracis* isolates for susceptibility to seven clinically relevant antimicrobials. This is the first report to use confirmed genetically diverse field isolates
Lightfoot et al. used 70 isolates, 33 of which were considered by the authors to be unrelated. Doganay and Aydin used 22 isolates collected from the same region in Turkey within a period of seven years. The most recent paper, Mohammed et al., used 50 historical isolates and 15 recent isolates. The 50 historical isolates collected between 1937 and 1997 were selected as representatives of temporally and spatially diverse strains. Together these studies have utilized 182 isolates to test antimicrobial susceptibilities. Unfortunately, the detailed isolate information from previous studies could not be obtained and therefore no correlations or comparisons can be made, genetically, spatially, or temporally.

Cefuroxime is a 3rd generation cephalosporin and is reported to be effective against gram-positive bacteria. The older studies showed a high rate of resistance with cefuroxime, though the MIC\(_{90}\)s were lower in this study. The MIC\(_{90}\) for the other studies was 64 µg/ml and for this study, it was 32 µg/ml. This could be an artifact of the

### Table 2.2. MIC\(_{50}\)s and MIC\(_{90}\)s with antimicrobial susceptibilities of 25 *B. anthracis* isolates.

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Range</th>
<th>MIC (µg/ml)</th>
<th>Categorical interpretation</th>
<th># of isolates</th>
<th>Staphylococcal breakpoints (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50%</td>
<td>90%</td>
<td>S</td>
<td>I</td>
<td>R</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>0.125 - 0.75</td>
<td>0.38 - 1.65</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>6 - 48</td>
<td>21.33 - 32</td>
<td>1</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>0.38 - 2</td>
<td>1.5 - 1.5</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.032 - 0.38</td>
<td>0.094 - 0.94</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.094 - 0.38</td>
<td>0.23 - 0.34</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin</td>
<td>&lt;0.016 - 0.50</td>
<td>0.042 - 2.36</td>
<td>22</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.25 - 1.5</td>
<td>0.75 - 0.97</td>
<td>25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( ^{a} \) S – susceptible; I – intermediate; R – resistant

\( ^{b} \) NCCLS M100-S12
methodologies or a true difference in the isolates, but in any case, the reason for this resistance is unknown and the mechanism of the resistance needs to be investigated further.

Penicillin and ciprofloxacin were also listed in all studies and all showed good activity against the isolates of *B. anthracis*. Differences in the MIC\textsubscript{90}s are noted between the reports.

Penicillin resistance was reported in two of the other studies. Lightfoot et al. reported resistance, MIC >0.25 µg/ml, in two isolates which originated from the same fatal case, and Mohammed et al. reported three resistant isolates including the same isolate from the Lightfoot et al. study\cite{6,7}. Using the *Staphylococcus* breakpoints, our study demonstrated three resistant isolates. These isolates were tested for beta-lactamase production and found to be non-producers as were all the isolates. Since the highest concentration was within a doubling dilution of the 0.25 µg/ml it is possible that the *Staphylococcus* breakpoints are not appropriate for comparison in bacillus species. There could be other explanations for this observation. Strains of *B. licheniformis* have been shown to produce large amounts of a beta-lactamase yet be sensitive to penicillin\cite{9}. In another study, a strain was shown to be highly susceptible to penicillin and still actively produce a beta-lactamase\cite{10}. Penicillin resistance in *Bacillus* spp. is an area that needs more research than a cursory susceptibility panel every few years.

Ciprofloxacin, doxycycline and the remaining antimicrobial agents, cefaclor, cephalexin, and tobramycin, showed all isolates tested highly susceptible. This was in agreement with the other studies that tested the corresponding antimicrobial agent.
Now that a reliable genotyping system is available, more genotypes should be tested against these and other antimicrobials to verify the trends noted here and in other studies. Beta-lactamase production or non-production should not be considered as an alternative for testing penicillin resistance. Hopefully this study will contribute to the impetus for a more thorough study into beta-lactamase production and *Bacillus* spp.

Continued surveillance of *B. anthracis* field isolates is recommended to monitor antimicrobial susceptibility.

2.5. References


CHAPTER THREE

BACILLUS ANTHRACIS VIRULENCE IN AVA VACCINATED GUINEA PIGS IS LINKED TO PLASMID QUANTITIES AND CLONALITY

3.1. Introduction

The etiologic agent of anthrax, *Bacillus anthracis*, is a gram positive, rod-shaped, spore forming bacterium. The disease primarily affects ungulate herbivores, occasionally carnivores, and less frequently humans (29). Anthrax is a disease well documented throughout human history with suggestive reports in the Bible and Sanskrit manuscripts (5). Koch, Pasteur, and others revolutionized the field of microbiology with their studies using *B. anthracis* as a model organism (27). Though anthrax has not been eradicated, the development of effective animal and human vaccines has reduced its importance for humans and animals in developed countries over the last century. Recently, however, anthrax research has become increasingly important due to this pathogen’s central role in biological warfare and biological terrorism (14). The importance of the disease in humans has been underscored by the bioterrorism events of October 2001 in the United States. Therefore, the need for basic techniques that are rapid and reliable, yet exhibit high specificity have taken on great importance in the development of detection assays.

Identification and detection systems based on real-time quantitative PCR (QPCR) have been described for several bacteria including *B. anthracis* (1, 2, 13, 23). These techniques are easy to design, simple to carry out and are commonly applied to monitor and detect the microbial status of sample specimens. With proper design and careful application, diagnostic quantitative PCR systems can be applied to reliably estimate and
in this case absolutely enumerate target organisms as well as nucleic acid elements within those organisms, such as plasmids (21).

The virulence of *B. anthracis* has been associated with two megaplasmids, pXO1 and pXO2 (29). Plasmid pXO2 (60 MDa) carries the genes required for synthesis of an antiphagocytic poly-D-glutamic acid capsule facilitating host immune system evasion (9, 16, 22, 30, 31, 34). The 110-MDa plasmid pXO1 is required for synthesis of the three anthrax toxin proteins, edema factor (EF), lethal factor (LF), and protective antigen (PA) (30). These proteins act in binary combinations to produce the two anthrax toxins: edema toxin (PA and EF) and lethal toxin (PA and LF) (17). Although the role of capsule and toxin in disease pathogenesis have been well characterized, copy numbers of these plasmids have not been investigated (7, 18). Recently, a study has been published alluding to the copy number of each plasmid per cell of the ‘Ames’ isolate of *B. anthracis* (26). While differences have been observed between the virulence of various isolates of *B. anthracis*, only a portion of these can be attributed to plasmid related effects (34).

Previous studies have shown that the virulence of *B. anthracis* can vary between isolates or strains (6, 8, 20). Some variation in virulence can be related to the presence or absence of the plasmids. Isolates lacking either the pX01 or pX02 plasmid are considered either avirulent or significantly attenuated (24, 32). However, this does not explain the variation in virulence observed in studies comparing fully virulent isolates such as the Ames and Vollum 1B strains (11, 12). Explanations for these differences in virulence have never been fully substantiated. Possible mechanisms responsible for the modulation of virulence exist including copy number of plasmids per cell, transcription regulation of the anthrax toxins and capsule, and additional variation may be mediated by
generation time and germination efficiency. These factors are likely associated with mutations that could be clonally heritable.

In this study, we have shown that *B. anthracis* virulence is related to clonality and pXO1 and pXO2 copy number. We chose 20 genetically diverse *B. anthracis* isolates as defined by the multi-locus VNTR analysis (MLVA) (89 distinct genotypes and seven major clusters) and performed survival trials in guinea pigs vaccinated with the current licensed U.S. human vaccine, anthrax vaccine adsorbed (AVA). Absolute quantitative PCR analysis was used to determine the plasmid copy number present per cell (pXO1, pXO2) for each of the isolates. Our model describing the virulence of *B. anthracis* isolates in AVA vaccinated guinea pigs using MLVA cluster association, clonality, and plasmid copy number was then verified using a randomly chosen group of 12 isolates.

3.2. Materials and Methods

3.2.1. Bacterial Isolates

A total of 36 isolates of *B. anthracis* were included in this study (Table 3.1). All of these isolates have been previously genotyped using multi-locus VNTR analysis (MLVA) (Keim). Initially, twenty isolates were chosen for animal challenge studies and plasmid copy number characterization based upon their representation of genetic diversity from a worldwide collection. Genetic diversity was determined by MLVA cluster assignment and was based upon genotypic allele patterns described previously for eight VNTR loci (15). The isolates used in this study are representative of all seven major MLVA clusters. Twelve isolates were chosen at random from the worldwide collection that were previously genetically characterized using MLVA.
Table 3.1. *B. anthracis* isolates used by country and cluster with the survival percentage of AVA vaccinated guinea pigs and the calculated number of plasmids, pXO1 and pXO2. Ratio of plasmid to chromosome is in parentheses.

<table>
<thead>
<tr>
<th>Id</th>
<th>Country</th>
<th>Cluster</th>
<th>% Survival</th>
<th>pXO1 (rpXO1)</th>
<th>pXO2 (rpXO2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model Isolate Set</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>China</td>
<td>A3b</td>
<td>0</td>
<td>185 (30.9)</td>
<td>9 (1.5)</td>
</tr>
<tr>
<td>2</td>
<td>Australia</td>
<td>A3a</td>
<td>0</td>
<td>121 (20.2)</td>
<td>32 (5.4)</td>
</tr>
<tr>
<td>3</td>
<td>Namibia</td>
<td>A3a</td>
<td>19</td>
<td>242 (40.3)</td>
<td>5 (0.9)</td>
</tr>
<tr>
<td>4</td>
<td>Ames</td>
<td>A3b</td>
<td>38</td>
<td>65 (10.8)</td>
<td>2 (0.3)</td>
</tr>
<tr>
<td>5</td>
<td>Germany</td>
<td>A3a</td>
<td>25</td>
<td>113 (18.9)</td>
<td>5 (0.9)</td>
</tr>
<tr>
<td>6</td>
<td>Turkey</td>
<td>A3a</td>
<td>31</td>
<td>131 (21.8)</td>
<td>11 (1.8)</td>
</tr>
<tr>
<td>7</td>
<td>France</td>
<td>B2</td>
<td>31</td>
<td>243 (40.5)</td>
<td>4 (0.6)</td>
</tr>
<tr>
<td>8</td>
<td>Turkey</td>
<td>A1b</td>
<td>38</td>
<td>236 (39.4)</td>
<td>27 (4.5)</td>
</tr>
<tr>
<td>9</td>
<td>Pakistan</td>
<td>A2</td>
<td>38</td>
<td>120 (20.0)</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>10</td>
<td>South Africa</td>
<td>B1</td>
<td>44</td>
<td>66 (11.0)</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>11</td>
<td>Zambia</td>
<td>A3a</td>
<td>44</td>
<td>242 (40.4)</td>
<td>15 (2.5)</td>
</tr>
<tr>
<td>12</td>
<td>Turkey</td>
<td>A1b</td>
<td>44</td>
<td>172 (28.7)</td>
<td>6 (1.0)</td>
</tr>
<tr>
<td>13</td>
<td>USA</td>
<td>A3a</td>
<td>44</td>
<td>109 (18.1)</td>
<td>4 (0.7)</td>
</tr>
<tr>
<td>14</td>
<td>South Africa</td>
<td>A3b</td>
<td>50</td>
<td>212 (35.3)</td>
<td>16 (2.6)</td>
</tr>
<tr>
<td>15</td>
<td>Argentina</td>
<td>A3a</td>
<td>50</td>
<td>104 (17.4)</td>
<td>7 (1.2)</td>
</tr>
<tr>
<td>16</td>
<td>Mozambique</td>
<td>B1</td>
<td>75</td>
<td>95 (15.9)</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>17</td>
<td>Canada</td>
<td>A1a</td>
<td>69</td>
<td>24 (40.5)</td>
<td>18 (3.0)</td>
</tr>
<tr>
<td>18</td>
<td>USA</td>
<td>A1a</td>
<td>69</td>
<td>128 (21.4)</td>
<td>5 (0.8)</td>
</tr>
<tr>
<td>19</td>
<td>Pakistan</td>
<td>A4</td>
<td>75</td>
<td>191 (31.9)</td>
<td>14 (2.3)</td>
</tr>
<tr>
<td>20</td>
<td>Vollum</td>
<td>A4</td>
<td>94</td>
<td>69 (11.5)</td>
<td>10 (1.6)</td>
</tr>
<tr>
<td><strong>Test Isolate Set</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Indonesia</td>
<td>A4</td>
<td>13</td>
<td>44.4 (7.4)</td>
<td>4 (0.6)</td>
</tr>
<tr>
<td>B</td>
<td>South Korea</td>
<td>A3a</td>
<td>19</td>
<td>62 (10.4)</td>
<td>3 (0.5)</td>
</tr>
<tr>
<td>C</td>
<td>USA</td>
<td>A3a</td>
<td>19</td>
<td>89 (14.8)</td>
<td>3 (0.5)</td>
</tr>
<tr>
<td>D</td>
<td>South Africa</td>
<td>A4</td>
<td>25</td>
<td>94 (15.6)</td>
<td>11 (1.9)</td>
</tr>
<tr>
<td>E</td>
<td>India</td>
<td>A3a</td>
<td>25</td>
<td>33 (5.5)</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>F</td>
<td>USA</td>
<td>A3b</td>
<td>31</td>
<td>44 (7.3)</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>G</td>
<td>Mozambique</td>
<td>B1</td>
<td>44</td>
<td>86 (14.4)</td>
<td>10 (1.6)</td>
</tr>
<tr>
<td>H</td>
<td>Norway</td>
<td>B1</td>
<td>44</td>
<td>218 (36.3)</td>
<td>1 (0.2)</td>
</tr>
<tr>
<td>I</td>
<td>Croatia</td>
<td>B1</td>
<td>44</td>
<td>49 (8.2)</td>
<td>3 (0.5)</td>
</tr>
<tr>
<td>J</td>
<td>Canada</td>
<td>A1a</td>
<td>50</td>
<td>83 (13.9)</td>
<td>5 (0.9)</td>
</tr>
<tr>
<td>K</td>
<td>USA</td>
<td>A1a</td>
<td>50</td>
<td>65 (10.8)</td>
<td>4 (0.7)</td>
</tr>
<tr>
<td>L</td>
<td>USA</td>
<td>A1a</td>
<td>56</td>
<td>115 (19.2)</td>
<td>9 (1.5)</td>
</tr>
</tbody>
</table>
3.2.2. DNA Preparation

DNA from each isolate was obtained by heat lysis of a single colony. The isolates were streaked onto blood agar plates and then incubated at 37°C overnight. A single colony from each plate was transferred into a 0.22 µm microcentrifuge tube containing 200 µl of TE (Tris-HCl [pH 8.0], 1.0 mM EDTA). The colony was resuspended by repetitive pipetting. The cellular suspension was heated to 95°C for 20 minutes and then cooled to room temperature. Cellular debris and any possible contamination by spores were removed by centrifugation at 6,000 x g for 1 min. Centrifugation was conducted inside a biosafety cabinet to contain any aerosols. The supernatant was then transferred to a new tube for storage. One microliter of the lysate contained sufficient template to support a single PCR.

3.2.3. Oligonucleotide Design of Probes and Primers

The loci sequences used for the QPCR assay primer and probe development on the chromosome, and plasmids pXO1, and pXO2 have previously been published (25). Probe and primer sequences (Table 3.2) were designed with the software program Primer Express (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) according to the manufacturer’s instructions. The sequence of the probe was selected based on previously described criteria: predicted cross-reactivity to B. anthracis; melting temperature of the probe at 69°C; lack of predicted dimer formation with corresponding primers and of self-annealing; a 10°C higher melting temperature of the probe than of the primers; and no stretches of identical nucleotides longer than four and no G at the 5’ end of probe (10).
The fluorescent reporter dye at the 5’ end of the probe was 6-carboxy-fluorescein (FAM); the quencher at the 3’ end was 6-carboxy-tetramethyl-rhodamine (TAMRA).

Table 3.2. 5’ nuclease assay primers and probes.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pXO1 plasmid</strong></td>
<td>BApXO1-PR</td>
<td>6FAM-TCGAATTACTAATCTGTCAGATACACTCCACC-TAMRA</td>
<td>84 bp</td>
</tr>
<tr>
<td></td>
<td>BApXO1-FP</td>
<td>AATGATCAATTGCGACCCTACTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BApXO1-RP</td>
<td>TGCATGCGTCGTCTTTGATA</td>
<td></td>
</tr>
<tr>
<td><strong>pXO2 plasmid</strong></td>
<td>BApXO2-PR</td>
<td>6FAM-TACTGCTTCTGTAGTTGTACCATGTCGC-TAMRA</td>
<td>131 bp</td>
</tr>
<tr>
<td></td>
<td>BApXO2-FP</td>
<td>TCACCAACCATCGTCTCGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BApXO2-RP</td>
<td>CGTTATGTAGCAATCGTATTACCTCTTAT</td>
<td></td>
</tr>
<tr>
<td><strong>Chromosomal</strong></td>
<td>BA813-PR</td>
<td>6FAM-AATGCCAGGTTCTATACCCTACAGCTATTC-TAMRA</td>
<td>123 bp</td>
</tr>
<tr>
<td></td>
<td>BA813-FP</td>
<td>GGAGGGAATACAGCAACACAGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BA813-RP</td>
<td>TGCAACTGATGGGATTTCTTCT</td>
<td></td>
</tr>
</tbody>
</table>

3.2.4. Standard for Absolute Quantitation

Standards for absolute quantitation of each primer/probe grouping (pXO1, pXO2, BA813) were prepared as follows. The PCR amplified fragment obtained with the primers for each grouping was cloned using pcDNA3.1/V5-His-TOPO TA and transformed into *Escherichia coli* TOPO10 (Topo TA cloning kit; Invitrogen, NV Leek, The Netherlands). Purification of the plasmid DNA was carried out with a commercial plasmid DNA isolation kit (Qiagen, Basel, Switzerland). The DNA concentration was determined with a spectrophotometer and was diluted to $10^{10}$ templates/5 µl. Dilutions were made equivalent to $10^9$, $10^7$, $10^5$, $10^3$, 10 and 1 copy of template per 5 µl. For bidirectional DNA sequencing of the insert, the forward primer and reverse primer were
used. The nucleotide sequence was determined using a fluorescence-based automated sequencing system (ABI 377A DNA sequencer) by Microsynth, Balgach, Switzerland. The plasmid insert was sequenced to confirm its identity with *B. anthracis*.

3.2.5. Test of Detection Limit for 5’ Nuclease Assay

The detection limit of the QPCR assays for *B. anthracis*, was determined by making serial dilutions of overnight cultures of two isolates, isolate 4 and isolate 17 (Table 3.1), suspended in 0.9% NaCl. The suspension was adjusted to a density corresponding to that of a McFarland 1.0 standard and from this serial dilutions were made. Plate counts from each dilution were performed in triplicate on 5% sheep blood agar (Remel). A total of 200 µl of culture from each dilution was heat lysed as described above, and 5 µl was used for the 5’ nuclease assay. Each test was repeated three times.

3.2.6. Specificity of the QPCR Assays

Ten isolates of closely related *Bacillus* spp. were used to examine the specificity of the QPCR assay. These isolates were *B. cereus* isolates, ATCC 4381 and ATCC 14579, *B. subtilis* ATCC 6051, *B. megaterium* ATCC 14580 and four confirmed environmental isolates, and two environmental isolates of *B. thuringensis*. The environmental isolates were confirmed by 16S rRNA sequencing.

3.2.7. 5’ Nuclease Assay

Each 25-µl PCR reaction contained 1X TaqMan® Universal PCR Master Mix (Part number 4304437, Applied Biosystems), 16 µM each primer, 100 nM fluorogenic probe, and 5 µl of template or plasmid standard. DNA amplification was carried out in MicroAmp® Optical 96-well reaction plates (part number N801-0560, Applied
Biosystems) sealed with MicroAmp® optical caps (part number N801-0935, Applied Biosystems). The cycling program consists of heating for two minutes at 50°C then 10 minutes at 95°C followed by a two stage temperature profile of 95°C for 15 seconds and 60°C degrees for one minute, repeated for 40 cycles. Amplification, data acquisition, and data analysis were carried out with an ABI 7700 Sequence Detector (Applied Biosystems). All 5’ nuclease tests were repeated at least three times for all isolates listed in the tables.

3.2.8. Vaccination and Challenge

All data associated was provided by Patricia F. Fellows, USAMRIID, Ft. Detrick, Md. Hartley guinea pigs (Charles River, Wilimington, MA) were vaccinated at 0 and 4 weeks intramuscularly (i.m.) with 0.5 ml AVA (Bioport, Michigan, etc.). At 10 weeks after the first vaccination, the guinea pigs were challenged i.m. with 10,000 spores of virulent *B. anthracis*. Survival was noted for 14 days post challenge.

3.2.9. Absolute Copy Number of Loci, Statistical Analysis and Modeling

All means, statistical analyses, and modeling were calculated using SPSS™ version 10.2 software. The mean absolute copy number of chromosomal loci per cell was determined by back calculation using total number of cells in a series of dilution trials analyzed with the QPCR assay. Cross-validation of the mean number of plasmid loci per cell was performed using the same methodology. The mean number of cells per trial were determined using most probable number methods and confirmed with direct spore counts. The ratios of plasmid to chromosomal DNA copy were derived by first dividing the total estimated copy number of chromosomal loci determined using the QPCR assay.
by the mean absolute copy number of chromosomal loci per cell (determined above) then, dividing the estimated total copies of plasmid loci (for each of the two plasmids individually) by the adjusted chromosomal copy number.

After assembling the data set and calculating the ratios of plasmids to chromosome copies, means of the MLVA clusters were estimated concurrent with a one-way analysis of variance. The percent survival of guinea pigs was modeled using a univariate general linear model. The dependent variable, percent survival, was modeled with the two covariates ratio of pXO1 to chromosome (rpXO1) and ratio of pXO2 to chromosome (rpXO2) and the factor MLVA cluster which had seven levels corresponding to the seven major MLVA clusters (15). The model examined main effects only of the covariates and factor. No interactions were examined for the model. Parameter estimates and 95% confidence intervals were calculated for each covariate, factor level and model intercept term. Statistical significance levels for evaluation of the model were set \textit{a priori} at \( \alpha = 0.05 \). Post hoc tests were conducted using the Student-Newman-Keuls method.

3.3. Results

3.3.1. Specificity and Sensitivity of the 5’ Nuclease Assay

The three unique primer/probe sets designed for the specific identification and target quantification of \( B. \) \textit{anthracis} correctly discriminated all isolates tested (Table 3.2). The analytical sensitivity of the QPCR assays was comparable to that of the previously described nested PCR (3). The assay system resolution was sufficient to detect one copy of the standard plasmid. The samples containing no template gave a negative result.
3.3.2. Chromosome, pXO1, pXO2 Loci per Bacteria and Plasmid Ratios

The average number of chromosomal loci detected per cell was approximately 6 for all of the isolates examined. The number of loci detected per cell for each of the virulence plasmids, however, varied greatly across isolates, ranging from 33 (isolate E) to 243 (isolate 7 and 17) for pXO1, and from 1 (isolates 9, 16, and H) to 32 (isolate 2) for pXO2. The plasmid:chromosome loci ratios are listed for each isolate in Table 3.1.

The mean plasmid:chromosome loci ratios calculated over the seven MLVA clusters were significantly different as indicated by the results of an ANOVA (p<0.05) (Figure 3.1). Post hoc tests indicated that for pXO1 there were three overlapping groups. Group 1 included MLVA clusters A2, A3a, A3b, A4 and B1. Group 2 included MLVA clusters A1, A2, A3a and A3b. Group 3 included MLVA clusters A1 and B2. Post hoc tests indicated that for pXO2 there were two overlapping groups. Group 1 included MLVA clusters A2, A3a, A3b, A4, B1 and B2. Group 2 included MLVA clusters A1, A3a, A3b, B1 and B2.

3.3.3. Vaccination Challenge and Survival

Survival of the vaccinated guinea pigs ranged from 0 to 94 percent. The percent survival of the vaccinated guinea pigs challenged with each of the isolates used in this study, are listed in table 3.1. The average survival overall was approximately 40%. The average time to death was 5 days for all isolates tested and ranged from 3 to 7 days. The mean percent survival for each MLVA cluster is shown in Figure 3.1. The results of an ANOVA indicated that the means were different (p<0.05). Post hoc testing showed four groups, two of which had overlapping membership (Figure 3.2). The most virulent
MLVA cluster, indicated by lowest survival, had a mean survival of 21.3% (cluster A3b) and included an isolate of the Ames strain.

3.3.4. Model of Virulence

We analyzed the combined data set of percent survival, ratio of plasmid:chromosome (rpXO1 and rpXO2) and MLVA cluster assignment using a general linear univariate model. Overall model statistics indicated that rpXO1 was not a significant predictor of survival ($p>0.05$) but that rpXO2 and MLVA cluster were significant ($p<0.05$ for both). The parameter estimate obtained for rpXO1 was not significant however the estimate obtained for rpXO2 was ($rpXO1 = 0.06$, $p>0.05$; $rpXO2 = -1.93$, $p<0.05$). The magnitude of the parameter estimates obtained for each of the seven MLVA clusters indicate a much greater influence on survival outcome than either plasmid ratio ranging from 0.00 (A3b) to 75.41 (A4) (Figure 3.2). As previously mentioned, post hoc testing revealed two overlapping and two distinct statistical groups (Figure 3.2) indicating that there are potentially as many as five or as few as three separate groups among the MLVA clusters. Graphs of the observed and predicted percent survival for both the isolates with which the model was constructed and a test set of 12 randomly chosen isolates indicate a good model fit (Figure 3.3). The raw residual values are also shown for each set of predicted and observed comparison.

Figures 3.1 and 3.2 show a grouping of the MLVA clusters into three distinct groups. The least virulent group, A4, contains isolates from the United States, Norway, Europe, and Asia (15). This group also contains ‘Vollum’ which was used in the UK biological warfare program (15). The middle group is made up of clusters A1 and B1
and is of medium virulence. Clusters A1 and B1 are similar in virulence but are geographically diverse. Isolates in the A1 cluster are found predominantly from Wood Bison National Park (Canada), south to Texas in the United States. Southern African isolates make up the majority of the B1 cluster (15). The most virulent group consists of four clusters, A2, A3a, A3b, and B2. This grouping is also geographically diverse. B2 is found mainly in southern Africa and has been loosely associated with the A cluster (15). A3a is associated with isolates from around the world especially Turkey and Namibia. A3b is dominated by isolates from China, yet this cluster also contains ‘Ames.’

![Bacillus anthracis MLVA Dendrogram (Keim, et al, 2000)](image)

**Figure 3.1.** Means of plasmid to chromosome ratios and percent survival of AVA vaccinated guinea pigs by MLVA cluster used to build model.
3.4. Discussion

In this study, we have presented results demonstrating that *B. anthracis* virulence is related to clonality (as indicated by MLVA genotype cluster) and pXO1 and pXO2 copy number. By combining data collected from challenge studies of AVA vaccinated guinea pigs, pXO1 and pXO2 plasmid copy number with a genetically diverse set of isolates we developed a model predictive of virulence. We then used this model to compare the predicted and observed survival of AVA vaccinated guinea pigs when challenged with a randomly chosen set of isolates. It is likely that factors affecting the variation in virulence we observed are due to mutations that are heritable and clonally passed on to daughter cells. This could also include a predisposition for stable plasmid copy number configurations of pXO1 and pXO2.
Figure 3.3. Observed and predicted survival of AVA vaccinated guinea pigs challenged with diverse *B. anthracis* isolates. Twenty isolates were used to construct the model (A) and an additional set of twelve isolates were used to the test the model (B).
While we can offer no explanation for the association of higher or lower virulence with respect to specific MLVA clusters the relationship is plausible from the standpoint of mutations and clonality. Other examples of genetic typing based group differences in pathogen virulence have been observed (4). Further investigation to determine the underlying reasons for variation in virulence should center on the genetic differences between representative isolates from different MLVA clusters such as A3b and A4, that showed extreme differences in mean survival and model parameter estimates. Although rpXO2 was found to be a significant contributor in the virulence model, there was no distinct difference in the number of plasmids per cell for clusters A3b and A4; therefore, we can conclude that the differences were due to factors other than the number of plasmids per cell.

Our observations have revealed tremendous variation in pXO1 and pXO2 copy number per cell using a genetically diverse collection of isolates. These observations were made possible by the use of a new method for molecular detection of *B. anthracis* using QPCR. Using QPCR, we were able to detect one copy of the standard plasmids for the chromosome, pXO1, and pXO2. A common belief among the research community has been that *B. anthracis* contains only one copy of each plasmid. Here, using a QPCR method, we have shown that there is more than one copy of each plasmid per cell and that there is tremendous variation among genetically diverse isolates. Our results indicate that there may be up to 243 copies of pXO1 and 32 copies of pXO2 per cell. While these numbers may seem extreme, this could be appropriate because the longer a cell survives the more toxin it can produce.
It has long been known that different isolates have exhibited varying levels of virulence (11, 19, 28). The factors responsible for the different levels of virulence in these isolates are unknown. We have shown that the number of pXO2 plasmids in each bacterial cell contributes to the level of virulence associated with that isolate. Although the model that we developed indicated that pXO1 copy number does not contribute significantly to virulence, it is likely that in an experimental challenge of unvaccinated animals would find that the number of copies of pXO1 would also contribute to variations in virulence. However, the results of our studies presented here indicate that pXO2 plays a significant role in virulence and can contribute to observed variation in virulence. In the mouse model, isolates that only contain pXO2 remained lethal at low doses (33). Welkos showed that the pXO2 plasmid contributes significantly to the virulence of the organism (32) and that mutants producing greater amounts of capsule exhibited a higher level of virulence than the parental strain. Figure 3.4 shows an increasing gradation of virulence associated with an increase of pXO2.

Virulence studies of anthrax are an important source of information especially considering the organisms use as a bioterror/biowarfare agent. This study has put forward some interesting and significant information concerning virulence of different isolates of *B. anthracis* and the testing of these same isolates in an animal system. Trends in the data with respect to plasmid copy number have revealed clues that will take us into the next paradigm of virulence testing. Hypotheses have been suggested that can now be tested due to technological advances in molecular biology techniques. Additional animal studies using unimmunized animals utilizing more representative isolates of all clusters
are recommended to validate the model and cluster effect observed in this study. Furthermore, validation of the model could lead to its use as a screening tool and complement vaccine efficacy testing.

Figure 3.4. Effect of pXO2:genome ratio on percent survival across MLVA clusters holding pXO1:genome ratio constant.

3.5. References


4.1. Introduction

Anthrax, an acute bacterial disease of primarily herbivores, is transmissible to humans. The etiologic agent, *Bacillus anthracis*, is a gram-positive spore forming rod shaped bacterium. Animals become infected by ingesting spores or possibly by being bitten by flies that have fed on an infected animal or carcass (8). Infected animals are usually found dead as death can occur within 24 hours. (25).

It is generally thought that *B. anthracis* is an obligate pathogen and that little propagation occurs in soil (4, 5, 12). With the exception of scavengers, anthrax is almost never transmitted directly from victim to victim but is rather ingested by herbivores while grazing or browsing. In such a model, the environmental spore reservoir becomes very important to the ecology and evolution of this pathogen. Survival in the soil is crucial for initiating subsequent anthrax epidemics.

Although environmental contamination with *B. anthracis* spores occurs because of wildlife and domestic livestock cases, the degree or level of contamination from each case is unknown. Anthrax spores are known to persist in the environment for years and are resistant to environmental factors (20). Spores may be found in soil contaminated by diseased animals or in diseased animal products such as hair, wool, hides, and bones (2).

To prevent or decrease the number of spores contaminating an area, several methods of carcass disposal have been suggested and utilized. These methods, including
incineration, burial, or rendering of the carcass, are recommended with no scientific data to back them. Once a site becomes seeded with spores, the disease becomes enzootic or at a constant risk of a persistent series of outbreaks, and sporadic occurrences of the disease occur in susceptible animals (24). The Office International Des Epizooties (OIE) and World Health Organization (WHO) Anthrax Guidelines recommend incineration of infected carcasses as the most desirable method of disposal followed by rendering and deep burial of the carcass after covering it with quicklime (1, 14, 21).

Spores are known to survive in the environment for years (19). The worldwide distribution of anthrax and the difficulty encountered in combating it is because of the marvelous tenacity of the anthrax spore (17). Spores prepared by Pasteur in 1888 were found to be viable 68 years later. Bones recovered in the Kruger National Park and estimated by carbon dating to be $200\pm50$ years old are reported to have yielded $B. anthracis$ on culture (4). Recent studies on the ecology of anthrax have reported a correlation between the disease and specific soil factors, such as alkaline pH, high moisture, and high organic content (16, 22, 23). Review of the properties of spores of $B. anthracis$ suggests that the specific soil factors linked to epidemic areas reflect important environmental conditions that aid the anthrax spores in causing epidemics (5).

A search of the literature shows little work has been done to ascertain the level of contamination from a carcass regardless of the manner of carcass disposal, and only three studies have been done concerning contamination from anthrax infected carcasses. Dragon et al. collected environmental samples from known burial and cremation sties in Wood Buffalo National Park and Mackenzie Bison Sanctuary in the Northwest
Territories of Canada after several epizootics had occurred in the region over the past ten years. Another study was conducted in the Etosha National Park, Namibia, by Lindeque et al (12). The park leaves all carcasses exposed on the surface therefore, there was not a disposal method comparison but they did look at spore levels in soil contaminated by the body fluids of animals that had died of anthrax. The Etosha study also detected *B. anthracis* in scavenger feces and water.

Turnbull et al. described a case in North Devon, England, where two ponies died in the field in which they had been grazing in 1986 (19). The ponies were disposed of by rendering and an investigation of how they may have become infected led to the discovery that a young bull had died of anthrax around 1938 and had been buried in the field. A bacteriological survey of the field in 1991 revealed isolates could be recovered from the immediate area which corresponded to the burial site of the bull. This area was surveyed again in 1992 with the same results. After two unsuccessful efforts at chemical disinfection with formalin being pumped into the ground and applied to the surface a decision was made to place a concrete cap, 9m X 5m X 20 cm, over the burial site to provide a permanent barrier between viable spores and humans or animals.

The samples obtained for these 3 studies ranged in time from immediately after death to 50 years later. The time between death and sampling for this study ranged from two to thirteen months. The sampling sites where these samples were taken were diverse in disposal method and environment. They included carcasses that had been left exposed on the surface to be scavenged and to decay with time, buried, burned and some were a combination of these methods. The environment from which these samples were
collected, ranged from the far North to the South, Wood Buffalo National Park (WBNP) in the Northwest Territories of Canada; Washoe County, Nevada; and Cuidad Acuna, Mexico, directly across the border from Del Rio, Texas.

Nothing definitive exists in the literature quantifying or comparing environmental contamination by carcass disposal methods. To study the risks of the environmental contamination posed by anthrax infected carcass disposal methods, the soil around carcasses which had been treated by the different recommended disposal methods was sampled. Utilizing methods to concentrate the *B. anthracis* spores found in a soil sample and extracting the DNA from these spores for identification, it was possible to use statistical methods to determine the efficacy of the disposal methods.

4.2. Materials and Methods

4.2.1. Site Selection

The locations were selected based on the carcass disposal method employed. Sampling sites were selected on accessibility and consisted of a confirmed anthrax carcass that had been left exposed, buried, and/or burned. Once the site was selected, a sampling area was created.

4.2.2. Surface Soil Sampling

A survey stake was driven into the ground directly over the buried carcass or in the center of the sampling area. Using the stake as the center, a five-meter radius circle was marked off using flags. With the use of a compass, the cardinal directions, north, south, east and west, were marked with a red flag at north and pink flags at the other directions (Appendix 1). The boundary of the circle was marked with a different colored
flag. The flags broke the circle into quadrants and five random samples were selected in each quadrant by tossing five tennis balls into the quadrant and taking 10 cm core samples with a Hoffer soil sampler where each ball had landed. The approximate location of each sample was noted on the data collection diagram with the sample number. Twenty surface samples were collected at each site. The soil sampler was cleaned with a sporacide between sites.

4.2.3. Deep Core Soil Sampling

Burial sites were sampled using a mud auger and the appropriate extensions. One deep core hole was made per buried carcass site. The soil was collected in the auger, and, as the auger would fill with soil, it was removed and the soil collected in a separate container and marked with the depth and site information. Soil collecting continued until the buried carcass was reached and tissue was recovered. The sampling equipment was cleaned with a sporacidal solution between sites.

4.2.4. Spore Concentration Method

From each soil sample collected, 4 grams of soil was placed into a 50ml centrifuge tube. To the sample, 30 ml of a wash solution (1.22 gm/ml sucrose, 0.5%Triton X-100) was added. The sample was then vortexed and placed on a shaker for 30 minutes. The top 3ml was transferred to a tube containing 6 ml of BSA/PBS (bovine serum albumin, with phosphate buffered saline, pH 7.4 [PN: P3688 Sigma Chemical Company, St. Louis, Mo.]), vortexed and centrifuged (6000 g) for 10 minutes. The supernatant was discarded, 5 ml of 90% ethanol was added to the pellet, vortexed, and placed on a shaker for 30 minutes. The sample was centrifuged (6000 g) for 10 minutes,
the supernatant was discarded, and the pellet was resuspended in 1ml of the BSA/PBS solution. Samples were stored at 4°C.

4.2.5. DNA Extraction Method

In a 2 ml bead-beater tube (BioSpec Products, Inc., Bartlesville, Okla.) containing 900 mg of a mixture of glass beads (450 mg each of 425-600 µm and 106 µm and finer glass beads [Sigma Chemical Company, St. Louis, Mo., or BioSpec Products, Inc., Bartlesville, Okla.]) and 0.5 ml of 2X TENS buffer (1X TENS is 50 mM Tris HCl [pH 8.0], 20 mM EDTA, 100 mM NaCl, 1% [wt/vol] sodium dodecyl sulfate [SDS]), 0.5 ml of liquid product from the spore concentration method was added. The tubes were incubated at 70°C for 30 minutes, removing the samples every 10 minutes to vortex. Samples were homogenized for three minutes at 5,000 rpm in a mini-beadbeater-8 cell disruptor (catalog no. 693; BioSpec Products, Inc., Bartlesville, Okla.) and were then centrifuged at 12,000 X g for 10 min at 4°C to pellet the bead mix. The supernatant containing DNA was collected into a 15 ml centrifuge tube and stored on ice. Under a vented safety hood, 1 ml of chloroform:isoamyl alcohol (24:1 volume/volume) was added to the 15 ml tube and placed on a tube rocker for 20 minutes, then centrifuged at 7000 X g at 4°C for 10 minutes. The upper aqueous phase of the mixture was transferred to a sterile micro-centrifuge tube and Rnase (1 µg/ml or 1:100) was added, inverted by hand to mix, and incubated at 37°C for 1 hour. Under a vented safety hood, an equal volume (500 µl) of chloroform:isoamyl alcohol (24:1 volume/volume) was added and the sample was placed on a tube rocker for 20 minutes, then centrifuged at 7000 X g at 4°C for 15 minutes. The upper aqueous phase of the mixture was transferred to a sterile micro-
centrifuge tube, and 0.6 volumes of isopropyl alcohol was added, then inverted by hand to mix. The samples were frozen at -20°C from 2 hours to overnight, and the nucleic acids were precipitated by centrifugation at 4000 X g for 5 minutes. The supernatant was carefully poured off, and the DNA was washed with 500 µl 70% ethanol and centrifuged at 800 X g for five minutes to re-pellet. The supernatant was carefully poured off, and the pellet briefly dried. The pellet was dissolved in 500 µl sterile 1XTE buffer (10 mM Tris pH 7.6, 1 mM EDTA). Samples were stored at -80°C.

4.2.6. Control Soil

Soil from Falaise lake, Northwest Territories, Canada, was used for a control study. One kilogram of the soil was sterilized by autoclaving at 121°C for 30 minutes on the wrapped cycle. Sterility was verified by mixing 0.1 gm of the sterile soil with 10 ml of nutrient broth and incubating at 37°C. The broth was checked for growth every 24 hours for three days with no growth noted. Four grams of sterile soil and unsterile soil was spiked with 100, 500, 1000, and 10000 spores of *B. anthracis* in triplicate. These controls were processed through the spore concentration and DNA extraction methods. From the 1 ml liquid product of the spore concentration method, 100 µl was plated on sheep blood agar (SBA, [Remel, Inc.]) in triplicate for all samples. After overnight incubation at 37°C, the plates were removed and colonies of *B. anthracis* were counted and recorded.

4.2.7. Miscellaneous Environmental Samples

Samples other than soil were collected in the area of the sampling site. These samples included parts of the carcasses, flies, carnivore scat, and water. These samples
were processed through the spore concentration method. From the 1 ml liquid product of the spore concentration method, 10 µl was plated on SBA. This was done in triplicate for all miscellaneous samples. After overnight incubation at 37°C, the plates were removed and visually inspected for colonies of *B. anthracis*. All suspect colonies were sub-cultured onto SBA, and a 5 µl drop of *B. anthracis* specific gamma phage was applied for confirmation. After overnight incubation, at 37°C the plates were inspected for a clear zone where the gamma phage had been applied indicating phage sensitivity. The gamma phage sensitive isolates were sub-cultured onto a nutrient agar slant and were placed in the incubator at 37°C for four weeks which allowed for full sporulation and drying of the agar, then stored at room temperature.

4.2.8. Spore Viability

Random samples from the WBNP 2000 and Nevada soil samples were selected and from the liquid product of the spore concentration method, 10 µl was plated on SBA. This was done in triplicate for all selected samples. After overnight incubation at 37°C, the plates were removed and visually inspected for colonies of *B. anthracis*. All suspect colonies were sub-cultured onto SBA, and a 5 µl drop of gamma phage was applied for confirmation. After overnight incubation at 37°C, the plates were inspected for a clear zone where the gamma phage had been applied indicating phage sensitivity. The number of positives was recorded.

4.2.9. Oligonucleotide Design of the Probe and Primers

PCR primer sequences for loci on the chromosome, plasmids pXO1, and pXO2 have previously been published (15). These primers were used as the basis of the primers
and probes developed for the TaqMan assays (Table 4.1). The TaqMan probe and the primer sequences were designed with the software program Primer Express (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) according to the manufacturer’s instructions. The sequence of the probe was selected based on previously described criteria (9): predicted cross-reactivity to *B. anthracis*, melting temperature of the probe at 69°C; lack of predicted dimer formation with corresponding primers and of self-annealing; a 10°C higher melting temperature of the probe than of the primers; and no stretches of identical nucleotides longer than four and no G at the 5’ end of probe. The fluorescent reporter dye at the 5’ end of the TaqMan probe was 6-carboxy-fluorescein (FAM); the quencher at the 3’ end was 6-carboxy-tetramethyl-rhodamine (TAMRA).

Table 4.1. 5’ nuclease assay primers and probes

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers</th>
<th>Sequence (5’-3’)</th>
<th>Amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>pXO1 plasmid</td>
<td>BApXO1-PR</td>
<td>6FAM-TCGAATTACTAAATCTCTGAGATACACTCCCACC-TAMRA</td>
<td>84 bp</td>
</tr>
<tr>
<td></td>
<td>BApXO1-FP</td>
<td>AATGATCAATTTGCAGACGTACTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BApXO1-RP</td>
<td>TGCATGCGTCGTCTCTTGATA</td>
<td></td>
</tr>
<tr>
<td>pXO2 plasmid</td>
<td>BApXO2-PR</td>
<td>6FAM-TACTGCTTTCTGACGTGTACCCATGTCGAC-TAMRA</td>
<td>131 bp</td>
</tr>
<tr>
<td></td>
<td>BApXO2-FP</td>
<td>TCACCAACCACGTTCATCCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BApXO2-RP</td>
<td>CGTTATGTAGCAATCGTATTACCTTTAT</td>
<td></td>
</tr>
<tr>
<td>Chromosomal</td>
<td>BA813-PR</td>
<td>6FAM-AATGCCAGGTTCTATACCGTACAGCAAGCTATCC-TAMRA</td>
<td>123bp</td>
</tr>
<tr>
<td></td>
<td>BA813-FP</td>
<td>GGAGGGAATACAGCAAAACAGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BA813-RP</td>
<td>TGCATTGATGGGATTCTCTT</td>
<td></td>
</tr>
</tbody>
</table>
4.2.10. Plasmid Standard for Absolute Quantitation

A plasmid standard for absolute quantitation of each primer/probe grouping (pXO1, pXO2, BA813) was prepared as follows. The fragment obtained with the primers for each grouping was cloned using pcDNA3.1/V5-His-TOPO TA and transformed into *Escherichia coli* TOPO10 (Topo TA cloning kit; Invitrogen, NV Leek, The Netherlands). Purification of the plasmid DNA was carried out with a commercial plasmid DNA isolation kit (Qiagen, Basel, Switzerland). The DNA concentration was determined with a spectrophotometer and was diluted to $10^{10}$ templates/5 µl. Dilutions were made equivalent to $10^9$, $10^7$, $10^5$, $10^3$, 10 and 1 copy per 5 µl. For bidirectional DNA sequencing of the insert, the forward primer and reverse primer were used. The nucleotide sequence was determined using a fluorescence-based automated sequencing system (ABI 377A DNA sequencer) by Microsynth, Balgach, Switzerland. The plasmid insert was sequenced to confirm its identity with *B. anthracis*.

4.2.11. Quantitative PCR Assay

Each 25-µl Quantitative PCR (QPCR) reaction contained 1X TaqMan® Universal PCR Master Mix (Part number 4304437, Applied Biosystems), 16 µM each primer, 100 nM fluorogenic probe, and 9 µl of template or 5 µl of plasmid standard. DNA amplification was carried out in MicroAmp® Optical 96-well reaction plates (part number N801-0560, Applied Biosystems) sealed with MicroAmp® optical caps (part number N801-0935, Applied Biosystems). The cycling program consisted of heating for two minutes at 50°C, then 10 minutes at 95°C, followed by a two stage temperature profile of 95°C for 15 seconds and 60°C degrees for one minute, repeated for 40 cycles.
Amplification, data acquisition, and data analysis were carried out with an ABI 7700 Sequence Detector (Applied Biosystems). All QPCR tests were repeated at least four times for each sample tested.

4.2.12. Statistical Analysis

All means, statistical analyses, and modeling were calculated using SPSS™ version 11.0.1 software. After assembling the dataset, the means of the QPCR results for each sample was calculated. Descriptive statistics, bivariate correlations, univariate general linear models, and linear regression analyses were performed. Statistical significance levels were set a priori at alpha = 0.05.

4.3. Results

4.3.1. Site Selection

A total of 77 sites were sampled (Table 4.2). No disposal method was used in WBNP; all of the carcasses were left exposed on the surface. Mixtures of disposal methods were utilized in Nevada. Six of the sites were of single carcasses that had only been burned, two sites consisted of one carcass each that had been burned and then buried, 14 were buried only, 15 were buried with lime poured over the carcass before backfilling and one carcass was limed and left exposed on the surface. The five cases in Mexico were all burned.

4.3.2. Soil Sampling

A total of 1538 surface and 241 burial soil samples were collected and worked through the spore concentration and DNA extraction protocols (Table 4.2.).
mean for the surface samples was $1.5 \times 10^7$ spores and $2.5 \times 10^7$ for the burial samples.

The difference was not statistically significant (p=0.792).

Table 4.2. Location of samples with types and number of samples collected.

<table>
<thead>
<tr>
<th>Location</th>
<th>Sites Sampled</th>
<th>Surface Samples</th>
<th>Deep Core Samples</th>
<th>Miscellaneous Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBNP 2000</td>
<td>19</td>
<td>388</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td>WBNP 2001</td>
<td>15</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nevada</td>
<td>38</td>
<td>760</td>
<td>241</td>
<td>58</td>
</tr>
<tr>
<td>Cuidad Acuna, Mexico</td>
<td>5</td>
<td>100</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>77</strong></td>
<td><strong>1538</strong></td>
<td><strong>241</strong></td>
<td><strong>165</strong></td>
</tr>
</tbody>
</table>

4.3.3. Control Soil

A comparison of the CFU counts and QPCR results is shown in Figure 4.1. These results revealed a Pearson correlation coefficient of .974, (p<0.05) for the sterile and unsterile soil and a linear relationship ($r^2 = .949$).

4.3.4. Miscellaneous Environmental Samples

Miscellaneous samples were collected from WBNP, Nevada, and Mexico (Table 4.2). No isolates were recovered from the 17 miscellaneous samples collected in Mexico, possibly because the carcasses were completely burned within hours of finding them. The samples collected from the WBNP and Nevada sites revealed many positives, 64.5% and 37.5% respectively (Figure 4.2.). Out of all the samples tested, the scat from both locations showed the highest percentage of recovery, 72% from WBNP and 42% from
Nevada. The scat samples were from wolf (*Canis lupis*) and black bear (*Ursus americanus*) in WBNP and coyote (*Canis latrans*) in Nevada and showed the highest levels of contamination per recovery plate. Most of the SBA plates revealed high numbers of *B. anthracis* colonies with low contamination from other bacteria.

All of the water samples from WBNP and half of the Nevada water samples tested positive. These water samples were taken from near a carcass and within the sampling area.

Figure 4.1. A comparison of *B. anthracis* CFU’s on sheep blood agar and QPCR absolute counts with the number of spores spiked in sterile and unsterile soil.
The carcass samples consisted of swabs of body fluids, tissue samples, hair, hooves, and bone. These samples were intimately associated with the carcass, yet the yield of recovery was low, only 53% for WBNP and 33% from the Nevada carcasses.

![Graphical comparison of the CFU and QPCR results.](image)

**Figure 4.2.** Miscellaneous environmental samples taken from sites in Wood Buffalo National Park, Canada, and Nevada.

4.3.5. Spore Viability

Figure 4.3 is a graphical comparison of the CFU and QPCR results normalized to 1000 µl. This 1000 µl is the product of the spore concentration method with four grams of soil. The Pearson correlation coefficient between the CFU’s and QPCR was positive (0.326, p=0.017) indicating a linear relationship. A linear trendline was calculated and overlaid for each line on Figure 4.3 to further point out the linear relationship between the colony counts and QPCR results. A linear regression analysis revealed that the lower
threshold of sensitivity was 7500, meaning there must be at least 7500 pXO1 copies before viable spores can be recovered.

Figure 4.3. Comparison of colony forming units on sheep blood agar and quantitative PCR.

4.3.6. Test of Detection Limit for Quantitative PCR Assay

The analytical sensitivity of the TaqMan PCR was comparable to that of the previously described nested PCR (2). The assay system detected one copy of the standard plasmid. The samples containing no template gave a negative result. The amplification of a plasmid standard dilution over nine 10-fold dilutions showed linearity over the whole range.
4.4. Discussion

Research methods have been a limiting factor to the isolation of *B. anthracis* from environmental samples. Bacteriological studies have been performed on a very small scale because of the difficulty of spore isolation involving environmental samples because of contamination by other soil organisms (6, 18). The spore concentration method developed for this study utilized the hydrophobic and specific gravity properties of the spore and showed a clear correlation between plate counts and quantification by PCR. This study has shown that large scale environmental bacteriological examination for *B. anthracis* is now possible. These improved methods have enabled the first analyses of carcass sites in regards to environmental contamination, disposal method comparison, and decay over time of *B. anthracis*.

The assessment of the environmental contamination by carcasses and scavenging was complex. Scavenging of the carcasses did contribute to the environmental contamination with numbers frequently high and potentially able to produce new foci of infection (21). Levels of anthrax spores in scavenger feces depend largely on the state of anthrax sporulation in the carcasses as vegetative *B. anthracis* probably do not survive transit through the digestive system of the scavengers (7, 10).

Environmental contamination by the carcass was observed by both soil and miscellaneous samples. The miscellaneous samples were surprising as a higher percentage of positives were expected since the samples were intimately associated with the carcass. Factors that could have affected the sporulation and the spores is that an acidic environment produced by a decaying body inhibits the vegetative bacteria from
sporulating (25). It has been shown that only a small proportion of bacilli released from a carcass by hemorrhage, natural and otherwise caused, sporulate successfully. Temperature, moisture, and UV radiation are also known to affect sporulation of *B. anthracis* (3, 11, 13). These factors, along with rain, wind, and snow, could also affect the spores over time.

Considering these factors, a significant difference was detected (*p*=0.17) between the samples taken from WBNP in 2000 and 2001 (Figure 4.4).

![Figure 4.4. Difference in QPCR counts over time in Wood Buffalo National Park in the years 2000 and 2001.](image)

The samples were taken one year apart at the same sites sampled in 2000. The time between death and sampling also proved to be significant (*p*=0.13) (Figure 4.5). This decrease in spores over time was also noted at carcass sites in the Etosha National Park. The differences seen here address the need to widen the sampling to include sites from
historical anthrax areas, present outbreak sites, and a time series over these sites sampling at regular quarterly intervals.

![Graph showing QPCR counts](image)

**Figure 4.5.** Difference in QPCR counts between time of death and time of sampling.

When separating the sites by locality within the region, a significant difference (p=0.001) was noted (Figure 4.6). The differences noted here could be caused by many factors such as soil, rain, and other environmental factors. The two regions with the highest counts, Lake One Delta and the 91–92 Site, are very different in that the 91-92 Site localities have not had a known anthrax case since 1992, while the Lake One Delta is an area where some of the first cases are found and the last cases in the area were reported in 2001. Another difference between these localities is the 91-92 Sites are burial sites of an unknown number of buried carcasses and the Lake One Delta is a low lying area that drains into a lake. Only surface samples were taken at both locals, and isolates
were recovered from both in the range of 1000 per ml. The reason for the high counts associated with the 91-92 Sites is unknown, but speculation could involve the surface contamination by the buried carcasses. A resampling of this burial site including deep samples would be informative and may give clues as to where the spores are coming from.

Figure 4.6. Comparison of mean QPCR of samples from localities by region.

The purpose of this study was to determine if there was a difference in the environmental contamination by the different carcass disposal methods, nothing, burned, or buried, by themselves or in combination. Statistically, there was not a difference using
the method of disposal and the mean QPCR \(p=0.965\) in the environmental contamination from each disposal method. A statistically significant difference is seen between the method of disposal and the mean CFU per ml \(p=0.013\). Burning the carcass did prove statistically significant \(p=0.044\) when compared to leaving the carcass exposed with no treatment but was not statistically significant from the other methods of burying the carcass with or without lime before backfilling (Figure 4.7). The \(p\) values for the other two methods, buried and buried with lime, was 0.05, indicating that they were the same as leaving the carcass exposed to the environment. Figure 4.8 demonstrates the reason that no difference was significant between buried, buried with lime, and burned, the 95% confidence intervals for these methods each contain the other.
Figure 4.7. Mean of the colony forming units per milliliter from the spore extraction compared to the method of disposal.

A clearer difference between disposal methods would no doubt become evident with more sampling. These samples should be taken over more sites from areas not represented here, and, as stated before, time-series sampling would lend more to the argument of a difference existing.

![Graph showing mean CFU per milliliter with 95% CI for different methods of disposal.]

Figure 4.8. 95% confidence intervals of the CFU per milliliter and the method of carcass disposal.

4.5. References


11. **Howie, W. J.** Some factors governing the formation of spores of *Bacillus anthracis*. 3. 1949. Society for General Microbiology, Proceedings.


CHAPTER FIVE
GENERAL DISCUSSION AND CONCLUSIONS

5.1. Discussion of the Results

Three major studies concerning the properties of *B. anthracis* are discussed in this document. Each study revealed some new and exciting discoveries. These studies also provided information which made us pause and want to ask more questions. They also showed how little we know about the antimicrobial susceptibilities, lethality, dispersion, and survival of *B. anthracis*.

Antimicrobial susceptibility testing for *B. anthracis* has only previously been reported three times (2, 8, 10). These reports together tested 182 isolates for antimicrobial susceptibilities and unfortunately did not include enough detail about the isolates to make a comparison except by species. This study utilized 25 isolates of *B. anthracis* that are diverse in space, time, and genetics, and was reported as such in hopes that the results will be more relevant to later studies.

A comparison of the results between studies did show similarities in susceptibilities to most of the antimicrobials tested, such as ciprofloxacin, doxycycline, cefaclor, cephalexin and tobramycin. It was also noted that with cefuroxime, a 3rd generation cephalosporin, differences were noted between the studies. The older studies showed a higher rate of resistance than the present study which could be a reflection of different methodologies or a difference in the isolates used (2, 8). The methods used in these older studies utilized agar dilution which has...
been shown to have a high correlation with the E-test method (10, 12). The differences noted are probably because of different isolates, and unless more testing is done, this will remain a theory.

Penicillin resistance was noted in this study and has been reported by other investigators (2, 8, 16).

Beta-lactamase production is a questionable issue with *B. anthracis*. Penicillin resistant isolates have been found to be non-producers, and penicillin sensitive isolates have been shown to actively produce beta-lactamase (13). A theory is that all *B. anthracis* carry the beta-lactamase gene but expression is suppressed (16).

The anthrax research community has believed there was only one of each plasmid, pXo1 and pXO2, since the discovery of the plasmids in 1985 (6). With the use of new technologies in molecular detection, we have shown that there is more than one copy of each plasmid per cell and that there is tremendous variation among genetically diverse isolates. Though the chromosome did not reflect the variation in loci seen with the plasmids, the numbers could be appropriate from a biochemical and virulence viewpoint.

An assumption exists that pXO1 contributes the most to the organisms virulence. The model we developed indicated that pXO1 copy number does not contribute significantly to virulence but that pXO2 plays a major role in virulence and is a contributor to the observed variation in virulence. These results would agree with previous studies that showed that mutants producing greater amounts of capsule were more virulent (17).
Another profound observation from this study was that anthrax virulence is related to clonality as indicated by MLVA genotype cluster (7). The variation in virulence we observed are likely caused by mutations that are heritable and clonally passed on to daughter cells. This phenomenon of virulence related clonality has been associated with the cause of Rabbit Fever, *Francisella tularensis* (1, 5, 11).

Such a large scale study of the environmental contamination caused by a carcass, directly and indirectly, has not been attempted before this study. Studies on a much smaller scale have been reported, but none have compared disposal methods (3, 9, 15). The study reported here compared carcass that had been left exposed on the surface to decompose over time, buried, burned, and some were a combination of these. To accomplish this comparison, unique methods were developed to isolate *B. anthracis* spores and to extract DNA on a large scale.

This study with its unique methods and samples had to have checks and balances to validate the protocols and results. These consisted of a comparison between CFU counts on SBA and the QPCR results, a QPCR detection limit study and spore viability.

The test of the detection limit for the 5’ nuclease assay found the system detected one copy of the standard plasmid, and the samples containing no template gave a negative result. This sensitivity was needed for the comparison of the CFU counts to the QPCR and the spore viability tests.
Spiking sterile and unsterile control soils revealed a clear correlation between the CFU counts and the QPCR results. This gives credibility to the spore’s viability found within the field samples and to the study as a whole.

One of the most surprising results was the finding of no difference between the overall means of the surface soil samples (1.5 x 10^7 spores) and the burial soil samples (2.5 x 10^7 spores). This could have been caused by the method of backfilling, spore movement from the carcass to the surface, or the backfill dirt being grossly contaminated. Other causes could be postulated, but, until more samples are collected and analyzed from various burial sites, it seems that burial is a method that only disguises the contamination - out of sight, out of mind.

One of the questions for this study was the extent of environmental contamination by scavengers. These samples were represented by carnivore scat that was collected within 25 meters of a carcass or burial site. Most likely because the carcasses in Mexico were completely burned within hours of finding the carcass, no isolates were recovered.

Minimum infectious dose (MID) estimates established by British biological warfare work in the early 1940s found that the dose needed to ensure lethal infection by the oral route in sheep, horses, and cattle was 5 x 10^8 spores (16). Of the 25 scat samples collected from WBNP, 18 were positive for viable *B. anthracis*. From these positive samples, a third produced half of this MID in just four grams. Since on average the scat weighed over 20 grams, scavengers could be a source by potentially producing new foci or infection.
Only five of the 12 scat samples collected in Nevada were positive and only one of these produced enough spores to potentially produce a new focus of infection. The difference to be pointed out here is that the carcasses in WBNP were left exposed to be scavenged and the majority of carcasses in Nevada were buried. In Nevada, the one sample that had a high recovery was from a site where the carcass had been left exposed on the surface.

The objective for the soil study was to ascertain which carcass disposal method contributed the least to environmental contamination. Unfortunately the answer to this question was not readily apparent from the data.

No difference between the methods of disposal and environmental contamination when comparing the QPCR results was noted, but there was a difference when comparing the disposal method with the CFU counts per milliliter. This difference is attributed to the fact that the DNA is still detectable even if the spore or bacteria have been rendered non-viable. This has been demonstrated with autoclaved material including *B. anthracis* spores and vegetative bacteria (4).

Using the CFU results, a definite difference was noted between the disposal method, especially between burning and doing nothing to the carcass. Though the difference between burning and burying was not statistically significant, an inspection of the CFU count data reveals that the burned carcasses in Mexico did not produce any isolates and the burned carcasses in Nevada did produce a mean of $3 \times 10^3$ CFU/ml. This difference could be attributed to the time lapse between discovery of the carcass and burning. The carcasses in Mexico were found within hours of death.
and burned within hours of being found, and the Nevada cases were not discovered until the following day after death and then were not burned until the next day after discovery.

The time lapse difference between these locations is probably caused by the difference in man power and regulations. The ranch in Mexico has range riders who ride the pastures every day and know the cattle and their habits. These range riders can tell where the cattle are within a few meters at any time of day. This ranch also has the means and manpower available to burn carcasses immediately after they are found. In Nevada there was only the ranch manager who was unable to continually check on the cattle. When a dead cow was found, the ranch manager would have to call in the veterinarian who would send samples into the state diagnostic laboratory. If anthrax was confirmed, the state and federal veterinarians were contacted, and a decision had to be made on how to dispose of the carcass. This would leave one to believe that our own bureaucracy is helping to contaminate the environment.

5.2. Future Studies

More studies need to be done involving isolates that have been genotyped with the same method. Though there was no association noted between antimicrobial susceptibility and MLVA genotype, surely if more representatives of all 89 genotypes were tested, a pattern would emerge.

The antimicrobial susceptibility study revealed that all isolates of a species are not alike and should not be judged so. When testing the 25 isolates with doxycycline, two isolates did show resistance. Unfortunately this could not be verified on retest
with the same lot of E-test strips of doxycycline and tetracycline as these two isolates where shown to be susceptible. Upon inspection of the recently published *B. anthracis* genome and discussion with the author revealed that *B. anthracis* does carry the tetracycline resistance gene (14).

Another issue associated with antimicrobial studies is beta-lactamase production. This, too, should be considered to verify if beta-lactamase production is associated with penicillin resistance in *B. anthracis*.

An association was definitely demonstrated between the MLVA clusters and virulence in AVA vaccinated guinea pigs. The virulence model used to compare the predicted and observed virulence of the isolates needs to be tested on a much wider dataset, such as the 89 genotypes as defined by MLVA (7). Using animals that are unimmunized with a full representative dataset of the 89 genotypes would validate the model and cluster effect.

Since these studies were performed, a new generation of technology has evolved capable of absolute quantitative PCR. Verification of the chromosome and plasmid copy numbers is suggested using the primers and probes presented here and other primers that target different areas of the chromosome and plasmids.

More samples from various locations utilizing various methods of carcass disposal are needed. These samples should include surface and burial soil and miscellaneous environmental samples. Background surface soil samples also need to be collected. These samples would consist of random sites not associated with
anthrax carcasses. These samples would provide insight into the question of the ubiquitous distribution of the *B. anthracis* organism.

These sampling efforts will generate hundreds - if not thousands - of samples. The plating effort to ascertain *B. anthracis* isolate recovery is very labor intensive and requires trained personnel to select the probable Bacillus colonies. The results would indicate that more isolate recovery is required. A better and more streamlined procedure needs to be developed to determine spore viability.

Adapting the methods, spore concentration, DNA extraction, and viability to robotic technology is suggested. This would greatly reduce the personnel and labor involved, accelerate the laboratory process, and provide results quickly and efficiently.

5.3. Conclusions

The results of this research have contributed greatly to the knowledge base of *B. anthracis*. Additions to the antimicrobial susceptibility database with genotype knowledge will possibly help with gene identification involved in resistance. The virulence model is an important tool for the screening of isolates and vaccine testing. This is truly a paradigm in virulence testing.

The methodologies and insights gained from the soil study have provided the background and direction needed to realize the importance of such studies. They have provided an impetus to continue the effort of trying to understand the ecology and life cycle of *B. anthracis*. 
5.4. References


APPENDIX 1

SITE DATA COLLECTION WORKSHEET

Date: ________________
Site #: ________________
Lat: ________________
Lon: ________________
Comments:

Labelling X1-X2
X1 = site number
X2 = sample number (1-20)
B = burial site
APPENDIX 2

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I am requesting written permission to use article AAC 275-02, "Antimicrobial Susceptibilities of Diverse Bacillus anthracis Isolates." This article is in press at this time. I am the principal and corresponding author of this paper and am requesting permission to use this article in my dissertation.

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Pamala R. Coker DVM Ph.D.

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VITA

Pamala R. Coker had an unconventional academic beginning. Because of boredom with herself and school, she rarely attended classes and finally dropped out of school at age 16. For two years she lived a bohemian lifestyle with no goals except to have a good time. Suddenly she realized there had to be more to life. So with her GED in hand, she enlisted in the United States Army Reserve which was possibly the best thing she could ever have done. The military gave her a new sense of direction and the self discipline needed to continue through life as a contributing member of society.

Always up for a challenge, she enrolled at Cameron University in Lawton, Oklahoma, and, after five years of hard work, she emerged with a Bachelor of Science in Physics. The job market for physicists was as flat as the economy at the time so she taught high school and worked as a project manager for the Boeing Company in Seattle, Washington. Pamala was an unhappy woman and wanted more in her personal life and career. She also wanted skills that could take her anywhere in the world. Ever since she was a small child, she had wanted to be a veterinarian and that career could take her places she had only recently begun to dream of. This goal was realized in 1998 when she was awarded her Doctor of Veterinary Medicine from the School of Veterinary Medicine at Louisiana State University, Baton Rouge, Louisiana.

While in veterinary school she slowly began to realize that the world of veterinary medicine was more than having a clinic on the corner. After working summers in research laboratories and experiencing a bout with breast cancer, Pamala chose a life in research instead of practice. This high school dropout will become a member of an elite club, the Paradocs Club, when she is awarded her Doctor of Philosophy in December 2002.