Concentration and Extraction of *Bacillus anthracis* Spores and Ricin Toxin from Liquid Foods

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Dedication

This dissertation is dedicated to the professors, teacher, and mentors who inspired me to take this journey.

Abstract

Food is an essential part of life for every human and animal. In order to feed the world, food production has become a global industry. This globalization brings efficiency of production, transportation, and year round availability of many ingredients. However, mass production of food also means that any mistake made during production is magnified in scale and distribution. Recent incidents of food contamination have involved not only traditional food pathogens, such as *Salmonella* and *Escherichia coli* O157, but also have included chemical contaminants such as melamine. These incidents serve to highlight the inherent vulnerability of food to contamination.

Although the majority of foodborne illnesses are caused by a small group of pathogens, this does not preclude other bacteria or agents from being transmitted through food sources. Many potential bioterrorist agents have also the potential to be transmissible through food and water sources. Some of these agents, such as *Bacillus anthracis* spores and ricin toxin, are also resistant to the effects of existing food processing technologies such as pasteurization. Given the inherent vulnerability of the food production system, it seems a likely target for potential bioterrorism attack.

Many rapid and sensitive tests have been developed to detect biological agents in a variety of settings. However, the complex nature of food matrices often limits the application of these tests to food sources. In addition, the distribution of a select agent in a food source may not be homogeneous, and testing of small samples may not represent the whole batch.

The goal of this project was to design and test pre-analytical extraction techniques for two potential bioterrorism agents, *B. anthracis* spores and ricin toxin, from liquid

foods. The outcome of this project was the development of a rapid concentration and extraction protocol for milk and fruit juice potentially contaminated with *B. anthracis* spores. The resulting sample was compatible with detection via real-time PCR for both milk and fruit juice samples and juice samples were compatible with detection with a commercially available lateral flow immunoassay. This concentration and extraction procedure enhanced the limit of detection by 2 log CFU/ml spores, such that real-time PCR can consistently detect *B. anthracis* at a level of 10 spores/mL in the initial sample. This project also examined the application of immunomagnetic separation for extraction of ricin toxin from liquids. Results from this portion of the project suggested that immunomagnetic beads can specifically bind ricin in traditional immunomagnetic separation. However, recirculating immunomagnetic separation using the Pathatrix® system was not demonstrated to specifically bind ricin.

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Chapter 1

Introduction and statement of the problem

1.1 Introduction

Infectious disease has shaped human history in a multitude of different ways throughout the centuries. From the biblical plagues, to Black Death, cholera, smallpox, influenza, and malaria, infectious disease has changed people, populations, and nations. However, developments in antibiotics, vaccines, sanitation procedures, and public health systems have decreased both morbidity and mortality due to infectious disease across the globe. Despite these advances of modern science, the threat of pandemic disease, such as influenza or AIDS, still lurks. In addition to the threat of a naturally arising pandemic disease, new concerns have risen over the potential use of biological agents as weapons against humans, animals, and crops. Neither the agents themselves nor use of biological weapons against an enemy are new to history, but due to developments in molecular engineering, these agents can now be altered and used in ways previously unimaginable. Though the probability of a large scale biological attack may be low, hazard posed by such an event is extremely high.

Bacillus anthracis is a Gram-positive, rod-shaped, spore-forming bacterium, and the causative agent of anthrax. The primary habitat of *B. anthracis* is soil, although it is often spread through animal products, such as carcasses and hides, as well as by blowflies (128). Anthrax has been documented in humans and animals worldwide throughout much of history and is thought to be responsible for the fifth and sixth plagues of the bible, as well as the “Black Bane” of Europe in the 1600’s (40, 260). Although natural infections continue to occur in many parts of the world, the majority of current concern

over this pathogen is its use as a bioterrorist weapon that can be distributed through a number of different media, one of which is through food.

B. anthracis is a food safety concern in two different capacities. As a natural foodborne infection, *B. anthracis* can be transmitted from infected animal to human through the consumption of animal products containing viable spores. Meat from herbivores, such as cows and water buffalo, is the most common vehicle for natural transmission of gastrointestinal anthrax due to their habit for grazing close to the soil. Although no study on the prevalence of *B. anthracis* in cow or other milk has been conducted, it is believed that milk would not serve as a natural source of infection as lactation is expected to stop with the onset of illness (205). Consumed spores germinate in the intestinal tract causing gastrointestinal anthrax, leading to rapid dissemination throughout the body and often fatal consequences. Little is known about the natural occurrence of gastrointestinal anthrax, although large outbreaks have been documented in various parts of the world.

In addition to transmission through animal products, *B. anthracis* is also a food defense concern as a potential bioterrorist agent that could be intentionally introduced in the food supply. Spores of *B. anthracis* are highly resistant to heat, chemical agents, and desiccation, allowing them to survive many of the microbial hurdles commonly used in food processing (178, 262). Retort processing used in canning foods will kill *B. anthracis* as well as *Clostridium botulinum* spores. However, spores introduced into foods that rely on less intense heat treatment, like pasteurization of milk, may persist beyond the shelf life of the food. Consumption of food intentionally contaminated with *B.*

anthracis spores is likely to cause widespread cases of gastrointestinal anthrax, likely resulting in significant morbidity and mortality.

Ricin is a naturally occurring toxin found in the seeds of the castor bean plant, *Ricinus communis*. Ricin is a ribosomal inactivating protein type two toxin, consisting of two peptide chains (A and B) linked by a disulfide bond. The B chain binds to terminal galactose residues on eukaryotic cells, while the A chain is the enzymatic domain. Once inside the cell, the A chain of ricin specifically depurinates the 28s subunit of rRNA, leaving the ribosome highly unstable and unable to continue protein production. The inability to produce protein quickly leads to cell death, which can quickly cause systemic organ failure and death. No specific treatment for ricin poisoning is currently available.

Although ricin poisoning has historically been associated with the consumption of the whole castor bean, recent events have shown that purified ricin can be used as a potent biological weapon. In fact, ricin has a relatively extensive history of use as a bioweapon and is currently classified by the CDC as a category B bioterrorist agent. Ricin is a likely candidate as a bioterrorist agent not only because of its potent activity, but also because of the availability of raw materials and inexpensive, relatively simple production. Castor bean plants grow wild throughout the United States and are commercially grown for the production of castor bean oil and as an ornamental plant worldwide. Due to its hydrophilic nature, ricin is not found in castor bean oil itself, but rather the spent bean mash (181). The toxin can be precipitated from the mash and purified. The toxic protein is relatively heat stable and can be dried to produce a powder or be dissolved in a variety of media, including food and water.

Although there are a vast variety of agents, bacterial, viral, parasitic, or toxic, that could be used as biological weapons; *Bacillus anthracis* and ricin toxin are two likely candidates due to their availability, dispensability, and heat stability. There are also any number of mechanisms and settings for dispersing these agents, including aerosolization, and contamination of food or water supplies. Though any of these scenarios are possible, contamination of the food supply has been identified as significant target and one that both the government and the food industry must be prepared to deal with.

1.2 Statement of the problem

The rapid detection of a highly pathogenic agent in foods is one of the most effective strategies to minimize the public health impact of an intentional attack on the food supply. To date, however, there is no existing analytical platform capable of screening foods continuously and immediately. The development and application of effective detection technologies for both natural and intentional pathogens has been largely limited by the complex nature of food matrices and the uneven distribution of microorganisms. Because of these limitations, the majority of bacterial pathogen detection methods rely on one or more enrichment steps that can last from 6 to 72 h to increase the number of target bacterial cells and thus enhance analytical capability. Toxins represent additional problems for detection, as they can be lethal at low levels and their number cannot be enriched prior to detection because they do not multiply. In the event of a bioterrorist attack, every hour of delay in detecting an agent could translate into thousands of lives affected. Although current methods are available to detect toxin at low levels in some foods, many of these methods are time consuming, require trained

technical personnel, and not suited to continuous monitoring of the food supply. The development of alternative and simple amplification methods for both bacteria and toxins is extremely urgent.

Amplification protocols should be based on quick separation and concentration techniques coupled to a rapid detection platform such as real time-PCR, lateral flow assay, or immunosensors. A variety of alternative amplification techniques have been tested for the recovery of foodborne pathogens, but their limited success with real food matrices has prevented their practical application. Some of these techniques include immunoseparation, metal hydroxide precipitation and two-phase aqueous extraction (16, 226). None of the methods investigated so far have provided a feasible replacement for the enrichment step. This major weakness of our nation's food protection system should be addressed by supporting research on novel and improved amplification methods.

1.3 Research goals, hypotheses (H_o), and sub-objectives

Research Goals:

The overall goal of this project is to define pre-analytical techniques that can be used to concentrate and separate *Bacillus anthracis* spores and ricin toxin from liquid foods, thereby lowering the detection limit of the agent in the food and eliminating interference from the food matrix. This technology should also be compatible with in-line sampling techniques and result in a less then 3 hour total time to detect.

Null Hypothesis I:

The percentage of *B. anthracis* spores recovered from sterile water is independent of the percent hydrophobicity as determined by the Microbial Adherence to Hydrocarbons (MATH) assay.

Null Hypothesis II:

Distribution of *B. anthracis* spores in raw whole milk following centrifugation is independent of prior heat treatment.

Null Hypothesis III:

The concentration of *B. anthracis* spores added to raw milk and then separated is independent of milk fraction.

Null Hypothesis IV:

Detection of *B. anthracis* spores in milk and fruit juice is independent of prior processing.

Null Hypothesis V:

Ricin extraction from phosphate buffered saline via immunomagnetic separation is independent of magnetic bead specificity.

Objectives:

1. Determine the hydrophobicity of *B. anthracis* spores using the microbial adherence to hydrocarbons (MATH) assay.
2. Assess if *B. anthracis* spores can be separated and concentrated from water, milk, and orange juice into a small volume of hexadecane.
3. Determine spore distribution following centrifugation of various commercial, raw heated, and raw non heat treated milk.

4. Determine the distribution of *B. anthracis* spores after separation of raw whole milk
5. Design a system for effective concentration and separation of *B. anthracis* spores from milk and fruit juice based on the results of separation experiments.
6. Assess the compatibility of PCR, real-time PCR, and lateral flow assays for *B. anthracis* with samples extracted from milk and fruit juice.
7. Determine if the Pathatrix ® system is capable of capturing and concentrating ricin from a large volume of buffer.

Chapter 2

Review of *Bacillus anthracis*, ricin toxin, the history of bioterrorism, milk processing, and orange juice processing

Illnesses caused by *Bacillus anthracis* and ricin toxin have long effected human populations. Pathogens such as *Clostridium botulinum, Salmonella, Escherichia coli,* and *Shigella* have also been commonly associated with food borne illness. Although both *B. anthracis* and ricin have been associated with food, in terms of consumption of contaminated meats (*B. anthracis*) and of the castor bean (ricin), these agents have not been generally associated with food safety concerns in the United States until recently. These agents, among others, are potential bioterrorist agents that could be used to contaminate the food supply.

Contamination of enemy food and water supplies has been used as a tactic by warring factions for centuries to weaken opponents, as described in section 2.9. As a result of the terrorist attacks on the United States on September 11, 2001 and the following anthrax mail attack, the US government and its people became more aware of the threat of potential biological weapons attack. Recent risk assessments of vital infrastructure in the U. S. revealed that the food supply is a particularly vulnerable target. Former Secretary of Health and Human Services, Tommy Thompson, described this threat in the following statement: “I, for the life of me, cannot understand why the terrorists have not, you know, attacked our food supply because it is so easy to do".

Bacillus anthracis and ricin toxin have been identified as two likely agents to be used as biological weapons against the food supply. This is due to their potent biological

activity, inherent heat resistance to standard food safety treatments (i.e. pasteurization), the ability to survive in a food matrix, and resistance to many standard sanitation techniques. Current technology is inadequate to detect these agents within food matrices at the minimum lethal dose. If a bioterrorist attack against the food supply with either of these agents were to occur, it could cause substantial morbidity and mortality in those consuming the affected product, as well as significant economic loss.

Two products that have been identified as potential targets for bioterrorist attack are milk and orange juice. Both of these products are pasteurized at time-temperature combinations that are inadequate to inactivate either ricin or *B. anthracis* spores. The milk supply is particularly vulnerable due to the accessibility of the product prior to processing. In contrast, the orange juice supply is mainly vulnerable to intentional contamination after production and prior to packaging. Contamination of either of these food sources has the potential to impact hundreds of thousands of individuals across the nation due to product distribution patterns. An additional consideration for protecting milk and orange juice is their level of consumption by children, who may be disproportionately affected by these agents.

The food supply is currently threatened by the potential of a bioterrorist attack with any number of agents. Although the government has emergency plans in place to handle such an event, it would preferable to stop such an event before the affected product was distributed to the general public by detecting the agent at the production site through rapid testing. In order to achieve this goal, it is imperative to understand the characteristics of these agents, the diseases they cause, disease prevention and treatment measures, the history of such events, and the production process potential points of

product contamination. Understanding of these properties can facilitate the development of new pre-analytical methods to couple with rapid detection technologies in order to detect and prevent a potentially devastating outbreak.

2.1 *Bacillus anthracis*

Bacillus anthracis is a Gram positive, facultative anaerobic, spore-forming rod in the family *Bacillaceae* and the etiological agent of anthrax. Anthrax has historically been a disease primarily found in herbivores and those in contact with products from these animals. Although animal anthrax remains a major concern, *B. anthracis* is also feared for its potential to cause human disease. The threat of using *B. anthracis* as a biological weapon against humans has been highlighted in recent years following the discovery of large stocks of weaponized spores in the former Soviet Union, admission by Iraq that it had filled warheads with anthrax, attempted anthrax attacks by the religious cult Aum Shinrikyo on Tokyo, and the anthrax mail attacks that followed September 11, 2001. Although the attacks in the fall of 2001 were carried out by distributing powdered anthrax spore through the mail system, anthrax could also be used to target other infrastructure systems such as food distribution. Food has previously been a target of biological weapons and could be targeted in the future with an agent such as anthrax. This fact makes it crucial to understand the properties *B. anthracis*, its importance as a biological weapon, the potential food systems that could be targeted, and how to detect the spores in a number of different food matrices.

2.1.1 Physiological properties of *Bacillus anthracis*

2.1.1a Physical structure

The overall structure of *Bacillus anthracis* is similar to many Gram-positive bacteria. At the core of the vegetative bacterial cell is the cytoplasm, bound by the cytoplasmic membrane, containing the chromosome, ribosomes, and plasmids. Virulent *B. anthracis* contains two plasmids, pXO1 (181 kb) and pXO2 (94 kb) (85). Outside of the plasma membrane is a thick layer of peptidoglycan, composed of alternating chains of *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked by oligopeptides, separated by a periplasmic space from the cytoplasmic membrane. Different from some other Gram-positive bacteria, the *B. anthracis* peptidoglycan layer is covered by the S-layer, composed of the proteins EA1 and Sap in crystalline layers (71, 167). The outermost layer of virulent *B. anthracis* vegetative cells is the capsule. Although not entirely unique among *Bacillus* species, the capsule is different from other polysaccharides produced by bacteria in that it is composed of poly-D-glutamic acid and is essential for virulence (19). Regulation of capsule production and specific function are discussed in section 2.2.1.1.

2.1.1b Defining characteristics

Bacillus anthracis is a member of the *Bacillus cereus* group (*Bacillus cereus senso lato*) and other members of this group are *B. cereus, B. mycoides,* and *B. thuringiensis*. Although members of this group have many characteristics in common, *B. anthracis* has a number of defining features.

B. anthracis is a Gram-positive rod which, unlike many other species, produces a capsule (fully virulent strains) and may be seen as a clear zone during Gram staining.

Unlike *B. cereus* and *B. thuringiensis*, *B. anthracis* does not produce β-hemolysis when grown on blood agar (109). *B. anthracis* also differs in motility from other *Bacillus* species in that it is non-motile (109). These tests, Gram stain, morphology, motility, hemolysis, in addition to catalase activity (*B. anthracis* is positive) are preliminary tests used to differentiate *anthracis* from other *Bacillus* and other bacterial species.

In addition to the preliminary tests, secondary testing of isolates can be used to further identify the organism. Differently than other *Bacillus* species, wild type isolates of *B. anthracis* are sensitive to both penicillin and gamma phage (1, 59). *B. anthracis* also be defined using molecular methods, such as PCR to detect toxin genes present on the plasmids (14, 26, 66). PCR can also be used to detect known differences in chromosomal genes, such as the gene *plcR* which is active in *B. cereus* but contains a non-sense mutation in *B. anthracis* (169). Protocols for laboratory identification of *B. anthracis* are available through the Centers for Disease Control and Prevention (CDC).

2.1.1c Morphology

Bacillus anthracis is a relatively large (1 μm x 3-5 μm) gram positive spore forming rod-shaped bacteria (109, 122, 236). Microscopic examination often shows bacilli in short chains of 2-4 cells (36). A zone of clearing around the organism may be seen in fully virulent strains due to the presence of the poly-d-glutamic acid capsule (36). Spores can be differentiated from vegetative cells by phase-contrast microscopy, in which the spores appear refractile, or through the use of malachite green to stain the endospore (36).

Colony morphology of *B. anthracis* can vary based on plasmid content. Strains containing the plasmid pXO1 plated on laboratory media produce large white/off-white colonies, with a ground glass texture and have slightly undulations (36, 236). Strains containing pXO1 can also produce 'medusa head' colonies, in which the colony has a comma like projection to one side (36). Strains devoid of plasmid pXO1 are generally smaller and rounder than those containing the plasmid. Strains containing pXO2 produce capsule leading to a shiny, mucoid colony appearance as opposed to those without the plasmid (73, 236).

2.1.1.1 The vegetative cell and germination

As a spore-forming bacterium, *B. anthracis* can exist in two states, the growing, multiplying vegetative cell and the metabolically dormant spore state. Conflicting evidence exists about the ability of *B. anthracis* to exist in the vegetative state outside of the host (128, 177, 208). Conventional thinking describes the lifecycle of *B. anthracis* as in the vegetative state only in the host (or grown using lab media) and that only the spore is found in nature. However recent evidence suggests that germination can occur in the soil in association with specific conditions (208). Although the spore is the infectious form, the vegetative cell is responsible for causing disease in the host. Disease in the host is caused by both bacterial multiplication and the expression of the numerous virulence factors discussed in section 2.2.1.

The transition from spore to vegetative cell has been divided into three general stages, activation, germination, and outgrowth by Keynan and Halvorson and further described by Moberly et al (137, 170). Activation of the spore germinant receptors is a

reversible process preparing the spore for germination and can be accomplished in vitro using mild heat treatment, although the specific conditions for activation have been debated (237). Germination of *B. anthracis* is accomplished through any of five varying pathways comprising of different amino acids at varying concentrations. These five amino acid stimulated or co-stimulated germination pathways are: L-alanine alone above 30 mM (alanine pathway), L-alanine and L-proline (alanine proline pathway), L-alanine and L-proline with either L-tryptophan, L-histidine, or L-tyrosine (enhanced aromatic amine pathway), and the amino acid and inosine dependant (AAID) pathways. The AAID pathways consist of inosine paired with either L-alanine, L-serine, L-valine, L-methionine, or L-proline, (AAID-1), and inosine paired with L-histidine, L-tyrosine, L-tryptophan, or L-phenylalanine (AAID-2) (83, 123).

Activation of specific germination pathways is dependant on the presence of genes in the *gerA* family, comprised of six germination genes located on the chromosome and one, *gerX* located within the pathogenicity island on plasmid pXO1 (201). Mutation in any one of these genes can be detrimental to germination efficacy depending on the specific stimulating compounds (83). In addition, null mutants of *gerX* not only display decreased germination rates, but also show decreased virulence from the parent strain (83, 103). Germination leads to the release of molecules that protect the DNA, such as calcium, dipicolinic acid, and small acid soluble proteins, from the cell (170). During germination the dormant state of the cell is terminated, and the spore swells in size and becomes non-refractile under phase contrast microscopy (170). Following germination the cell can grow and divide as a vegetative cell under appropriate nutrient and temperature conditions. The growth requirements of *B. anthracis* are relatively minimal,

requiring only methionine, thiamine, and glucose, although additional amino acids allow for more rapid growth (82). *B. anthracis* can be grown on tryptic soy agar, nutrient agar, and brain heart infusion agar, among other common laboratory media. The doubling time of *B. anthracis* has been reported as low as 30 minutes in some studies up to 61 minutes in others (45, 82).

2.1.1.2 The spore and sporulation

The spore of *B. anthracis* is most commonly found in the soil environment. Although it is the infectious form of the bacteria, it is dormant and neither grows nor divides. The spore form of *B. anthracis* is resistant to many mechanisms which can inactivate non-spore forming bacteria, as well as the vegetative form of *B. anthracis*. Spores are resistant to a relative extent to heat, UV light, desiccation, cold, chemical disinfectant (up to 0.05%, sodium hypochlorite at 30 min; 500 mg/L ethylene oxide at 30 min; or 0.88 mol/L hydrogen peroxide) and radiation up to 10 kGy (111).

Conversion of the vegetative cell to the spore occurs through the multi-stage process of sporulation. Sporulation can be triggered by any one of a number of events including mechanical stress, dehydration, pH change, heat, or lack of specific nutrients. Although other *Bacillus* species are able to convert between spore and vegetative cell in the soil environment, evidence is conflicting over the pre-requisite of a host (or lab media) for *B. anthracis* to begin germination and multiplication and only the spore form has been naturally found in the environment (128, 177).

Sporulation begins in *B. anthracis* cells following completion of logarithmic growth (stage 0/I), as the cell begins to sense nutrient deprivation or other environmental

stress. This triggers the activation of a vast number of genes and sigma factors, not limited to those directly associated with sporulation, to be upregulated by the gene family *spo0* (109). Stage II is the first stage in which the spore can be microscopically differentiated from a vegetative cell by the presence of an asymmetrical septum. Formation of the septum is directed by *spoII* genes, which also control further spore development and a variety of sigma factors, in turn controlling expression of serine proteases and spore coat precursor proteins. During stage III the smaller forespore fragment is engulfed by the larger mother cell. The cortex of the spore is then formed between the inner and outer membranes of the forespore in stage IV. During stage IV dipicolinic acid production, which is essential for resistance to heat, chemical agents, and desiccation by lowering the core water content, is upregulated in the mother cell through the action of *spoIV* genes (109, 214). Stage V is characterized by the deposition of multiple protein layers as both the inner and outer spore coats are formed, as well as an increase in small acid soluble proteins that saturate spore DNA and protect it from UV and other damage (74, 109, 214). Finally, in stage VI the spore is released by the mother cell complete with exosporium. The entire sporulation process takes 6-8 hours to complete and the resulting spore is highly resistant to desiccation, heat, light, and numerous chemical agents.

The resulting *B. anthracis* spore is composed of numerous different layers. The innermost layer of the spore is the core, containing DNA and cytoplasmic contents. Surrounding the core is the cortex, composed of an inner layer of tightly linked peptidoglycan and an outer layer of loose peptidoglycan. This is covered by the spore coat, which is composed of a keratin-like protein and helps protect the spore from the

outside environment. The outermost layer is a balloon-like structure of protein, carbohydrates, and lipids synthesized by the parent cell referred to as the exosporium. The exosporium has a hexagonal lattice structure consisting of a paracrystalline basal layer and a hair-like outer layer (95). The exosporium serves as the initial site of interaction between spore and host and includes adhesive proteins such as BslA, although its exact role is not yet understood (136, 167). This structure contains immunogenic proteins and confers adhesive and hydrophobic properties, although the exact role of the exosporium in pathogenicity is still unclear (145, 228, 233).

2.1.2 Virulence factors

Bacillus anthracis virulence is mediated mainly by four gene groups located on the two plasmids, designated pXO1 and pXO2. The lack of plasmid pXO2 (which encodes the capsule) increases the LD_{50} to 10^6 times that of strains containing both plasmids (34). Strains lacking pXO1 are unable to produce either of the major toxins and are essentially avirulent. The four major virulence products are the capsule, protective antigen, edema factor and lethal factor, although other factors such as adhesins and anthrolysin also play important roles in pathogenesis.

Capsule

The capsule of *B. anthracis* is composed of poly-D-glutamic acid and is present in fully virulent strains as the outermost layer of the vegetative cell. The capsule is essential to vegetative cell survival in the host, as it is poorly immunogenic and can mask other antigens on the cell surface (97). This allows vegetative cells to evade the immune

response and reach their target macrophages. The proteins responsible for capsule formation are encoded in five genes, *capA, capB, capC*, *capD* (formerly known as *dep*), and *capE* as the biosynthetic operon *capBACDE* located on pXO2 (31, 61, 62, 100, 241). *CapBCAE* genes encode capsule synthesis proteins, whereas the product of *capD* is a glutamyl transpeptidase that anchors the capsule as well as depolymerizes it into smaller fragments (30, 31, 241). Capsule synthesis is controlled by positive regulation at the level of transcription by carbon dioxide/bicarbonate and the gene *atxA* located on pXO1, as well as *acpA* and *acpB* located on pXO2 (9, 61, 62, 116, 143, 220, 241, 246). *AcpA, acpB* and *capB* have both *atxA* dependant and independent transcriptional start sites (62).

The capsule is essential for virulence in mouse models for inhalation anthrax and thought to protect vegetative cells from phagocytocis (62). The presence of capsule is apparent in laboratory media by shiny, mucoid appearance of the colony. Loss of the pXO2 plasmid and capsule production can occur spontaneously in the environment, or induced in the laboratory through the use of media with added novobiocin (73, 163). Strains of *B. anthracis* lacking pXO2 are significantly less virulent and are used in vaccine production (34).

Lethal and Edema Toxins

The lethal and edema toxin are comprised of three elements; protective antigen, lethal factor, and edema factor. All are encoded for by genes on plasmid pXO1 and contained within a pathogenicity island (8, 180). Both lethal and edema toxins are A-B type toxins. The edema toxin (EdTx) is comprised of a combination between the edema factor and protective antigen, and the lethal toxin is a combination of the lethal factor and

protective antigen (171). In both cases, the protective antigen is responsible for mediating binding to host cells and transport of the toxins across the cell membrane, while the lethal factor and edema factor actively cause damage to host cells during an infection (17).

Protective antigen

The protective antigen (PA) protein (Fig. 1) is encoded by the gene *pagA*, positively regulated by *atxA* and negatively regulated by *pagR*. For both toxins, PA serves as the B subunit, and is responsible for binding to the cell and mediating entry of the enzymatically active A subunit. PA has a mass of 83 kDa and is organized as four domains, mainly containing anti-parallel β-sheets (190). Domain 1 contains two calcium ions and a site for proteolytic cleavage (190). Domain 2 contains regions essential for membrane insertion and heptamerization (190). The function of domain 3 is currently unknown. Domain 4 allows PA to bind to two type 1 membrane protein with extracellular von Willenbrand factor A domains known as the anthrax toxin receptor (ATR) and capillary morphogenesis protein 2 (CMG2) (23, 190).

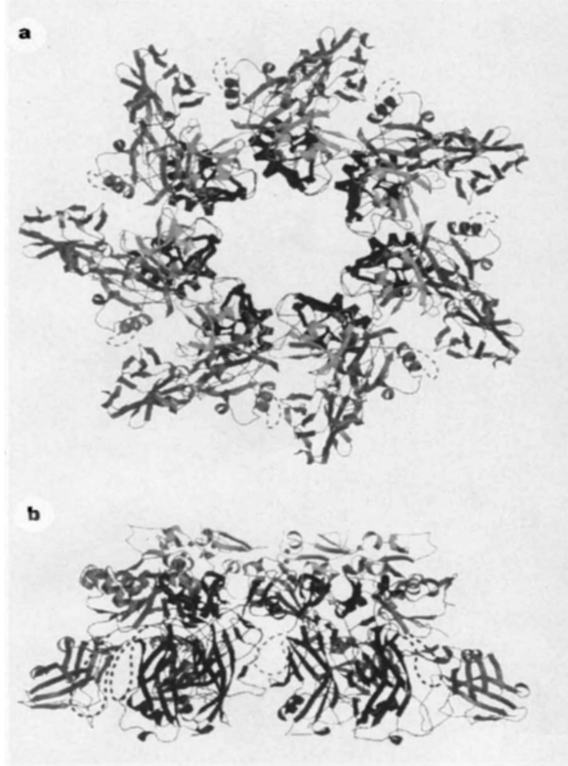

Figure 1. *Bacillus anthracis* protective antigen. A: axial view, B: side view. Reprinted by permission from Macmillan Publishers Ltd from Petosa et al, 1997, Nature © (190).

Following attachment of PA to either ATR or CMG2, domain 1 of PA is cleaved by a host cell surface furin protease at residues 164-167 to form 20 kDa and 63 kDa portions (98). The 20 kDa portion is then released from the molecule, while the 63 kDa portion (termed PA63) remains intact on the cell surface. Complex interactions between lipid rafts and multiple ATRs allow PA to heptamerize and form a prepore structure (198). Edema or lethal factor can then competitively bind to the heptamer at the PA63 site using a conserved sequence at the amino terminal of each protein. The toxin complex is then endocytosed into the cell. Following endocytosis, the PA heptamer converts from a prepore to a β-barrel pore through which either edema factor or lethal factor is introduced into the cytosol (Fig. 2). Although it was initially thought seven

edema or lethal factor molecules could bind to the PA heptamer, recent studies demonstrated that a hypothetical maximum of three toxin molecules could bind the heptamer, although only one edema or lethal factor molecule bound per heptamer has been observed (204).

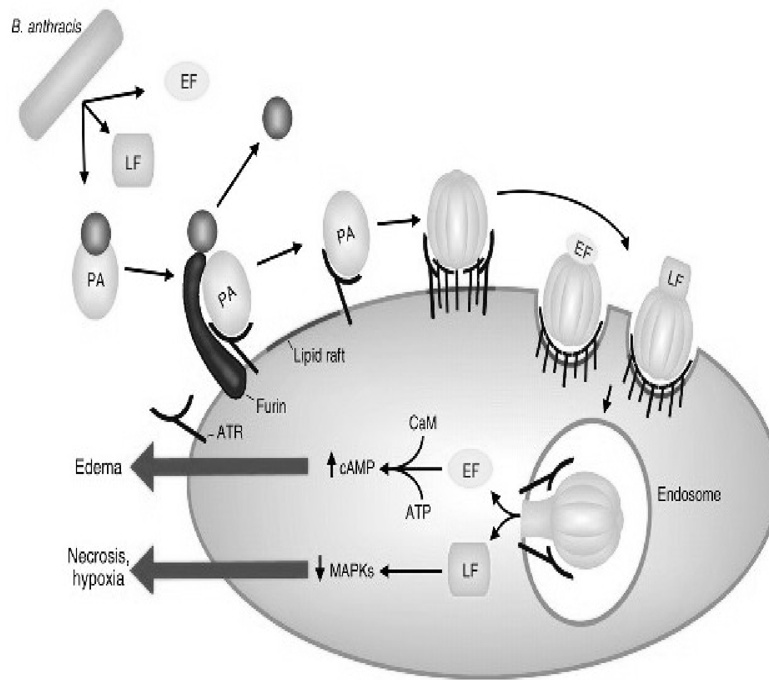

Figure 2. Binding and entry of anthrax toxin into a cell. Reprint by permission from Prince, 2003, The American Society for Clinical Investigation © (198).

Edema factor

The edema factor (EF) is a 89 kDa protein, which is secreted by *B. anthracis* as an inactive adenylate cyclase (Fig. 3) (60, 114, 127). Similar to the adenylate cyclase produced by *Bordetella pertussis,* activation of the enzymatic properties of the protein requires calcium and the host molecule calmodulin (114, 153). The protein is composed of three globular domains which act in concert to bind calmodulin (155). The catalytic

center of the molecule is termed Ca and Cb, and is located at the interface between domains 1 and 2 (60). The amino terminal of EF is highly homologous to that of lethal factor (LF) and involved in PA63 binding (60).

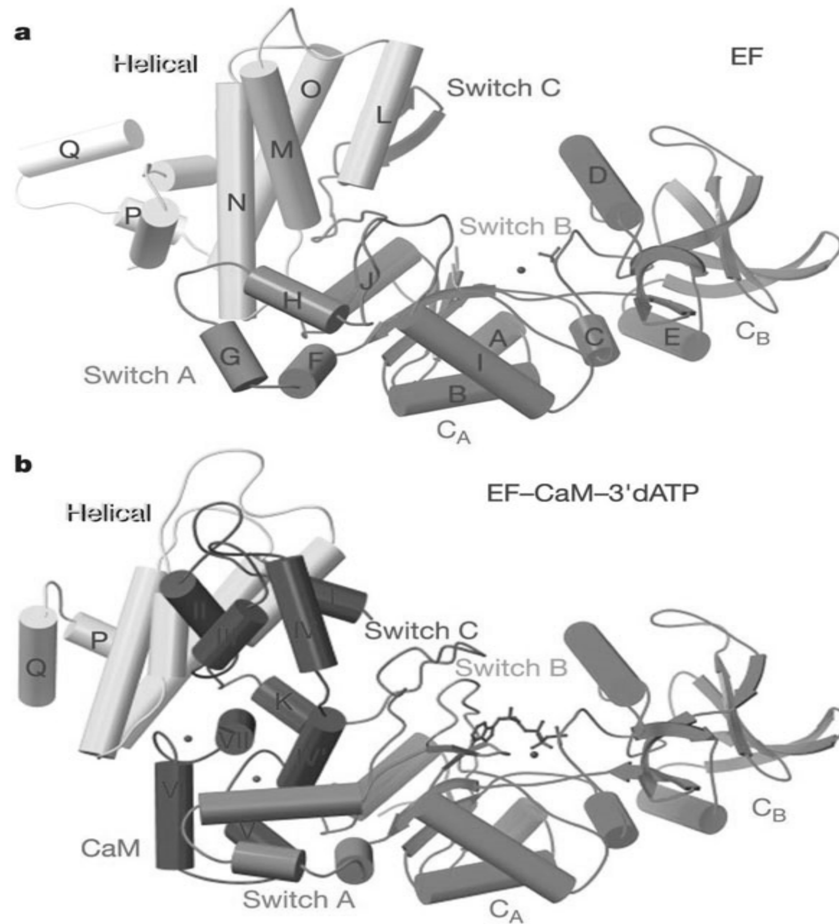

Figure 3. *Bacillus anthracis* edema factor. A: free EF, B: EF complexed with calmodulin. Reprinted by permission from Macmillan Publishers Ltd Drum et al 2002, Nature© (60).

Following translocation to the cell cytosol via PA, the native EF structure is altered by complex interactions with calmodulin (CaM). The structural changes induced by calmodulin complexation involve a 15 Å translocation and 30° rotation of a 15 kDa

helical domain, which in concert with binding of a single metal ion (calcium) and 3'dATP, opens and activates the catalytic site of the molecule (60). The edema factor-calmodulin complex (EF-CaM) enzymatically converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The enzyme attacks cellular ATP with rapid speed, achieving a V_{max} of up to 1.2 mM cAMP/min/mg protein and a K_m value between 0.16 and 0.21 mM (60, 154). Following exposure to EF-CaM, cellular levels of cAMP can reach up to 10^3 times normal levels, representing a conversion of up to 50% of the cells ATP stores (114). This increase in cAMP triggers water and ions to be lost from the cell en masse, causing the characteristic edema associated with anthrax disease. Although the effect is not cytotoxic, EF-CaM contributes to virulence by altering the immune response, inducing interleukin (IL)- 6 and chemotaxis of neutrophils, while inhibiting phagocytosis, tumor necrosis factor alpha (TNF- α), IL-12p70, interferon gamma (INFγ) and the oxidative burst of neutrophils (119, 234)

The EF protein is encoded by the gene *cya*, which is expressed and secreted into the host during vegetative cell growth. EF (via *cya*) is regulated through the *atxA* network (Fig. 5), as well as by bicarbonate concentration. The presence of 5% carbon dioxide incubation or 0.8% bicarbonate added to media have both been shown to increase expression of both edema factor, lethal factor, and protective antigen between 5-and 20-fold relative to growth in air (9, 116, 117, 143).

Lethal factor

The lethal factor (LF) of *B. anthracis* is a 90 kDa zinc-metalloprotease (Fig. 4), which proteolytically inactivates mitogen activated protein kinase kinases (MAPKKs)

(63). Similar to EF, LF is expression is controlled via the *atxA* network and similarly influenced by the effects of bicarbonate (72, 116, 143, 185, 220). The structure of LF contains four domains, three of which (domains 2, 3, and 4) are responsible for holding and then cleaving the N-terminal end of MAPKKs. Domain 1 is highly homologous to domain 1 of EF, and similarly functions to mediate interaction with PA (185).

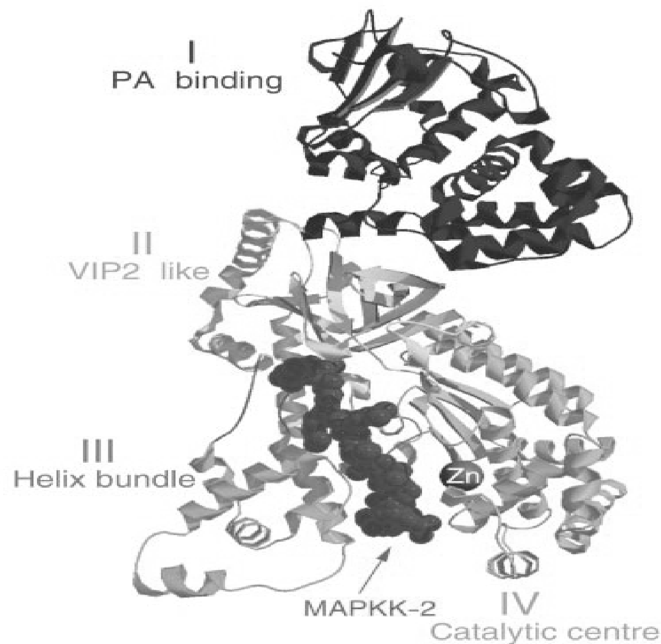

Figure 4. *Bacillus anthracis* lethal factor. Reprinted by permission from Macmillan Publishers Ltd from Pannifer et al, 2001, Nature © (185).

Unlike the edema toxin, purified LeTx (LF+PA) can cause death in as little as 60 minutes in laboratory animals (106, 153, 191). Kinetic studies of LF cleavage of MAPKKs yielded K_m values of 19 μM and V_{max} of 1.1 μmol/min/mg (72, 184). Strains with the LF gene (*lef)* deleted have been shown to have a $>10^3$ increase in LD_{50} relative to strains without the deletion (191). In contrast, deletion of the EF gene (*cya*) resulted in

only a 10-fold increase in LD_{50} in mice (191). This evidence suggests that although both toxins are important for virulence, LeTx is the major determinant of lethality.

The primary source of LF toxicity has been demonstrated to be a result of specific and drastic effects on macrophages in the host. Although other cell types are affected by LeTx activity, only macrophages are lysed by the toxin. Additionally, laboratory animals lacking macrophages are not sensitive to the effects of LeTx, suggesting that they play a central role in *B. anthracis* pathogenesis (106). Specifically within macrophages, LF is known to cleave the MAPKKs: MEK1, MEK2, MKK3, MKK6, and MKK7 (187, 247). A number of different macrophage cell lines have been demonstrated to be resistant to cytolysis when exposed to LeTx alone *in vitro*, however these results do not correlate to resistance *in vivo* (139, 187). This suggests that macrophages may require sensitization to experience cytolytic effects of LeTx, with TNF-α and calyculin A suggested as a possible sensitizers (133, 139). *In vitro* resistance to LeTx has also been mapped to the presence of three specific allele variants, all of which must be present to make the cell line resistant (164).

LeTx disruption of the MAPKK signaling pathway interrupts a number of significant cell functions, including cytokine production, macrophage activation, neutrophil function, and barrier function of lung epithelial cells (43, 217). LeTx inhibits IL-10 and TNF-α in dendritic cells (234). In CD4+ cells, LeTx inhibits IL-2 production (41, 76). In macrophages, LeTx has been demonstrated to inhibit TNF-α, IL-1β, interferon regulatory factor 3, and nitric oxide production (47, 187, 234). Additionally, the cytolytic effects of LeTx have been attributed to inability of cleaved MAPKKs to activate p38 MAP kinase. Currently, p38 is thought to be essential for induction of genes

that prevent apoptosis of activated macrophages (130). Overall, LeTx functions to significantly suppress the host innate and adaptive immune response to infection, and disrupts cell signaling and function to the point where the host experiences lethality due to its effects.

2.1.3 Regulatory pathways relating to virulence

In all biological systems, coordination of growth, replication, and gene expression are regulated through complex signaling pathways. *B. anthracis* is no exception to this, with many pathways, gene functions, and signaling mechanisms yet to be fully elucidated. However, due to the importance of toxin in disease, the signaling pathways governing expression of these virulence factors in *B. anthracis* has been relatively well characterized.

Both toxin expression and capsule synthesis are regulated by the action of the protein AtxA. The *atxA* gene is located on plasmid pXO1 and produces the protein AtxA. This protein has been demonstrated to be a *trans*-acting master regulator of many cellular functions, including growth, sporulation, virulence expression, and control of over 70 other genes (21, 117, 240, 246). AtxA has been demonstrated by numerous studies to be vitally important to the direct transcription of all three toxin genes (*cya, lef, pagA*), with null-mutants demonstrating significantly lower level of transcription and expression (25, 45, 46, 104). AtxA activates transcription of both *pagA* (protective antigen) and its repressor, *pagR*. The protein produced by *pagR* in turn negatively regulates both *atxA* and *pagA*, controlling toxin synthesis (117). PagR also affects the S-layer of the bacteria by negatively regulating the *sap* gene, while activating the *eag* gene,

effectively changing the composition of the bacterial surface (85, 168). AtxA also positively regulates capsule synthesis through the activation of *acpA* and *acpB* located on plasmid pXO2, although these genes can also be up regulated through the action of bicarbonate (61, 104).

Although toxin production is carried out at some level at all times in the vegetative cell, expression varies throughout the stages bacterial growth. Experimental evidence has shown that toxin production is highest at the late stages of logarithmic growth and in early stationary phase (209). This is due to the negative regulation of *atxA* by the transition state regulatory gene, *abrB* , which is active throughout most of bacterial growth (209, 227). However, as the vegetative cell begins to sense nutrient deprivation the *spo0* family of genes is activated (109). The activation of these genes, *spo0A* specifically, acts as a negative regulator of *abrB*, thus de-repressing the transcription of *atxA* and increasing toxin and capsule synthesis (Fig. 5) (209).

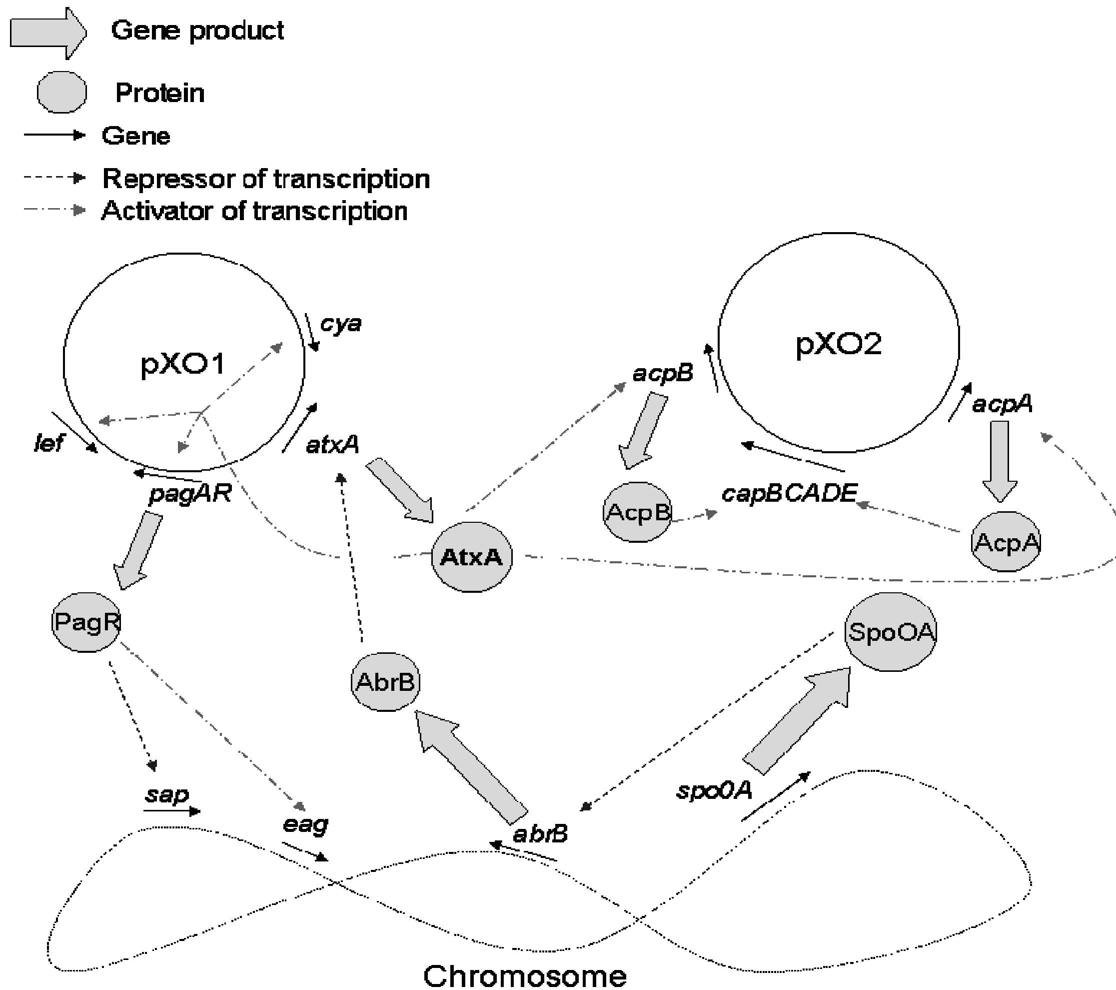

Figure 5. Regulatory network governing *B. anthracis* toxin expression. Adapted from Fouet and Mock, 2006 (85).

2.1.4 Diseases caused by *Bacillus anthracis*

Bacillus anthracis is the causative agent of anthrax disease in both humans and animals worldwide. The majority of data relating to the course of disease and pathogenesis has been collected by using laboratory animals and examination of human case reports. Although there are discrepancies between some animal models and human

disease, it is assumed that the majority of disease pathology is the same between humans and animals.

2.1.4.1 Animal disease

In animals, anthrax is most common in herbivores that graze low to the ground, such as cows, sheep, bison, goats and moose. The animals are infected by inhaling or possibly ingesting spores laying dormant in the soil. Anthrax spores can concentrate at the soil surface when a prolonged dry period has followed several seasons of flooding. Anthrax is either endemic or epidemic in many parts of the world (Fig. 6), but is relatively uncommon in North America and Western Europe.

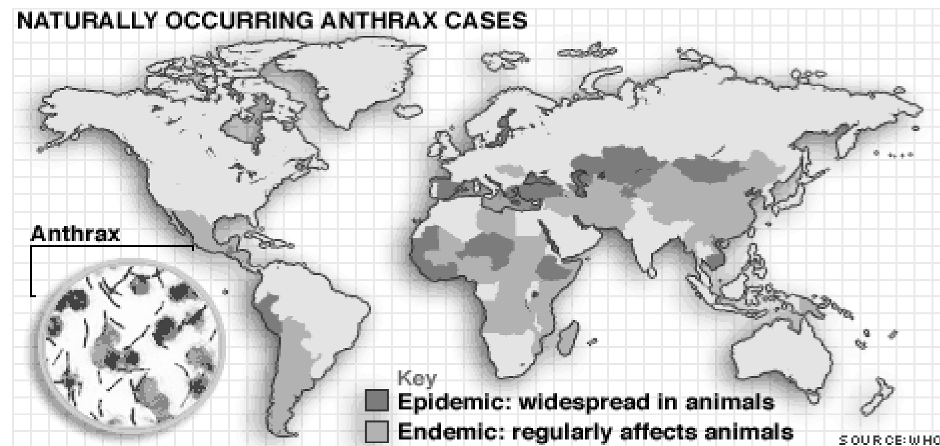

Figure 6. Naturally occurring cases of animal anthrax throughout the world. Source: World Health Organization (WHO) (258).

Although animal anthrax is relatively uncommon in North America as a whole, anthrax outbreaks in Canadian bison populations are a notable exception. Prior to 1952, when animal hide disinfection procedures were established, the majority of anthrax cases

reported in Canada could be traced back to infected imported animal products used in textile production (57). However, since 1962 there have been ten confirmed epizootic outbreaks of anthrax in northern Alberta and the Northwest Territories that killed over 1,300 bison (56, 57). Although the exact origin of these outbreaks remains unclear, genetic analysis revealed that the *B. anthracis* strain affecting bison in these outbreaks is a distinct strain which is relatively closely related to those which occasionally cause anthrax in western Canadian cattle (57).

Another recent notable outbreak of animal anthrax occurred in Australia in 1997. This outbreak affected a total of 83 properties, resulting in the deaths of 228 cattle, 4 sheep, and one horse in the Goulburn Valley of North Central Victoria, Australia. Although few sporadic cases of anthrax had been reported in Victoria during the 1890's, this was the first major outbreak in the area (238). The initial case was diagnosed in January of 1997, and was subsequently diagnosed in cattle on a further 82 farms in the area (238). Australian authorities acted rapidly to stem the further spread of disease by vaccinating animals on the infected properties and those in a 20 km by 30 km area around the center of the initial outbreak, as well as by enforcing strict procedures for carcass, milk and other product disposal, increasing farm sanitation standards, and quarantining animals on infected farms (238). This outbreak of anthrax in cattle demonstrates the rapid and potentially destructive impact of this disease even in modern farming communities. The measures taken by the Australian government were highly effective in controlling the spread of disease. These actions may serve as a template for controlling future animal outbreaks, although many of these measures may not be financially or

physically enforceable in many developing nations where animal anthrax occurs most often.

2.1.4.2 Cutaneous anthrax

Cutaneous anthrax is the most common form of disease, comprising approximately 95% of all anthrax cases. Cutaneous disease results from skin contact with the spores of *B. anthracis*. The lesion starts between 1-12 days after exposure as a painless papule at the sight of inoculation. The papule rapidly enlarges with edema surrounding the site. The infected site then progresses into a black necrotic eschar (Fig. 7) covering the edema and original papule (254). This black lesion is the hallmark characteristic of cutaneous anthrax. In many cases the bacteria remains localized at the sight of infection, and the disease is generally self-limiting (258). However in 5-20% of untreated cases, the bacteria are carried to regional lymph nodes where they can spread throughout the body causing systemic disease (254). Death in these cases is usually due to malignant edema in the head and neck, interference with respiration, or septicemia (254).

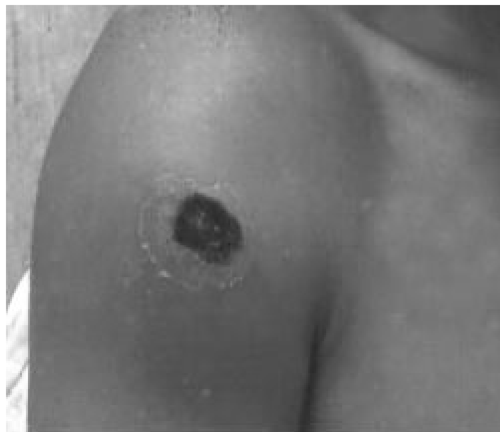

Figure 7. Necrotic black lesion caused by cutaneous anthrax. Source: Centers for Disease Control (CDC).

Those most at risk for contracting cutaneous anthrax are people in contact with animals, animal products, and lab workers. In the United States, cutaneous anthrax was most commonly found among persons employed in wool and hide production prior to the introduction of animal product sanitation standards introduced in the 1950's, although a few sporadic cases are reported each year (15). Despite most infections being self-limiting any suspected case of cutaneous anthrax should be treated with antibiotics, generally with oral ciprofloxacin or doxycycline.

2.1.4.3 Inhalation anthrax

Inhalation anthrax occurs rarely in nature, but is the most fatal infection caused by *B. anthracis*. Inhalation anthrax occurs when the spores are inhaled and become lodged in the lungs. The most common route of natural infection of this nature occurs during contact with hides, pelts, hair, or fur of animals that are exposed to the bacteria. Historically, inhalation anthrax has also been referred to as "wool-sorters' disease" (15, 254). A total 18 cases of inhalation anthrax had been reported in the US over the last 100 years prior to the 2001 bioterrorist attacks (see Section 2.9) (254). Recently, inhalation anthrax was also reported in 2006 in New York man, who worked with untreated animal hides used for drum making (250).

In order for inhalation anthrax to occur, spore particles must be a specific size, between 1.5 and 5 μm in diameter. If the spores are too small, they are readily exhaled and if too large, they do not penetrate the lung alveoli. The infectious dose of spores to

necessary to cause disease via inhalation is estimated at 6,000-8,000 spores (254). Once the spores of *B. anthracis* have lodged into the lung, they are ingested by alveolar macrophages and transported to the lymph nodes. The spores germinate between 1-60 days after initial introduction into the lungs to produce vegetative cells. Only after the spores have germinated does disease onset begin.

Initial stages of inhalation anthrax resemble influenza, with non-specific symptoms such as fever, headache, chills and nausea. The bacterial cells quickly produce large amounts of anthrax lethal and edema toxins, leading to massive hemorrhage. Inhalation anthrax cases in the early stages of disease often present with a characteristic abnormal chest x-ray, demonstrating mediastinal widening with infiltrates or pleural effusions (82). Inhalation anthrax is generally fatal within 3 days of disease onset unless treated with antibiotics. Anthrax can be treated using intravenous ciprofloxacin or doxycycline (35). With treatment the mortality rate of inhalation anthrax is approximately 45-80%, without treatment the disease is almost 100% fatal. Person to person spread is not a significant threat as disease is mainly toxin mediated and, the bacteria remains in the vegetative state in the host and only sporulates following death of the host and exposure to air. Sporulation of the bacteria following host death presents a hazard to those involved in treatment and care of anthrax patients. Strict isolation and decontamination procedures, as well as personal protective gear, must be used during post-mortem care to prevent secondary spread of disease.

2.1.4.3a Inhalation anthrax outbreak in Sverdlosk, USSR 1979

In April and May of 1979 a number of anthrax cases were reported in the city of Sverdlosk, United Soviet Socialist Republic (now Ekaterinaberg, Russia), approximately 1400 km east of Moscow. Initially, the Soviet government claimed this outbreak was due to gastrointestinal anthrax following consumption of contaminated meat and cutaneous anthrax from contact with the infected animals. However, following intense epidemiological scrutiny from American and Russian scientists, the outbreak was attributed to accidental aerosol release of *B. anthracis* spores from a military microbiology facility located in the city, resulting in cutaneous and inhalation anthrax.

In total 96 cases of human anthrax were reported between March and May of 1979 (166). Of the 96 cases, 79 cases were initially claimed to be gastrointestinal anthrax, although later shown to be inhalation anthrax, and 17 cases of cutaneous anthrax (166). A total of 64 deaths were reported, all of which resulted from the inhalation cases. This incident is the single largest outbreak of inhalation anthrax reported, and no case of human anthrax has been reported from Sverdlosk since 1979 (166).

The epidemiological investigation of this outbreak yielded highly pertinent data as to the incubation period and disease course of inhalation anthrax, as well as demonstrated the distance and impact an aerosol release of *B. anthracis* spores could have on a population. The outbreak investigation demonstrated the most likely day of accidental release of spores from the military facility was April 2, 1979 (166). Assuming this is correct and that there was a single date of accidental release, the range of incubation time was 2 to 43 days. In fatal cases, the mean time between onset and death was 3 days

(166). In addition to the human cases of disease, animals as far as 50 km from the release site were infected and lost to the disease.

The Sverdlosk anthrax outbreak in 1979 was caused by accidental release of aerosolized *B. anthracis* spores from a USSR military microbiology facility. This incident illustrates the danger posed by the development of potent biological weapons, as well as the danger posed by those who are now in possession of the stockpiles developed by the USSR. Further details of the USSR bioweapons program can be found in section 2.9. Although the amount of spores released in this incident is unknown, this outbreak graphically illustrates the potentially devastating impact an intentional release of aerosolized *B. anthracis* spores could have on a major city.

2.1.4.4 Gastrointestinal anthrax

Gastrointestinal anthrax is the result of ingesting *B. anthracis* spores, most commonly through the consumption of meat from infected animals. Unlike cutaneous and inhalation anthrax, gastrointestinal (GI) anthrax is relatively poorly studied and uncharacterized. The incidence of disease is unknown due to the fact that most cases occur in the developing world and may be either unrecognized or unreported. The infectious dose is unknown, but estimated to be greater than 10^8 spores using evidence from primates (11). However, several major outbreaks have recently documented human infection and deaths from GI anthrax. These cases have been reported in Thailand, India, Gambia, Turkey and Uganda. Illness and death has been attributed to the consumption of meat from herbivores infected with anthrax in all cases.

Consumed *B. anthracis* spores can either germinate in the oral cavity, causing the oral-pharyngeal form of disease (Fig. 8), or further down the intestinal tract, resulting in infection initiating in the intestines (Fig. 9). The extent of disease varies based on the human host and quantity of spores consumed, causing disease ranging from asymptomatic to death resulting from shock and sepsis.

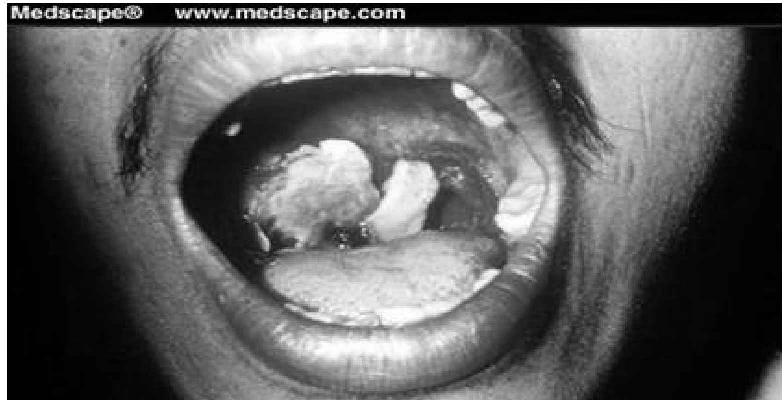

Figure 8. Oropharyngeal anthrax. From Sirisanthana and Brown, 1984 (222), CDC.

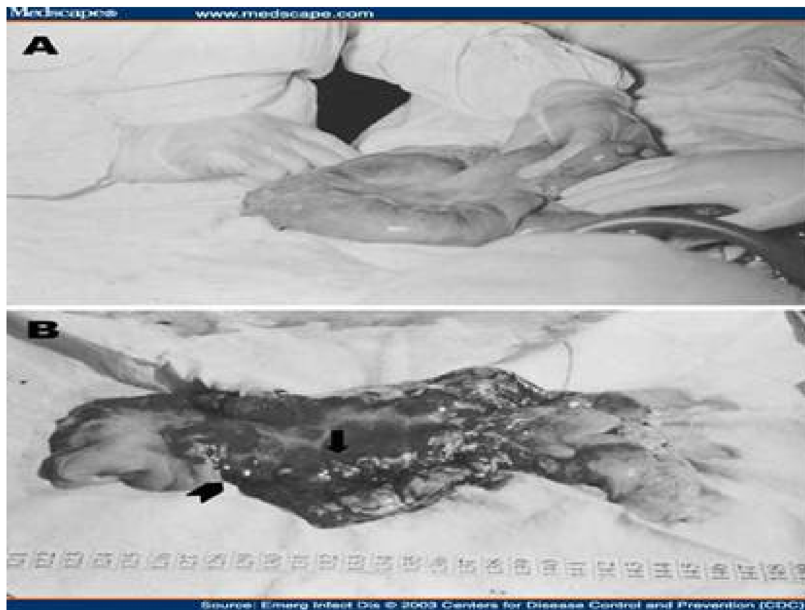

Figure 9. Gastrointestinal anthrax. A: small intestines removed at autopsy from a patient with GI anthrax, B: same small intestines as in A following dissection. From Kanafani et al, 2003 (131), CDC**.**

Although few reports documenting oropharygeal anthrax are available, one case study exists of twenty-four cases of the oropharyngeal form occurred in Thailand 1983, documenting the course of disease. In this incident, the outbreak occurred concurrently with the cases of cutaneous form and were attributed to consumption of cattle and water buffalo (222). Case reports indicate that spores germinating in the oral cavity most commonly caused ulcerative lesions on the tonsils (222). Successive disease progression resulted in fever, septicemia, and marked swelling of the lymph nodes in the neck, which resulted in airway obstruction in some cases (222). The mortality rate in this outbreak was 12.5%.

Case reports indicate that the intestinal form of GI anthrax is more common then the oropharyngeal form. Reports of the intestinal form indicate that ingested spores pass through the stomach and germinate in the intestines, causing ulcerative lesions along the intestinal tract. The lesions may lead to death by causing a combination of hemorrhage, shock, sepsis, intestinal obstruction or intestinal perforation. The mechanisms of disease are not fully understood for GI anthrax, however evidence suggests that the mucosa and the region lymph nodes are always involved (221). Gastrointestinal anthrax is treated with the same antibiotic regimen as inhalation anthrax with the addition of rehydration therapy. The diagnosis of gastrointestinal anthrax can be made by culture taken from the lesion in the oral cavity (if present) or from rectal swabbing and confirmed by testing serum antibody to anthrax antigens.

Large outbreaks of gastrointestinal anthrax have been reported in Uganda and Thailand. In 1983, 155 people became ill in Uganda after consuming meat from an infected zebu (Asian Ox). Symptoms developed in the majority of cases between 15-72

hours after exposure. Nine deaths occurred in this outbreak, all in children. Of the remaining 146 people exposed, 12 were asymptomatic and 134 complained of gastroenteritis and were treated. In January to June 1982, an outbreak of anthrax animal and human occurred in the Udon Thani province of Thailand. The outbreak killed 36 water buffalo, 7 cows, caused 28 cases of cutaneous anthrax and 74 cases of gastrointestinal anthrax with three deaths. Other reports of smaller outbreaks of gastrointestinal anthrax come from Turkey in 1986 and 1993 yielded 50% mortality (221). Determining average mortality rate from gastrointestinal anthrax is difficult as it is likely that so many cases undetected or unreported. Research in this area is needed both to document disease prevalence, infectious dose, mortality rate, and further to elucidate the mechanisms of pathogenesis.

2.2 Population structure of *B. anthracis*

The natural genetic population structure of *B. anthracis* is one of the most homogenous bacterial species known to date. Analysis of both chromosomal and plasmid markers via amplified fragment length polymorphism yielded extremely few points of polymorphism between 87 different *B. anthracis* isolates (135). Both gene (protective antigen) and genome sequencing identified very few single nucleotide polymorphisms (SNPs) across strains, providing further evidence to the genetic homogeneity of *B. anthracis* (199, 243). Van Ert et al used SNPs along with variable number tandem repeat analysis (VNTR) to describe the genetic structure of over 1,000 *B. anthracis* strains collected from different parts of the world (243). The results of this analysis again demonstrated the genetic homogeneity of *B. anthracis*, but were also used

to describe the relative geographic distribution of closely related strains, as well as an evolutionary timeline. In particular this study showed that each continent, with the exception of Europe and Asia, is dominated by a different set of 1 to 3 sub-groups of *B. anthracis* (243). The evolutionary timeline put forth by Van Ert et al also suggests the relatively recent rise of *B. anthracis* and that the small differences between lineages arose less then 7,000 years ago (243).

Although the reasons behind the genetic homogeneity of *B. anthracis* are unknown, a few theories have been put forth to explain this phenomena. These theories include evolution from a single recent ancestor, a reduced rate of molecular evolution, and that the *B. anthracis* population size and transmission cycle restrict the introduction of new genetic material (135). Of these theories, most evidence supports the recent evolution from *B. cereus*.

Genetic analysis of various *Bacillus* species indicates that *B. anthracis* is most closely related to *Bacillus cereus*, followed by *Bacillus thuringiensis* and more distantly by *Bacillus mycoides* (135). If *B. anthracis* did evolve recently, as evidence described above suggests, it is most likely that it evolved from *B. cereus*. This line of evolution is supported by data from number of different sources. Strong evidence from full genome sequencing of *B. cereus* and *B. anthracis* demonstrate that the two species are incredibly similar, differing mainly by plasmid content and the activity of the *plcR* gene (124). The *plcR* gene of *B. cereus* functions as a global regulator of many cell functions, but is inactivated in *B. anthracis* by a nonsense mutation. This mutation is considered a defining characteristic of *B. anthracis* and is essential for maintenance of plasmids pXO1 and pXO2 (169). In fact the similarities between the two species, despite the difference

in functionality of the *plcR* gene, has led to the argument by some scientists that *B. cereus* and *B. anthracis* are one species differentiated by plasmid content (112). However, *B. anthracis* is still considered a separate species from *B. cereus* despite their genetic similarity.

Other strong evidence for the recent evolution of *B. anthracis* from *B. cereus* comes from the isolation and characterization of *B. cereus* G9241 from an unusual case of pneumonia with clinical and laboratory findings similar to those caused by *B. anthracis* (118). *B. cereus* G9241 contains a large plasmid, pBCXO1, highly similar to *B. anthracis* plasmid pXO1 and is able to produce a polysaccharide capsule, although it lacked homologues of the anthrax biosynthetic *cap* genes (118). Although other features of this strain, such as motility, hemolysis, 16s RNA, resistance to gamma phage and penicillin were typical of *B. cereus*, the presence of the anthrax toxin gene homologues, capsule, and disease characteristics demonstrated a distinct similarity between species (118). In addition to this isolate, other *B. cereus* strains related to similar incidents of fatal pneumonia have been found to harbor *B. anthracis* capsule biosynthetic genes, although they did not produce a poly-d-glutamic acid capsule (115). Although no *B. cereus* isolate has been found to harbor all of the virulence genes of *B. anthracis*, these genes do naturally occur in the *B. cereus* population lending support to the possibility of recent evolution from *B. cereus* leading to the current homogeneous population.

2.3 *B. anthracis* vaccines

Development of effective and safe vaccines against disease is one of the classical public health methods that can be used to prevent wide spread illness. In an ideal setting,

a vaccine against *B. anthracis* would be safe, effective, and easily and rapidly administered in the case of an emergency. However, both current and potential human vaccines against *B. anthracis* present numerous challenges to all of these points.

Currently, there is only one anthrax vaccine licensed for use by the U.S. Food and Drug Administration for use in humans. This vaccine is known as the anthrax vaccine adsorbed (AVA) and produced by BioPort, Rockville, MD, under the trade name BioThrax. The vaccine is a cell free filtrate derived from a toxigenic, but non-encapsulated strain of *B. anthracis*, known as V770-NP1-R. The major antigenic component of the vaccine is the protective antigen, and is thought to be critical for immune response by preventing the uptake of the lethal and edema toxins into the cell (5). The original version of the vaccine was developed in 1954, but has since been improved by using microaerophilic growth conditions, selection of a strain that expresses higher amounts of protective antigen, change of adjuvants to aluminum hydroxide from alum, and the development of a protein free media (5). The AVA is currently used by the United States Department of Defense for military purposes and is not available for use in the general population.

Though the AVA has been effectively used in the military, the vaccine does not meet the requirements listed above to be used in the general population as either an emergency or preventative measure against potential bioterrorist attack using *B. anthracis*. As with any vaccine, one of the first concerns is safety. The AVA has been demonstrated to be relatively safe to administer to health individuals between the ages of 18 and 65, although more than 8% of those receiving the vaccine in pre-license clinical trials developed mild local erythematic lesions and over 1% of the study population

developed moderate or severe erythema at the site of injection (5). All systemic effects of the vaccine were reported as transient. Data from post-license surveillance has indicated similar patterns of reaction among those receiving the vaccine in the US military (5).

Despite the relative safety and usefulness of the AVA as a military tool, many questions have been raised concerning its efficacy and administration. As a cell free filtrate containing mainly the protective antigen, the vaccine has been criticized for failing to sensitize patients to other components of the bacteria which may be more effective in elucidating an immune response that would result in long lasting immunity. This shortcoming of the vaccine is apparent in experimental trials conducted in animals by multiple scientists, indicating that AVA does not protect against certain strains of anthrax (7, 157, 251).

One hurdle to creating a more effective cell free vaccine is the lack of other conventional vaccine targets. Other cell free vaccines, such as those for *Neiserria meningiditis,* are based on the capsule as opposed to the toxigenic component. However, the capsule essential for *B. anthracis* virulence is non-immunogenic and actually helps the bacteria to evade the immune response – thus is not a candidate for vaccine target. Although killed cell and live attenuated vaccines have proven to be more effective as animal vaccines against a variety of different strains *B. anthracis*, the FDA will not license such a vaccine for human use due to the potentially lethal effects (157). Improvements to vaccine efficacy must be made in order to provide full protection against all potential strains of *B. anthracis* that could be used as a biological weapon. This is particularly important when considering that many strains of *B. anthracis* used as

a biological weapon would likely be engineered to be resistant to both antibiotics and the effects of the vaccine.

An additional concern over general use of the AVA is the demanding immunization schedule required to impart and maintain immunity. A full course of AVA consists of an initial set of 3 subcutaneous injections two weeks apart, followed up with subsequent vaccinations at 6, 12, and 18 months, as well as annual boosters (5). This rigorous vaccination course may be useful in a highly structured organization, such as the military, but is simply not feasible for administration to the general population. Although the vaccine has not been studied for efficacy or safety in a post-exposure setting, it has been authorized by the FDA for emergency use in this situation (77).

Although the AVA has been used effectively by the US military for a number of years, the problems with vaccine efficacy, long term administration, and relatively high rate of local adverse reaction makes the AVA a less then ideal vaccine. New recombinant protective antigen vaccines, including those expressed by adenovirus, have been developed by various research groups, although none have been licensed for use (156). Continued research is essential to the development of more useful vaccine against anthrax.

2.4 Current detection methods for *B. anthracis*

Numerous detection methods have been developed and are currently marketed for the detection of *B. anthracis* both from patients and the environment. Rapid detection methods can be broadly classified into two groups, based on either antibody reaction (i.e. enzyme linked immunoassay and lateral flow assays) or molecular detection. Antibody

based assays developed for direct detection from environmental samples generally either target the protective antigen or use proprietary antibody targets (211). Commercial lateral flow assays are designed to detect a minimum of 10^5 spores/sample, although performance of these assays at this level and compatibility with food samples has been questioned (140). Molecular detection methods for *B. anthracis* center on PCR and real-time PCR detection of protective antigen and capsule genes, and have been developed into standardized kits used by the CDC and World Health Organization (WHO) (14, 38, 39, 66, 188, 200). Another real-time PCR methodology under development is the ruggedized advanced pathogen identification device (RAPID), which detects the *vrrA* and *lef* (LF) genes and has demonstrated compatibility with milk samples following DNA extraction to a level of 2.5×10^3 CFU/mL (188).

Although a number of procedures have been developed to make these detection methods compatible with various sample types, such as blood and soil, little has been published on direct detection from liquid foods such as milk or orange juice. Current literature suggests that milk cannot be directly used as a media for PCR reaction without prior DNA extraction to eliminate calcium and other potential reaction inhibitors (18, 197). No current literature was found on detection of *B. anthracis* from orange juice. Current published real-time PCR and lateral flow assay detection limits (2.5×10^3 CFU/mL and 10^5 CFU/mL, respectively) are relatively high and may fail to detect milk or orange juice contaminated at a low, but still potentially infectious level. The inability of these techniques to reliably detect low levels of *B. anthracis* spores from liquid foods is a major weakness in our nation's food protection system. One method of increasing reliability and lowering detection limits, without a significant increase in time to detect, is

the application of rapid pre-analytical procedures that both concentrate spores, as well as separate them from the food media.

2.5 Ricin

Ricin is a naturally occurring toxin found in the seeds of the castor bean plant (*Ricinus communis*) and is composed of two polypeptide chains (A and B) linked by a disulfide bond (Fig. 9). This water soluble toxic compound is part of the group of proteins known as type II ribosomal inactivating proteins (RIP II), which include abrin and viscumin among other members. Ricin and other RIP II proteins belong to the A-B type family of toxins and have demonstrated relatively high heat stability (125).

Ricin polypeptide chain B (34 kDa) binds galactose on the cell surface, allowing the A chain to be translocated within the cell. Once inside the cytosol of the cell, the A chain (32 kDa) enzymatically removes an adenine residue from 28S ribosomal RNA (Fig. 10) (6, 52, 181). The depurination itself does not physically break the rRNA loop, but it leaves the rRNA prone to hydrolysis at alkaline pH and at acid pH in the presence of aniline (20, 158). Hydrolyzed 28S rRNA is unable to bind elongation factors essential to further protein synthesis, leading to cell death (181).

The castor bean plant and its seeds have long been recognized by humans as poisonous and unfit for consumption, with the exception of a few medicinal uses, dating back many centuries prior to modern protein science. In fact ricin is not the only potentially toxic molecule produced by the castor bean plant. Closely related to ricin and found in the castor bean is *Ricinus communis* agglutinin (RCA), which is composed of four polypeptide chains. The four polypeptide chains of RCA are composed of two sets

of molecules each highly similar to the ricin A and B chains (29). RCA toxicity differs from ricin in that it is a strong hemagluttinin, but only weakly cytotoxic (158). An additional toxin of the castor bean plant is ricinine, which is found in the leaves and pericarp of the plant, as well as in small quantities in the beans (6). Ricinine acts differently then either RCA or ricin, and primarily affects the nervous system acting by increasing release of glutamate and inhibiting of the postsynaptic γ-aminobutyric acid receptor subtype A in the brain (81). Large amounts of ricinine can be fatal or cause severe seizure reactions, although it has potential for therapeutic use as a cognition enhancer for treating amnesia at low concentrations (81).

Ricin has a relatively extensive history of use as a bioweapon and is currently classified by the CDC as a category B bioterrorist agent. In addition to its use by terrorist groups, ricin was also produced as part of offensive bioweapons programs in the United States and Iraq. Ricin is a likely candidate as a bioterrorist agent not only because of its potent activity, but also because of the availability of raw materials and inexpensive, relatively simple production. Castor bean plants grow wild throughout the United States, and are commercially grown for the production of castor bean oil and as an ornamental plant worldwide. Due to its hydrophilic nature, ricin is not found in castor bean oil itself, but rather the spent bean mash (181). Toxin can be precipitated from the mash and purified. The toxic protein can be dried to produce a powder or can be dissolved in a variety of media, including food and water.

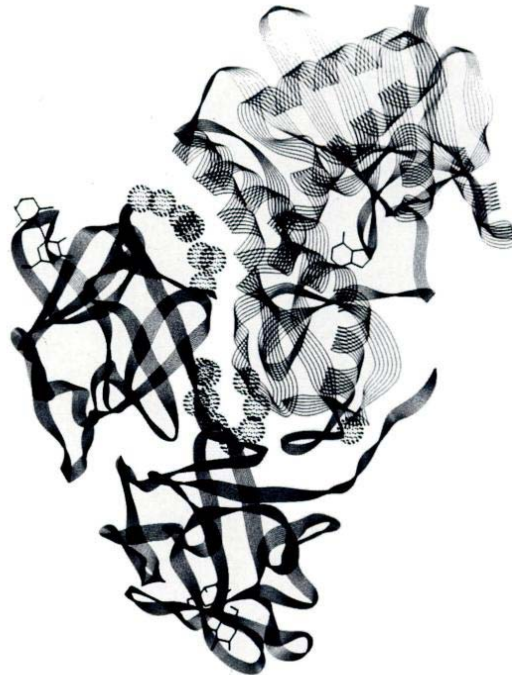

Figure 10. Ricin structure. A chain denoted by the striped ribbon, B chain denoted by solid ribbon. Reprinted by permission from Lord et al, 1994, The Federation of American Societies for Experimental Biology © (158).

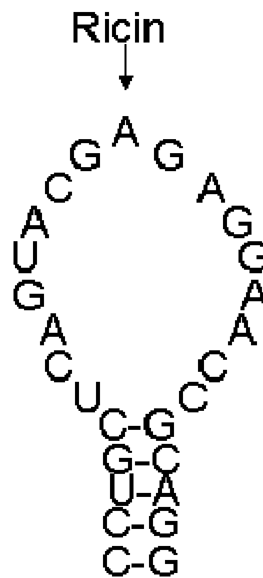

Figure 11. Ricin loop and cleavage site within the 28 S ribosomal RNA. Adapted from from Olsnes, 2001 (181).

2.5.1 Ricin toxicity and treatment

The toxicity of ricin varies based on route of administration. Inhaled or injected ricin is extremely toxic with an LD_{50} (lethal dose 50) in mice of 3 to 10 μg/ kg (89). LD_{50} estimates for ingested ricin range from 30 μg/kg to 20 mg/kg in humans and are thought to be higher due to decreased absorption via the gastrointestinal tract and potentially enzymatic breakdown by gastric enzymes (6, 89, 212). The no observed adverse effect level (NOAEL) of ricin consumed in water is estimated 30-225 μg/day (28). The variation between oral ricin toxicity estimates is likely due to differences in

methodology between labs and purity of ricin toxin, some using whole beans or crude extracts from them, while others have used highly purified toxin.

Signs of ricin poisoning vary by the route of exposure, but generally occur from 6 to 8 hours after ingestion. Death can occur in as little as 36 to 72 hours after exposure, depending on route and dose, and is generally due to organ failure of the kidneys, liver, or spleen (52). In the case of Georgi Markhov, assassinated via ricin injection, an estimated 500 μg of ricin were administered. This dose resulted in immediate local pain and resulted in death within three days due to cardiac failure and shock.

Treatment of ricin poisoning is generally limited to supportive care and symptomatic treatment of hypotension. In cases where ricin is known to have been recently ingested, treatment with gastric lavage or activated charcoal may decrease absorption of the toxin (6). Vaccines against ricin poisoning are currently under active development, although their practical use may only be limited to military or persons using the toxin for medical research. Current vaccines under development include a formalin treated toxoid vaccine and a recombinant A-chain protein (89, 248). Passive immunity against ricin via administration of an anti-ricin antibody has been demonstrated to only be effective for few hours after administration (194). This method of prevention would only be effective in cases where exposure was anticipated, as administration of antibody following exposure has been shown to be of little value in preventing biological effects (89). Other potential methods of prevention and treatment for ricin poisoning, such as anti-ricin aptamer, are under development (75). Despite efforts to develop more effective preventive and treatment measures for ricin poisoning, ingestion, inhalation, or injection of very small quantities of ricin remains highly fatal.

2.5.2 Potential therapeutic uses of ricin

Although ricin is toxic in small quantities, its ability to inactivate the ribosome and kill cells has been explored for therapeutic use in cancer treatment. In order to avoid the toxic effects of ricin, researchers couple a deglycosylated ricin A-chain (enzymatic moiety) of the toxin to an anti-tumor antibody creating an immunotoxin. In the immunotoxin, the anti-tumor antibody acts as the site of cellular recognition instead of the B-chain. This directs the action of the A-chain almost solely against cells displaying the tumor antigen (146, 249). Although the A-chain can potentially be taken up in minute quantities by healthy cells, the majority of toxic activity is directed towards the tumor (249). Application of ricin immunotoxins in mouse and human clinical trials has yielded promising results (88, 102, 148, 224, 229). The majority of clinical trials have been directed at leukemias and lymphomas, although some trials have been carried out with solid tumors. In most studies the toxicity of the immunotoxin treatment was low, although some moderate toxicity has been reported. Although some early studies had mixed efficacy results, continued technological improvement in production of the immunotoxins has lead to very positive anti-cancer results in many numerous types of disease (88, 102, 146, 148, 224). Immunotoxins, utilizing ricin or other ribosomal inactivating proteins, continues to be an area of active cancer research and clinical trials.

2.6 Current methods for ricin detection

The current rapid methods for detection of ricin are mainly antibody based, although new assays based on the enzymatic activity of ricin are also under development (12, 165). The majority of antibody based assays target the A or B chain specifically,

although some use polyclonal antibody raised against the whole molecule. Commercial enzyme linked immunoassays (ELISA) and lateral flow assays are available and rated for detection of ricin to 100 pg/mL (195, 218). Both ELISA and lateral flow assays have variable compatibility for direct use from food samples, and generally require dilution or extraction from the food media prior to testing to achieve accurate results (27, 93). As with detection of *B. anthracis* from food, both detection limits and reliability may be improved using a pre-analytical procedure to extract and concentrate the ricin out of the food media.

The majority of ricin detection assays based on activity are either based on the release of adenine from ribosomal cleavage or by protein expression array. Although these systems have not yet been commercialized, they represent a potential new wave of rapid detection. These systems are potentially less sensitive to the effects of food media, as they are based on activity rather than detection of the molecule itself. However, due the variability in end-product measured in these systems, the effects of food media would need to be determined would need to be determined individually for each assay.

2.7 Pathatrix® use for detection of foodborne pathogens

Pathatrix® is a recirculating immunomagnetic separation system for extraction and concentration of pathogens from large samples of food developed by Matrix MicroScience Inc (Fig. 11). Pathatrix® operates by continuously circulating the food sample with antibody coated magnetic beads. The antibody binds the target antigen and the beads are collected via a magnetic platform. The beads with antibody bound target are eluted from the magnet and resuspended in a small volume of buffer for detection.

The target can then be detected via ELISA, agar plating, PCR, or other methods. Pathatrix® has been approved by the Association of Analytical Chemists (AOAC) for detection of *Listeria* and *Salmonella* from a variety of foods and *E. coli* O157 from raw ground beef. Numerous other applications are currently being studied for extraction of bacteria from food samples; however the application of this system to toxins has not been tested.

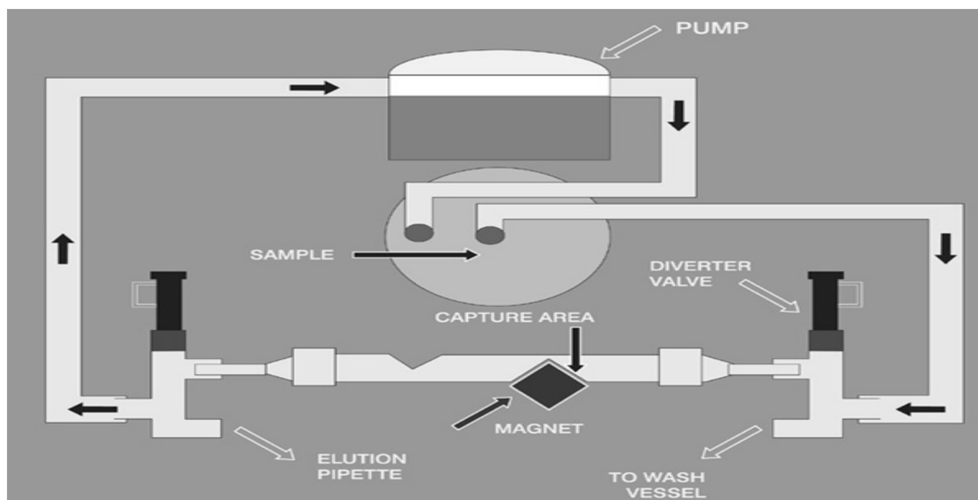

Figure 12. Schematic diagram of Pathatrix® system. Used with permission from Matrix MicroScience, Inc.

2.8 Brief history of biological and chemical warfare

Biological and chemical weapons have a long history of use by nations, armies, and terrorists, as a means of accomplishing political, military, or economic goals. Biological and chemical weapons have a different connotation then conventional weapons, in both the eyes of the user and the target. The fear inspired by the potential threat of such an attack is enough to cause significant economic loss, public outcry, and political turmoil, enough to lead many individuals and groups to start rumors of attack as

a hoax. Although many attacks have been unsuccessful throughout history, the threat of effective production and use of biological and chemical weapons remains a concern for United States and other nations worldwide.

Biological and chemical weapons were used on multiple occasions in antiquity during warfare. Despite the rudimentary nature of these weapons, they were highly effective in many cases in expediting the military venture. Documented instances of the effective use of chemical and biological weapons include: the use of helleborus roots by the Athenian dictator Solon to contaminate water supplies at Kirrha and the contamination of wine with mandrake roots by the Carthaginians to sedate their enemies (230). The basic, yet effective, contamination of food and water supplies with naturally occurring plant toxins demonstrates that even a rudimentary chemical or biological attack on these vital systems can significantly affect a population and the course of history.

As history progresses, so does the nature and sophistication of biological and chemical weapons. During the middle ages, it was recognized by many different groups that the bodies of those who had succumbed to infectious disease could also spread the disease. Successful use of infected corpses as biological weapon is documented most notably in the siege of Caffa by the Mongols from 1346-1347 A.D. In this incident, documented by de Mussis, the Mongols catapulted plague-infected corpses over the city walls (256). As a result, many of the city's Genoese defenders were sickened and fled the city, spreading plague with them across the Mediterranean basin (230, 256, 257).

The successful use of chemical weapons during the middle ages was exemplified by the use of arsenic smoke to distract and sicken enemies during military sieges and has been documented both in China and Europe (230). Other less successful biological

warfare strategies of the time included contamination of wine with the blood of leprosy patients in Spain, and the use of saliva from rabid dogs in hollow munitions shells (230).

The arrival of the British to North America signals a change in the nature of use of chemical and biological weapons, not only to as a military tactic, but to significantly deplete specific susceptible populations. Substantial evidence exists that British forces intentionally infected the Native American population with smallpox on multiple occasions through the use of infected fomites, particularly blankets and handkerchiefs (80, 257). The disease proceeded to decimate the native population, virtually assuring both military victory and submission the remaining people. A similar set of events is thought to have taken place with the arrival of Pizarro to South America, although there is less direct evidence of intentional disease spread (257).

Scientific advancements prior to the First World War also lead to an increase in biological and chemical weaponry. For the first time scientists had an understanding of the chemistry and microbiology that could be harnessed for use against their enemies. The most commonly used agents during the war were chemical toxins and irritants that could be used in gas form. Of the gas agents, the most common was sulfur mustard gas, which acts both via the lungs and skin as a cytotoxic agent (230). Other chemical gasses used included irritants ethylbromoacetate and chloroacetone, and lung damaging agents chlorine and phosgene (230).

Biological warfare during World War I was increased in sophistication. During the war, Germany was alleged to have contaminated numerous horses and cattle with both *Burkholderia mallei* (glanders) and *Bacillus anthracis* (anthrax) to weaken supply lines of the allied forces (151, 230, 257). However, these claims have not been

substantiated due to the endemic nature of both diseases. The allegations against German forces also include attempts to spread anti-personnel agents, such as cholera and plague, through the use of contaminated food stuffs and toys (151).

Following World War I, the Geneva Protocol of 1925 was drawn up to prohibit the use in war of asphyxiating, poisonous, or other gases and bacteriological methods of warfare. However, this had little effect on the progressive use and development of biological agents during World War II, and at this point in history many nations began or expanded their capacity for developing biological and chemical agents.

The most prodigious user of biological and chemical weapons during World War II was Japan. The Japanese program included research on a number of agents including *B. anthracis*, *Salmonella* Typhi (typhoid), *Vibrio cholera* (cholera), *Yersinia pestis* (plague) and numerous anti-crop and anti-animal agents (257). Japanese research also included the use of thousands of human test subjects, of whom 10,000 are though to have been killed or allowed to die after deliberate infection (151, 257). In the field, numerous attacks using biological agents were carried out during the war. The most successful of these attacks included air drops of plague infected fleas, contamination of wells with intestinal pathogens, and aerial spraying of cultures (257). The majority of these attacks were carried out in China, with casualty estimates of several hundred thousand.

Although Japan is the only confirmed mass user of biological agents during World War II, the development of offensive chemical and biological weapons programs was rampant in many countries, including Great Britain, Canada, Russia (USSR), and the United States. Each of these nations researched and produced biological and/or chemical weapons, although programs in Great Britain and Canada were scaled back not long after

the conclusion of World War II (151). In contrast, the United States and USSR both escalated their programs with the rise of the Cold War.

The United States chemical and biological weapons program performed extensive research on a number of potential weapons however; available information suggests that only 10 of these agents were fully weaponized. The list of weaponized agents includes anti-personnel agents (*B. anthracis, Franciscella tularensis, Coxiella burnetti*, yellow fever, botulinum toxin, and staphylococcal enterotoxin), anti-livestock agents (*Brucella suis* and Venezuelan equine encephalitis (VEE)), and anti-crop agents (*Pyricularia oryzae* and *Puccinia graminis tritci*) (151). In addition, the US also developed a number of means for effectively distributing these agents, including spray tanks, loading in warheads, and as cluster munitions. Although China, North Korea, and Cuba alleged that the US used biological weapons against them, investigation of all three incidents yielded little evidence of such an attack (257). Development of offensive weapons ceased with President Nixon's orders in 1969, although stockpiles were not destroyed until 1972 when the international biological weapons and toxins convention (BWTC) and chemical weapons convention (CWC) were signed. The BWTC barred the development and transfer of biological and toxin agents not used for peaceful research purposes, although these agreements were later violated by both the USSR and Iraq. The United States is not currently involved in offensive biological weapons development, although peaceful research on these agents continues at both military and academic laboratories. The CDC has also compiled a list of potential bioterrorist agents based on agent transmissibility, virulence, and human health effects.

In contrast to the United States program, the USSR program was covertly operated both before and after it signed the BWTC and CWC. Extensive biological weapons research was carried out by various government ministries and the Biopreparat programs. Biopreparat research included some of the same agents as the US program, such as VEE, *B. anthracis,* and *Brucella suis*, but also included highly contagious anti-personnel agents such as smallpox (*Variola major*), *Y. pestis,* and Marburg virus (3).

Table 1. Centers for Diseases Control (CDC) classification of potential biological weapons agents. Adapted from www.cdc.gov

Category	Agent (disease)	Agent Characteristics
A – High Priority	*Yersinia pestis* (plague) *Bacillus anthracis* (anthrax) *Clostridium botulinum* toxin (botulism) *Francisella tularensis* (tularemia) Variola major (smallpox) Viral hemorrhagic fevers (i.e. ebola, lassa fever)	Result in high mortality rates and have the potential for major public health impact May cause public panic and social disruption Requires special action for public health preparedness Can be easily disseminated or transmitted from person to person
B- Second Priority	*Brucella* spp. (Brucellosis) *Clostridium perfringens* epsilon toxin Food safety threats *(E. coli* O157:H7, *Shigella)* *Burkholderia mallei* (Glanders) *Burkholderia pseudomallei* (Melidoidosis) *Chlamydia psittaci* (psitticosis) *Coxiella burnetii* (Q fever) Ricin toxin Staphylococcal enterotoxin B *Rickettsia prowazekii* (Typhus fever) Viral encephalitis Water safety threats (e.g., *Vibrio cholerae* (cholera) *Cryptosporidium parvum (*cryptosporidiosis))	Are moderately easy to disseminate Result in moderate morbidity rates and low mortality rates Require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance.
C – Third Priority	Emerging infections i.e. Nipah virus and hantavirus	Available Easy to produce and disseminate Potential for high morbidity and mortality rates and major health impact.

The USSR programs not only optimized pathogens through the use of genetic engineering and improving antibiotic resistance, but also stockpiled agents, including *B. anthracis* spores. In addition to enormous stockpiles of weaponized agents, Biopreparat also constructed numerous production facilities with the ability to rapidly increase capacity (3, 50). No direct intentional attack by the USSR using these agents has ever been confirmed, although an unintentional release of anthrax spores from a plant in Sverdlovsk (Ekaterinaberg) in 1979 killed approximately 64 people (see section 2.2.3.3a) (166). Although the USSR did not carry out any large scale attack using biological weapons, in 1978 the Bulgarian communist secret service assassinated exiled Bulgarian journalist Georgi Markov using ricin toxin, likely of Soviet origin, injected via an umbrella. A similar attack on another Bulgarian exile, Vladimir Kostov, was unsuccessful.

Following the collapse of the Soviet Union several high ranking Biopreparat member defected to the US, including Ken Alibek. Alibek's testimony as to the inner workings of and developments made as part of the Biopreparat program lead many to believe that many weaponized agents still exist in large quantity (50). If these agents are still in existence it is likely that many of them can be purchased on the black market, representing a venue for distribution of these agents to terrorist groups worldwide.

An additional biological and chemical weapons program of note in recent history is that of Iraq. Under Saddam Hussein, the Iraqi program produced botulinum toxin, *B. anthracis*, aflatoxin, ricin, *Clostridium perfringens,* wheat smut, rotavirus, and camel pox, with the capability to distribute these agents through a number of different systems

(50). During the 1990's the United Nations investigators attempted to ascertain the status of the Iraqi biological and chemical weapons program, although they were expelled on more then one occasion. Fears of biological and chemical weapon capability and use by Hussein are partially responsible for the US invasion of Iraq in 1993 and 2001 (50). Currently the Iraqi program is believed to be dismantled and the majority of biological weapons destroyed.

In the past the majority of biological and chemical weapons threat was mainly attributed to the fear of an attack by one nation upon another. Following the dismantling the of the USSR and Iraqi biological and chemical weapons programs, the threat has shifted from state-sponsored efforts to independent groups using these agents for terrorist purposes. A number of terrorist groups have both attempted and succeeded at using biological and chemical weapons against civilian populations.

Aum Shinrikiyo, a Japanese apocalyptic cult, is the most prominent user of biological and chemical agents for terrorist purposes. In June 1994 cult followers released sarin gas in the residential area of Matsumoto, a city 322 kilometers northwest of Tokyo. The attack was intended to incapacitate or kill three judges currently presiding over a real estate dispute in which Aum Shinrikyo was involved (183). The attack resulted in seven deaths and five hundred injured. In 1995 Aum Shinrikyo again attacked the Japanese people, this time releasing sarin gas on five trains throughout the Tokyo subway system. The second sarin release killed twelve and injured approximately 3,800 people (183). Aum Shinrikyo also attempted to release botulinum toxin and anthrax spores in 1993 (183). In addition to the aforementioned agents, the cult has also attempted to develop cholera, Q fever, and ebola as weapons (183). Unlike some other

terrorist groups, Aum Shinrikyo counts a number of graduate level biologists and chemists among its members. The cult is also extremely well funded, with an estimated net worth of approximately $1.5 billion dollars in 1995. Although the cult has since fractionated, the resources, education, and coordination of the attacks raised serious concerns about biological and chemical terrorism.

Biological and chemical weapons attacks have also been successfully executed by various organizations and individuals in the United States. The most notable use of biological weapons in the US was the anthrax attacks of 2001. In September and October of 2001 approximately six letters containing weaponized anthrax spores were mailed to media representatives and US senators (129). During the mail sorting process, both automatic sorting machines and thousands of postal workers were exposed. The attack resulted in eleven cases of each cutaneous and inhalation anthrax, leading to five deaths and thousands of individuals given emergency prophylactic treatment (129). Current evidence suggests that Dr. Bruce Ivins, a notable anthrax researcher at the United States Army Medical Research Institute of Infectious Disease, was the perpetrator in these attacks. On August 8, 2008 federal prosecutor, U.S. Attorney Jeffrey Taylor, officially stated that Dr. Ivins was the "sole culprit" in the 2001 anthrax attacks. However, Dr. Ivins committed suicide prior to being formally charged with the crime. Both the direct and indirect impact of this attack and upwards of five thousand false alarm and hoax incidents have cost the US government an estimated $100 million dollars (151).

In addition to the anthrax mail attacks, a number of other bioterrorist incidents have also occurred and many more have been thwarted by authorities or exposed as hoaxes. In 1984 the Ranjneesh Sect developed and distributed *Salmonella* Typhimurium

by spraying it on salad bars near The Dalles, Oregon (235, 257). The attack resulted in 750 cases of salmonellosis, although the group was not able to achieve their intended goal of swaying a local election.

A number of groups and individuals have been arrested on the suspicion of developing and planning to use various biological weapons. The most popular agent found as part of these potential threats is ricin toxin. Upwards of twenty incidents have been documented involving plans to extract ricin or possession of the toxin itself (263). Although much of the ricin found as part of these investigations was in the form of a crude extract, pure ricin has also been confiscated in at least one incident (263). The most recent ricin incident occurred in 2008, when ricin was found in a Las Vegas, Nevada hotel room (92).

Biological and chemical weapons have been used on countless occasions throughout history. Much of the research and development of these agents was done by state sponsored programs, however, given the current international agreements, it is relatively unlikely that an attack using these agents would be carried out by a national government. More likely, a biological or chemical weapons attack would come from an individual or terrorist group. Recent events involving these agents demonstrate that attacks can range from crude, uncoordinated events to highly coordinated, complex attack plans using highly refined agents. These attacks can also be directed against any number of targets including general populations, specific persons, crops, livestock, or food and water resources. Although it is likely that biological or chemical agents will be used in the future, the timing, sophistication, extent, perpetrators, targets, and impact of a

potential attack are highly variable, making detection of the agents from a wide variety of media and preparedness for any situation crucial.

2.9 Cow's Milk

2.9.1 Milk composition and definition

Milk is defined by the United States milk standard of identity as the lacteal secretion from a complete milking of healthy cows. Milk must contain not less then 8.25% solids-non-fat, not less then 3.25% milk fat, and must be practically free of colostrum (79). In 2006, the United States produced over 82 million tons of cows milk, with an average consumption of nearly 84 liters of fluid milk per person (121).

Milk is a highly complex food media, comprised of a number of intricate sub-unit systems. Milk is approximately 88% water, 5% carbohydrate, 4% fat, 3% protein, and contains a variety of different minerals including calcium. Virtually all of the carbohydrate contained in milk is in the form of lactose. Milk contains a variety of different types of fat including triglycerides, phospholipids, mono- and di-glycerides, and sterols. At least 95% of these fats are contained within the milk fat globule, which is enclosed by a thin membrane (Fig.13). The protein content of milk is primarily composed of various caseins, with whey proteins (β-lactoglobulin, α-lactalbumin, and bovine serum albumin) comprising the remainder of the protein. Caseins are typically found in a micelle structure (Fig. 14), while whey proteins are free within the aqueous phase of the matrix.

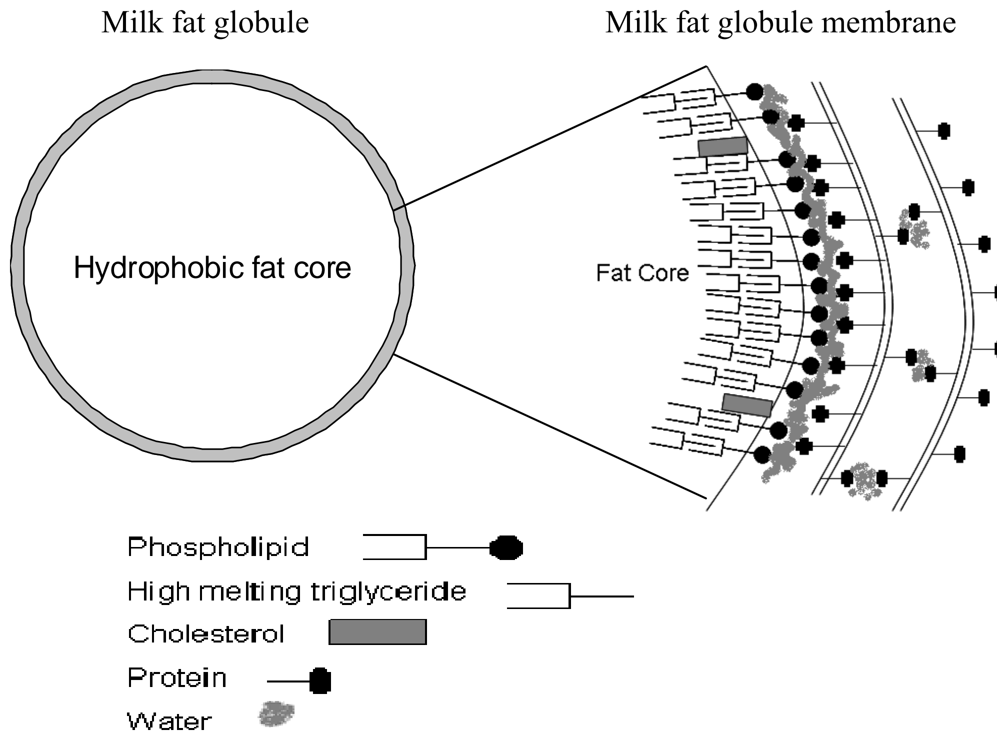

Figure 13. Schematic diagram of milk fat globule and milk fat globule membrane. Adapted from King, 1955 (141).

Raw milk is collected from cows on dairy farms daily and stored in tanks on the premises. Milk must be cooled to 10°C (50°F) or less within four hours or less of the start of milking and to 7°C (45°F) or less within two hours after the completion of milking (79). In addition, each individual producer's milk must contain less then 10^5 bacterial CFU per mL and less then 750,000 somatic cells per mL prior to commingling with other producer milk (79). Milk is collected from dairy farms on a daily basis by a bulk milk hauler using an insulated truck, which then transports the raw product to either the processer or other receiving station. At the point of collection from the farms and prior to comingling, milk samples from individual producers are taken and saved for later analysis at the processor. Each bulk milk truck collects from multiple farms per day and

can typically carry between 3,000 and 6,000 gallons. Milk haulers then deliver the raw milk to processing facilities, which tests the milk temperature, total acidity, flavor, odor, tanker cleanliness, and the presence of antibiotics. After passing these tests, milk is transferred to milk silos, which typically hold between 20,000 and 60,000 gallons of milk. Following delivery and clearance of the above described tests, milk can be stored in the silo for up to 72 hours from the time of receipt at the processing plant (79).

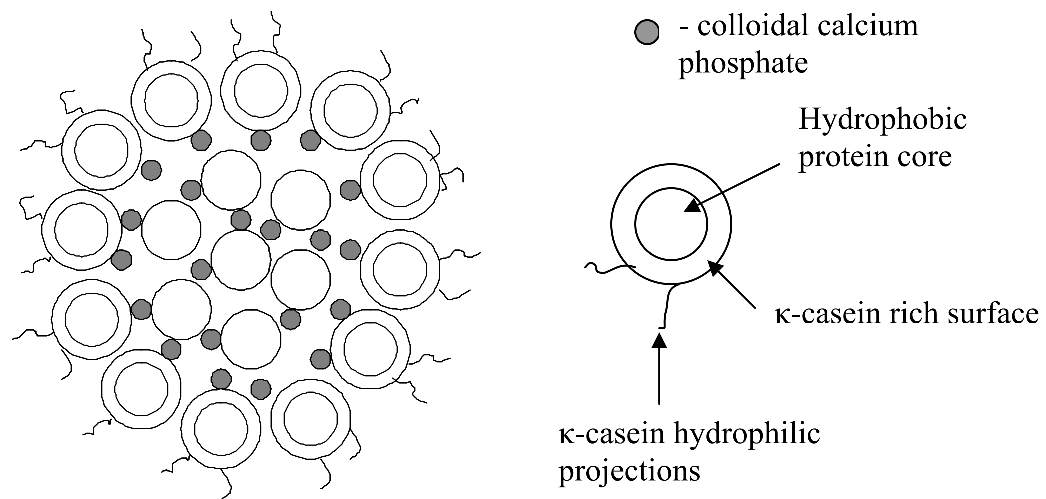

Figure 14. Casein micelle. Adapted from Dr. Carole Tranchant, Ph.D. Dissertation, Univ. of Guelph, 1999.

2.9.2 Milk Processing

Fluid raw milk can be processed into a vast variety of different products including cream, buttermilk, ice cream, cheese, butter, dried milk, evaporated milk, condensed milk, yogurt, sour cream, whipping cream, eggnog, and pasteurized fluid milks of

numerous types. All of these products have different production processes, heat treatments, protein, and fat content. For the purposes of this project only fluid milk processing will be discussed, although it is recognized that if the raw milk supply were contaminated with ricin, *B. anthracis*, or other bioterrorist agent that products other then fluid milk are also likely to be affected. Terminology used in the description of processing will use the United States standards of identity for the following products: skim milk- less then 0.5% milk fat, light cream – milk fat between 18-30%, heavy cream – greater then 36% milk fat (79).

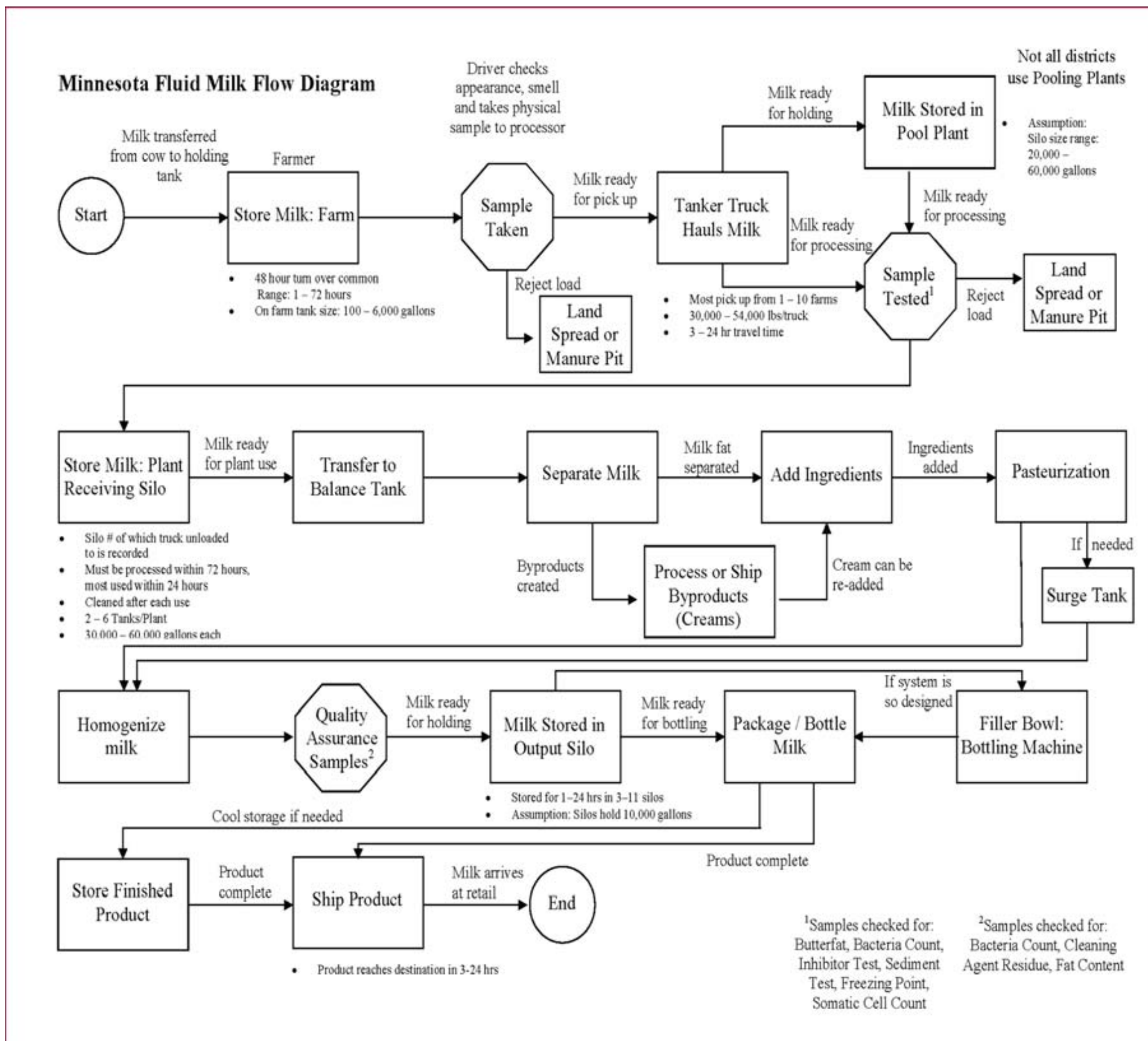

Figure 15. Minnesota milk production schematic. Courtesy of Ryan Newkirk.

Raw milk enters the processing line from the silo and enters either in to a milk separator or standardizer-clairifier (Fig. 15). Separators are essentially continuous centrifugation systems utilizing disks and operating at a fixed speed. Separators have a single inlet for raw milk and two outlets, one low fat stream (skim milk) and one high fat stream (light or heavy cream). Using a separator, processors must re-blend separated milks to produce products such as 1% and 2% milk fat milks. Milk standardizer-clarifiers operate on the same principle as separators; however it allows the operator to control the back-pressure in the system, thus allowing different low and high fat streams to be produced without the need to re-blend.

Separation and standardization both cause significant damage to the milk fat globule membrane, leaving it susceptible to natural bovine lipase. Thus milk that has been separated or standardized must be either kept extremely cold to slow enzyme activity or must be pasteurized immediately to inactivate bovine lipase as to preserve the quality of the milk and prevent rancidity. Pasteurization is also essential to reduce the total number of microorganisms in the milk and eliminate pathogens. The most prevalent human pathogenic organisms found in milk are *Coxiella burnetti, Mycobacterium tuberculosis, Listeria monocytogenes, Brucella abortus* and *Salmonella* species. Other microorganisms, which are generally non-pathogenic but can cause off flavors in milk include *Pseudomonas spp., Streptococcus spp.,* and *Lactococcus*. Minimum standards for pasteurization time and temperature are set by the inactivation kinetics of *C. burnetti*, as it is the most heat resistant of these potential pathogens. Pasteurization can be monitored

by the activity of alkaline phosphatase, which has inactivation characteristic highly similar to *C. burnetti*.

Pasteurization can be accomplished by a number of different methods, including low temperature-long time (LTLT or batch pasteurization), high temperature-short time (HTST), and ultra high temperature pasteurization (UHT). Batch pasteurization is more often used by smaller processors, as it is more time consuming and does not allow for continuous production. US federal standards for batch pasteurization require holding the raw milk at a minimum of 145° F (63°C) for 30 minutes (79). HTST pasteurization is most commonly used by producers today, as requires less time and energy the LTLT, as well as allows for continuous product flow and can be cleaned-in-place. HTST pasteurization requires less energy than LTLT due to the regenerative heating mechanism which transfers heat from the already pasteurized to the incoming raw product (Fig. 16). US federal regulations standards require that HTST processed milk be held at a minimum temperature of 161° F (72°C) for 15 s (79). UHT technology is not widely used for fluid milk destined for retail sale in the United States. UHT processing pasteurizes milk by treating it at high temperature (exceeding 135°C (275°F)) for 2-3 seconds (96). Milk treated by UHT can be made shelf stable by aseptic packaging. It is also noteworthy to mention that UHT processing is capable of inactivating both *Bacillus anthracis* spores and ricin toxin, as opposed to LTLT and HTST (262).

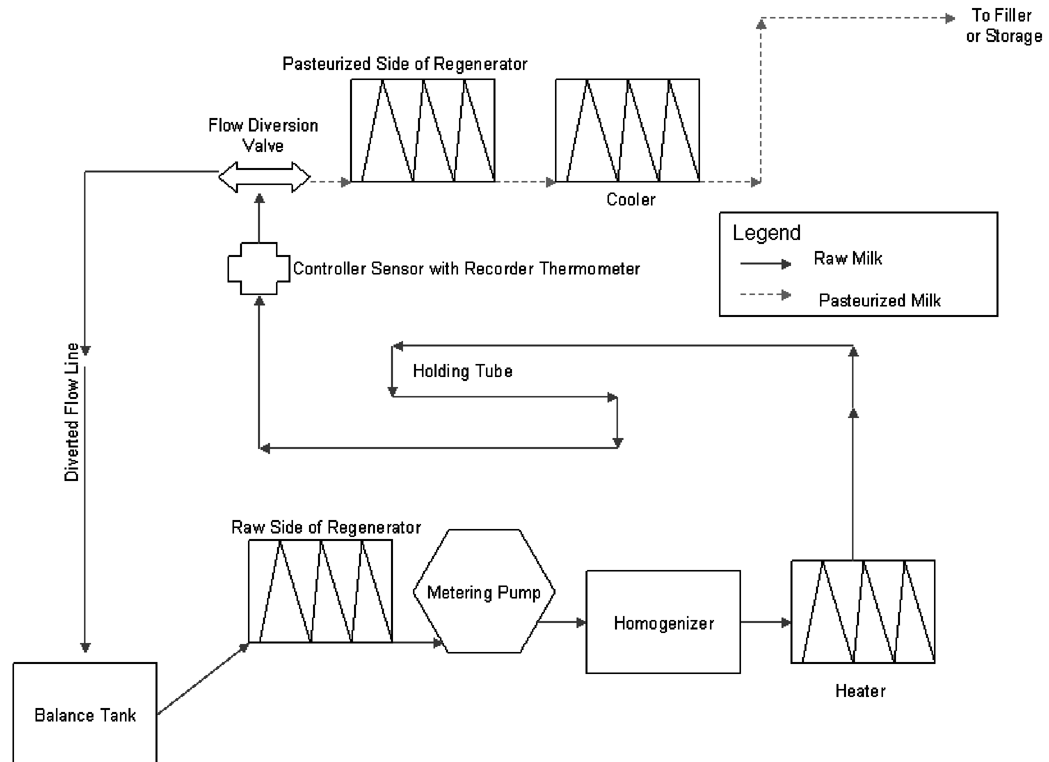

Figure 16. Schematic diagram of HTST pasteurizer. Adapted from US FDA (79).

The pasteurization unit operation is also where vitamins are added to fluid milk. Whole milk contains substantial amounts of both calcium and vitamin A. However, much of the vitamin A is lost during separation to produce lower fat milk products (skim, 1%, and 2%) due to its high solubility in fat. In addition, vitamin D is need for the human body to properly absorb calcium from the milk. To resolve these issues, both vitamins A and D are added to lower fat milks, while whole milk need only be supplemented with vitamin D (96). In either case, the vitamin pre-mix is added to the milk at the balance tank prior to pasteurization.

Following pasteurization, almost all milk goes through the additional process of homogenization. Most milk processed in the United States is homogenized, a process which breaks up the larger fat globules to make them less then 2 μm in diameter. This

allows the fat to remain suspended in the milk matrix much longer then non-homogenized milk, in which the cream would rise relatively quickly. Homogenization breaks down fat globules by forcing them through very small valves at a high pressure, of approximately 2000 psi. These forces cause the fat globule to undergo shearing, shattering, attenuation, explosion, and compression. Milk intended for ice cream process is subjected to this process twice, the first to reduce fat globule size and the second to break up any remaining clusters, whereas most fluid milk is processed with a single stage (96).

After milk leaves the homogenizer, it enters the filling line. Milk can be packaged in a wide variety of container shapes, sizes, and materials, including paperboard, plastic jugs, glass, and plastic bags. Common problems associated with filling are microbial contamination (mainly through the de-foaming process), leaky containers, and light associated degradation. An additional packaging concern is the migration of off-flavors from the packaging to the product, which most often occurs in small packages, such as the half-pints used in school lunches. However, this can be combated by using separate coolers for milk, minimizing the times the cooler is opened and maintaining the milk at 4°C at all times. The defoaming apparatus is located just prior to sealing and is essential for removing milk foam generated during filling in order to get a proper package seal. This step can be a microbiological concern as the equipment is not sterile and is liable to contamination from the surrounding environment. Light associated defects occurs in clear packages such as glass and plastic, where sunlight or fluorescent light activates riboflavin, which leads to the conversion of methionine to methanal (96).

2.9.3 Vulnerability of the milk processing system

The milk processing system includes a number of points that a potential bioterrorist could target and possibly contaminate large volumes of milk. From the time milk is produced on the farm, all points of fluid transfer and storage present opportunities for intentional contamination. Potential for milk contamination in the farm tank, in the hauler truck, and at the processor are all dependant on the security applied to each area. On the farm, the milk tank can be secured by locking the tanks and the area they are stored in, as well as general farm securing including employee background checks and control over those entering the farm. The hauler truck may be one of the most vulnerable points for milk contamination. This is due to the fact that many trucks are not equipped with electronic locks and many truckers park their truck at home overnight. Limiting this risk would require all trucks to be locked and monitored at all times, especially overnight when parked at a trucker's residence, in addition to through background checks on the truckers themselves.

Contamination at the processing plant could be accomplished by two major routes. The first one is direct contamination of the milk stored in the holding silo. Small amount of milk containing a high concentration of spores could be added or pumped into the silo. This type of event has the potential to contaminate large volumes of milk, but would likely not result in uniform distribution of the agent in the final product. Another possible point of contamination may be through the addition of other ingredients such as vitamins or flavoring (for flavored such as chocolate or strawberry milks). Contamination of these ingredients may be a highly effective way for a potential terrorist to impart large volumes of milk with a consistent level of the agent. Measures that can be

taken to prevent such an event include employee background checks, improved lighting at the facility, restricted access to raw ingredients, and routine or random select agent testing. Routine testing is more likely to catch such a contamination event, however random testing may be more fiscally responsible, while still deterring potential terrorists. Although select agent testing of milk would be ideal, many of the current rapid detection technologies are not compatible with milk matrices and require further research to determine more effective strategies.

2.10 Orange juice

2.10.1 Orange juice composition and definition

Orange juice in the United States is produced as a number of different products including fresh juice, pasteurized juice, frozen juice concentrate, and canned juice. Processing from fresh fruit varies slightly for each of these products, although many processing steps are similar or the same. Processing requirements and product standards in the United States are regulated by the Code of Federal Regulations, Title 21, Chapter 1, although definitions and requirements in the European Union and other regions of the world may vary slightly.

The standard of identity for generalized orange juice is found in the Code of Federal Regulations Title 21, Chapter 1 Section 146.135. Orange juice is defined as unfermented juice obtained from mature oranges of the species *Citrus sinensis* or of the citrus hybrid commonly called ‘‘Ambersweet’’ (1/2 *Citrus sinensis* × 3/8 *Citrus reticulata* × 1/8 *Citrus paradise* (USDA Selection:1–100–29: 1972 Whitmore Foundation

Farm)). Seeds (except embryonic seeds and small fragments of seeds that cannot be separated by current good manufacturing practice) and excess pulp are removed. The juice may be chilled, but not frozen.

Pasteurized orange juice (21 CFR 146.140) is defined by the above description, but also must be heat treated to substantially kill microorganisms and reduce enzyme activity. This juice may be chilled, however not frozen if producing chilled pasteurized orange juice. Certain sweeteners may be added to increase the Brix (refractive index) and total solids content. The finished product must have a minimum of 10.5% orange soluble solids by weight exclusive of added sweetener, and a Brix to acid (g of anhydrous citric acid) ratio of not less then 10:1 per 100 mL juice. Pulp and orange oil content may be adjusted in accordance with good manufacturing practices. However any added pulp cannot be either washed or spent type. The solids may be adjusted by the addition of one or more of the optional concentrated orange juice ingredients specified by the 21 CFR 140.146 (78).

Canned orange juice is defined by 21 CFR 140.141 and is defined by the general orange juice definition, which is sealed in containers and so processed by heat, either before or after sealing, as to prevent spoilage. The finished canned orange juice must be more than 10° Brix, and the ratio of the Brix hydrometer reading to the grams of anhydrous citric acid per 100 milliliters of juice is not less than 9 to 1.

Frozen concentrated orange juice is defined by 21 CFR 140. 146 and is the food prepared by freezing after removing water from a product complying with the generalized orange juice definitions, to which the unfermented juice obtained from mature oranges of the species *Citrus reticulata* (tangerine), other *Citrus reticulata* hybrids, or of *Citrus*

aurantium (bitter or Seville orange), or both added. However, in the unconcentrated blend, a maximum of 10% of the juice by volume may be from *Citrus reticulata* or *Citrus reticulata* hybrids (with the exception of the "Ambersweet") and juice from *Citrus aurantium* at a maximum of 5% by volume. Orange oil, orange pulp, orange essence (obtained from orange juice), orange juice and other concentrated orange juice for manufacturing provided in 21 CFR 146.153 (when made from mature oranges), water, and one or more of the optional specified sweetening ingredients may be added to adjust the final composition. Any ingredient of the frozen concentrate may be heat treated to substantially reduce microorganisms and limit enzymatic activity. The frozen concentrate, when diluted according to manufacturer's instructions, must not contain less than 11.8 percent by weight of orange juice soluble solids, exclusive of the solids of any added optional sweetening ingredients. The maximum dilution ratio is three parts of water to one part concentrate. Orange juice, whether fresh, pasteurized, or reconstituted from concentrate, has approximately the same nutritional value and composition.

2.10.2 Orange juice processing

All orange juice processing begins with the harvest of mature oranges from the farm, at which point they are graded and then shipped to a processing facility. Following arrival at the processing facility, the fruit is washed, juiced, and pulp and seed materials extracted by filtration. After the seeds and excess pulp have been removed, each product is processed differently to meet the desired properties (Figs. 17 and 18).

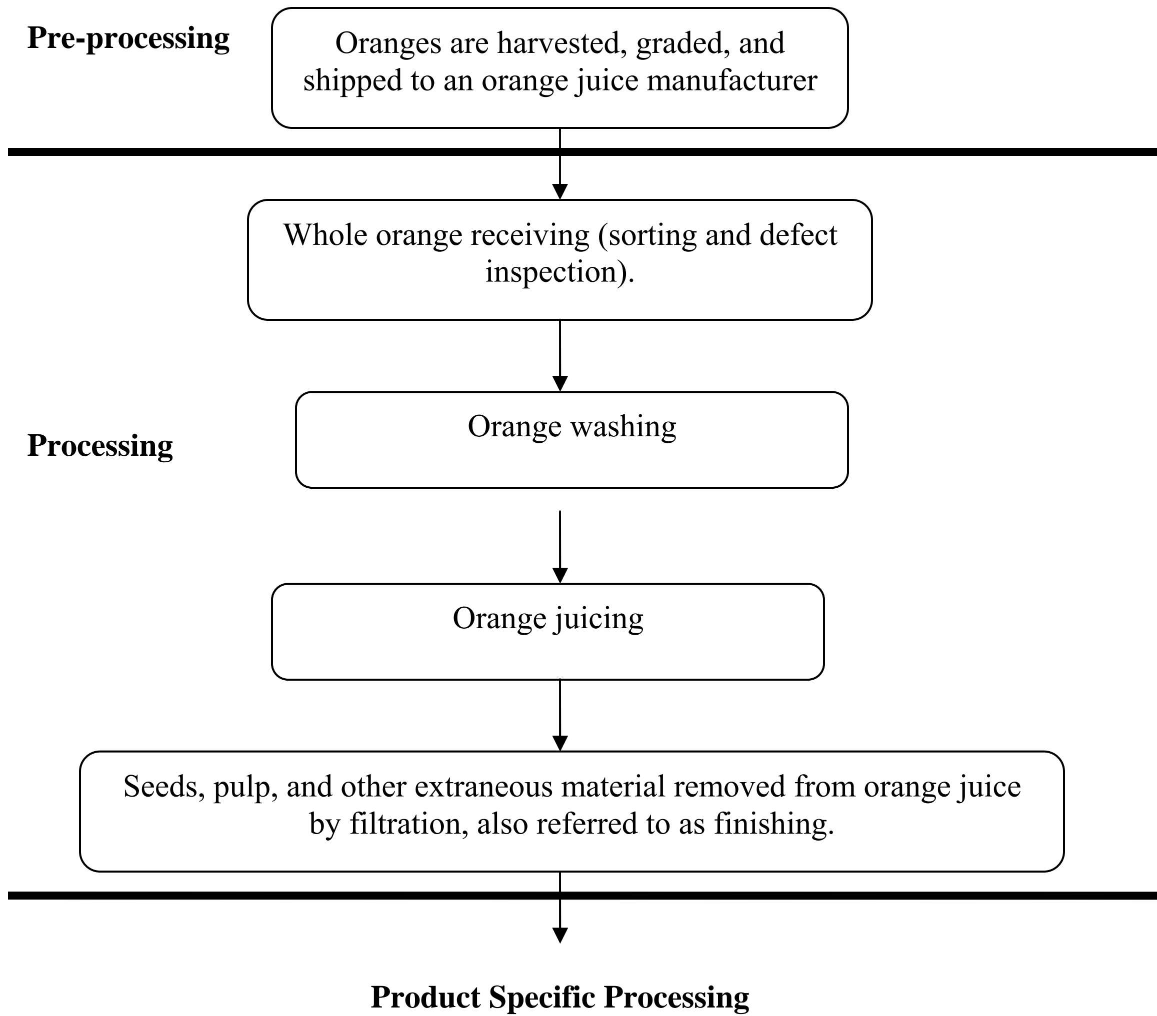

Figure 17. Generalized orange juice processing

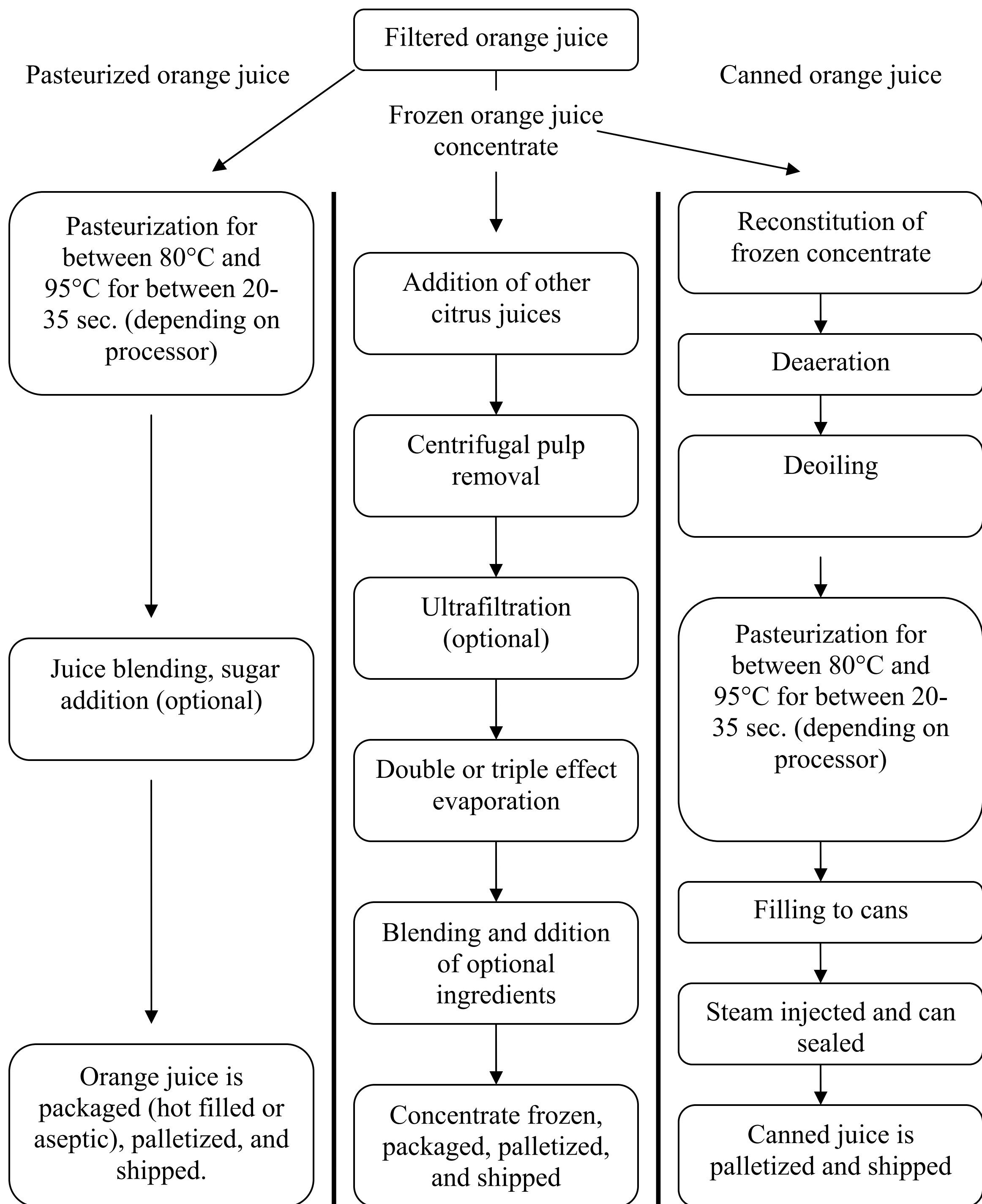

Figure 18. Specific processing steps for production of pasteurized orange juice, frozen orange juice concentrate, and canned orange juice.

2.10.3 Vulnerability of the orange juice processing system

In contrast to milk, the most vulnerable points for orange juice contamination are found during post processing. Although it may be possible to contaminate the orange juice directly at the juicing step, the system is relatively closed until after pasteurization when other ingredients can be added or when the juice is stored prior to packaging, as either juice or concentrate. Similar to the milk system, other ingredient addition may present a potential bioterrorist with the best opportunity to contaminate the juice. If any of the added ingredients were contaminated with a biological agent, it would be added in consistent metered doses to every batch, potentially contaminating thousands of gallons of juice. Contamination of the juice during storage is not likely to be as evenly distributed within the product as through the addition of contaminated ingredients, although it may be similarly effective in causing the intended harm.

In order to prevent such an event from affecting the orange juice industry the security of both raw ingredients and final product should be tested for potential bioterrorist agents. Other options to deter potential bioterrorists is to increase security measures, such as employee background checks, improved locking systems, and random raw ingredient and finished product testing for select agents. Although random select agent testing will not be applied to every batch and could potentially miss a contaminated lot of product, it may serve as enough of a deterrent to prevent an attack while being fiscally conservative. However, accurate testing methods for many select agents from orange juice have not been developed.

Chapter 3

Hydrophobic properties and extraction of *Bacillus anthracis* spores from liquid Foods

The hydrophobic properties of bacterial cell surfaces have been demonstrated to be important for understanding interactions between bacteria and their environment. Previous studies have described the hydrophobic properties of various *Bacillus* species spores, however little data exists for *Bacillus anthracis*. As concern has risen over the use of *B. anthracis* as a bioterrorist agent, particularly in the food supply, this property may be useful to separate spores from a complex matrix. This study characterized the hydrophobic properties of three strains of *B. anthracis* using the Microbial Adherence to Hydrocarbons (MATH) assay and determined the recovery of spores in the hexadecane fraction after mixing with water, milk and orange juice using a modified assay. In water mixtures, the hydrophobicity of *B. anthracis* spores ranged from 5 to 80% as the concentration of hexadecane and the mixing time increased. Strain 7702 was significantly less hydrophobic than strain ANR-1. The hydrophobicity of spores did not change significantly during storage at 4°C, or with increased temperature. The recovery of spores from the hexadecane fraction in aqueous mixtures was always less than 3% even at conditions in which the hydrophobicity values were higher than 40%. The recovery of spores in the hexadecane fraction increased to almost 20% when the hexadecane was mixed with milk or orange juice, but most of the spores remained in the aqueous phase. These findings indicated that for *B. anthracis* the MATH assay was not a good predictor

of the partition of spores to hexadecane, and suggested that separation of *B. anthracis* from food matrices based on hydrophobicity was ineffective.

3.1 Introduction

Bacillus anthracis is a Gram-positive spore forming bacteria, which can cause anthrax and may be used as a biological weapon. Gastrointestinal anthrax is caused by the consumption of *B. anthracis* spores, most commonly by consuming undercooked meat from an infected animal (69, 221, 254). Although meat is the most common vehicle for consumption in natural infections, similar effects may be occur through the consumption of spores regardless of food or beverage media. Gastrointestinal anthrax is less well characterized than the inhalation and cutaneous disease, but can cause similar mortality rates as inhalation anthrax in outbreak scenarios (69, 221, 254, 260). The actual infectious dose needed to cause disease via ingestion is unknown, but experimental evidence suggests that it is greater than 10^8 spores (11).

The potential use of dried airborne *B. anthracis* spores is considered as the most likely scenario for its use as a bioterrorism agent, although other routes of exposure, such as foods have been identified as feasible vehicles. Previous attempts to intentionally contaminate the food supply with other agents have been of limited scale, but accidental contamination incidents have demonstrated the impact scale that an intentional contamination event could have on the population (134, 161). Intentional contamination events have been modeled by a number of researchers for different events and contaminants, such as botulinum toxin contamination of the milk supply (24, 252). Intentional contamination of food with *B. anthracis* at a large processing facility, such as

those for milk and orange juice, could have devastating effects on the population and the food supply chain.

The complex and heterogeneous nature of food are major hurdles for effective detection of pathogens in a food matrix. Although many sensitive, rapid tests have been developed to detect *B. anthracis* and other pathogens, most are incompatible with food samples due to interference from various food components (27, 69). The heterogeneous distribution of the pathogen within a product further complicates detection and often requires sample enrichment for 8-72 hours prior to testing, in order to increase the concentration in a sample prior to detection (16, 226). This is not an acceptable timeline in the case of a bio-terrorist attack on the food supply, where each hour of detection delay could increase the number affected by hundreds or thousands. One strategy to avoid both interference from the food matrix and time consuming enrichment is the use of pre-analytical procedures that both separate the target organism from the food and concentrate the organism in a sample.

One such pre-analytical technique could be based on hydrophobic properties. Hydrophobic properties of various bacterial species have been extensively studied under various food processing and biological conditions (54, 110, 144, 152, 174, 179, 203, 219, 225, 244, 245, 255, 259). Separation of cells by hydrophobic characteristics has previously been used to effectively decrease the detection limit for a number of bacteria in foods, such as *Listeria monocytogenes* from blue cheese, and *Salmonella* from sausages (16, 149, 186, 226). Previous reports have indicated that *Bacillus* species spores have unique hydrophobic properties, and that this separation technique may be applicable to separating *B. anthracis spores* from foods (54, 145). Some studies have examined a

modified Microbial Adherence to Hydrocarbon (MATH) assay as an alternative to a polyethylene glycol-dextran system for separating spores from their environment due to the tendency for debris to build up at the phase interface (226, 259).

The MATH assay is based on partitioning of cells or spores between aqueous hydrocarbon phases, most commonly hexadecane, octane and xylene (54, 55, 207). The hydrocarbon is mixed with the aqueous spore suspension (of known absorbance at 440 nm) and then monitored for the change in absorbance of the aqueous layer after separation. Percent hydrophobicity is calculated as the fractional decrease in absorbance between initial and final aqueous phases, and is often calculated at different hexadecane to spore suspension ratios. The assumption of this assay is that spores partition to the hydrocarbon fraction due to their hydrophobicity. However, the fraction of spores physically portioning to the hydrocarbon fraction has rarely been reported and the recovery of viable spores from the hydrocarbon fraction has not been demonstrated.

This study was undertaken to determine the hydrophobicity of three strains of *Bacillus anthracis* using the MATH assay, and to determine the effectiveness of spore recovery in the hydrocarbon layer from sterile distilled water, commercial whole milk, and commercial pulp-less orange juice. Other factors such as spore strain, mixing time, prior heat treatment, and the age of the spores were also examined for their influence on hydrophobicity via the MATH assay.

3.2 Materials and methods

Spores and spore preparation

Bacillus anthracis strains 9131, 7702, and ANR-1 were kindly provided by Theresa Kohler, University of Texas- Houston Medical School. The Sterne strain 7702 harbors pXO1 that encodes the anthrax toxins, but lacks pXO2 (33, 262). Strain ANR-1 is a pXO2-cured non-encapsulated variant of the Ames strain that harbors pXO1 and pXO2 (253). The plasmid-less strain 9131 was obtained in Dr. M. Mock's laboratory of the Pasteur Institute by curing strain RP31 from the pXO1 plasmid (192). Spores were prepared following the protocol outlined by Xu et al and verified to contain >95% spores by phase contrast microscopy (262).

Microbial Adherence to Hydrocarbon (MATH) assay

The MATH assay for hydrophobicity measurements were performed using a modified version of Rosenberg's original assay design, similar to those used by others (54, 55, 145, 174, 207, 219, 225, 259). Spore suspensions were adjusted to an A_{440} (Spectronic 20 Genesys, Spectronic Instruments, Rochester, NY) between 0.8-1.0 (A_I) with sterile water. Spore suspensions were then incubated at 37°C or 75°C for 15 minutes. Following pre-incubation, hexadecane was added to the aqueous spore suspension in varying volumes as outlined by Weincek et al (259). The volume of spore suspension used was 3 mL in all experiments with the exception of changes in hydrophobicity over time, where 1.5 mL spore suspensions were used with 0.5 mL hexadecane. Mixtures were vortex mixed at high speed (VWR Vortex Genie 2, Scientific Industries Inc., Bohemia, NY) for 1 to 4 minutes and allowed to separate for 15 minutes or until completely separated. Following phase separation, 1 mL of the aqueous phase was carefully removed and measured for absorbance (A_F). All assays were performed at

minimum in duplicate trials, with three replicates per trial, and were performed in 13 × 100 mm glass tubes. Percent hydrophobicity was calculated at four different ratios of hexadecane to spore suspension (v/v) as done by Weincek et al (259) as follows:

Equation 1: $[(A_I - A_F)/ A_I] \times 100$ = Percent hydrophobicity

Spore germination and growth after suspension in hexadecane

Germination and growth of *B. anthracis* spores suspended in hexadecane was compared to spores suspended in an aqueous phase. Volumes of 100 µL of spore suspension, containing approximately 10^4 spores/mL, were added to 900 µL of either sterile water or hexadecane in a microcentrifuge tube, to obtain approximately 10^3 spores/mL. Mixtures were vortex mixed for 30 s from which 3-100 µL aliquots were removed and immediately plated on tryptic soy agar (TSA; Neogen, Inc., Lansing, MI) from each tube and incubated overnight at 37°C. Resulting colony forming units from duplicate trials were compared between hexadecane and water samples using Students T-test.

Spore recovery from liquid foods with hexadecane extraction

A modified version of the MATH assay was used to evaluate spore recovery in the hexadecane and aqueous fractions following mixing with food matrices. *B. anthracis* spores were diluted to approximately 10^3 spores/mL in sterile distilled water, commercial whole milk (pasteurized and homogenized), and pasteurized orange juice. Aliquots of 3 mL of each spore suspension were heated to 37°C for 15 minutes, and then vortex mixed in 13 × 100 mm glass test tubes with varying ratios of hexadecane (v/v) for 1 min.

Following 15 min of separation, samples (1 mL from the water, milk, or orange juice layer, and as much as possible from the hexadecane layer) were taken from each phase to determine CFU/mL in the aqueous and hydrocarbon phases. Volumes of 100 μL of each phase were plated in duplicate or triplicate on TSA and incubated overnight at 37°C. The number of spores recovered in each fraction was compared using the Student's T-test.

3.3 Results

Hydrophobicity percentage for all strains increased as much as 4-fold when the fraction of hexadecane increased from 0.03 to 0.33 (v/v) in the mixtures (Fig. 19). The mean hydrophobicity at all hexadecane ratios of strain ANR-1 was statistically different from that of strain 7702 ($p = 0.03$).

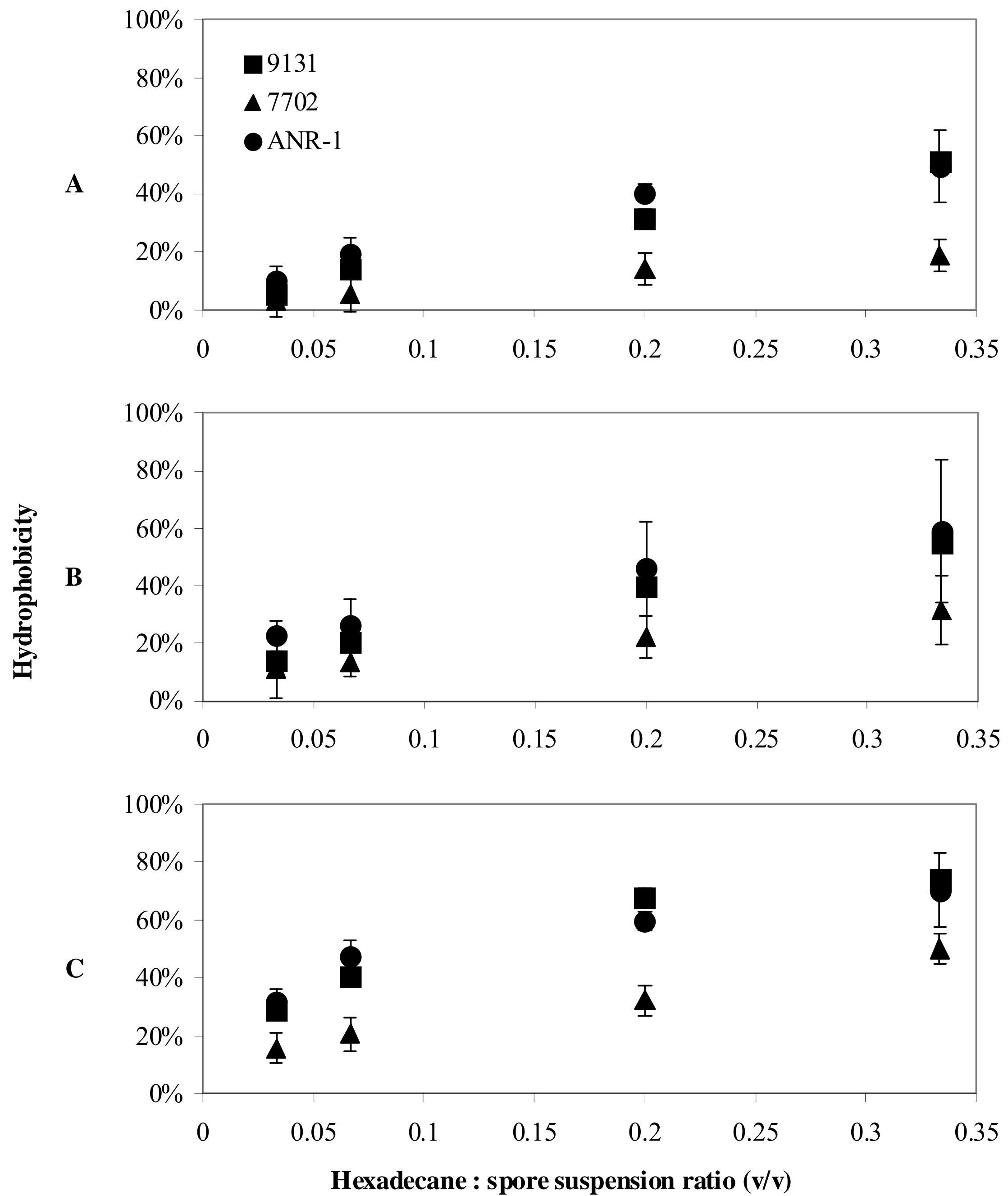

Figure 19. Hydrophobicity of 9131, 7702, ANR-1 using the MATH assay, with pre-incubation at 37°C at 1(A), 2(B), and 4(C) minute mixing times. Error bars represent 95% confidence intervals.

Increase in vortex time also increased mean hydrophobicity for all three strains (Fig. 19). Significant differences exist at each time point for strain 9131 ($p<0.01$ between 1 and 2 minute mixing periods and $p=0.05$ between 2 and 4 minutes), but only between 1 and 2 minute mixing times for strain 7702 ($p=0.04$) and only between 2 and 4 minutes of mixing for strain ANR-1 ($p=0.02$).

Increased pre-incubation temperature prior to hexadecane addition increased the hydrophobicity values differently for all three strains (Fig. 20). No strain differed significantly in hydrophobicity at 75°C pre-incubation from 37°C pre-incubation. Spore suspension age did not appear to affect the hydrophobicity values (Fig. 21). The spore suspensions did not significantly vary in hydrophobicity over a twelve-day time period after spore suspension preparation in a single trial.

The presence of hexadecane had no significant influence on the germination and growth of spores on TSA plates for any of the three strains (Table 2). Student's T- test p-values for 9131, trial 1= 0.57, trial 2=0.27; 7702 trial 1 $p=0.21$, trial 2=0.90; ANR-1 trial 1 $p=0.26$, trial 2 $p=0.79$.

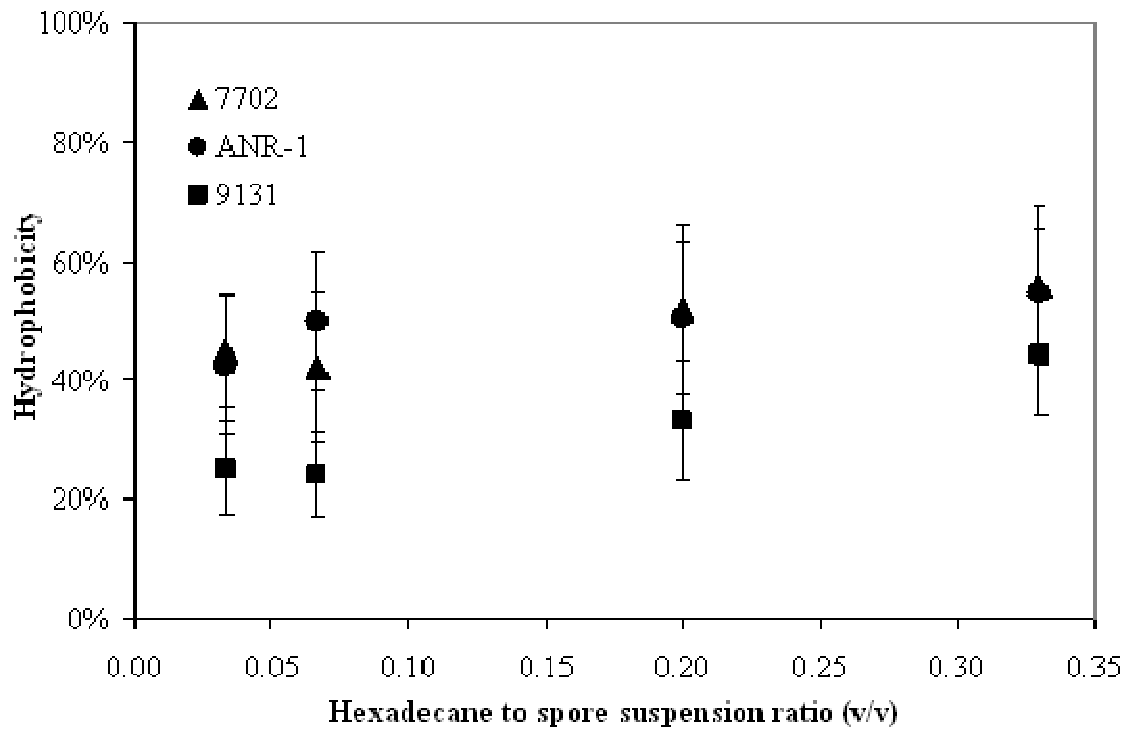

Figure 20. Hydrophobicity of *B. anthracis* strains 9131, 7702, and ANR-1 using MATH assay with pre-incubation at 75°C and 1 min mixing time. Error bars represent 95% confidence intervals.

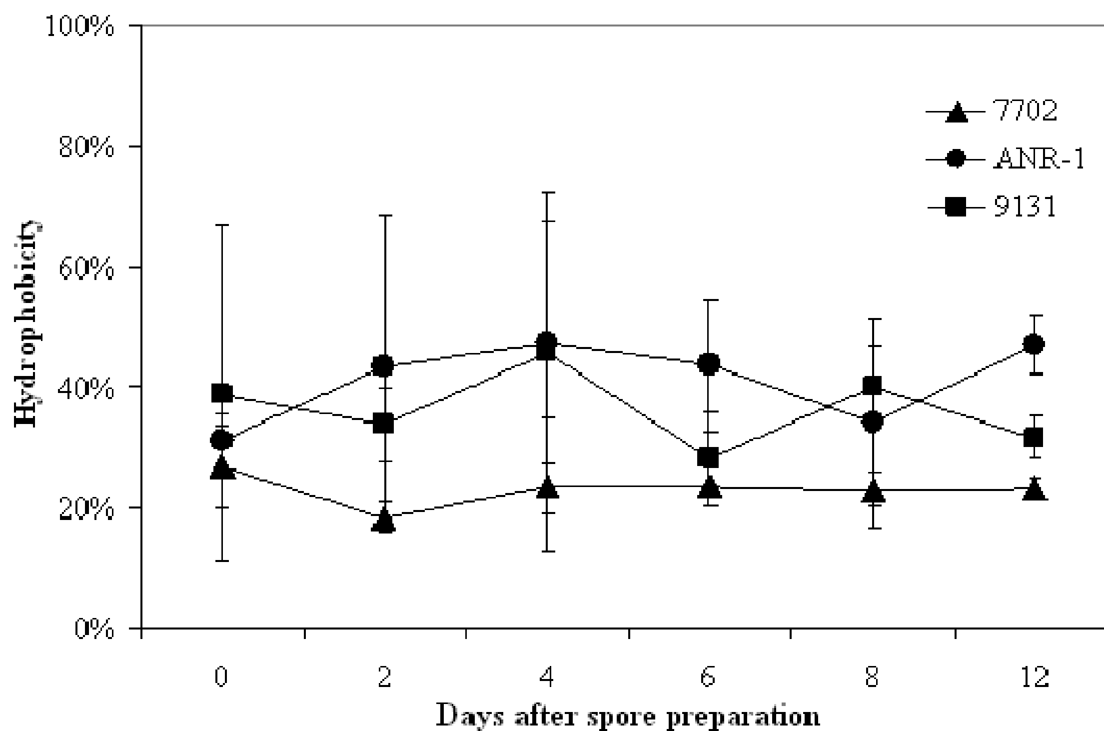

Figure 21. Stability of hydrophobicity values over time using the MATH assay. Mixture included 1.5 mL spore solution suspended in sterile water and 0.5 mL hexadecane. Error bars represent 95% confidence intervals.

Table 2. Viability of spores after mixing hexadecane compared to water

Strain	Average viable spore count (CFU/mL) in hexadecane (± 95% confidence)	Average viable spore count (CFU/mL) in water (± 95% confidence)	p-value
9131 - trial 1	1315.6 ± 297.6	1206.7 ± 163.8	0.57
9131 - trial 2	1608.3 ± 480.2	2008.3 ± 205.8	0.27
7702 - trial 1	771.1 ± 394.6	485.6 ± 59.4	0.21
7702 - trial 2	793.3 ± 80.1	785.0 ± 11.8	0.90
ANR-1 – trial 1	754.4 ± 398	511.1 ± 53.9	0.26
ANR-1 – trial 2	913.3 ± 241.7	951.7 ± 29.4	0.79

Less than 2% of any strain's spores were recovered in the hexadecane layer after hydrophobic extraction of water spore suspensions (Fig. 22). The mean percentage of the initial inoculum recovered in both aqueous and hexadecane phases were less then expected from the results of the MATH assay (Fig. 23). When the hexadecane extraction was applied to milk, the spore recovery was more than 10 times greater than from water, but it was always less than 20%. Similar results were obtained when spores had been suspended in orange juice before extraction, but in both milk and orange juice the majority of spores were found in the aqueous phase (>70%). For spore recovery from water, the mean percentage of the original inoculum recovered in both aqueous and hexadecane phases were less then expected from the results of the MATH assay observed in Fig. 23.

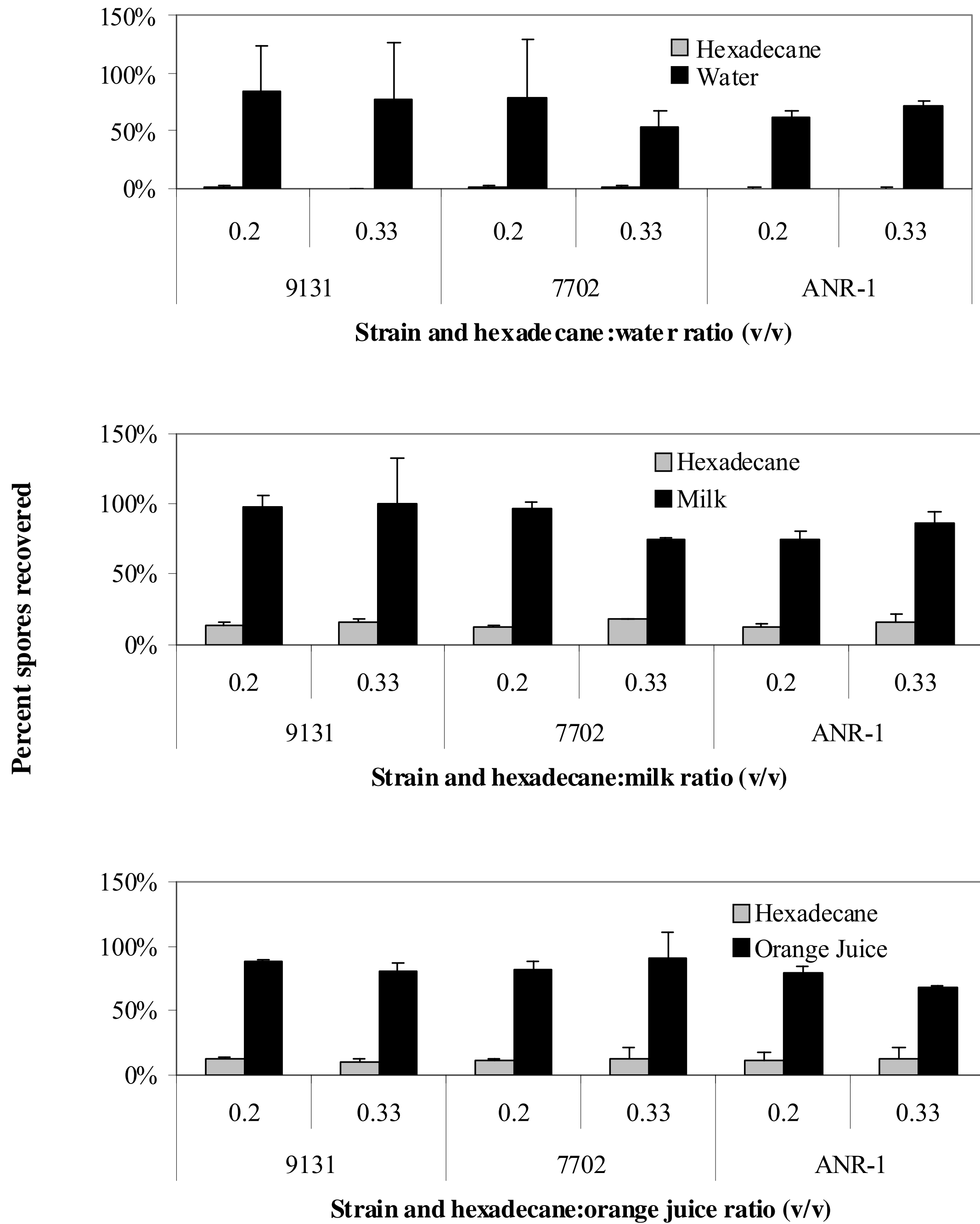

Figure 22. Spores recovered after in original spore suspension media (water, milk, or orange juice) and hexadecane following mixing and subsequent separation. Error bars represent 95% confidence intervals.

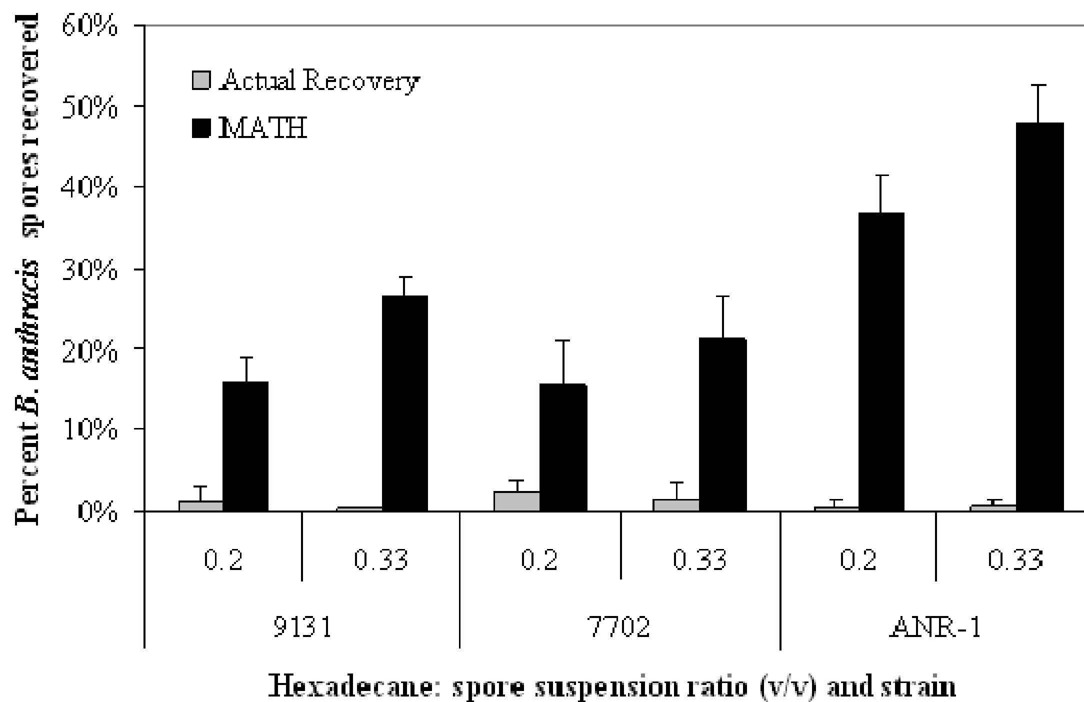

Figure 23. Total spore recovery as estimated by the MATH assay compared with actual recovery when suspended in sterile water. Error bars represent 95% confidence intervals.

3.4 Discussion

In the present study, hydrophobic properties of three strains of *Bacillus anthracis* and the potential for extraction from food using this property were examined. All three strains showed moderate hydrophobicity, although notably less than some other *Bacillus* and *Clostridium* spores (145, 259). The MATH assay is based on the disappearance of absorbance in the water phase, which had originally led us to assume that the spores would be migrating to the hexadecane phase. However, the low spore recovery in the hexadecane phase indicated that the concept of hydrophobicity as defined by the MATH

assay did not correlate well to spore recovery using hydrophobic extraction with hexadecane.

MATH assay measured hydrophobicity did not seem to be affected by storage and aging over a short period of time. This indicates, that at least in terms of hydrophobic properties, the spore coat and exosporium proteins likely remain stable once the spore has formed. This result is concurrence with previously published data, indicating that the spore surface is established during sporulation and does not change until germination is triggered (58, 138, 202).

Previously published data suggests that pre-heating during the MATH assay can significantly alter hydrophobicity measurements of some *Bacillus* species spores (259). The current study indicates that *B. anthracis* spores had no significant change in hydrophobicity following heat treatment. Previous studies have examined this effect only in *B. subtilis, B. globigii,* and *B. sterothermophilus*, none of which are closely related to *B. anthracis* (259). Further study is needed to elucidate the relationship between heat treatment of specific *Bacillus* strains and hydrophobicity. Although previous evidence from Weincek et al (259) shows that heat affects hydrophobicity more dramatically at higher temperatures, more extreme time-temperature combinations may also alter components within the food matrix further complicating potential extraction. The lack of significant change in *B. anthracis* hydrophobicity after 75°C pre-incubation lead to the exclusion of high heat treatment in the further development of an extraction procedure.

Mixing time significantly influenced the results of the MATH hydrophobicity assay for all three strains of *B. anthracis* spores. This effect was most likely a product of the creation of smaller hexadecane droplets in the mixture. This in turn may have

increased the hexadecane surface area for potential hydrophobic interactions, the number of hexadecane droplets, and may have slightly increased the stability of the hydrocarbon/aqueous system. Although spore count measurements were only made in the hexadecane and aqueous phases following separation of the system, it is possible that the decreased hexadecane droplet size and increased droplet number may have increased the proportion of spores which are trapped between droplets in the interphase. This result emphasized the need for use of standardized procedures when comparing hydrophobicity measurements between studies and methodologies.

A modified version of the MATH assay was used to evaluate the potential for hexadecane extraction of spores suspended in water, milk, and orange juice. Results from these experiments supported the conclusion that spores adhere to the hydrocarbon in the MATH assay, but did not partition into it. As the interphase was carefully avoided during the collection of both hydrocarbon and aqueous phase samples, this fraction still remained a likely location for spores not accounted for in the other two fractions. This notion is also supported by the results of spore extraction from milk and orange juice, in which the hexadecane layer did not separate well from the food product. Incomplete separation may have lead to inclusion of some interphase material in the extracted hexadecane sample, yielding misleadingly higher recovery rates.

A complicating factor in the current study may be the effect of gravity on the distribution of spores between the hydrocarbon and aqueous phases. *B. anthracis* spores suspensions have a natural tendency to settle at the bottom of aqueous solutions due to the force of gravity. This effect may also be present in spores suspended in hexadecane (773 kg/m^3), as it is less dense than either water (1000 kg/m^3) or *B. anthracis* spores

(1162-1186 kg/m^3) (32). Both the MATH assay hydrophobicity and the modified recovery assay include a 15 minute wait time to allow for hydrocarbon and aqueous phase separation, and this may have affected the results of the hydrophobicity assay and recovery assay differently. During the separation time, the spores noticeably settled to the bottom of the aqueous phase during hydrophobicity experiment and required sample mixing prior to spectrometric measurement. Although the hydrophobicity and recovery assays were essentially the same design, the recovery assay relied directly on measurement of the upper hexadecane phase. In the recovery experiments, there is the possibility that spores initially partitioning into the hexadecane phase may have settled during the separation period either into the bottom of the hexadecane layer, the interphase, or possibly the aqueous phase. This may have contributed to the low recovery of spore in the hexadecane phase despite their hydrophobicity.

Previous studies have examined the relationship between microbial hydrophobicity measurements and adherence to various surfaces, including foods and food processing equipment (120, 206, 242). In contrast to the results of the current study, previous investigation showed that hydrophobicity correlated well to adhesion to hydrophobic surfaces (22, 206, 242). One notable difference between the experiments is that the majority of previous studies have examined attachment to solid surfaces, rather than partitioning into a bulk hydrophobic liquid. This difference in methodology may explain the difference in study results, as the current study did not examine the spore population at the interphase, which may be most akin to a hydrophobic surface. The results of hydrophobicity experiments conducted as part of this project in conjunction with previous evidence present above suggest that if a food processing facility were

contaminated with *B. anthracis* spores, the spores may adhere to hydrophobic food processing surfaces. This could potentially cause persistent equipment contamination and subsequent adulteration of multiple production runs, leading to a large amount of affected product and increasing the potential health affects on the population.

Evidence from this study suggests that despite moderate hydrophobicity, hydrophobic extraction with hexadecane is ineffective for recovery of *B. anthracis* spores from liquid foods. However, hydrophobic extraction may still be a viable if used with a hydrophobic phase denser than water or in conjunction with other concentration or extraction measures. Although the current procedure appears to be inefficient, the need for a continuous, rapid pre-analytical concentration and extraction procedure for potential bioterrorist agents from food remains vital to the security of the food supply.

Chapter 4

Concentration, extraction, and detection of *Bacillus anthracis* from milk

Milk and other foods are likely potential targets for bioterrorists seeking to contaminate the food supply as they are mass produced at few facilities and widely distributed. Although most milk in the United States is pasteurized to eliminate bacterial pathogens, a number of potential bioterrorism agents including *Bacillus anthracis* spores can survive this heat treatment. Current methods of detecting *B. anthracis* are either not sensitive enough to detect the infectious dose or are not compatible with food samples. This project was undertaken to develop a rapid pre-analytical concentration and extraction (PACE) procedure using centrifugation for *B. anthracis* in skim and whole milk. Prior to method development, the distribution of spores during milk separation was characterized, resulting in the majority of spores in the skim fraction. These results, in conjunction with high levels of spore recovery into the pellet fraction during simple centrifugation, were used as the basis for the PACE method. The PACE centrifugation method was developed using factorial experiments varying centrifugation speed and time for optimum recovery of spores at the minimum centrifugation conditions, which were determined to be 1500 × *g* for 10 minutes. The procedure developed was compatible with PCR and real-time PCR methods, but not with a commercially available lateral flow immunoassay. The total time to detect using any of these three rapid detection methods was less then 3 hours. Real-time PCR was the most sensitive of these methods allowing for the potential detection of 10 spores/mL milk. This pre-analytical procedure increased the spore concentration in the sample by 2 log CFU/mL. The PACE method developed

offers a first step for the adoption of rapid in-line process detection compatible with molecular identification platforms.

4.1 Introduction

The food supply is one of the most critical points of infrastructure and also one of the most vulnerable. Intentional contamination of the food or water supplies as a means of attacking an enemy has been documented in many instances throughout history, from ancient Greece to World War II Japan to 1980's Oregon (230, 257). Food is an attractive target for intentional contamination due to its centralized production and wide reaching, rapid distribution systems. This vulnerability has been effectively highlighted by recent accidental contamination events, in which biological agents at a relatively low level caused hundreds of illnesses across the country and around the world (134, 161, 261). Although most current food processing systems include steps intended to reduce or eliminate bacterial pathogens, such as pasteurization, there are a number of potential bio-terrorist agents that may survive this process and enter the food supply, including *Bacillus anthracis* spores (188, 262).

Bacillus anthracis is a Gram-positive spore forming bacteria and the etiological agent of anthrax. Consumption of *B. anthracis* spores can cause gastrointestinal anthrax, which can lead to shock and death in some cases. Gastrointestinal anthrax naturally occurs in many parts of the world with endemic animal anthrax, and is most commonly by consuming undercooked meat from an infected animal (69, 221, 254). Although little is known about the prevalence of gastrointestinal anthrax, outbreaks of the disease in Thailand, Lebanon, Turkey, and Uganda have demonstrated relatively high case-

fatality rates between 5 and 50% (53, 131, 175, 221, 222). The infectious dose needed to cause anthrax via ingestion is unknown, but experimental evidence from primates suggests that it is equal or greater than 10^8 spores (11). The inherent resistance of *B. anthracis* spores to many food processing techniques and environmental factors, as well as the potential severity of disease related to consumption makes this an agent likely to be used for intentional contamination of food (178, 262).

Milk is a particularly sensitive food to contamination due to its processing and distribution. Fluid milk has the potential to be contaminated both before and during processing. Prior to arrival at the processing plant, milk has already been stored or transferred a minimum of two times on the farm and in the milk hauler (113). Milk could be contaminated at either of these points by a potential bioterrorist. In addition, once at the processing facility a number of other opportunities to contaminate the milk are presented, from in the silo to vitamin addition steps (252).

Milk contaminated with *B. anthracis* has the potential to cause substantial morbidity and mortality. The potentially fatal affects of *B. anthracis* spores consumed via milk are heightened by the rapid distribution of the contaminated product, ability of spores to survive pasteurization, and consumption by susceptible individuals, particularly children (90, 262). The potential effects of *B. anthracis* contamination of the milk system make it essential to secure our food supply through both proactive security measures as well as rapid pathogen detection techniques.

The complex and heterogeneous nature of food are major hurdles for effective detection of pathogens in a food matrix. Although many sensitive rapid tests have been developed to detect *B. anthracis* and other pathogens, most are incompatible with food

samples due to interference from various food components (27, 69). The heterogeneous distribution of the pathogen within a product further complicates detection and often requires sample enrichment for 8-72 hours prior to testing, in order to increase the concentration in a sample prior to detection (16, 226). This is not an acceptable timeline in the case of an intentional attack on the food supply, where each hour of detection delay could increase the number affected by hundreds or thousands. One strategy to avoid both interference from the food matrix and time consuming enrichment is the use of pre-analytical procedures that both separate the target organism from the food and concentrate the organism in a sample.

Although pre-analytical techniques of centrifugation and filtration have been applied to a variety of biological separation and concentration applications, they have not been tested for compatibility with extraction of spores from different milk samples (226). The goal of this project was to develop a pre-analytical protocol for concentration and extraction of *B. anthracis* spores from milk and to determine the compatibility of these samples with PCR, real-time PCR, and lateral flow immunoassays. The purpose of this procedure was ultimately to develop a more sensitive and effective method for detection of *B. anthracis* spores intentionally added to the milk supply, thereby increasing the security of the milk supply against bio-terrorism.

4.2 Materials and Methods

Spores and spore preparation

Bacillus anthracis strains 9131, 7702, 7702p610, UT258, UM44-1tr203-1, UM23C1-2, and ANR-1 were kindly provided by Theresa Kohler, University of Texas-Houston Medical School. The Sterne strain 7702 harbors pXO1 that the anthrax toxins, but lacks pXO2 (33, 262). Strains 7702p610 and UT258 are derivatives of strain 7702, containing additional plasmids conferring antibiotic resistance markers and green fluorescent protein (208). Strains UM44-1tr203-1 and UM23C1-2 are derivatives of the Weybridge strain marked with antibiotic resistance elements (10, 208). Strain UM44-1tr203-1 harbors pXO1 only and UM23C1-2 contains neither pXO1 or pXO2 (4, 10, 208). Strain ANR-1 is a pXO2-cured non-encapsulated variant of the Ames strain that harbors pXO1 and pXO2 (253). The plasmid-less strain 9131 was obtained in Dr. M. Mock's laboratory of the Pasteur Institute by curing strain RP31 from the pXO1 plasmid (192). Spores were prepared following the protocol outlined by Xu et al and verified to contain >95% spores by phase contrast microscopy (262). *B. thuringiensis* ATCC 35696 was obtained from the American Type Culture Collection.

Milk samples

Raw whole milk was obtained from the University of Minnesota dairy barn and was used within 24 hours of receipt. Commercial skim and whole milk were purchased at local retail outlets.

Concentration of B. anthracis *spores from small volumes milk using silicone oil*

The procedure used to recover *B. anthracis* spores via centrifugation through silicone oil was adapted from Ahmed et al (2). Milk (commercial whole milk or raw

whole milk) was inoculated with *B. anthracis* spores to approximately 10^3 spores/mL. Volumes of 1 mL of inoculated raw milk (with or without heat treatment as specified) were overlaid on to 0.25 mL high density silicone oil (Sigma-Aldrich, St. Louis, MO) contained in microcentrifuge tubes and centrifuged at either 16,250 × *g* for 2 minutes (to determine impact of commercial processing and heat treatment) or for the time and force specified (optimization of heat treatment and centrifugal force) in a Hettich Mikro 20 microcentrifuge (Beverly, MA). Following centrifugation, tube bottoms were clipped off below the level of silicone oil and resuspended in 1.0 mL of buffered peptone water, then plated in triplicate on tryptic soy agar (TSA; Neogen, Inc, Lansing, MI). The percentage of spores recovered was calculated as (CFU/mL final) / (CFU/mL initial milk solution).

Distribution of B. anthracis *spores to cream and skim fraction following continuous separation via lab scale milk separator*

Raw whole milk samples were divided into three 2 L bottles and heated to 37°C in a water bath. One 2 L bottle of milk (non-inoculated control) was immediately separated in a mini scale electric cream separator (9,000 RPM fixed speed, mini electric cream separator MC60, Coburn Inc., Whitewater, WI) The remaining 2 bottles (4 L of milk) were held in a 37°C incubator until separation of the first bottle was complete (~ 5 minutes). Prior to separation of the last 2 bottles, each was inoculated with spores to approximately 10^3 spores/mL milk. From each separation three 10 mL samples were collected from each milk fraction. Additionally, 50 mL samples of skim and cream from non-inoculated milk were collected for fat analysis via infrared detection. All samples from inoculated and non-inoculated milk were mixed thoroughly prior to plating 3 × 0.1

mL samples on the appropriate antibiotic TSA. TSA was supplemented with 5 μg/mL erythromycin for strain 7702p610 and UT258, 10 μg/mL rifampin for strain UM23C1-2, or 5 μg/mL tetracycline for strain UM44-1tr203-1. Separation experiments with each strain were performed in triplicate. Strains not marked with antibiotic resistance elements were not used in this portion of the study due to the abundance of other bacterial flora, including other spore-forming organisms, in the raw milk samples.

Concentration of B. anthracis *spores from commercial milk*

Small scale (1 mL) concentration and recovery of *B. anthracis* was performed by inoculating commercial skim milk to approximately 10^3 CFU *B. anthracis* spores/mL. One mL samples of inoculated milk were aliquoted into sterile microcentrifuge tubes and centrifuged at either 4,700 × *g*, 9,600 × *g*, or 16,300 × *g* for 1 min. Following centrifugation, supernatants were decanted and the pellet resuspended in 1 mL sterile water. Non-inoculated milk, inoculated milk, and resuspended pellet fractions were plated 3 × 0.1 mL on the appropriate TSA to calculate concentration. Recovery was calculated as the fraction of the total initial spores recovered in the pellet.

Large scale concentration and recovery of *B. anthracis* spores from commercial milk (skim or whole) was performed similarly as small scale recovery. Milk was inoculated with *B. anthracis* spores (10^3 CFU *B. anthracis* spores/mL for optimization, between 10^6 CFU *B. anthracis* spores/mL and 10 CFU *B. anthracis* spores/mL, using serial dilutions for validation studies). Both inoculated and non-inoculated milk samples were plated 3 × 0.1 mL on TSA (or antibiotic TSA as strain appropriate) to determine baseline levels in milk and initial spore concentration. Samples of 100 mL of the

inoculated milk were aliquoted into two 250-mL sterile Corning centrifuge bottles. Centrifuge conditions were optimized using strains UT258 and 7702p610 by centrifugation at varying time and centrifugal force combinations using a Sorvall RC-5 centrifuge (Waltham, MA) and GSA rotor at room temperature. Following optimization, validation of conditions was performed on strains 9131, 7702, ANR-1, 7702p610, and UT258. For all trials the concentrate recovered was standardized to 1 mL volume with sterile water. The concentrate was then serially diluted and plated to determine the total spores recovered, anticipating at 2 log CFU/mL increase in concentration.

Sensitivity and compatibility of a lateral flow assay

Tetracore© BioThreat Alert (Rockville, MD) *B. anthracis* lateral flow immunoassays were purchased from the manufacturer. Samples of *B. anthracis* ANR-1 spores suspended in water or skim milk were enumerated by plating 3 × 0.1 mL on TSA. Samples were diluted 1:1 by volume with assay buffer as described by the manufacturer and applied to the port. Milk samples following both single concentration alone and concentration followed by secondary centrifugation were processed as described above. Single concentrated samples were diluted 1:1 by volume with assay buffer according to the manufacturer and applied to the port. Samples processed using the additional secondary centrifugation were resuspended in 0.5 mL of lateral flow assay buffer after decanting supernatant. Assay results were both recorded and photographed at 15 and 30 minutes after inoculation. All experiments were performed in duplicate, with two replicates per trial.

Extraction of B. anthracis *spore DNA*

DNA extraction efficacy was determined using three methods: germination followed by boiling lysis, the UltraClean© kit (MoBio Labs, Carlsbad, California), and germination followed by lysis using the same extraction kit. Germination-boiling lysis: *B. anthracis* spores were germinated by centrifuging 1 mL spores at 16,250 × *g* for 2 min to collect spores, followed by resuspension in an equal volume of germination solution (10 mM Tris-HCl (pH 8.0), 10 mM l-alanine, and 10 mM $CaCl_2$) (13). Spores were then heat shocked in an 80°C water bath for 20 min, then incubated at 37°C for 60 minutes. Following germination, the spore suspension was boiled for 30 min to lyse germinated spores. Spore lysis efficacy was calculated as the difference between the mean initial concentration of the spore suspension following heat shock and the mean concentration in the final spore suspension following boiling, both by plating three 100 µL samples of appropriate serial dilution on TSA and overnight incubation at 37°C. The UltraClean kit was used as described by the manufacturer (see appendix G) and was chosen over other DNA extraction methodologies due to its ability to effectively extract DNA from *B. anthracis* spores, as demonstrated by Dauphin et al, 2009 (48).

Compatibility of concentrated and extracted samples with PCR and real-time PCR

Spore-containing samples of milk with and without prior concentration were extracted via the optimized methodology (UltraClean kit). Concentration was carried out as described above and the concentrate was standardized to a volume of 1 mL with sterile water. Following concentration, volumes of 0.1 mL were removed from each tube of concentrate and serially diluted as appropriate to validate the efficacy of concentration.

The remaining 0.9 mL concentrate volumes were extracted using the UltraClean kit. Aliquots of 0.9 mL of non-concentrated milk (from each dilution and a negative control without added spores) were extracted similarly.

Standard PCR was performed using Go-Taq Green PCR Master Mix (Promega, Madison, WI) to amplify a 210 bp fragment of the protective antigen gene, *pagA*. Primers used were PA6 (5'- ACCAATATCAAAGAACGACGC-3') and PA7 (5'-ATCACCAGAGGCAAGACACCC-3') at a final concentration of 0.8 μM each primer. 2 μL of extracted DNA was used in a total reaction volume of 25 μL. The following PCR cycle was used for quantification: 1× 95°C for 2 min; 35 × (95°C for 15 s followed by 60°C for 15 s followed by 72°C for 30 s); 1× 72°C for 5 min (162). Following PCR amplification, 10 μL of each reaction was run on a 1.5% agarose gel for 60 minutes at 100 V.

Real-time PCR was carried out using the Light Cycler 2.0 system (Roche, Hague, IN) and the a Fast Start DNAMaster SYBR Green (Roche Hague, IN) to target the same 210 bp fragment of the protective antigen gene *pagA* as used in standard PCR using the same primers, PA6 and PA7. Primers were used at a final concentration of 0.5 μM each and an $MgCl_2$ concentration of 4 mM as previously published (162). Two μL of extracted DNA were used in a total reaction volume of 20 μL, using capillary tubes. The following PCR cycle was used for quantification: 1× 95°C for 10 min; 45 × (95°C for 10 s followed by 60°C for 7 s followed by 72°C for 9 s), with single fluorescence quantification during the 72°C extension step. Data collected from real-time PCR was analyzed by the second derivative maximum to calculate mean cycle threshold (C*t*) values.

4.3 Results

Relatively high recovery rates (80 to 100%) of *B. anthracis* spores from commercial whole milk were observed using the silicone oil method (Fig. 24). Use of this method resulted in a small spore containing pellet at the bottom of the silicone oil fraction that could be easily resuspended in the buffer of choice. However, when the spores were mixed with raw whole milk the recovery of spores into the silicone oil pellet was in average less than 40% (p-values = 0.01 and 0.03, for strains 9131 and 7702, respectively). Recovery of spores was restored to comparable levels to those of commercial whole milk when raw whole milk was heated at 85°C for 45 s in a static water bath following the addition spores, but prior to silicone oil processing (Fig. 24). Additionally, qualitative evidence from cream samples centrifuged with and without heat pre-treatment demonstrated an increase in spores in the cream fraction in unheated samples (see appendix D). Heating parameters and centrifugation conditions for maximum recovery of *B. anthracis* strain 9131 spores from raw whole milk were optimized to include heating at 85°C for 60 s, followed by centrifugation at 3,500 × *g* for 1 minute (Tables 3 & 4).

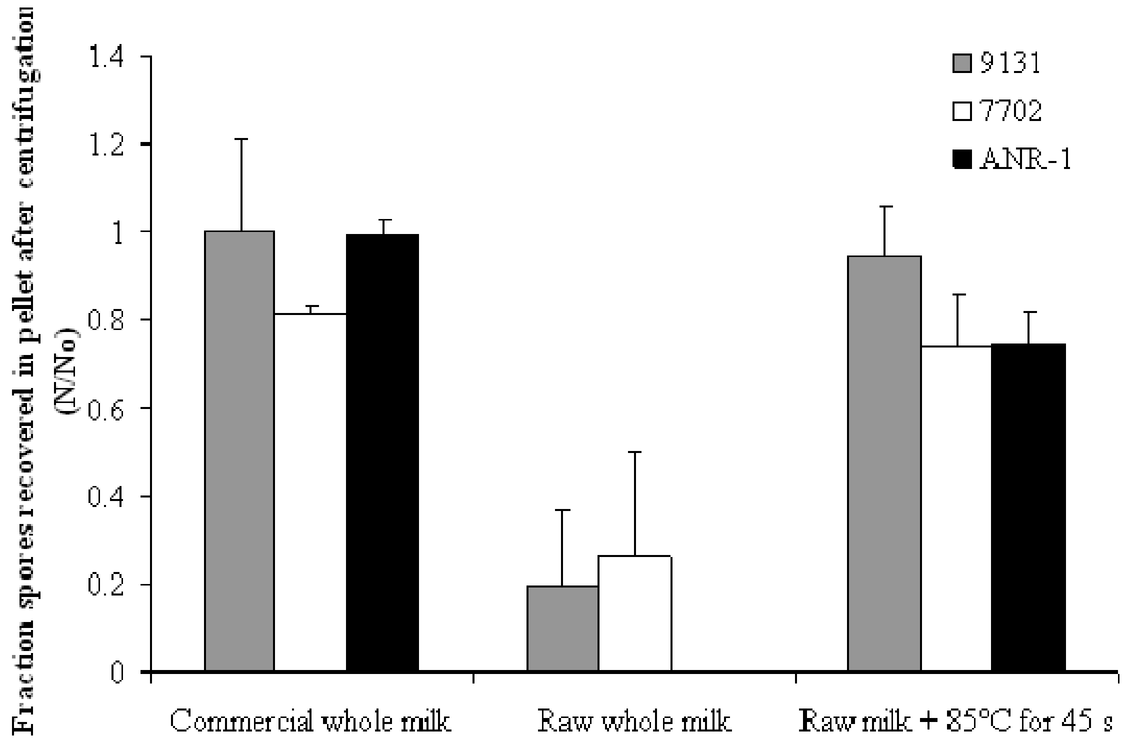

Figure 24. Recovery fraction of *B. anthracis* spores in pellets of milk collected via the silicone oil method following centrifugation for 1 minute at 16,300 × g. Milk types were inoculated with 10^3 spores/mL. Error bars represent 95% confidence intervals. N: spores recovered from pellet, and No: initial spore count. Note: Recovery of ANR-1 from raw whole milk was not calculated due to high background flora of raw milk samples.

Table 3. Percentage of spores recovered (± 95% confidence interval) from raw whole milk heated at different temperatures for 45 s using the silicone oil method at varying centrifuge speeds for 1 minute. The initial spore concentration was an average of 10^3 9131 spores/mL.

Temperature (°C)	Centrifuge Speed (× g)				
	3,500	**4,700**	**7,800**	**11,700**	**16,300**
75	8.8±0.6	9.1±2.7	26.8±2.5	29.9±3.6	ND
78	10.1±0.2	16.1±6.1	47.9±4.4	55.5±1.8	ND
81	2.9±2.7	16.6±4.0	46.1±4.3	64.5±4.3	ND
83	25±11.1	48.8±5.1	61.8±2.7	61.9±3.6	66.1±4.4
85	33.3±7.4	57.9±3.7	71.3±3.3	72.2±6.1	73.3±5.6

Table 4. Percentage of spores recovered (± 95% confidence interval) from raw whole milk heated at different temperatures for 60 s using the silicone oil method at varying centrifuge speeds for 1 minute. The initial spore concentration was an average of 10^3 9131 spores/mL.

Temperature (°C)	Centrifuge Speed (× g)				
	3,500	**4,700**	**7,800**	**11,700**	**16,300**
75°C	ND	ND	ND	ND	ND
78°C	5.6 ±3.45	31.0±3.5	39.3±3.9	48.7±3.8	54.5±4.0
81°C	15.4±10.7	61.4±3.2	65.1±2.6	71.6±6.2	78.2±1.9
83°C	75.7±7.4	76.3±3.4	77.9±1.8	87.2±3.4	77.9±1.8

85°C	101.6±4.3	106.2±4.4	97.0±8.6	104.0±3.6	100.1±9.3

In contrast to the results of simple centrifugation processing, milk separation via continuous centrifugation using a lab scale cream separator yielded a significantly different outcome. For all strains the skim fraction contained at least 4-fold more *B. anthracis* spores than the cream fraction (p-values = 0.04, 0.01, <0.01, and <0.01 for strains 7702p610, UT258, UM44-1tr203-1 and UM23C1-2, respectively) (Fig. 25). Non-inoculated milk samples run with each trial contained less then 10^2 CFU/mL of naturally present spores (less than 10 CFU/plate). Strains not marked with antibiotic resistance elements were not used for this experiment due to the abundance of bacteria in the raw milk samples. Cream resulting from the lab scale milk separation had a slightly higher fat content then commercial cream (Table 5). However, this could not be adjusted due to the nature of the separator.

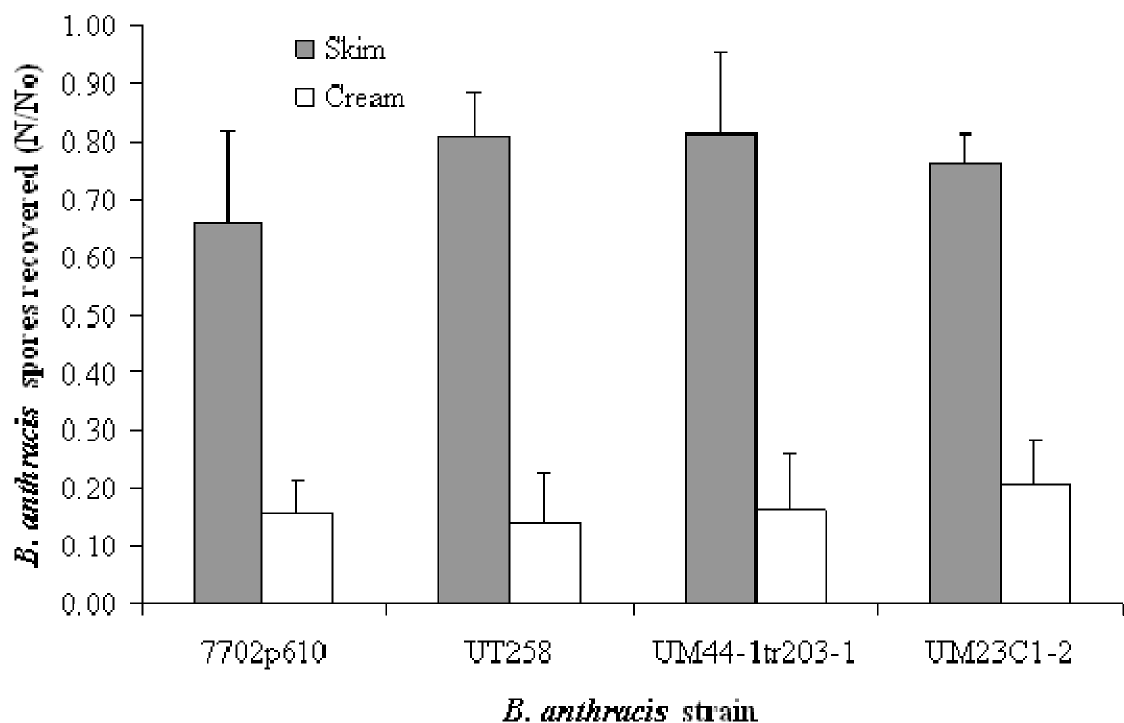

Figure 25. Recovery fraction of *B. anthracis* spores in skim milk and cream following separation of inoculated raw whole milk. Error bars represent 95% confidence intervals. N: spores recovered from pellet, and No: initial spore count.

Table 5. Percent fat in cream and skim fractions resulting from lab scale milk separator. *Skim milk curdled prior to analysis.

Separation Date	% Fat in Cream	% Fat in Skim Milk
3/11/08	59.00	0.12
3/26/08	56.19	N/A*
3/28/08	56.84	N/A*
4/16/08	57.10	0.19
4/18/08	55.72	0.15

Mean ± 95% CI	56.97 ± 1.10	0.15 ± 0.04

The silicone oil method previously described was not easily adapted to recover *B. anthracis* spores from larger volumes of milk. Instead, recovery and concentration of spores from milk was performed via centrifugation without the addition of silicone oil in an RC-5 centrifuge. Although this eliminated the additional extraction effect, it reduced the possibility of silicone oil interference with downstream detection.

Centrifugation conditions using 1-mL volumes were tested using six strains of *B. anthracis* (Table 6). All conditions tested yielded high recovery (>95%) of spores into the pellet, indicating successful concentration. Several centrifugation time and speed combinations were tested to identify a centrifugation method capable of recovering *B. anthracis* spores from 100 mL milk volumes. At any of the speeds tested (1,500, 2,600 and 4,100 × g), centrifugation for 10 min caused more 95% recovery of strains 7702p610 and UT258 (Fig. 26). Based on those results, a speed of 1,500 × g for 10 min was selected for further experiments. The effectiveness of this centrifugation combination was then tested with strains 9131, 7702, ANR-1, 7702p610, and UT258 at initial spore concentrations ranging between 10^2 spores/mL (10^4 total spores in 100 mL) and 10^6/mL (10^8 total spores in 100 mL). Centrifugation at these conditions yielded nearly 100% recovery of all strains and spore concentrations from both commercial skim and whole milk (Figs. 27 and 28).

Table 6. Recovery fraction *B. anthracis* spores recovered (N/N_0) from 1 mL volumes of commercial skim milk via simple centrifugation for 1 minute with 95% confidence intervals. N: spores recovered from pellet, and No: initial spore count.

***B. anthracis* strain**	**Centrifuge Speed (× *g*)**		
	4,700	**9,600**	**16,300**
9131	0.98 ± 0.03	1.00 ± 0.01	0.99 ± 0.00
7702	0.95 ± 0.08	0.95 ± 0.08	0.95 ± 0.08
ANR-1	0.98 ± 0.03	0.99 ± 0.03	0.99 ± 0.02
UT258	0.98 ± 0.05	1.00 ± 0.01	1.00 ± 0.04
UM44	0.98 ± 0.01	0.99 ± 0.02	0.99 ± 0.02
UM23C1-2	0.99 ± 0.01	1.00 ± 0.01	1.00 ± 0.01
Mean recovery across strains	0.98 ± 0.02	0.99 ± 0.01	0.99 ± 0.01

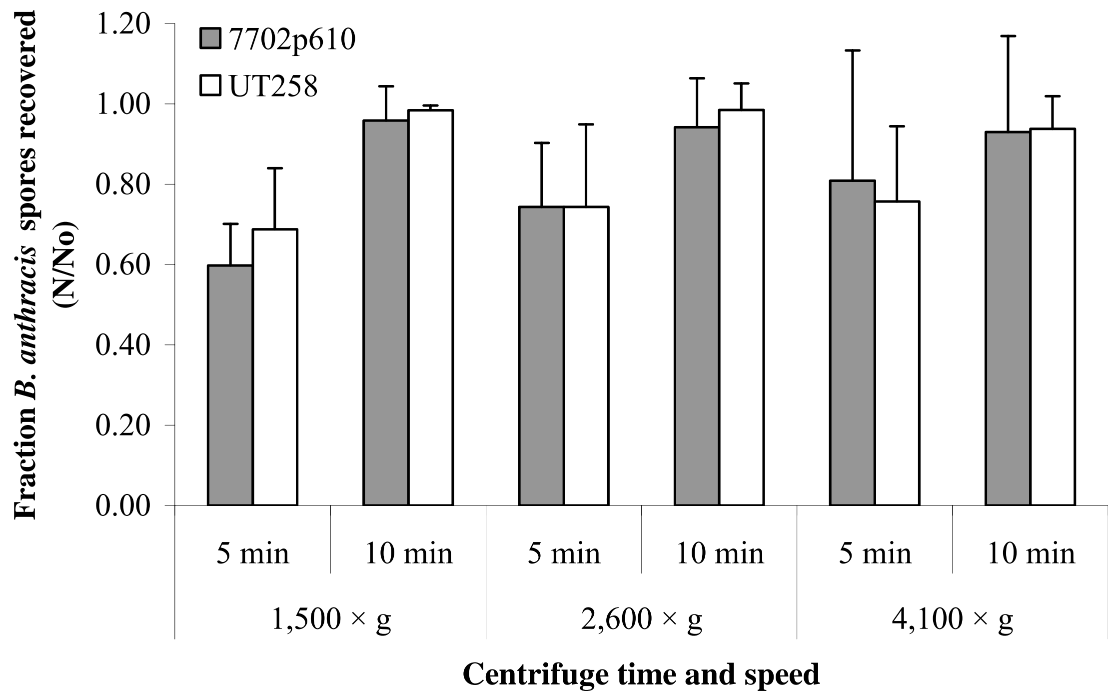

Figure 26. Recovery fraction of *B. anthracis* spores after centrifugation of inoculated commercial skim milk at varying centrifugal speeds and times from 100 mL total volume. Error bars represent 95% confidence intervals. N: spores recovered from pellet, and No: initial spore count.

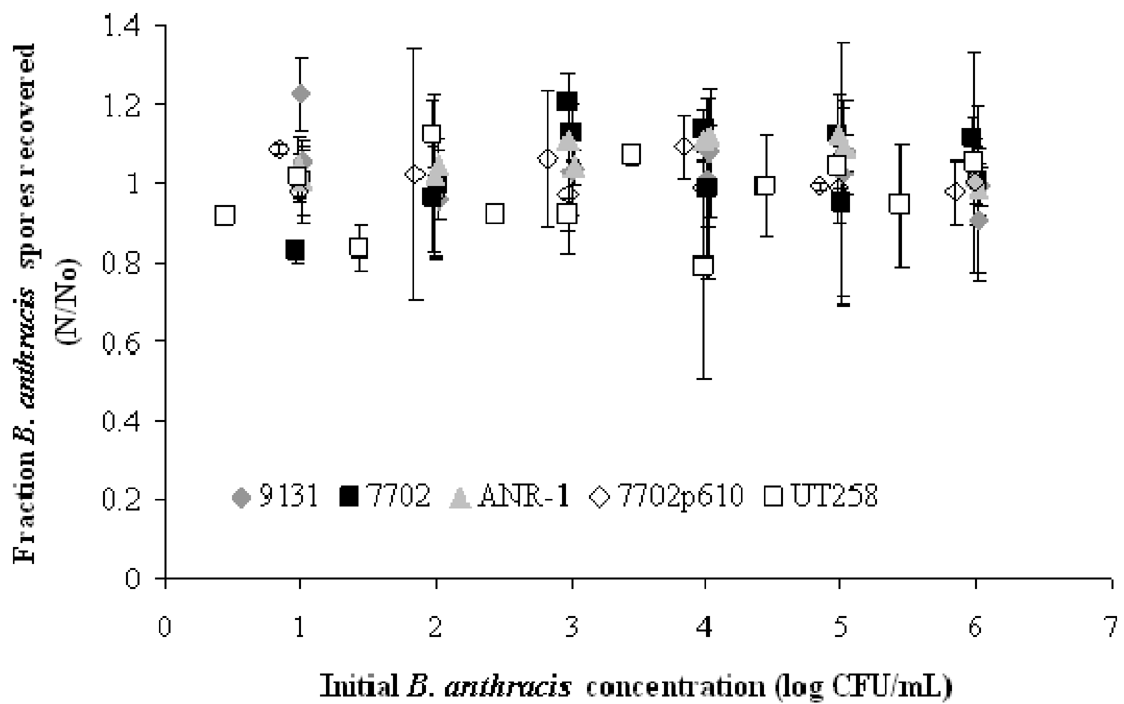

Figure 27. Recovery fraction of *B. anthracis* spores after centrifugation of inoculated commercial skim milk for 10 min at 1,500 × *g* (100 mL total volume). Error bars represent 95% confidence intervals. N: spores recovered from pellet, and No: initial spore count.

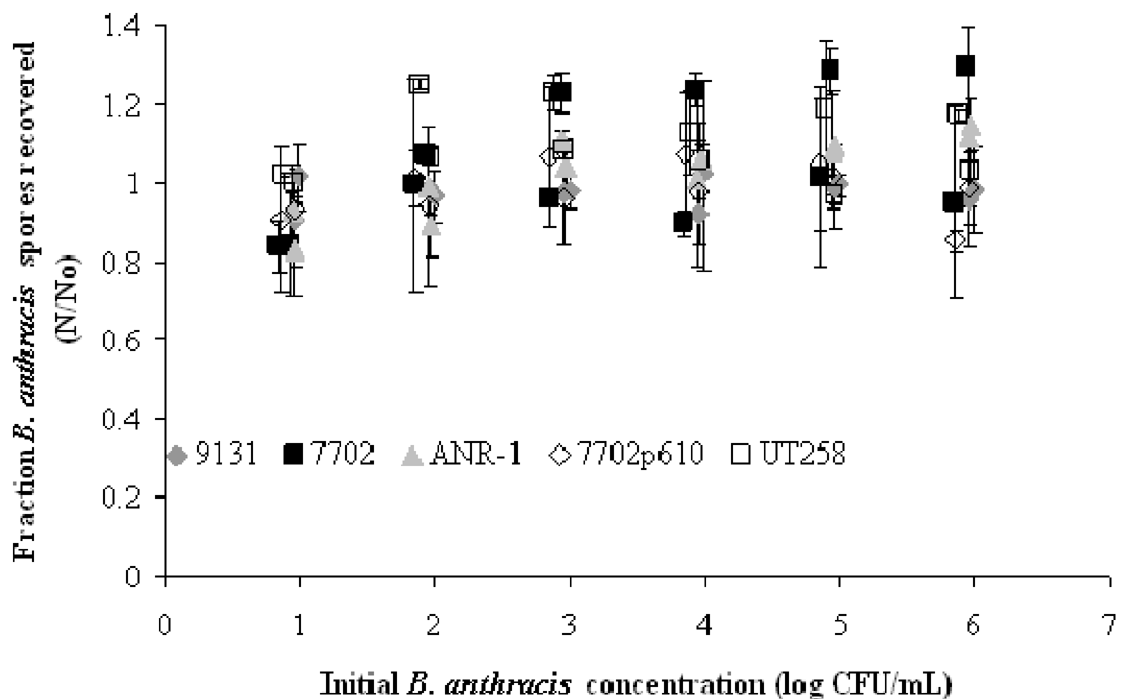

Figure 28. Recovery fraction of *B. anthracis* spores after centrifugation of inoculated commercial whole milk for 10 min at 1,500 × *g* (100 mL total volume). Error bars represent 95% confidence intervals. N: spores recovered from pellet, and No: initial spore count.

The sensitivity of Tetracore© *B. anthracis* lateral flow assay was determined using strain ANR-1 suspended in sterile water and in commercial skim milk. The assay was 100% sensitive to concentrations of 10^7 spores/mL or greater after 30 min incubation both in water and skim milk (Table 7). However, at spore concentrations less than 10^6 spores/mL in milk or water, the assay rarely yield a positive result (Table 7).

Table 7. Sensitivity of Tetracore © *B. anthracis* lateral flow assay using ANR-1 spores suspended in either sterile water or commercial skim milk at varying concentrations. Samples diluted 1:1 by volume in assay buffer. Performed in two trials, two replicates per trial.

Spore suspension matrix	Target spore concentration (spores/mL)	Actual spore concentration (CFU/mL)	Positive results at 15 minutes (%)	Positive results at 30 minutes (%)
Sterile distilled water	10^8	Trial 1: 7.7×10^7 Trial 2: 9.2×10^7	4/4 (100%)	4/4 (100%)
	10^7	Trial 1: 7.7×10^6 Trial 2: 9.2×10^6	3/4 (75%)	4/4 (100%)
	10^6	Trial 1: 7.7×10^5 Trial 2: 9.2×10^5	1/4 (25%)	2/4 (50%)
	10^5	Trial 1: 7.7×10^4 Trial 2: 9.2×10^4	0/4 (0%)	0/4 (0%)
Commercial skim milk	10^8	Trial 1: 9.8×10^7 Trial 2: 1×10^8	4/4 (100%)	4/4 (100%)
	10^7	Trial 1: 9.8×10^6 Trial 2: 1×10^7	4/4 (100%)	4/4 (100%)
	10^6	Trial 1: 9.8×10^5 Trial 2: 1×10^6	0/4 (0%)	0/4 (0%)
	10^5	Trial 1: 9.8×10^4 Trial 2: 1×10^5	0/4 (0%)	0/4 (0%)
	Non-inoculated control (Skim milk only)	Trial 1 and 2: 0	0/4 (0%)	0/4 (0%)

Despite to relative compatibility of the lateral flow assay with spores suspended in milk, the assay was not directly compatible with ANR-1 spores recovered in the milk concentrate, and was not sensitive to the spores despite levels of greater then 10^7 spores/mL in the concentrate (Table 8). Excessive smearing was also noted on lateral flow assays run with concentrated samples. This result led to the utilization of a secondary concentration/extraction step of centrifugation at 10,000 × *g* for 1 min, in accordance with the data previously collected from concentration of spores from small volumes (1 mL) of skim milk. After secondary concentration, the remaining milk in the sample was decanted and the pelleted spores were resuspended directly in lateral flow assay buffer. This step not only further concentrated and extracted milk from the sample, but also avoided additional dilution of the sample. Despite this step to further eliminate protein and concentrate the sample while avoiding additional dilution in assay buffer, the sample was not compatible with the lateral flow assay. This procedure did not lower the detection limit of ANR-1 spores from 10^7 to 10^5 using the concentration and extraction procedures as anticipated from the concentration increase and concentrated samples were found to be incompatible with the lateral flow assay (Table 8).

Table 8. Sensitivity of Tetracore© *B. anthracis* lateral flow assay using ANR-1 spores suspended in 100 mL commercial skim milk following centrifugal treatment with and without secondary centrifugation. Samples not treated with secondary centrifugation were diluted 1:1 by volume in assay buffer. Performed in two trials, two replicates per trial.

Milk treatment	Initial target spore concentration (spores/mL)	Actual initial spore concentration (CFU/mL)	Final spore concentration (after processing) (CFU/mL)	Positive results at 15 minutes (%)	Positive results at 30 minutes (%)
Centrifugation at 1,500 × *g* for 10 minutes (1 mL volume)	10^6	Trial 1: 9.4×10^5 Trial 2: 9.2×10^5	Trial 1: 9.1×10^7 Trial 2: 9.3×10^7	0/4 (0%)	0/4 (0%)
	10^5	Trial 1: 9.4×10^4 Trial 2: 9.2×10^4	Trial 1: 1×10^7 Trial 2: 9.1×10^6	0/4 (0%)	0/4 (0%)
	10^4	Trial 1: 9.4×10^3 Trial 2: 9.2×10^3	Trial 1: 9.2×10^5 Trial 2: 9.4×10^5	0/4 (0%)	0/4 (0%)
Centrifugation at 1,500 × *g* for 10 minutes followed by secondary centrifugation	10^6	Trial 1: 6.1×10^5 Trial 2: 6.5×10^5	Trial 1: 1.0×10^8 Trial 2: 1.1×10^8	0/4 (0%)	0/4 (0%)
	10^5	Trial 1: 6.1×10^4	Trial 1: 1.0×10^7	0/4 (0%)	0/4 (0%)

at 9,600 × *g* for 1 minute (0.5 mL volume)		Trial 2: 6.5×10^4	Trial 2: 1.1×10^7		
	10^4	Trial 1: 6.1×10^3 Trial 2: 6.5×10^3	Trial 1: 1.1×10^6 Trial 2: 9.5×10^5	0/4 (0%)	0/4 (0%)

Efficacy of DNA extraction from *B. anthracis* spores via UltraClean kit, germination followed by UltraClean kit, and by germination-boiling lysis methodologies was compared by standard PCR targeting the *pagA* gene (210 bp, located on pXO1). DNA was extracted from ANR-1 spores at an initial concentration of approximately 10^9 spores/mL in duplicate trials via each method. The detection limit of each method was determined by serial dilution of template DNA in sterile water. Both extraction via UltraClean kit and germination followed by use of the UltraClean kit yielded high concentrations of template DNA. Both methods were sensitive to a template dilution of 10^{-3}, representing a detection limit of approximately10^6 spores/mL (Fig. 29). Germination-boiling lysis DNA extraction yielded lower amounts of DNA, only sensitive to PCR detection only in the undiluted sample (Table 9). Ultimately the use of the UltraClean kit alone was selected for extraction of spore DNA from milk samples and for real-time PCR, as it bypasses the time-consuming germination procedure without any loss of extraction efficacy.

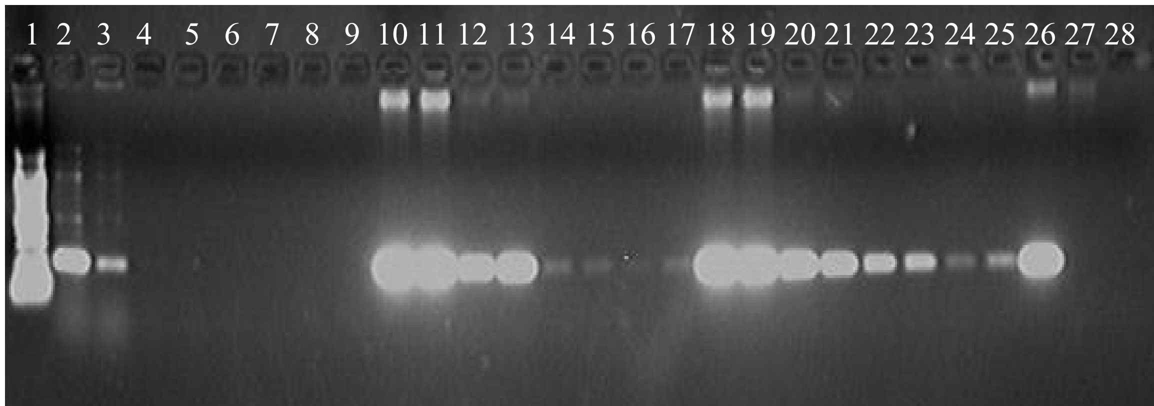

Figure 29. Picture of a gel resulting from electrophoresing PCR products based on *pagA* (210 bp) detected in *B. anthracis* ANR-1 spores extracted via different methodologies after template dilution.

Lanes- 1: 100 bp ladder; lanes 2 to 9 from germination-lysis extraction (2&3, undiluted DNA template; 4&5, 10^{-1} dilution; 6&7, 10^{-2} dilution; 8&9, 10^{-3} dilution); lanes 10 to 17 from germination followed by UltraClean extraction (10&11, undiluted DNA template, 12&13, 10^{-1} dilution; 14&15, 10^{-2} dilution; 16&17, 10^{-3} dilution); lanes 18 to 25 from UltraClean extraction (18&19, undiluted DNA template;, 20&21, 10^{-1} dilution; 22&23, 10^{-2} dilution; 24&25, 10^{-3} dilution) 26: ANR-1 vegetative cells extracted with UltraClean kit, 27: *B. thuringiensis* vegetative cells extracted with UltraClean kit, 28: Sterile distilled H_2O

Table 9. Summary of PCR results establishing detection limits for spores extracted via different methodologies after template dilution. Undiluted DNA extract was obtained from 10^9 spores/mL. Total positives/total reactions (% positive).

Dilution of extracted DNA	**Germination-lysis**	**UltraClean Kit**	**Germination followed by UltraClean Kit**
Undiluted DNA extract	4/4 (100%)	4/4 (100%)	4/4 (100%)
10^{-1} dilution	2/4 (50%)	4/4 (100%)	4/4 (100%)
10^{-2} dilution	0/4 (0%)	4/4 (100%)	4/4 (100%)
10^{-3} dilution	0/4 (0%)	4/4 (100%)	4/4 (100%)

Following DNA extraction efficacy trials establishing the UltraClean kit as the most effective method for *B. anthracis* spore DNA extraction, the kit was used to extract DNA from milk inoculated with spores with and without prior concentration. Results of standard PCR indicated that concentration facilitated consistent detection of the *pagA* gene (*B. anthracis* pXO1) at initial spore concentrations of 10^4 CFU/mL milk or greater. In contrast, standard PCR failed to consistently detect spore concentrations lower than 10^6 CFU/mL when directly extracted from milk without pre-analytical concentration. This represented a 2-log decrease from the limit of detection of spores extracted directly from milk (Fig. 30).

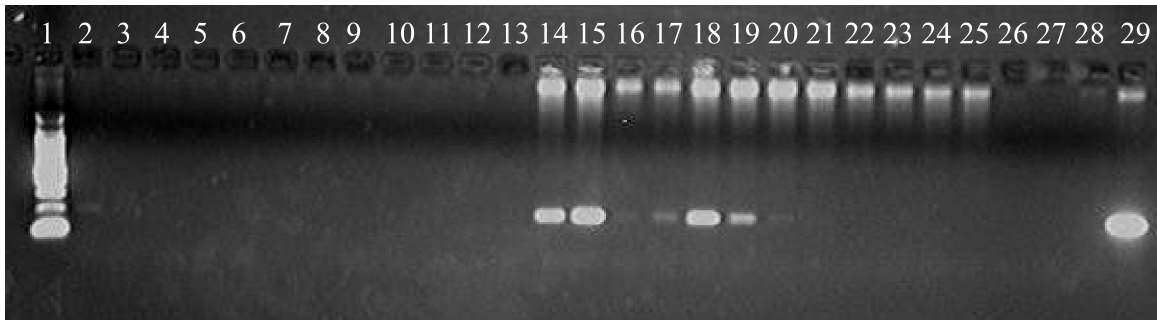

Figure 30. Picture of gel of PCR products using primers against *pagA* (210 bp) in *B. anthracis* ANR-1 spores at varying concentrations in skim milk, with and without prior centrifugal concentration, following extraction with the UltraClean kit.

Lanes- 1: 100 bp ladder, lanes 2 to13 DNA extraction from milk (2&3, 10^6 spores/mL; 4&5, 10^5 spores/mL; 6&7, 10^4 spores/mL; 8&9, 10^3 spores/mL; 10&11,10^2 spores/mL; 12&13, 10^1 spores/mL), lanes 14 to 25 DNA extraction from concentrated milk (14&15, 10^6 spores/mL; 16&17, 10^5 spores/mL; 18&19, 10^4 spores/mL; 20&21, 10^3 spores/mL; 22&23,10^2 spores/mL; 24&25, 10^1 spores/mL), 26: non-inoculated milk extracted with UltraClean kit, 27: *B. thuringiensis* vegetative cells extracted with UltraClean kit, 28: Sterile distilled H_2O, 29: *B. anthracis* ANR-1 vegetative cells extracted with UltraClean kit.

Prior to detection of spores from skim and whole milk using real-time PCR, a standard curve of number of spores vs. cycle threshold (C_t, defined as the point at which amplification crosses a set fluorescence threshold) was constructed using DNA extracted from 1 mL spore suspensions at an approximate concentration of 10^9 CFU/mL via serial dilution of the template. Results of both standard curve trials were highly linear, with an $R^2 = 0.97$ (see appendix D). Dilutions of the DNA template (representing an initial

concentration of 10^8-10^3 ANR-1 spores/mL) were used in all subsequent sample runs to validate results across trials.

Real-time PCR was used to compare efficacy of extraction, limits of detection, and C*t* values between milk samples inoculated with *B. anthracis* spores, with or without concentration prior to DNA extraction. All trials were validated using the internal amplification standards, and a negative control of skim milk extracted with the UltraClean kit. Additionally the internal standard (10^8 spores/mL) was amplified using standard PCR was found to be the size expected, approx 210 bp. Despite variable extraction efficacy, results showed that *B. anthracis* DNA from extracted directly from milk samples was consistently detectable at a levels of 10^3 CFU/mL or greater for all four strains. The addition of the pre-analytical concentration procedure prior to DNA extraction lowered the limit of detection by 2-log units, such that 10 CFU/mL or greater could consistently be detected. Results for all strains demonstrated higher levels of *B. anthracis* detected and lower C_t values for samples from both skim and whole milk concentrated prior to extraction (Figs. 31 and 32, Tables 10, 11, and 12).

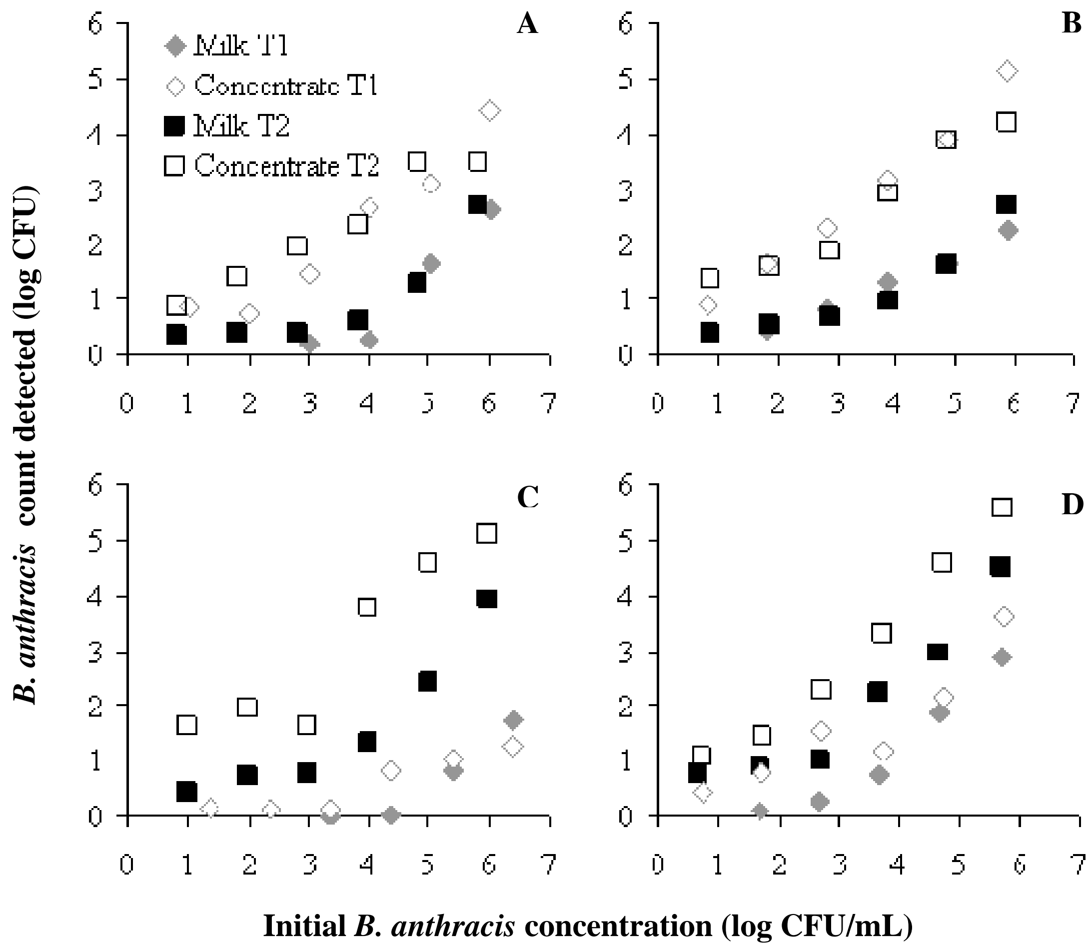

Figure 31. Real-time PCR data comparing the initial and detected counts of *B. anthracis* from skim milk samples containing various levels of spores, 7702 (A), ANR-1 (B), UT258 (C) and 7702p610 (D), with and without prior concentration. T1 and T2 denote trial 1 and 2.

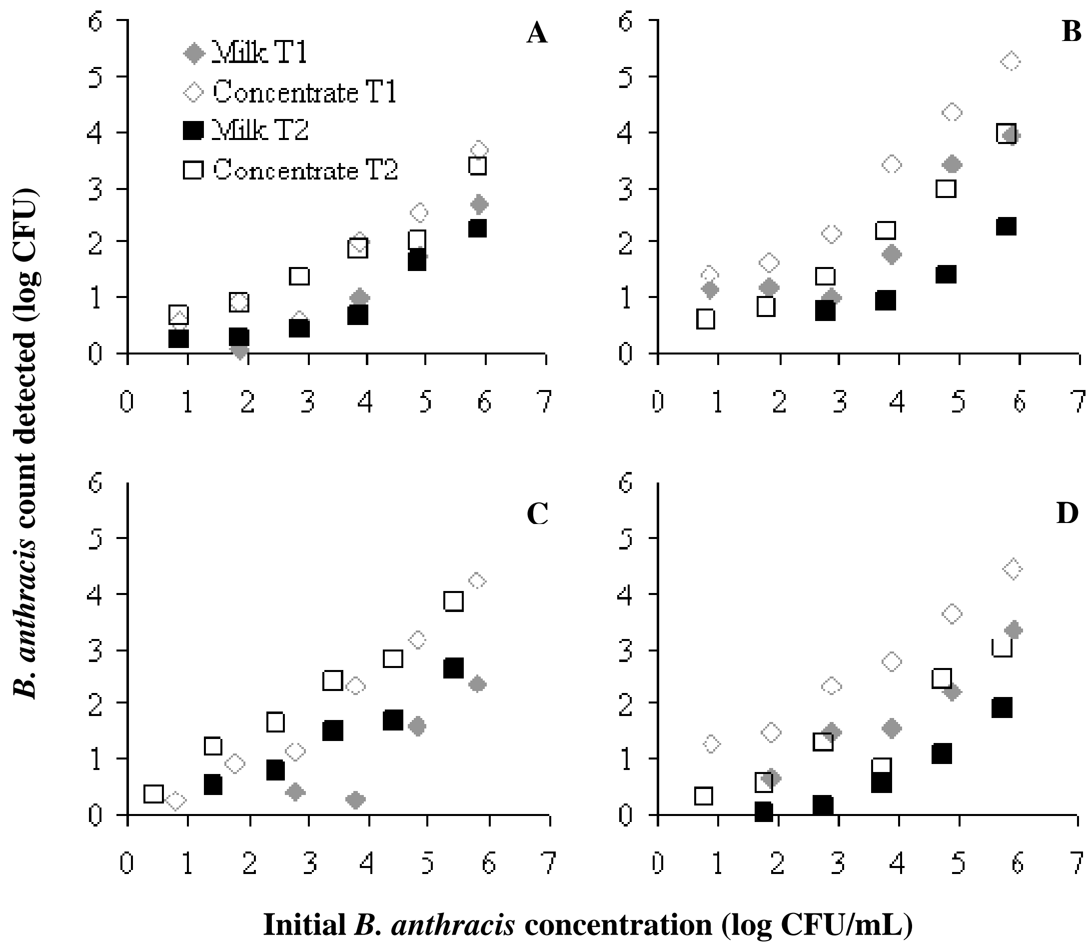

Figure 32. Real-time PCR data comparing the initial and detected counts of *B. anthracis* from whole milk samples containing various levels of spores, 7702 (A), ANR-1 (B), UT258 (C) and 7702p610 (D), with and without prior concentration. T1 and T2 denote trial 1 and 2.

Table 10. Limits of detection using real-time PCR targeting the *pagA* gene located on plasmid pXO1 using DNA extracted skim and whole milk samples via UltraClean kit, with or without prior concentration

Strain	Limit of detection (LOD)[a] *B. anthracis* spores/mL milk			
	Directly from skim milk (no concentration)	**Skim milk following concentration**	**Directly from whole milk (no concentration)**	**Whole milk following concentration**
7702	10^3	10^1	10^2	10^1
ANR-1	10^2	10^1	10^3	10^1
7702p610	10^2	10^1	10^2	10^1
UT258	10^3	10^1	10^3	10^1

[a] Limit of detection determined as minimum concentration at which all replicates were positive

Table 11. Mean C*t* value reduction of *B. anthracis* samples in skim milk that were pre-concentrated vs. non-concentrated samples.

Strain	Initial spore concentration (per mL)					
	10^1	10^2	10^3	10^4	10^5	10^6
7702	1.97[a]	4.02[a]	5.43	7.96	7.15	5.03
ANR-1	3.80[a]	4.42	5.20	7.34	8.79	8.56
7702p610	2.01[a]	2.15	2.23	5.57	5.36	5.53
UT258	4.75[a]	4.74[a]	1.99	6.26	4.61	1.43
Mean across strains (95% CI)	3.13 ± 0.95	3.83 ± 0.80	3.71 ± 1.28	6.78 ± 0.78	6.48 ± 1.30	5.14 ± 2.03

[a]C*t* difference based on only one pair of data points

Table 12. Mean C*t* value reduction of *B. anthracis* samples in whole milk that were pre-concentrated vs. non-concentrated samples.

Strain	Initial spore concentration (per mL)					
	10^1	**10^2**	**10^3**	**10^4**	**10^5**	**10^6**
7702	1.64[a]	2.91	2.03	4.29	2.26	4.11
ANR-1	3.13[a]	3.77[a]	3.48	5.58	4.88	5.90
7702p610	4.39[a]	2.61	3.94	2.80	5.39	4.39
UT258	3.33[a]	2.70[a]	3.01	5.76	5.19	5.98
Mean across strains (95% CI)	2.70 ± 0.90	3.00 ± 0.52	3.11 ± 0.80	4.61 ± 1.34	4.43 ± 1.43	4.95 ± 0.82

[a]C*t* difference based on only one pair of data points

4.4 Discussion

The aim of the current project was to design a method for pre-analytical concentration and extraction of *B. anthracis* spores from milk compatible with rapid detection techniques. The pre-analytical concentration was accomplished using simple centrifugation at low speed and was determined to be compatible with molecular detection method via standard and real-time PCR. However, this centrifugal concentration protocol was not compatible with a commercial lateral flow assay

The initial steps of this process used silicone oil to provide both concentration and extraction of spores from milk in a single step. However, this process was difficult to adapt to larger volumes of milk. The use of this procedure was effective for extracting spores from small volumes and was useful to illustrate recovery of spores from milk using different pre-treatments, as well as characterize spore distribution in various milk types. Similar to the results found by Perdue et al, 2003 spores suspended in raw whole milk were not recovered in the bottom fraction of the tube as would be expected by Stokes law, but were instead found in the cream fraction (188).

Both quantitative and qualitative evidence suggested that the 'sweep' effect of the cream rising through the milk column during simple centrifugation likely prevented the spores from effectively separating by density. However, it was found that this 'sweep' effect could be disrupted through the use of heat treatment, in both commercial milk and raw whole milk heat treated at 85°C for 45 s. Evidence from the literature indicated that heat alters the surface of the milk fat globule and prevents flocculation due to cryoglobin interaction which may be responsible for the differences between raw and heat treated milk (87). Homogenization of the commercial milk samples likely further disrupted fat

globule coalescence, further reducing the up-sweeping effect of the cream and allowing spores to accumulate in the pellet formed at the bottom of the tube.

Although both heating and centrifugation conditions for concentration and recovery of *B. anthracis* spores from raw whole milk were defined for small volumes of milk, the additional heating step may complicate application of this process in the field. In addition, it was found that a majority of spores partitioned to the skim fraction during milk separation as opposed to the cream as demonstrated during simple centrifugation of raw whole milk. The different results from the two separation techniques may also be related the sweeping effect of the cream through the milk column during simple centrifugation, as during separation the volume of fat being separated and separation area are significantly lower. This may decrease the ability of the cream to sweep up through the milk column, resulting in a lower concentration of spores entrapped in the cream. The results from milk separation suggest that skim milk with or without the reintroduction of fat could effectively be targeted for concentration and extraction as opposed to raw whole milk, without significant loss of spore concentration. Results from milk separation indicated that the most effective point of process application would likely be in the plant following separation and pasteurization. In most cases the milk would likely also go through homogenization prior to analysis due to the closed nature of the processing system. Testing of samples after commercial processing may also reduce the number of opportunities to intentionally introduce *B. anthracis* into large volumes of milk post-screening.

The concentration procedure defined in this project is very simple, requiring only centrifugation and decanting to reduce to total volume of milk containing *B. anthracis*

spores from 100 mL to 1 mL. This method was effective to concentrate the spores based on their density (1162-1186 kg/m^3) as opposed to the many components of milk (32). However, this procedure does not fully extract the spores from all milk components, including casein micelles (density estimates between 1114 kg/m^3 to 1434 kg/m^3) (84, 173, 232). This type of problem is not unexpected with non-selective separation based on particle radius and density. Other non-specific pre-analytical methods previously used separation of other bacteria from food, such as ion exchange, lectin binding, hydrophobic separation and metal hydroxides have also been reported to co-extract interfering compounds and have lower or less consistent rates of recovery than centrifugation (16, 44, 159, 160, 226). Many of these other pre-analytical techniques also require either sample pre-treatment or post-processing treatment to be compatible with rapid detection assays (16, 226). Bacteria-specific pre-analytical concentration and extraction techniques, such as immunomagnetic separation, may provide more selective extraction of the target bacteria, but may be less efficient in terms of recovery and cost (16, 226). Although the pre-analytical centrifugation method developed in this study is non-selective, it is an inexpensive, simple, and rapid method for concentration of *B. anthracis* spores in milk.

B. anthracis DNA was effectively extracted from the spores in the milk concentrate using the MoBio UltraClean kit for use in PCR and real-time PCR. The limits of detection vary between strains for non-concentrated milk samples, but can effectively be reduced by 1-2 log units by concentrating the sample prior to DNA extraction. The UltraClean kit uses both bead beating and silica gel spin columns for DNA extraction and recovery. This technique may be better suited for extraction of *B.*

anthracis spore DNA as it utilizes physical disruption via bead beating as opposed to other nucleic acid extraction kits that use chemical or enzymatic digestion, which have been shown to be not very effective for spore lysis (99, 215, 216). The lower limit of detection for real-time PCR was previously reported at 10^3 CFU *B. anthracis* spores /mL using the UltraClean kit (48). However the addition of the concentration procedure effectively lowered the detection limit to an initial concentration of 10 CFU *B. anthracis* spores/mL from commercial skim and whole milk samples, corresponding to the concentration increase due to the centrifugation process.

Although real-time PCR represents a highly sensitive method for detection of *B. anthracis* added to the milk supply, the cost and complexity of this analysis may be prohibitive in a plant or field setting. Lateral flow assays circumvent the need for complex instrumentation, requiring very simple sample preparation and training while yielding results in as little as 15 minutes. The downfall of lateral flow assays in the inherent insensitivity of the assay (140). This project determined an effective limit of detection of a lateral flow assay (100% sensitivity) at 10^7 CFU *B. anthracis* spores/mL in water or skim milk. Following centrifugal concentration and extraction of spores, the resulting concentrate was not compatible with lateral flow assays. The concentrated protein remaining in the milk/spore concentrate may be the reason for the incompatibility of the concentrate with lateral flow assays. Although little has been published on the direct effect of milk proteins on immunoassay efficacy, numerous other studies have documented the ability of protein to interfere with accurate detection using these assays (64, 108, 147, 176, 213). If excess protein in the sample is the cause of incompatibility between the concentrated spore/milk sample and the lateral flow assay, it may be possible

to eliminate the protein through digestion or precipitation. However, the addition of either of these steps may complicate the application of this type of detection in the field.

Detection of *B. anthracis* spores added to the milk supply is crucial to securing the safety of our food supply against bioterrorist action. The methods developed as part of this project represent the ability to achieve a 2-log decrease in the limit of detection via PCR and real-time PCR. Although the application of any of these detection systems remain cost prohibitive, the potential of pooling samples may decrease this cost to become financially feasible. The compatibility of concentrated and extracted milk samples processed using this method with rapid detection assays represents the successful development of a pre-analytical procedure to improve the safety of our milk supply against attack using *B. anthracis.*

Chapter 5

Survival and pre-analytical concentration, extraction, and detection of *Bacillus anthracis* in fruit juice

Foods have been identified as a potential target for bioterrorism due to their essential nature and global distribution. Foods produced in bulk have the potential to have large batches of product intentionally contaminated, which could potentially affect hundreds or thousands of individuals. Fruit juices and other products subjected to minimal or no heat treatment may support the survival of numerous potential bioterrorism for extended periods of time. *Bacillus anthracis* spores are one of the potential bioterrorism agents that may survive pasteurization and remain viable throughout the shelf life of fruit juices to cause disease if consumed. Many current methods of detecting *B. anthracis* are either not sensitive enough to detect the infectious dose or are not compatible with food samples. This project demonstrated that *B. anthracis* spores can survive, with little to no loss in population, for up to thirty days in fruit juices and wine. This project also successfully applied a pre-analytical concentration and extraction procedure using centrifugation to *B. anthracis* spores in apple and grape juice. The centrifugation procedure was not compatible with orange juice due to the high solids content. DNA was successfully extracted from *B. anthracis* spores suspended in juice using the MoBio UltraClean kit. The DNA extracted from inoculated juice samples was compatible with real-time PCR and lateral flow assays and effectively lowered the limit of detection 1-3 log units. Real-time PCR was the most sensitive of these methods

allowing for the potential detection of 10 *B. anthracis* spores/mL juice. The total time to detect using any of these three rapid detection methods was less then 3 hours. Although application of these detection techniques at the plant may be limited by fiscal and personnel constraints, the pre-analytical method developed allows for rapid concentration and extraction of spores compatible with multiple detection platforms.

5.1 Introduction

Anthrax is a disease of humans and animals that has been present since antiquity. First characterized by Koch and Pasteur, *B. anthracis* vegetative cells are susceptible to many environmental factors, such as heat and UV light (91, 171). However, the spore form of the bacteria has the ability to persist in the environment for extended periods of time in a metabolically dormant state. *B. anthracis* spores can be found in the soil of many parts of the world (258). Its primary lifecycle involves contact of the spore with herbivores, such as cows, which graze low to the ground, followed by germination and growth causing fatal toxin mediated disease in the animal, and finally sporulation upon death of the animal (70, 171). Although anthrax primarily affects animals, humans can also be affected through contact with the spore, via minor skin abrasion, inhalation or ingestion, with all types of infection potentially causing fatal systemic disease (8, 51, 91, 221, 254, 260).

Although anthrax is not common in humans, it does naturally occur in many parts of the world (221, 243, 254). Anthrax can also be used as a potential biological weapon due to the durable nature of the spore and ease of procurement from the soil. The 2001 anthrax mail attacks demonstrated the potential potency of an intentional attack on the

human population using this agent, causing 22 cases of disease and 5 deaths (129). Although anthrax initiating in the gastrointestinal tract has a relatively high infectious dose (estimated at 10^8 spores) when compared to inhalation anthrax (estimated at 10^3 to 10^4 spores) the ease of production, availability of the agent, and high mortality rates make this an attractive agent for potential bioterrorists (11, 91, 189, 193, 254).

One area of critical infrastructure particularly vulnerable to intentional contamination is food. Production facilities often produce large quantities of product with wide distribution patterns that may be attractive to a potential bioterrorist (252). Many of these production facilities also are minimally secured, such that an employee may be able to contaminate the product. One of many foods that may be potentially targeted for intentional contamination is liquid foods, such as milk and fruit juices, where the continuous mixing of the product throughout production may serve to spread the agent in large batches of product.

Fruit juices are potential targets for intentional contamination with biological agents as they are often produced in bulk and utilize pasteurization as the bacterial kill step. Although *B. anthracis* spores are able to survive the pasteurization process, little is known about their ability to survive, germinate, or grow in this type of acidic juice environment (262). Much of the acidity present in fruit juices is due to organic acids, which can have bacteristatic or bactericidal properties (49, 150). Wine has many of the same properties as fruit juices, but also contains alcohol and other fermentation products which have the potential to be inhibitory for spores. Fruit juices also naturally contain considerable amounts of carbohydrates which could possibly support germination and growth (223). Juice and wine products also have relatively long shelf lives, which makes

understanding of *B. anthracis* spore survival over long periods of time crucial to accurate risk assessment.

Due to the potentially severe effects of intentional contamination of the food supply, it is vital to be able to quickly identify these agents in high risk foods. Although current detection methodologies are able to rapidly detect many potential bioterrorism agents, few are compatible with direct detection from food matrices and have a limit of detection lower than the infectious dose (65). One potential method to harmonize food samples with rapid detection methods is pre-analytical extraction and concentration of the agent (16, 226).

The current project examined the *B. anthracis* spore survival in fruit juices and wine, as well as the use of a simple centrifugation protocol as potential concentration and extraction methodology for *B. anthracis* spores from apple, grape, and orange juice. The compatibility of three rapid detection platforms (lateral flow assays, real-time PCR, and standard PCR) was evaluated with each of the juices and the corresponding extracted samples.

5.2 Materials and Methods

Spores and spore preparation

Bacillus anthracis strains 7702, 7702p610, UT258, and ANR-1 were kindly provided by Theresa Kohler, University of Texas- Houston Medical School. The Sterne strain 7702 harbors pXO1 that encodes the anthrax toxins, but lacks pXO2 (33, 262). Strains 7702p610 and UT258 are derivatives of strain 7702, containing additional plasmids conferring antibiotic resistance markers and green fluorescent protein (208).

Strain ANR-1 is a pXO2-cured non-encapsulated variant of the Ames strain that harbors pXO1 and pXO2 (253). Spores were prepared following the protocol outlined by Xu et al and verified to contain >95% spores by phase contrast microscopy (262).

B. anthracis *spore survival in wine and orange, apple, and grape juices*

B. anthracis spore survival in pulpless orange juice, grape juice, apple juice, and wine was monitored as both total bacterial count and spore count. All juices and wine were purchased at local retail outlets. Wine used was cabernet sauvignon with 13% alcohol by volume. Only *B. anthracis* strains containing antibiotic resistance elements were used in this study due to the abundance of background flora in wine, apple and grape juices. Non-inoculated juice and wine controls were also tested to monitor background flora. *B. anthracis* spores were added to juice to a target level of 10^6 spores/mL. The total bacterial count was monitored by sample serial dilution and plating 3 × 0.1 mL on tryptic soy agar (TSA; Neogen, Inc, Lansing, MI) supplemented with 5 μg/mL erythromycin (ERM TSA). Spore count was monitored using the procedure outlined by the Compendium of Methods for the Microbiological Examination of Foods followed by plating on ERM TSA (196). Both total bacterial count and spore count were measured on days 0- 5, 10, 15, 20, 25, and 30. This experiment was completed in two trials, with 2 replicates per trial.

Centrifugal concentration and extraction protocol as applied to juices

The concentration/extraction protocol applied to these beverages was originally designed and developed for milk samples. Samples were centrifuged at room

temperature in a Sorvall RC-5 (Thermo Scientific, Waltham, MA) centrifuge with GSA rotor for 10 minutes at 1,500 × *g*. The supernatants were then decanted and the pellets resuspended and standardized to a volume of 1 mL using sterile water. Spore concentrations in the initial samples were determined through serial dilution and plating on TSA. Concentrations in the pellet (final) samples were determined by serial dilution and plating, anticipating a 2 log increase from the initial sample. This procedure was performed in duplicate trials with two replicates per trial. This procedure was only applied to antibiotic marked strains 7702p610 and UT258 in apple and grape juice, due to the high levels of background flora in these juices.

Optimization of centrifugal removal of large particulates from orange juice

Differential centrifugation was used to optimize removal of large particulates from orange juice while maintaining the *B. anthracis* spores in the supernatant. Pulpless orange juice was inoculated with 10^3 spores/mL juice with either strain UT258 or 7702p610. Inoculated and non-inoculated juice samples were plated 3 × 0.1 mL on either TSA or ERM TSA to determine background flora and initial spore concentration. Volumes of 100 mL of juice were then aliquoted into 250 mL centrifuge bottles. The bottles were centrifuged at either 165 × *g* (1,000 RPM) for 5 and 10 minutes or at 655 × *g* (2,000 RPM) for 5 or 10 minutes in a Sorvall RC-5 centrifuge with a GSA rotor at room temperature. Following centrifugation supernatants were decanted and collected into sterile bottles. After collection, the supernatants were mixed well and 3 × 0.1 mL volumes were plated on ERM TSA. Plates were incubated overnight at 37°C and counted to determine the fraction spores recovered in the supernatant.

Sensitivity and compatibility of a lateral flow assay

Tetracore© *B. anthracis* BioThreat Alert (Rockville, MD) lateral flow assays were obtained from the manufacturer. Samples of *B. anthracis* UT258 spores suspended in water, orange grape or apple juice were enumerated by plating 3 × 0.1 mL on ERM TSA. Inoculated water and juice samples were diluted 1:1 by volume with assay buffer as described by manufacturer and applied to the port. Following concentration/extraction processing, samples were standardized to 1 mL. The sample was split for the assay and for processing to determine concentration efficacy. Samples for lateral flow assay were diluted 1:1 by volume in assay buffer. The remaining sample was serially diluted and plated to validate processing efficacy anticipating a 1 to 2 log CFU/mL increase in concentration from the initial sample. Assay results were both recorded and photographed at 15 and 30 min after inoculation. All experiments were performed in duplicate, with two replicates per trial.

Compatibility of concentrated and extracted samples with PCR and real-time PCR

DNA from samples of apple and grape juice inoculated with *B. anthracis* spores, both with and without pre-analytical centrifugation processing, and orange juice without processing were extracted via UltraClean kit (MoBio Labs, Carlsbad CA). This kit was chosen over others as it has been previously demonstrated to be effective for extraction of *B. anthracis* both from water and suspended in milk (48). Pre-analytical concentration and extraction was carried out as described above and samples were standardized to a volume of 1 mL with sterile water. Following pre-analytical processing, 0.1 mL was

removed from each tube of concentrate and diluted as appropriate to validate the efficacy of concentration by serial dilution and plating, anticipating a 1 to 2 log increase in concentration from the initial sample. The remaining 0.9 mL concentrates were used for extraction using the UltraClean kit. Volumes of 0.9 mL of non-concentrated/extracted orange juice (from each dilution and a negative control without added spores) were also processed using the UltraClean kit.

Standard PCR was performed using Go-Taq Green PCR Master Mix to target a 210 bp fragment of the protective antigen gene, *pagA*. Primers used were PA6 (5'-ACCAATATCAAAGAACGACGC-3') and PA7 (5'-ATCACCAGAGGCAAGACACCC-3') at a final concentration of 0.8 μM each primer. 2 μL of extracted DNA was used in a total reaction volume of 25 μL (38, 39, 162). The following PCR cycle was used for quantification: 1× 95°C for 2 min; 35 × (95°C for 15 s followed by 60°C for 15 s followed by 72°C for 30 s); 1× 72°C for 5 min (162). Following PCR amplification, 10 μL of each reaction was run on a 1.5% agarose gel for 60 minutes at 100 V.

Real-time PCR was carried out using the Light Cycler 2.0 system (Roche) and the a Fast Start DNAMaster SYBR Green (Roche Diagnostics) to target the same 210 bp fragment of the protective antigen gene *pagA* as used for standard PCR, using the same primers PA6 and PA7 as described above (199). Primers were used at a final concentration of 0.5 μM each and an $MgCl_2$ concentration of 4 mM as previously published (162). Two μL volumes of extracted DNA were used in a total reaction volume of 20 μL, using capillary tubes. The following PCR cycle was used for quantification: 1× 95°C for 10 min; 45 × (95°C for 10 s followed by 60°C for 7 s

followed by 72°C for 9 s), with single fluorescence quantification during the 72°C extension step. Data collected from real-time PCR was analyzed by the second derivative maximum to calculate mean cycle threshold (C*t*) values.

5.3 Results

B. anthracis spores survived with less than 1 log CFU/mL loss in viable spore concentration during the 30 day study period in all juices. Spores suspended in orange or grape juice survived at approximately the same concentration throughout the study period, whereas viable spore populations in both apple juice or wine decreased slightly beginning between days 10 and 15 (Figs. 33 and 34) . Differences between total counts and spore counts were minimal, indicating that spores were not germinating during the study period.

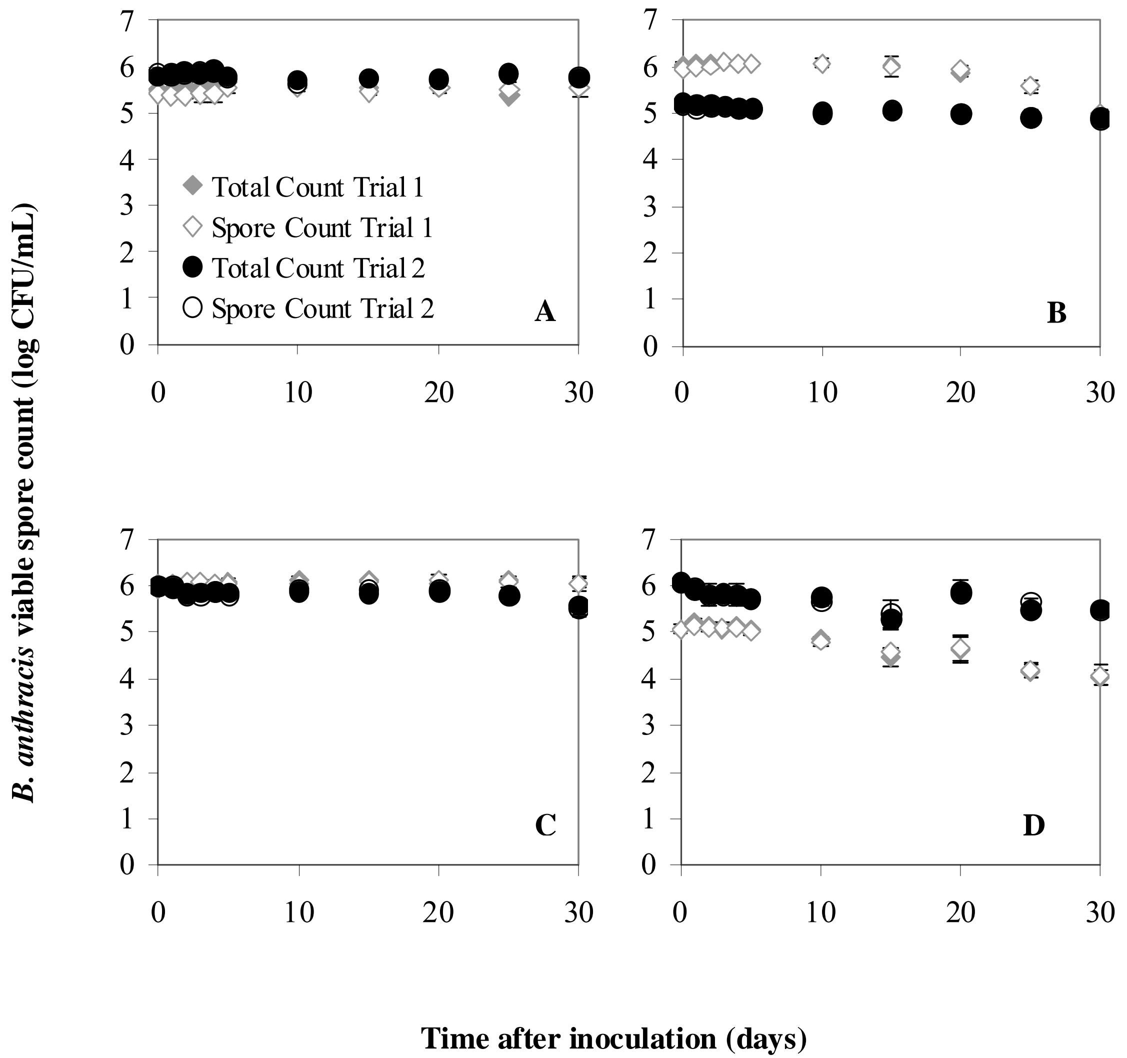

Figure 33. Spore survival of *B. anthracis* strain 7702p610 in orange juice (**A**) trial 1 pH 4.3, trial 2 pH 3.4; apple juice (**B**) trial 1 pH 3.4, trial 2 pH 3.37; grape juice (**C**) trial 1 pH 2.9, trial 2 pH 2.84; and wine (**D**) trial 1 pH 3.0, trial 2 pH 3.08

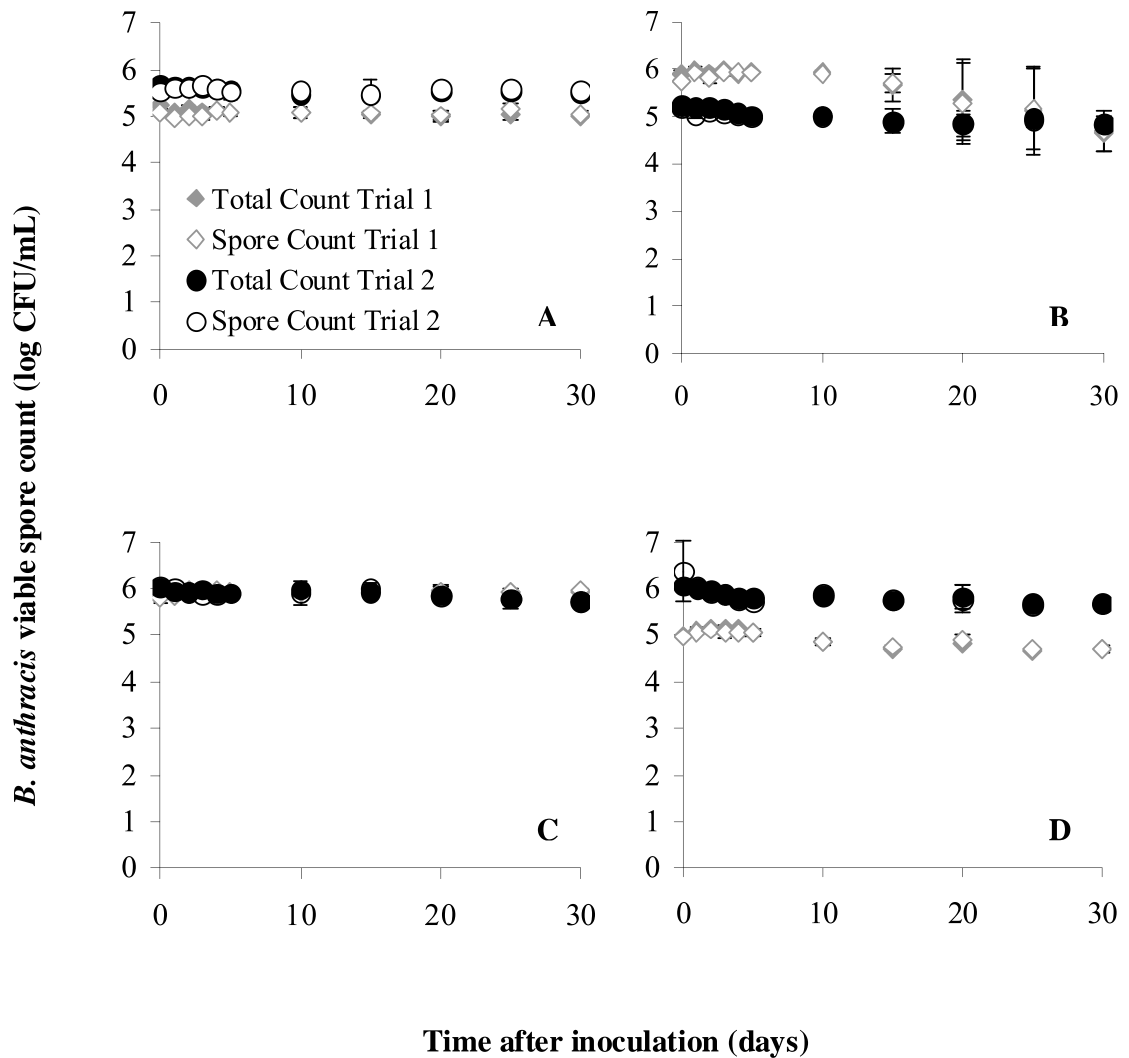

Figure 34. Spore survival of *B. anthracis* strain UT258 in orange juice (**A**) trial 1 pH 4.3, trial 2 pH 3.4; apple juice (**B**) trial 1 pH 3.4, trial 2 pH 3.37; grape juice (**C**) trial 1 pH 2.9, trial 2 pH 2.84; and wine **(D**) trial 1 pH 3.0, trial 2 pH 3.08

Following preliminary trials of the application of the centrifugal concentration and extraction to apple, grape, and orange juice it became clear that the juices presented extremely different challenges. Apple and grape juice both contained an extremely small

fraction of insoluble particulates that were collected during concentration. This resulted in the formation of a pellet that was not easily visible. Additionally, the spore pellet appeared be unstable during decanting, resulting in low recovery during preliminary trials. In order to stabilize the pellet during and after centrifugation, 0.1 g cornstarch was added to each 100 mL volume prior to centrifugation. The cornstarch formed a visible pellet in the centrifugation bottle. The spore recovery rate appeared to by highly variable from 25 to 90% and was not dependent on the initial spore count, but did effectively increase the concentration of spores by 1 to 2 log units in the extracted fraction (Fig. 35). Cornstarch was selected as it is relatively inert, insoluble at room temperature, and inexpensive.

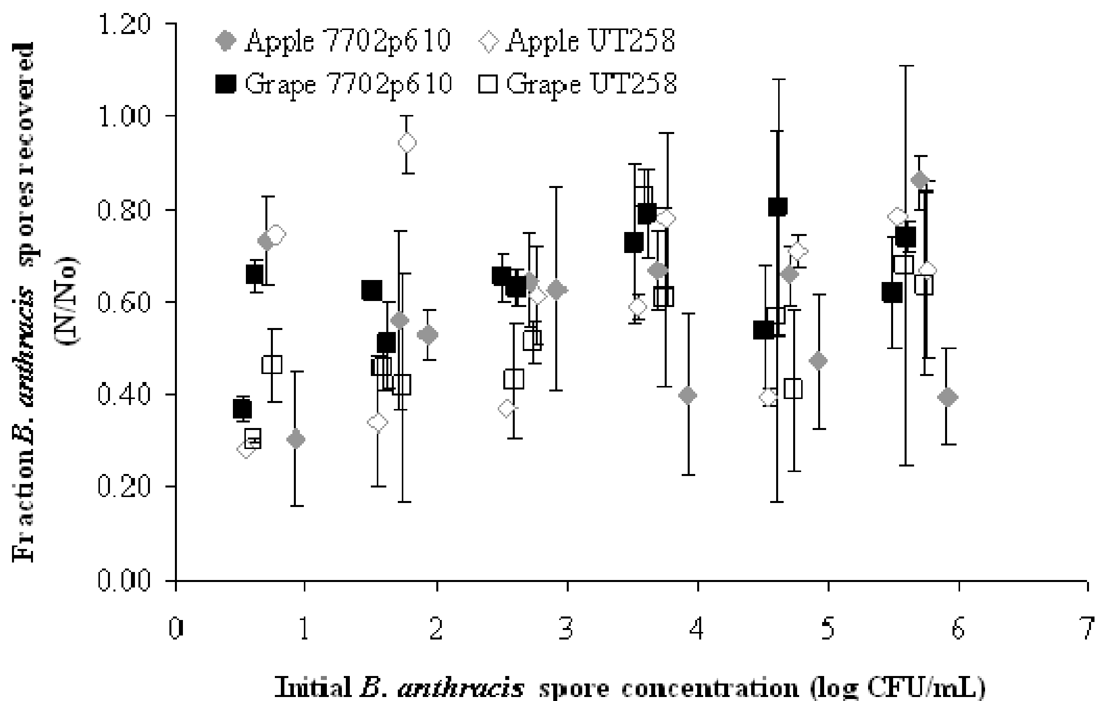

Figure 35. Recovery fraction of *B. anthracis* spores from apple and grape following the addition of 0.1 g cornstarch/ 100 mL juice prior to centrifugation at 1,500 × *g* for 10 minutes. N: spores recovered from pellet, and No: initial spore count.

Orange juice had high solids content despite being pulpless and produced extremely large, viscous, solids pellets following centrifugation. The pellet volume was over 10 mL and was extremely difficult to pipette. Preliminary trials clearly indicated a need to reduce the solids content of the juice prior to *B. anthracis* spore concentration.

Large particulate removal was optimized using centrifugation as opposed to filtration due to rapid membrane fouling during filtration attempts. Optimal conditions for large particulate removal were determined to be 5 minutes at 655 × *g*, due to the high fraction of spores remaining in the supernatant after centrifugation and the removal of a large amount of particulates (Fig. 36). Although centrifugation at speeds lower than 655 × *g* resulted in greater spore recovery, the juice solids did not form a stable pellet which was difficult to separate from the supernatant. Application of the spore concentration procedure to the supernatant directly resulted in the formation of an extremely large and viscous pellet, which was greater than 5 mL volume and difficult to pipette without prior dilution. This serious limitation led us to test various methods of combination filtration and centrifugation processes to further separate spores from orange juice solids.

Numerous combinations of filtration and centrifugation of the resulting supernatant were examined. In preliminary trials, differential centrifugation alone consistently resulted in co-extraction of spores and solids. The addition of filtration steps to separate spores from juice solids in the supernatant after large particulate removal continued to be hampered by rapid filter fouling, even in large pore filters (~20 µm).

These results precipitated the exclusion of orange juice as a target for pre-analytical extraction using centrifugation in this study.

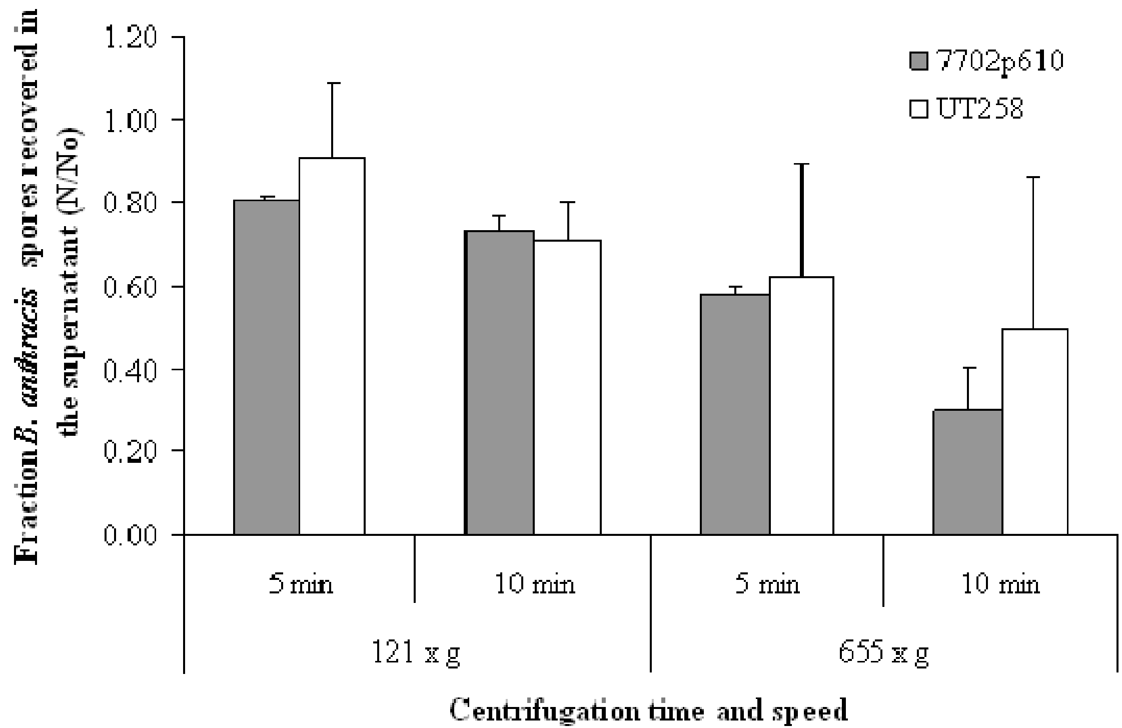

Figure 36. Recovery fraction for optimization of centrifugation conditions for large particulate removal from orange juice. Error bars represent 95% confidence intervals. N: spores recovered from pellet, and No: initial spore count.

Lateral flow assays were able to consistently detect concentrations of 10^7 spores/mL *B. anthracis* UT258 or greater suspended in sterile water, apple juice, and grape juice after 30 minutes (Table 13). Samples of orange juice were not compatible with the lateral flow assay due to membrane fouling in the sample port caused by the orange juice solids. Application of the centrifugal concentration procedure lowered the

limit of detection to 10^6 *B. anthracis* UT258 spores/mL in the initial sample of both apple and grape juices (Table 14).

Table 13. Sensitivity of *B. anthracis* lateral flow assay to *B. anthracis* strain UT258 suspended in various media. Performed as two experiments with two replicates each.

Spore suspension matrix	Target spore concentration (CFU/mL)	Actual spore concentration (CFU/mL)	Positive results at 15 minutes (%)	Positive results at 30 minutes (%)
Sterile distilled water	10^8	Trial 1: 7.6×10^7 Trial 2: 2×10^7	4/4 (100%)	4/4 (100%)
	10^7	Trial 1: 7.6×10^6 Trial 2: 2×10^6	2/4 (50%)	4/4 (100%)
	10^6	Trial 1: 7.6×10^5 Trial 2: 2×10^5	0/4 (0%)	0/4 (0%)
Apple juice	10^8	Trial 1: 5.2×10^7 Trial 2: 2.4×10^7	4/4 (100%)	4/4 (100%)
	10^7	Trial 1: 5.2×10^6 Trial 2: 2.4×10^6	0/4 (0%)	4/4 (100%)
	10^6	Trial 1: 5.2×10^5 Trial 2: 2.4×10^5	0/4 (0%)	0/4 (0%)
Grape juice	10^8	Trial 1: 5.4×10^7 Trial 2: 2.5×10^7	4/4 (100%)	4/4 (100%)
	10^7	Trial 1: 5.4×10^6 Trial 2: 2.5×10^6	0/4 (0%)	4/4 (100%)
	10^6	Trial 1: 5.4×10^5 Trial 2: 2.5×10^5	0/4 (0%)	2/4 (50%)

Table 14. Results of lateral flow assay with grape and apple juice after concentration processing. Performed as two experiments with two replicates each.

Fruit juice	Initial target spore concentration (CFU/mL)	Actual initial spore concentration (CFU/mL)	Final spore concentration (after processing) (CFU/mL)	Positive results at 15 minutes	Positive results at 30 minutes
Apple	10^7	Trial 1: 4.1×10^6 Trial 2: 6.1×10^6	Trial 1: 2.2×10^8 Trial 2: 4.5×10^8	4/4 (100%)	4/4 (100%)
	10^6	Trial 1: 4.1×10^5 Trial 2: 6.1×10^5	Trial 1: 1.9×10^7 Trial 2: 5.0×10^7	0/4 (0%)	4/4 (100%)
	10^5	Trial 1: 4.1×10^4 Trial 2: 6.1×10^4	Trial 1: 2.0×10^6 Trial 2: 3.3×10^6	0/4 (0%)	1/4 (25%)
Grape	10^7	Trial 1: 4.1×10^6 Trial 2: 5.3×10^6	Trial 1: 3.1×10^8 Trial 2: 3.2×10^8	4/4 (100%)	4/4 (100%)
	10^6	Trial 1: 4.1×10^5 Trial 2: 5.3×10^5	Trial 1: 3.4×10^7 Trial 2: 2.0×10^7	3/4 (75%)	4/4 (100%)
	10^5	Trial 1: 4×10^4 Trial 2: 5.3×10^4	Trial 1: 2.7×10^6 Trial 2: 3.0×10^6	1/4 (25%)	3/4 (75%)

An external standard curve was created prior to testing any sample using the Light Cycler system using serially diluted DNA extracted from a known amount of *B. anthracis* ANR-1 spores. Results of both standard curve trials were highly linear, with an $R^2 = 0.97$

(see appendix D). One set of these standards was also included in each Light Cycler trial to ensure that amplification is consistent between trials (see appendix E).

DNA samples extracted via the UltraClean kit from orange juice inoculated with *B. anthracis* 7702, ANR-1, 7702p610 or UT258 were detected using both standard and real-time PCR. Standard PCR was not able to consistently detect samples of orange juice containing 10^6 spores/mL, but did demonstrate a 210 bp product as expected when present (see appendix E). Real-time PCR results showed variability in extraction efficiency between trials. The limit of detection for real-time PCR from orange juice samples was 10^4 *B. anthracis* spores/mL (Fig. 38, Table 15).

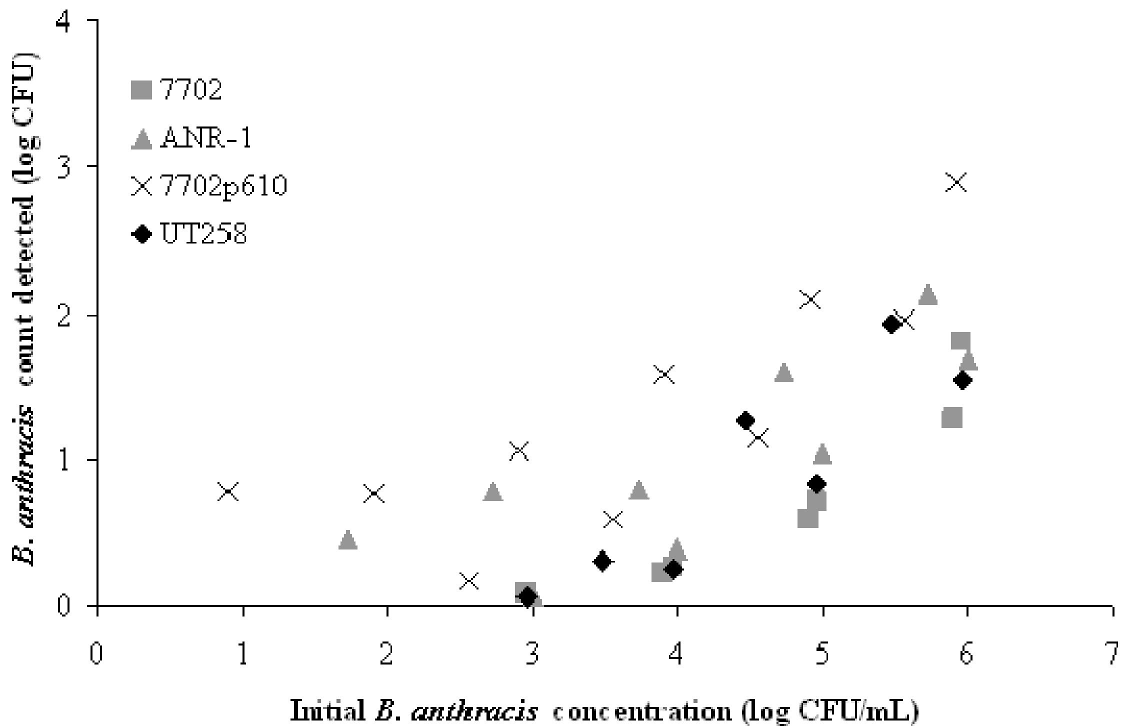

Figure 37. *B. anthracis* detection via real-time PCR directly extracted from pulpless orange juice.

Table 15. Limits of detection of *B. anthracis* spores from orange juice using real-time PCR and standard PCR. * Limit of detection greater than highest concentration tested (10^6 spores/mL)

	Limit of Detection (spores/mL)	
Strain	**Real-time PCR**	**Standard PCR**
7702	10^4	ND*
ANR-1	10^3	ND*
7702p610	10^3	ND*
UT258	10^4	ND*

The application of real-time PCR to juice samples containing *B. anthracis* spores following DNA extraction obtained a limit of detection of 10^3 *B. anthracis* spores/mL from apple juice and 10^4 *B. anthracis* spores/mL from grape juice (Fig. 38, Table 16). Samples from concentrated samples consistently were detected at higher levels of *B. anthracis* and had significantly lower C*t* values than the un-concentrated counterpart samples though the extraction efficiency varied between trials (Table 17).

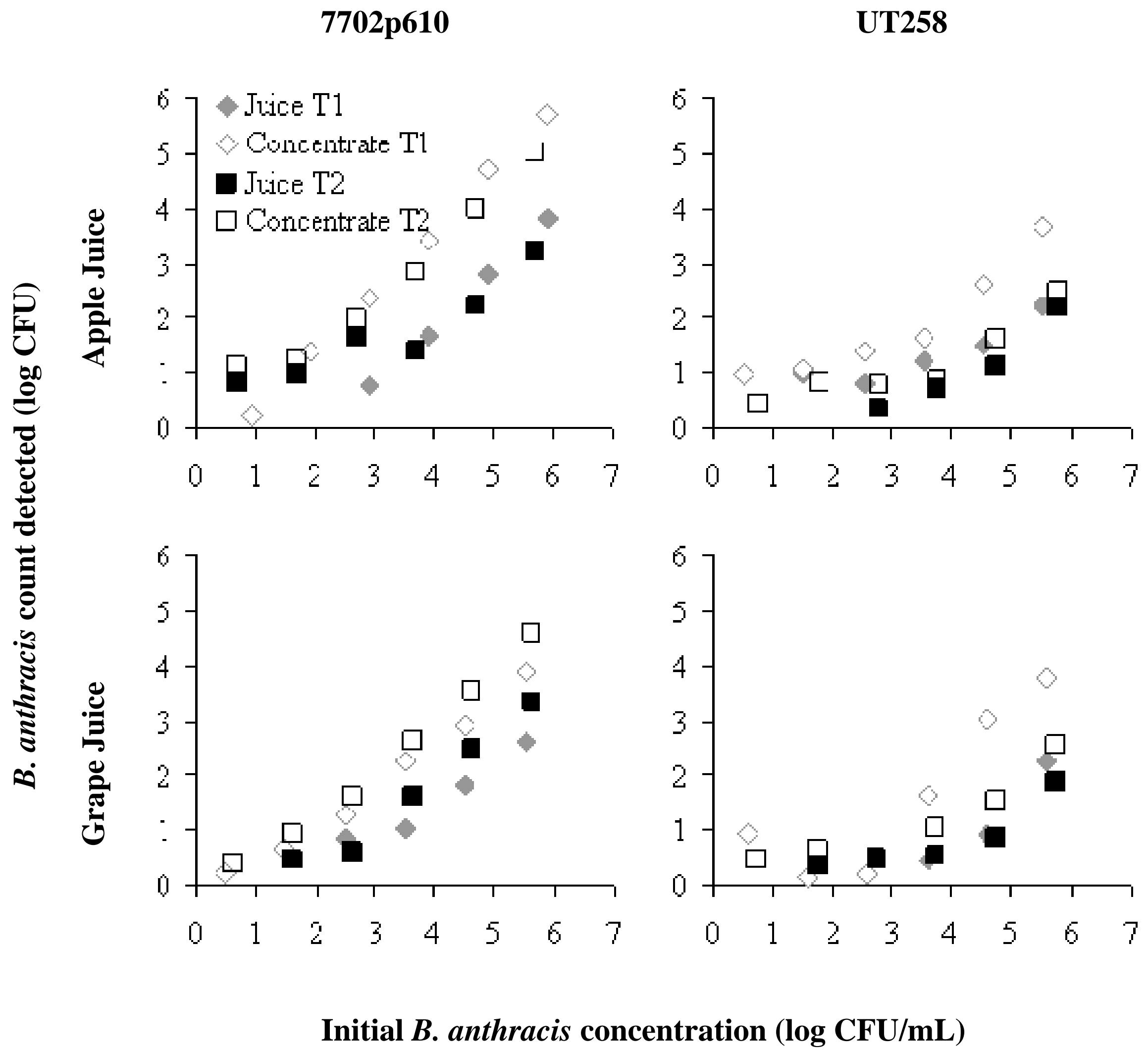

Figure 38. Detection of *B. anthracis* extracted from apple and grape juice via real-time PCR, with and without prior concentration. T1 and T2 denote trials 1 and 2.

Table 16. Limits of detection of *B. anthracis* spores extracted from apple and grape juice via real-time PCR, with and without prior concentration

Strain	Limit of detection (LOD)[a] *B. anthracis* spores/mL			
	Directly from juice (no concentration)		Juice following concentration	
	Apple Juice	Grape Juice	Apple Juice	Grape Juice
7702p610	10^3	10^3	10^1	10^1
UT258	10^3	10^4	10^1	10^1

Table 17. Mean cycle threshold (C*t*) difference between processed and unprocessed samples. Processed samples amplified at a lower C*t* than non-processed counterpart.

Juice	Strain	*B. anthracis* initial spore concentration per mL					
		10^1	10^2	10^3	10^4	10^5	10^6
Apple	7702p610	1.07[a]	1.00[a]	1.27	4.03	6.54	9.90
	UT258	ND[b]	2.92[a]	1.61	1.65	2.44	5.97
Grape	7702p610	ND[b]	1.86[a]	3.40	4.41	4.16	4.57
	UT258	ND[b]	1.13[a]	0.20[a]	2.40	4.98	4.24
	Overall mean *	1.07[a]	1.72 ± 0.61	1.77 ± 1.11	3.12 ± 0.91	4.53 ± 1.18	6.17 ± 1.80

[a] Only one pair of values compared

[b] Not detectable in any non-concentrated samples

* ± indicates 95% confidence interval

5.4 Discussion

The current project examined the survival of *B. anthracis* spores in four different liquid foods as well as the application of a centrifugation protocol for extraction and concentration of the spores from the food media prior to detection. This project obtained important information for the assessment of these liquid foods as potential targets for bioterrorist attacks utilizing *B. anthracis.* This project also addresses a key issue in food defense, compatibility of food samples with rapid detection technologies for efficient detection of bioterrorist agents in foods.

As a spore, *B. anthracis* is generally resistant to the influences of many environmental factors (178). Fruit juices contain potential deleterious factors to spore survival in the form of organic acids, some of which are known to have bactericidal properties, in addition to a low pH (49). Wine combines many of the same properties as grape juice with the addition of alcohol and other compounds generated by fermentation which also have potential to affect spore viability. Although this study did not address the cause of the slight loss of viable spores in apple juice and wine, it is not likely due to acidity alone, as grape juice was the most acidic product tested and demonstrated little to no loss of *B. anthracis* spore viability. The slight loss in viable spore population could have been due to the specific combination of organic acids in the apple juice and wine, or other components of the product. This study indicated that *B. anthracis* spores can survive extended periods in fruit juices and wine without more than 1 log loss in viable spore population. This finding suggested that *B. anthracis* spores added to fruit juice

may present a potential bioterrorism hazard and stressed the importance to rapidly detect *B. anthracis* in fruit juices is critical to food defense.

This study also examined the efficacy of a pre-analytical concentration and extraction centrifugation protocol for the rapid concentration and separation of the spores from fruit juices. The centrifugation protocol, designed and successfully applied to concentration of *B. anthracis* spores from milk, was effective for both apple and grape juice with the addition of cornstarch, but not effective for extraction from orange juice. Neither the apple nor grape juice contained enough other solids to form a stable spore containing pellet during centrifugation without the addition of starch. Extraction from samples of apple and grape juice inoculated with *B. anthracis* spores had the same limit of detection (10^7 spores/mL) as spores suspended in water. The lower limit of detection was reduced by 1 log, to 10^6 *B. anthracis* spores/mL in the initial sample, following the application of the centrifugal concentration protocol. This result corresponded with the concentration effect of the centrifugation protocol, with a 1 to 2 log CFU/mL increase in concentration from the initial sample.

The MoBio UltraClean kit showed variable efficiency, but effectively extracted *B. anthracis* DNA from spores suspended in fruit juices. The lower limit of detection for spores suspended in grape and orange juice using real-time PCR was 10^4 *B. anthracis* spores/mL and 10^3 spores/mL for spores suspended in apple juice. The previous limit of detection reported by Dauphin et at, 2009, was 10^3 spores/mL when spores were suspended in water (48). Following the application of the centrifugal concentration procedure to apple and grape juice, the lower limit of detection was reduced to 10 spores/mL.

The greater reduction in the limit of detection of real-time PCR versus the lateral flow assay may be attributable to the affect of juice compounds on the specific detection platform. In the case of the lateral flow assay, spores suspended in fruit juice had the same lower limit of detection as spores suspended in water, whereas the limit of detection for spores directly extracted from fruit juice was greater than that previously demonstrated in water or in milk (48). As the lateral flow assay appeared to be unaffected by juice, the extraction component of the procedure is less important than the concentration effect. Hence the decrease in the limit of detection of the lateral flow assay was limited by the increase in spore concentration in the sample. Detection of *B. anthracis* spores appeared to be affected by juice compounds following extraction from the product directly. In this case, the pre-analytical procedure may serve to both reduce the presence of juice compounds that may interfere with DNA extraction or detection, as well as concentrate the spores in the sample, leading to a greater reduction in the limit of detection.

B. anthracis spore extraction from orange juice was extremely difficult as opposed to apple and grape juice. This was caused by the high solids content of the juice, such that the solids could not readily be separated from the spores using filtration or centrifugation alone. Although modified procedures were tested, all were either inconsistent, impractical, or resulted in the continued presence of large quantities of orange juice solids. Preliminary trials demonstrated that direct filtration of the juice or an initial particulate removal step from orange juice using either vacuum or syringe filtration resulted in rapid membrane fouling. Although this problem could potentially be solved through the use of continuous filtration through a hollow fiber cartridge, this would

significantly increase the system cost and potentially hamper applicability. Other options for pre-analytical extraction and concentration of *B. anthracis* spores from orange juice include metal hydroxide, lectin, aptamer, and immunoassay separation (16, 226). These separation techniques may be more complex than simple centrifugation, but may be more effective and circumvent the complicating effects of the high solids content.

Results from this project suggest that spores of *B. anthracis* remained highly viable in the fruit juice environment following contamination for at least 30 days. The MoBio UltraClean kit may be effectively used to extract *B. anthracis* DNA from orange, apple, and grape juice directly, as well as from concentrated samples of apple and grape juice using the outlined centrifugation procedure. Concentration of samples prior to detection lowered the limit of detection using both lateral flow assays and real-time PCR by 1 to 3 log in the initial sample. Although application of either real-time PCR or lateral flow assays in a production facility at this time may be cost-prohibitive, sample pooling may be an option to reduce testing cost. This procedure represents a method for improving the security of the food supply by effectively screening fruit juices for potential *B. anthracis* contamination.

Chapter 6

Concentration, extraction, and detection of ricin via immunomagnetic separation

Ricin is a toxic protein derived from the castor bean, which has been previously used for bioterrorism purposes. Although many methods have been developed for rapid detection of ricin, few systems have been developed or tested for pre-analytical concentration and extraction of ricin from liquid systems. The successful application of a pre-analytical method would permit larger volumes of material to be screened for the presence of ricin. Immunomagnetic separation (IMS) systems are commonly used to effectively and specifically extract target bacteria from mixed cultures or food samples. This study evaluated the efficacy of a manual and a recirculating IMS systems for the novel application of extraction and concentration of ricin toxin from a liquid system. Immunomagnetic beads coated with anti-ricin antibodies and specifically prepared for this project were capable of binding ricin using a manual protocol. However, recovery of ricin by a recirculating IMS Pathatrix® system was confounded by the relatively high level of non-specific binding of ricin to the system components. Although difficulties eluting ricin from the beads prevented direct evaluation of ricin concentration, this study is the first provide proof of concept that a protein toxin can be specifically captured from a simple liquid system using manual immunomagnetic separation.

6.1 Introduction

Ricin is a ribosomal inactivating type II protein (RIP II) found in castor bean (*Ricinus communis*). The protein is a powerful toxin which acts by inhibiting protein synthesis through the specific depurination of 28s rRNA (6, 52, 89, 158, 212). Ricin can be fatal to humans and animals through injection, inhalation, and ingestion (52). The potent nature of ricin and other related plant derived protein toxins, such as abrin (derived from *Abrus precatorius*, the rosary pea) and modeccin (derived from *Adenia digitata*, wild granadilla), as well as their raw material availability make these compounds attractive for potential bioterrorism (182, 210, 231).

Ricin is 66 kDa molecule composed of two protein chains (A and B) linked together by a disulphide bond (158, 181). The B chains (34 kDa) acts as a lectin, binding to terminal galatose residues on cell surface (158). The A chain (32 kDa) is the enzymatically active portion of the molecule and is translocated into the cell cytosol where it reacts with 28s rRNA (67, 158). The enzymatic removal of adenine from the 28s rRNA is highly efficient, with a Km value of 2.6 μM (68). The enzymatic action of the A chain destabilizes the ribosome to the point were is can no longer produce protein, leading to cell death and potential systemic failure (101, 158, 181, 182).

Ricin is currently listed as a class B bioterrorist agent by the Centers for Disease Control and Prevention (37). It is also one of the most common plant toxins attempted or used for intentional poisonings (80, 126, 235). Ricin was also a popular agent developed and purified by a number of state-sponsored bioweapons programs, including that of the United States, the former USSR, and Iraq (105, 151). The most notable incident of ricin poisoning is the assassination of Bulgarian journalist Georgi Markov in 1978 by injection

of a ricin pellet through an umbrella (42, 151). More recently, in 2003 ricin was found in mail bound for the White House and in 2008 was found in a Las Vegas, NV hotel room (92, 235).

Ricin toxicity varies based on the route of exposure and is most potent via injection or inhalation. The LD_{50} (lethal dose 50) for injected or inhaled ricin in mice is approximately 10 μg/kg (89). LD_{50} estimates for ingested ricin range from 30 μg/kg to 20 mg/kg in humans and are thought to be higher due to decreased absorption via the gastrointestinal tract and potentially enzymatic breakdown by gastric enzymes (6, 89, 212). Despite the increase in LD_{50} via ingestion, ricin could potentially be used as a bioterrorism agent added to food or water due to the ease of production from spent castor bean mash (28). Other plant lectins, such as abrin and modeccin, are more toxic than ricin and could also be used as biological weapons in this manner, but may be more difficult to produce in large quantity (94, 101, 210).

Although numerous sensitive and specific assays have been designed to detect ricin, many of these systems can only be applied to small samples. Using small samples to represent a large volume may not be representative when screening for ricin or other toxins contamination. This may particularly be a problem for liquids, such as water or liquid foods which may be targeted for bioterrorist attack (28). This project examines the use of manual IMS and the recirculating IMS Pathatrix® system as potential pre-analytical methods to concentrate and extract ricin from a liquid samples. If effective, this method could be used to reduce the number of assays needed to screen large sample of food or water potentially contaminated with ricin, thus improving the safety and security of the food supply.

6.2 Materials and Methods

Ricin

Ricin was obtained from Vector Labs (Burlingame, CA). All ricin was stored at 4°C in a locked cabinet and disposed of in accordance with institutional biosafety committee guidelines.

ELISA assays

Ricin ELISA assays, with both positive and negative (+/-) wells, were obtained from Tetracore Inc. (Rockville, MD). Assays were performed as per manual instructions with the addition of a stop solution added after 30 minutes of the final ABTS reaction and read within five minutes. The reaction was stopped by adding an equal volume of 0.01% sodium azide in 0.1 M citric acid as per the Invitrogen ABTS MSDS (Camarillo, CA). The absorbance at 405 nm was read using a Synergy HT plate reader (Biotek Instruments Inc., Winooski, VT). All samples were run at minimum in duplicate on the ELISA plate and mean values used to calculate ricin concentration. Standards of 100 ng/mL, 50 ng/mL, 25 ng/mL, 12.5 ng/mL and 6.25 ng/mL were run in duplicates with each plate to generate a standard curve based on the mean absorbance. Samples with concentrations above the linear range of ELISA detection were diluted in phosphate buffered saline (PBS) prior to assay application.

Anti-ricin antibody used for magnetic bead coating

Polyclonal goat anti-ricin IgG antibody was obtained from Tetracore Inc. (Rockville, MD). Immunomagnetic beads were purchased from Matrix MicroSci (Golden, CO) were coated at a level of 1 mg anti-body/1 mL beads using the protocol specified by the manufacturer.

Immunomagnetic separation

Manual IMS was performed using the immunomagnetic beads with and without anti-ricin antibody coating. Standard solutions of ricin at varying concentrations were prepared and stored in sterile phosphate buffered saline (PBS, pH 7.2). One mL volumes of ricin solution were aliquoted into 2 mL screw cap tubes, and 50 µL of beads, with or without 1 mg/mL anti-ricin antibody coating were added to ricin samples. The tubes were closed and sealed with parafilm, then incubated at 37° C with end-over-end mixing. Following incubation, the immunomagnetic beads were extracted via magnet, supernatants were removed and saved, and the beads resuspended in an equal amount of PBS in new tube. Experiments examining the whole ricin samples plus immunomagnetic bead samples were split following incubation, only one of which was separated into beads and supernatant. Extracted immunomagnetic beads were then resuspended in a volume of PBS equal to the original split sample volume. Individual volumes of 100 µL the ricin sample, the ricin sample containing beads, the separated and resuspended beads, and supernatant were applied to sample wells, and then processed according to the ELISA protocol.

Bead beating

Samples (1 mL) of 100 ng/mL ricin in PBS were mixed with 50 µL of anti-ricin immunomagnetic beads for 60 min at 37°C as in standard IMS. Following mixing, supernatants were removed and saved for ricin quantification. The remaining beads were resuspended in 1 mL fresh PBS and transferred to new 2 mL screw cap microcentrifuge tubes containing 0.25 g 1-mm glass beads (Biospec, Bartlesville, OK). The new tubes were closed, sealed with parafilm, and bead beat for varying amounts of time using a mini bead-beater (WU-36270-07, Cole-Parmer, Vernon Hills, IL). Following bead beating, the supernatant was separated from the beads using a magnet. All samples were stored at 4°C when not actively being used. Ricin in initial supernatant and bead beat samples were measured using Tetracore +/- ELISA assay. All experiments were completed in duplicate, with 2 replicates per trial and a minimum of two ELISA measurements per sample.

Recirculating immunomagnetic separation via Pathatrix

The recirculating IMS system used was the Pathatrix Mini-Ultra (Matrix MicroScience, Golden, CO), utilizing a 50 mL initial sample size. The system was used with a 60 min run time, incubation temperature of 37°C, and the addition of 50 µL of either 1 mg/mL anti-ricin beads, unconjugated beads (negative bead control), or no beads at all (negative system control). In order to maintain an undiluted effluent sample, no additional PBS was added to the collection vessel and effluent samples were collected and stored in sterile 50 mL tubes at 4°C until used in the ELISA (within 12 h).

Experiments were run in duplicate trials, using three replicates per trial. All samples were measured in duplicate in the ELISA assay.

6.3 Results

Prior to any experimentation using IMS, a standard curve was prepared for the Tetracore ricin ELISA kit. Figure 39 shows the mean standard curve generated as part of this project. Both the shape and absorbance values were similar to the standard curve provided by the manufacturer. From the logarithmic scaled standard curve data, there appears to be a linear portion of curve between 78 ng/mL and 2.5 ng/mL. When linearly rescaled, the R^2 value of the linear function in this range is 0.66. However subsequent standard curves generated experiments were based on dilution in larger vessels, as opposed to the 'in-well' dilution described in the ELISA kit manual, and resulted in higher R^2 values for linear regression, as well as overall lower absorbance values (Fig. 40). Although each trial was relatively linear, the range of absorbance values was highly variable between assay plates resulting in large error bars. Subsequently, standard curves were included in all sample measurements and used to calculate ricin concentration in samples included in the same plate.

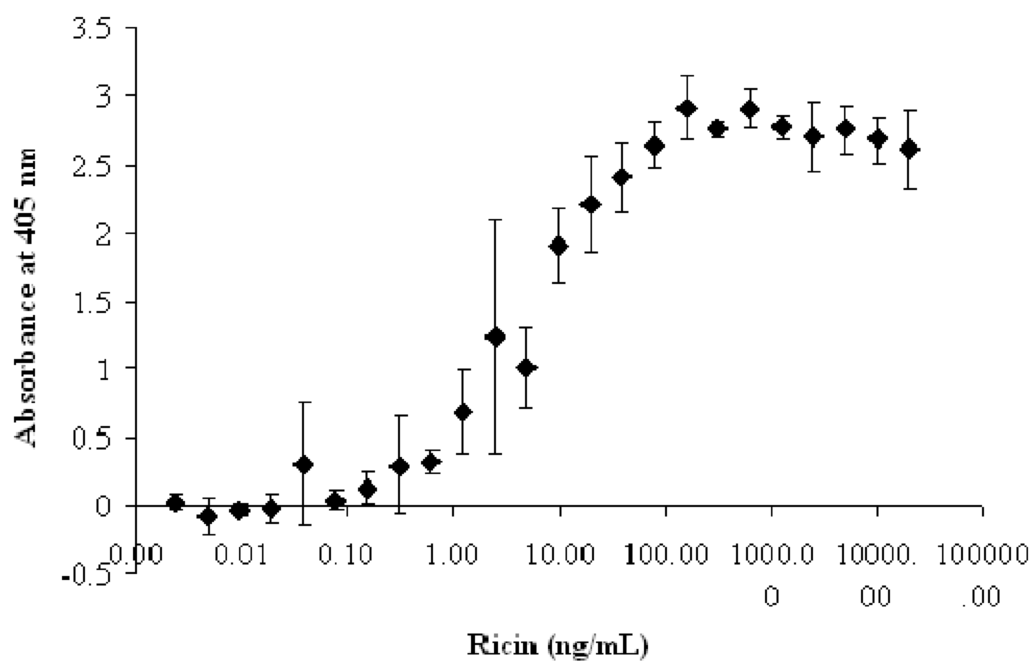

Figure 39. Ricin standard curve generated using Tetracore © ricin ELISA kit. Error bars represent 95% confidence intervals.

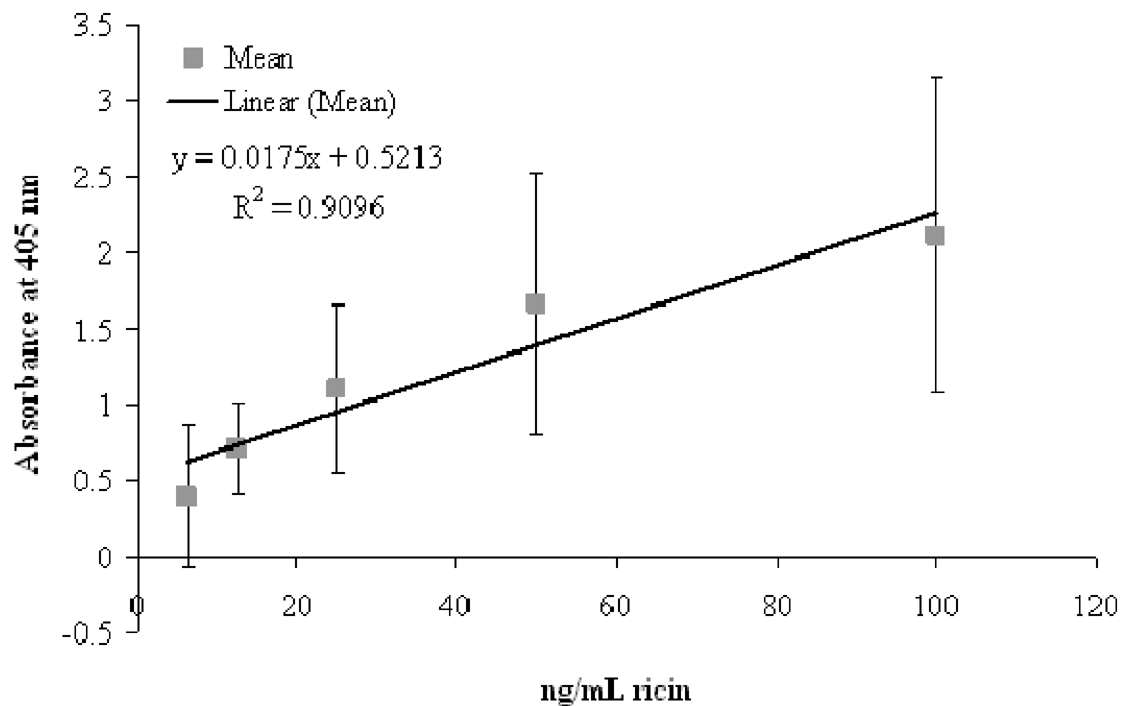

Figure 40. Standard curve generated from ricin dilution prior to sample application. Error bars represent 95% confidence intervals from replicate trials.

As both immunomagnetic beads and ricin ELISA kits used anti-ricin antibodies to capture the toxin, concern arose over potential competition to bind ricin between the beads and ELISA assay. In addition, Pathatrix beads used directly with the ELISA are washed out during plate washing and have the potential to reduce the amount of ricin bound to the ELISA plate. This concern was confirmed by the highly variable affect of beads on the results of the ELISA assay (Fig. 41). Subsequently, it was concluded that samples containing anti-ricin Pathatrix beads were not suitable for direct quantification via ELISA. Additionally, samples of anti-ricin bead separated from the supernatant were not measured to have as much ricin as expected from either measurements of the whole

sample or the bead-free supernatant (Fig. 41). Although these results are somewhat confusing, it is possible that magnetic separation and resuspension alters the binding of ricin to the beads, further complicating accurate measurement. However decreased ricin in the supernatant, which does not contain any beads, suggests that the ricin is bound to the beads despite measurement difficulties.

Bead binding experiments illustrated the time dependence of binding. Results indicated that the fraction of ricin concentration remaining in the supernatant was not significantly different at any concentration of ricin between 30 and 60 min incubation periods (p-values, =0.14, 0.74, 0.62 at 25, 50, and 100 ng/mL, respectively) (Fig. 41). Despite the lack of statistical difference, the 100 ng/mL sample incubated for 30 min contained an average ricin concentration in the supernatant almost twice that of samples incubated for 60 min. Thus the 60 min incubation time was chosen for all subsequent experiments including Pathatrix.

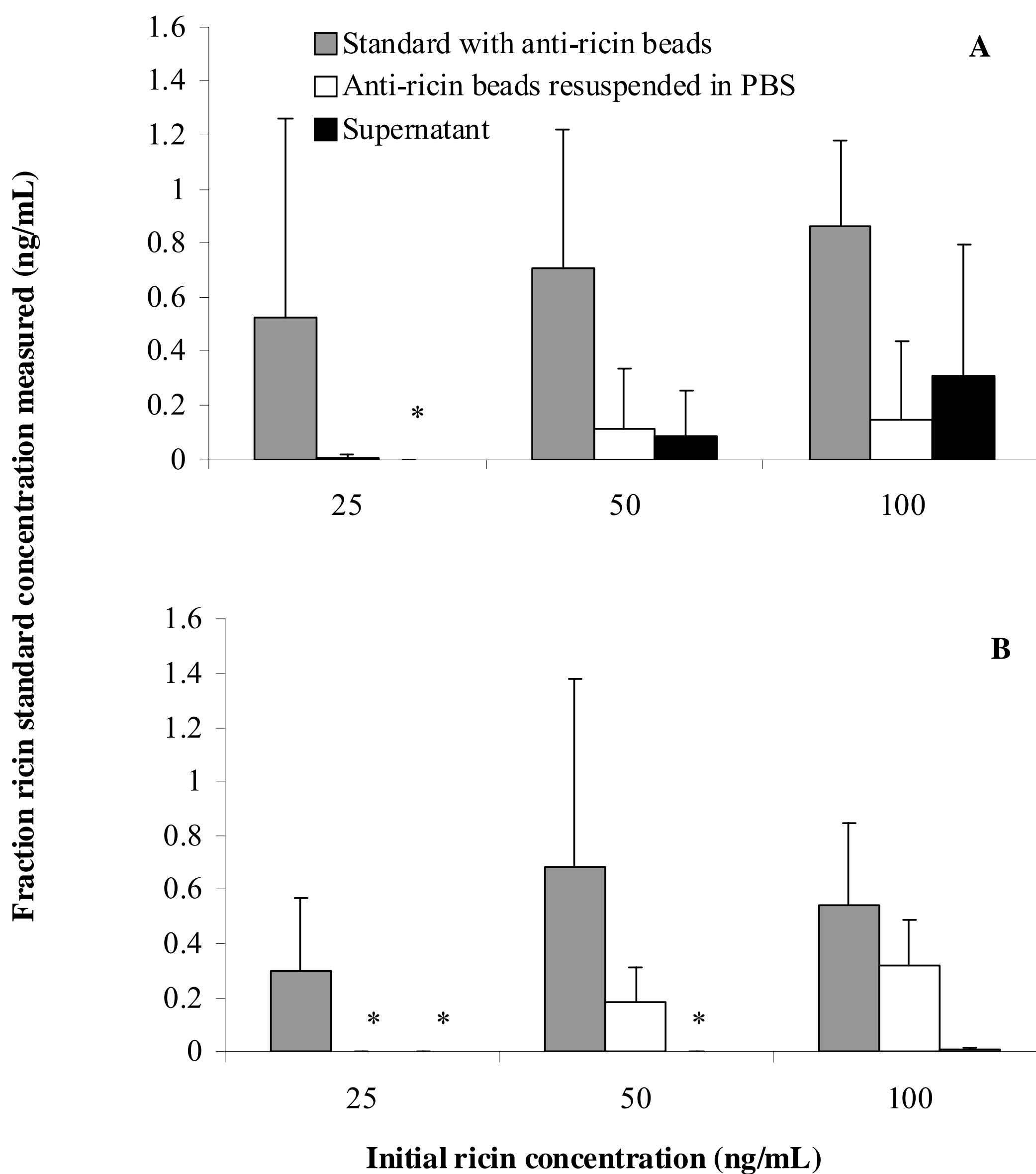

Figure 41. Ricin measured by ELISA after 30 or 60 min incubation at 37°C in standard samples containing 50 μL of anti-ricin beads, the anti-ricin beads extracted and resuspended in PBS, and the remaining supernatant after bead extraction, as a fraction of ricin in the standard. Error bars represent 95% confidence intervals. A, 30 min; B, 60 min. * Below linear range of detection. .

Although some ricin could be detected in the samples following bead beating, it did not correspond to the reduction of ricin concentration measured in the effluent (Fig. 42). This method was deemed ineffective and measurements of ricin remaining the in supernatant or effluent were subsequently used to measure ricin binding.

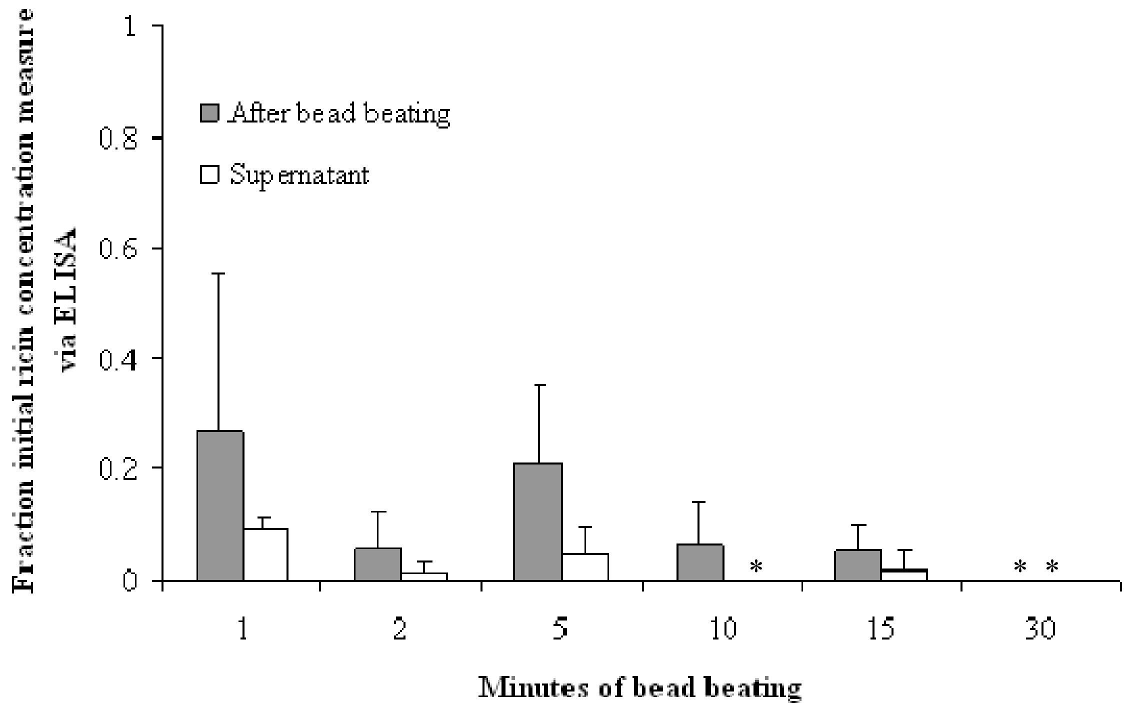

Figure 42. Fraction initial ricin concentration measured after bead beating and in supernatant after 60 min binding with 1 mL of 100 ng/mL ricin at 37°C with 50 µL anti-ricin beads. *Mean below linear detection limits. Error bars represent 95% confidence intervals.

The ricin binding capacity of the immunomagnetic anti-ricin beads was determined using increased concentrations of ricin in the standard IMS format. As anticipated, the fraction of total ricin remaining in the supernatant after bead binding

increased with increasing ricin concentration (Fig. 43). However, the fraction of the initial ricin remaining in the supernatant was not statistically different for any sample (ANOVA p-value=0.71).

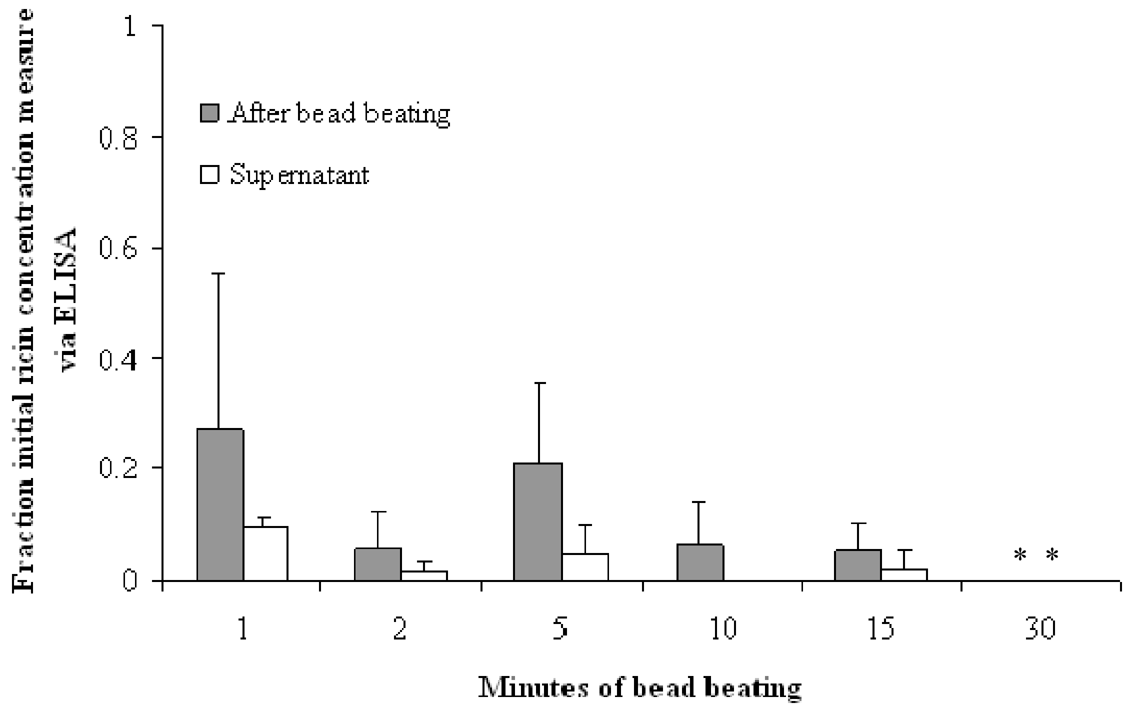

Figure 43. Fraction of initial ricin measured via ELISA in supernatant after 60 min of mixing with 50 μL of anti-ricin antibody coated beads. Error bars represent 95% confidence intervals.

The supernatant from ricin samples incubated with 50 μL of either anti-ricin antibody coated immunomagnetic beads or unconjugated immunomagnetic beads showed an antibody specific effect (Fig. 44). Significantly less ricin was measured in the

supernatant after incubation with anti-ricin antibody coated beads as opposed to the unconjugated beads (p-values of 0.01, and 0.03 for 100 and 500 ng/mL, respectively).

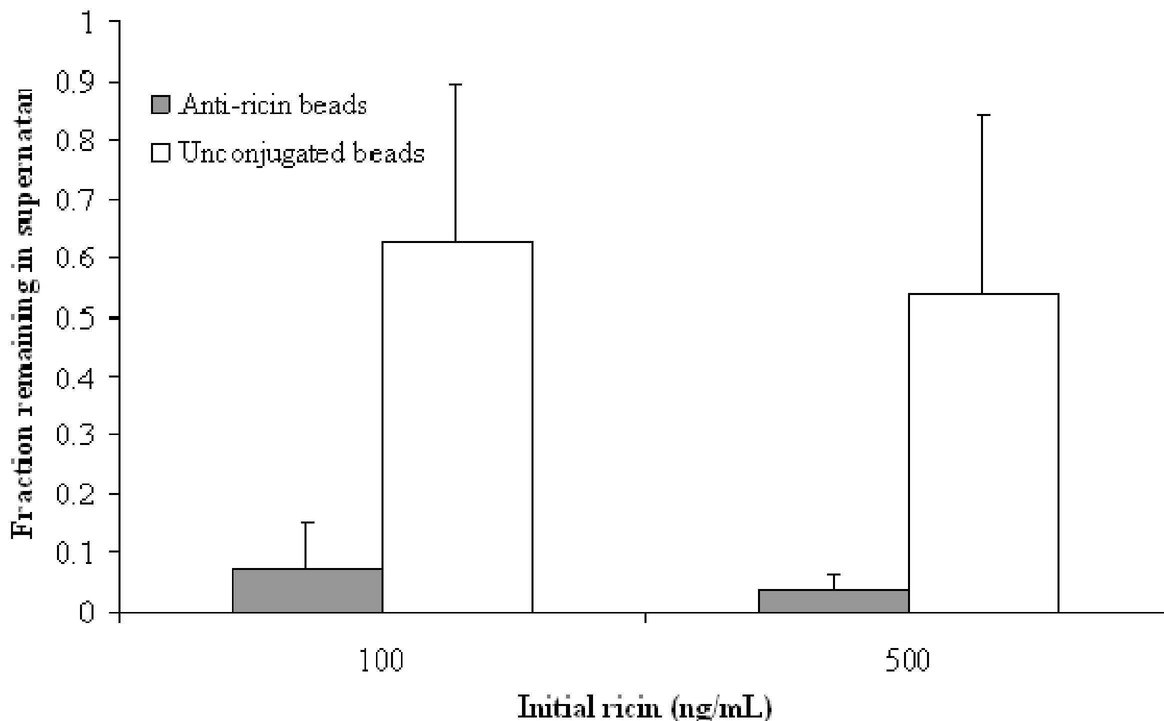

Figure 44. Fraction of initial ricin concentration measured in the supernatant after 60 min mixing with 50 µL of either beads coated with anti-ricin antibody or unconjugated beads. Error bars represent 95% confidence intervals.

The Pathatrix system demonstrated non-specific binding both in the system alone and with the addition of unconjugated beads (Fig. 45). The fraction of initial ricin remaining in the effluent was not statistically different between anti-ricin coated beads and unconjugated beads of 0.73 and 0.69, at 100 and 500 ng/mL, respectively). However, the addition of anti-ricin coated beads significantly decreased the concentration of ricin remaining in the effluent in both samples as compared the negative system

control (p-values of 0.02 and 0.05 at 100 and 500 ng/mL, respectively). In addition, the negative system control demonstrated significantly decreased ricin in the remaining in the effluent, indicating non-specific system binding (p-values of <0.01 at 100 and 500 ng/mL).

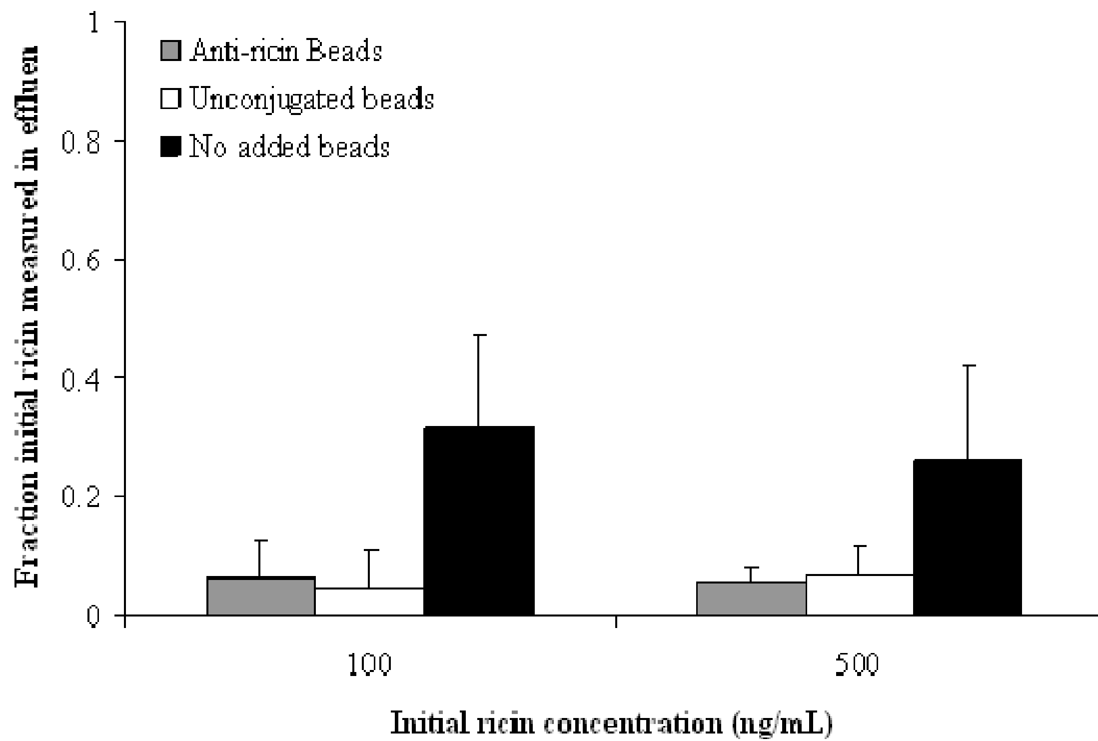

Figure 45. Fraction of initial ricin concentration measured by ELISA in effluent after 60 min Pathatrix binding with either 50 µl of 1 mg/mL anti-ricin beads, unconjugated beads, or no beads added to the system. Error bars represent 95% confidence intervals.

6.4 Discussion

The aim of the current project was to assess immunomagnetic separation as a possible pre-analytical concentration and extraction technique for ricin in liquids. However, this assessment was complicated by a number of factors. ELISA is not an ideal measurement tool for quantifying ricin from samples due to the variability incurred between assay plates, the multiple incubation/washing steps, and the small range of linear response. Despite the narrow range of linear response and variability between plates, the assay can be used to quantify ricin, with or without prior dilution, as long as internal standards are included.

Ideally, the sample obtained from immunomagnetic extraction would be directly compatible with the detection procedure without further processing. However, this project demonstrated that the immunomagnetic beads themselves had a highly variable affect on the ELISA assay. This is most likely due to the variability in how and when the beads are washed from the plate, as well as the competition between the assay and beads for ricin binding. In addition, separation of beads and supernatant after ricin incubation yielded less ricin detectable on the beads than expected based on the results from the whole sample prior to magnetic extraction of the beads or the ricin remaining in the bead-free supernatant. This result corroborates evidence that the presence of immunomagnetic beads interferes with ELISA assay detection of ricin. The variability and apparent decreased level of detection prevented accurate, direct quantification of the ricin bound to the beads.

Evidence from this study also showed the time dependence of bead-ricin binding, with more ricin remaining in the supernatant after 30 min of binding than after 60 min.

This result correlates to the 60 min binding time used for initial ricin binding in the ELISA assay.

Bead beating was tested as a method to elute ricin from the immunomagnetic beads, but was unsuccessful. Other possible elution techniques, such as papain digestion of the bead bound antibody, were considered but not used due to evidence of ricin digestion with the same enzyme treatment (29). This forced the use of indirect measurement of ricin binding through the supernatant (standard IMS) and effluent (Pathatrix recirculating IMS), which avoids the variable effect of the beads, but does not give direct evidence of concentration in the bead fraction. Other methods that could be considered for future work to release the ricin from the bead bound antibody, may include heat or pH denaturation of the antibody. However, no standard procedure exists for separating ricin from antibody using these methods. Additionally, the application of either of these methods would need to be verified to not alter the ricin molecule to preserve antigenic site for future immune-based detection assays. Another possibility may be to use the anti-ricin beads themselves as the primary phase for the ELISA using a 96 well magnet, although this method would not permit the use of an automated plate washer.

Anti-ricin antibody conjugated to immunomagnetic beads decreased the ricin concentration remaining in the supernatant at a wide range of concentrations when used in manual IMS. Manual IMS experiments found that the anti-ricin beads specifically bound ricin in the system. The anti-ricin beads appeared to have a binding capacity close to 20 µg of ricin. However, when applied to the Pathatrix system, the specific effect of the beads was no longer apparent. Non-specific binding of ricin in the system alone

decreased the concentration in the effluent, although the remaining ricin in the effluent was significantly less when anti-ricin beads were added. Although anti-ricin beads reduced the amount of ricin remaining in the supernatant significantly more than the unconjugated beads, some ricin was likely to be non-specifically bound. The combined effect of non-specific binding to the unconjugated beads and in the Pathatrix system may have overwhelmed the specific anti-ricin bead binding capacity.

Although the current results seemed discouraging for the future use of recirculating IMS for pre-analytical separation of protein toxins such as ricin, there were a number of factors not taken into account in this system based on ricin detection in the effluent. Any future work using IMS must first establish a methodology for rapidly and completely eluting ricin from the beads without altering the antigenic site, or develop an alternative quantification method directly compatible with the bead samples. Direct quantification of the ricin bound to the beads would be key to generate the proof of concept for using IMS as a method to extract ricin from liquid systems.

As the present study was only able to quantify the ricin remaining in the effluent, a wash step in the Pathatrix system was not utilized due to the need for an undiluted sample for quantification of ricin. Addition of this wash step to this type of indirect measurement would have diluted the effluent to a variable degree. However, if ricin bound to the beads could be measured directly, it is likely that the system wash step could decrease non-specific binding in the system. Additionally, after the system wash and bead collection from Pathatrix, the beads are washed and extracted a second time. This secondary wash would also help to reduce non-specific binding to give a better idea of the extraction efficacy of the antibody coated beads in a recirculating IMS system.

The findings of this project indicate that IMS may be suitable for extraction of protein toxins from simple liquid systems. However, prior to further investigation an alternative system of either toxin elution from the immunomagnetic beads or quantification method compatible with the beads is required to demonstrate active concentration in the extracted fraction to provide some proof of concept for this pre-analytical method.

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Appendix A. Development of a surrogate positive control for PCR detection of *Bacillus anthracis* plasmid pXO2

Recent incidents involving unreported laboratory infections have increased the concern to validate the virulence of attenuated and surrogate strains of infectious agents. Current U. S. Department of Health and Human Services' regulations for research on *Bacillus anthracis* allow an exemption from select agent certification for those laboratories that conduct research with strains that lack the pXO2 plasmid (capsule encoding and essential for virulence). Additional guidelines also restrict the presence of isolated pXO2 DNA in those laboratories. Due to these restrictions, the verification of the absence of pXO2 in working cultures is difficult. This work was undertaken to examine a plasmid isolation and digestion methodology and the development of a DNA construct that could be used as a positive control for pXO2. Plasmid isolation of pXO1 was successful, although subsequent digestion and characterization was not. A small fragment of the *capA* gene encoded in pXO2 was selected as a positive control surrogate for PCR. This DNA fragment was synthesized and cloned into *Escherichia coli* strain DH10b. A PCR protocol was optimized and tested with seven *B. anthracis* strains and three other *Bacillus* species. The genes *gyrB* and pagA were used as controls for *Bacillus* genera and *B. anthracis* species controls. . No strain was found positive for this portion of the *capA* gene with exception of DH10b. These results indicated that this strain could serve as a PCR positive control for *Bacillus anthracis* plasmid pXO2 to ensure the lab

protocols meet the current select agent guidelines and can be indefinitely propagated for future use.

A.1 Introduction

Bacillus anthracis is a spore-forming, Gram-positive bacterium that causes anthrax, and is classified as a category A select agent. Anthrax has three forms, cutaneous, inhalation, and gastrointestinal (51, 65, 221, 254). Virulent strains contain two plasmids, pXO1 and pXO2. Plasmid pXO1 (181 kb) encodes the genes of 3 major virulence factors, protective antigen (PA), lethal factor (LF), and edema actor (EF) (85, 103, 107, 172, 180) . The pXO2 (94 kb) plasmid encodes the genes involved in capsule production necessary for virulence (30, 31, 73, 100, 239).

As a category A select agent, laboratory use of fully virulent *B. anthracis* is strictly regulated. However, the use of strains lacking either pXO2 or both plasmids is exempt from select agent registration as these strains are estimated to be at least 10^6 times less virulent (34). Although many of these strains have been used previously without incident and reported not to contain pXO2, recent laboratory related infections with supposedly avirulent bacteria have heightened awareness of this issue among many institutional biosafety committees (IBC). As a result, IBC may require proof that none of the *B. anthracis* strains contain pXO2.

One solution to this problem may be the simple demonstration that strains used either contains no plasmids or that the single plasmid contained is pXO1. This may be accomplished by plasmid extraction and subsequent digestion using restriction enzymes. As both pXO1 and pXO2 have been sequenced, the pattern resulting from digestion with

various enzymes can be predicted. In addition, both Greene et al (100) and Kaspar and Robertson (132) demonstrated that different restriction enzymes can be effectively used to clearly distinguish pXO1 and pXO2.

Another simple solution to this problem would be the use of PCR for detecting the capsule encoding genes on pXO2. However, this cannot be demonstrated without a positive control. This causes a problem as potential positive controls, pXO2 containing and fully virulent *B. anthracis*, require select agent registration and the use of complete pXO2 plasmid DNA is not typically allowed by IBC so as to prevent accidental plasmid transfer. Although commercial kits are available to screen samples for capsule and toxin genes, they have a finite shelf life and can incur considerable expense when screening large numbers of strains or DNA samples over long periods of time.

This project sought to define a molecular methodology to identify strains containing the pXO2 plasmid without the use of a strain containing the plasmid or full pXO2 plasmid DNA. The two methods used to accomplish this goal were plasmid extraction and restriction enzyme digestion and the creation a PCR positive control using a small fragment of pXO2. These methods were tested on seven strains of *B. anthracis* as well as on other *Bacillus* species in order to demonstrate the lack of pXO2. The successful development of either methodology would result which could be indefinitely used for laboratory use for future screening of new strains and extracted DNA samples

A.2 Materials and Methods

Microorganisms

Bacillus anthracis strains 9131, 7702, 7702p610, UT258, UM44-1tr203-1, UM23C1-2, and ANR-1 were kindly provided by Theresa Kohler, University of Texas-Houston Medical School. The Sterne strain 7702 harbors pXO1 that encodes the anthrax toxins, but lacks pXO2 (33, 262). Strains 7702p610 and UT258 are derivatives of strain 7702, which contain additional plasmids conferring antibiotic resistance markers and green fluorescent protein (208). Strains UM44-1tr203-1 and UM23C1-2 are derivatives of the Weybridge strain marked with antibiotic resistance elements (10, 208). Strain UM44-1tr203-1 harbors pXO1 only and UM23C1-2 contains neither pXO1 or pXO2 (4, 10, 208). Strain ANR-1 is a pXO2-cured non-encapsulated variant of the Ames strain that harbors pXO1 and pXO2 (253). The plasmid-less strain 9131 was obtained in Dr. M. Mock's laboratory of the Pasteur Institute by curing strain RP31 from the pXO1 plasmid (192). Other bacteria used were *B. cereus* ATCC 14579, *B. mycoides* ATCC 6462, *B. thuringiensis* ATCC 35696, and *Escherichia coli* DH10b ATCC 31446 (Table 18).

Table 18. Bacterial strain characteristics

Strain	Relevant characteristics	Relevant plasmids	Reference
B. anthracis 9131	Sterne derivative, avirulent	None	(71)
B. anthracis 7702	Sterne derivative, avirulent	pXO1	(208)
B. anthracis ANR-1	Ames derivative, avirulent	pXO1	(86)
B. anthracis UT258	7702 derivative, GFP producing, erythromycin resistance, spectinomycin resistant	pXO1, pUTE610	(208)
B. anthracis UM44-1tr203-1	Weybridge derivative, avirulent, tetracycline resistant, spectinomycin resistant	pXO1, pXO12 (from *B. thuringiensis*), pBC16	(10)
B. anthracis UM23C1-2	Weybridge derivative, avirulent, rifampin resistant	None	(10)
B. anthracis 7702p610	7702 derivative, GFP producing, erythromycin resistant	pXO1, pUTE610	(208)
B. mycoides ATCC 6462			ATCC
B. cereus ATCC 14579			ATCC
B. thuringiensis ATCC 10792			ATCC
E. coli DH10b ATCC 31446			ATCC

Extraction and digestion of plasmids

Plasmid DNA was extracted using a modified version of the method outlined by Kaspar and Robertson (132). Strains of *B. anthracis* were incubated at 37°C in 50 mL tryptic soy broth (TSB; Neogen, Lansing, MI) overnight. The cells were collected by centrifugation at 10,000 × *g* for 10 minutes (RC-5 centrifuge, Sorvall; Waltham, MA, using a GSA rotor) and the pellet the cells. The cells were then resuspended in 5 ml E-buffer (0.04 M Tris-acetate, 2 mM EDTA). Cells were lysed by the addition of 10 ml of freshly-made lysis solution (50 mM Tris Base, 15% w/v sucrose, 3% sodium dodecyl sulfate [SDS], 0.5 M NaOH) and incubated at 55°C for 30 min. Cellular debris and unlysed cells were collected by centrifuging at 10,000 × *g* for 10 minutes. The plasmid-containing supernatants were removed to sterile tubes and extracted twice with an equal volume of unbuffered 1:1 phenol/chloroform. The aqueous phase fractions were incubated on ice for 10 min and neutralized with 1.25 mL ice-cold 2 M Tris-HCl (pH 7.0). Sodium acetate was then added to 0.3 M NaOAc total volume and followed by ethanol precipitated using two volumes of 95% ethanol. Nucleic acids were precipitated at -20° C for 30 minutes and the collected by centrifugation. Following 30 min incubation at -20°C, the nucleic acid was collected by repeated centrifugation and decanting at 13,000 RPM (16,250 × *g*) in a microcentrifuge. The pellet was dissolved in 50 µL TE (10 mM Tris-HCl, pH 7.4, 1mM EDTA).

The resulting DNA was run on a 0.75% agarose gel at 70 V for 120 minutes to visualize the plasmid or directly digested. Enzyme digestion with PvuI and KpnI (New England Biolabs, Ipswich, MA) were carried out individually according to manufacturer instruction and subsequently visualized in the same manner as the uncut plasmid (Table 19).

Table 19. Enzyme restriction sites. Adapted from New England Biolabs website www.neb.com.

Enzyme	Restriction site
PvuI	5′...CGAT▼CG...3′ 3′...GC▲TAGC...5′
KpnI	5′...GGTAC▼C...3′ 3′...C▲CATGG...5′

Selection of PCR target and cloning into E. coli *plasmid*

B. anthracis capA gene target was selected from GenBank accession number M24150. From this sequence, a 360 bp region (bp 481- 841) was identified as containing multiple PCR targets (Fig. 46). This sequence was constructed and inserted into vector pJ201 and transformed into *Escherichia coli* DH10b by DNA 2.0, Menlo Park CA, (Fig. 47). The plasmid was maintained using a kanamycin resistance element and maintained on Luria-Bertani agar containing 30 µg/mL kanamycin, as well as a frozen glycerol stock. Primer 3 was used to design a series of primers targeting various portions of the gene segment. Ultimately, the primers chosen amplify a 198 bp sequence of the *capA* target (Table 20). A BLAST search of this gene fragment revealed no homology to sequences found in bacteria other than pXO2 containing *B. anthracis* or *B. cereus* isolates containing pXO2 homologues.

5’aaatttcaaagatgtaaaaaatattgtgtatcaaaatgtaaatggtgttagggttgctactcttggatttacagatgcatttgtagcagg
agctat**tgcaacgaaagaacaaccaggttcgttaagtatgaacccagatgtattacttaagcaaattagtaaggcaaaggat
cctaaaaaaggtaatgctgatcttgtcgtagtaaatacgcactgggggaagaatacgataataaaccgagtcctagacag
gaagccttagcaaaagcaatggttgatgcaggggcagatat**tattgtgggacaccatccgcatgtacttcaatcttttgatgtgtat
aagcaagggattatcttcta -3’

Figure 46. Partial *capA* gene insert sequence (PCR targeted region in bold) derived from GenBank accession number M24150.

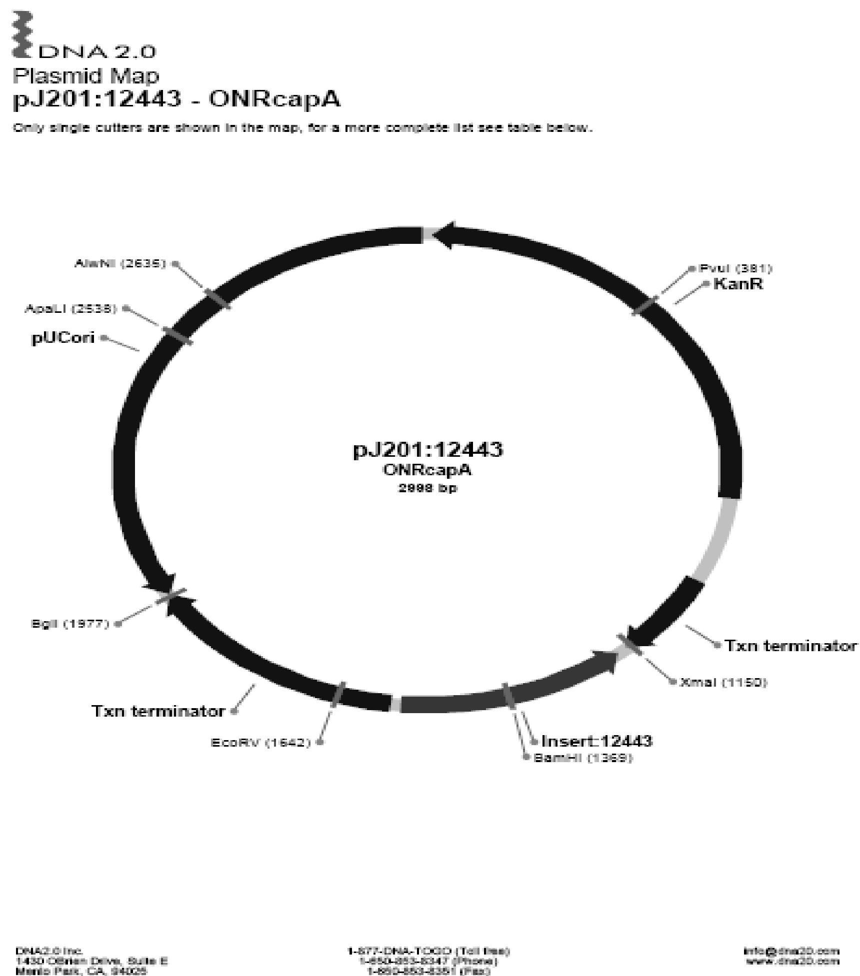


Figure 47. Plasmid map of pJ201 with *capA* insert and kanamycin resistance element.

DNA preparation for PCR

Vegetative cells were prepared for PCR in sterile 1.7 mL microcentrifuge tubes by suspension of a single colony grown on tryptic soy agar, in sterile 0.5 mL 5% chelex solution, incubation at 56°C for 45 min in a water bath, followed by boiling for 30 min.

Primers and PCR conditions

PCR cycling conditions for *capA* amplification (simplex) were generically based on primer melting temperature (Tm) and an annealing temperature gradient was used to determine optimal annealing conditions. Previously published PCR primers for *pagA* and *gyrB* were also screened using a temperature gradient to determine compatibility of PCR cycling conditions (Table 21)**.** Analysis of simplex PCR yielded appropriate amplification for all three genes using cycling conditions of 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. The optimized multiplex PCR mixture (*capA, gyrB,* and *pagA*) was a total of 25 µl containing $MgCl_2$ at 1.5 mM, 0.1 mM each dNTP, *capA* primers at 0.2 µM, *gyrB* primers at 0.5 µM, *pagA* primers at 0.6 µM, 0.2 units Amplitaq Gold, and 2 µL of extracted DNA sample. 10 µL of the resulting product was dyed with 6× loading buffer and analyzed by gel electrophoresis using a 1.5% agarose gel at 100 v for 60 min.

Table 20. Primers for *capA*, *pagA*, and *gyrB*

Gene	Gene Location and Function	Primers	Expected Size	Reference
capA	pXO2, capsule biosynthesis	OR198F (5'-3') tgcaacgaaagaacaaccag OR198R (5'-3') atatctgcccctgcatcaac	198 bp	This work
gyrB	Chromosome, *Bacillus* DNA gyrase	BA-gyrBF (5'-3') aaaacaaccrattcatgaag BA- gyrBR (5'-3') tcgcttcactattyccaagt	300 bp	(142)
pagA	pXO1, protective antigen (PA)	PA5 (5'-3') tcctaacactaacgaagtcg PA8 (5'-3') gaggtagaaggatatacggt	596 bp	(162)

A.3 Results

Although plasmid DNA was clearly visible (Fig. 48) following the extraction procedure, enzymatic digestion proved problematic. Digestion with PvuI should have resulted in a single band for pXO1 or three bands for pXO2. However, results of digestion with PvuI showed no visible bands other then chromosomal DNA (Fig. 49), although it was predicted to produce one large band for pXO1. Digestion with KpnI should have resulted in thirteen DNA fragment bands for pXO1 or three bands for pXO2.

The results of digestion with KpnI yielded a single band of the same approximate size as the uncut plasmid indicating digestion failure (Fig. 49). Despite the use of extended digestion times and increased enzyme concentration, no digestion reveled any of the expected products from either pXO1 or pXO2 plasmids. Following the results of plasmid extraction demonstrating low plasmid yield and ineffective restriction enzyme digestion, strategy for accomplishing plasmid identification was changed to focus on PCR.

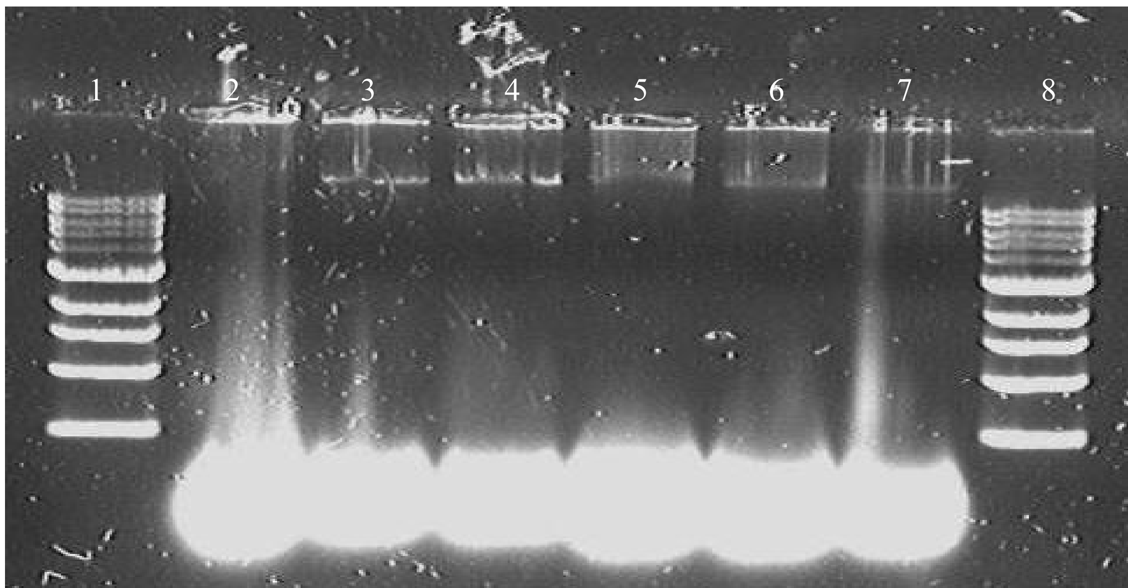


Figure 48. Plasmid after ethanol precipitation. Lanes – 1 and 8: 1 kb ladder, 2: 9131, 3: 7702, 4: ANR-1, 5: 7702p610, 6: UT258, 7: UM44-1tr203-1

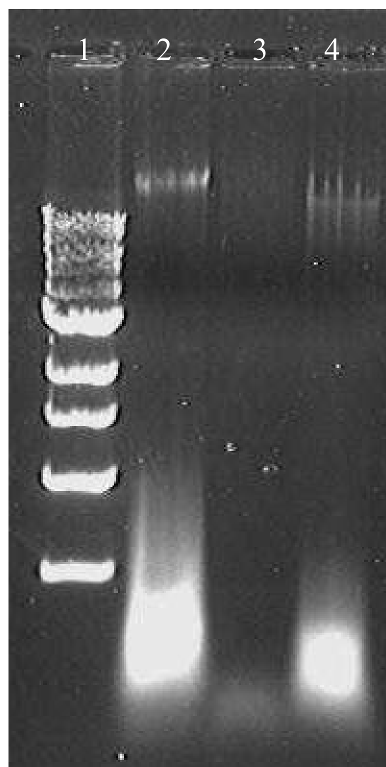


Figure 49. Plasmid preparation and digestion of UT258. Lane 1) 1kb ladder, 2) Original plasmid preparation, 3) PvuI digestion, 4) KpnI digestion.

The *capA* fragment was successfully amplified in the constructed positive control (Fig. 50). The *capA* and *pagA* primers were effective at a wide temperature range, although amplification of *gyrB* was only seen at 56°C or less (Fig. 51). Multiplex of all three genes at an annealing temperature of 53°C was successful. *CapA* was only found in the positive control, and not found in any strain of *B. anthracis*, other *Bacillus* species, or the plasmid background strain *E. coli* DH10B (Fig. 52). The PCR product was also sequenced and found highly homologous to the target. The *gyrB* gene was found in all *Bacillus* strains analyzed. The *pagA* gene was only found in strains known to contain the pXO1 plasmid.

Figure 50. Simplex PCR reaction for *capA*. Lanes – 1: 100 bp ladder, 2: *CapA* insert *E. coli*, lanes 3- 9 *B. anthracis* (3, 9131; 4, 7702; 5, ANR-1; 6, 7702p610; 7, UT258; 8, UM44-1tr203-1; 9, UM23C1-2;) 10: *B. cereus* ATCC 14579 (negative *Bacillus* control), 11: water (negative control)

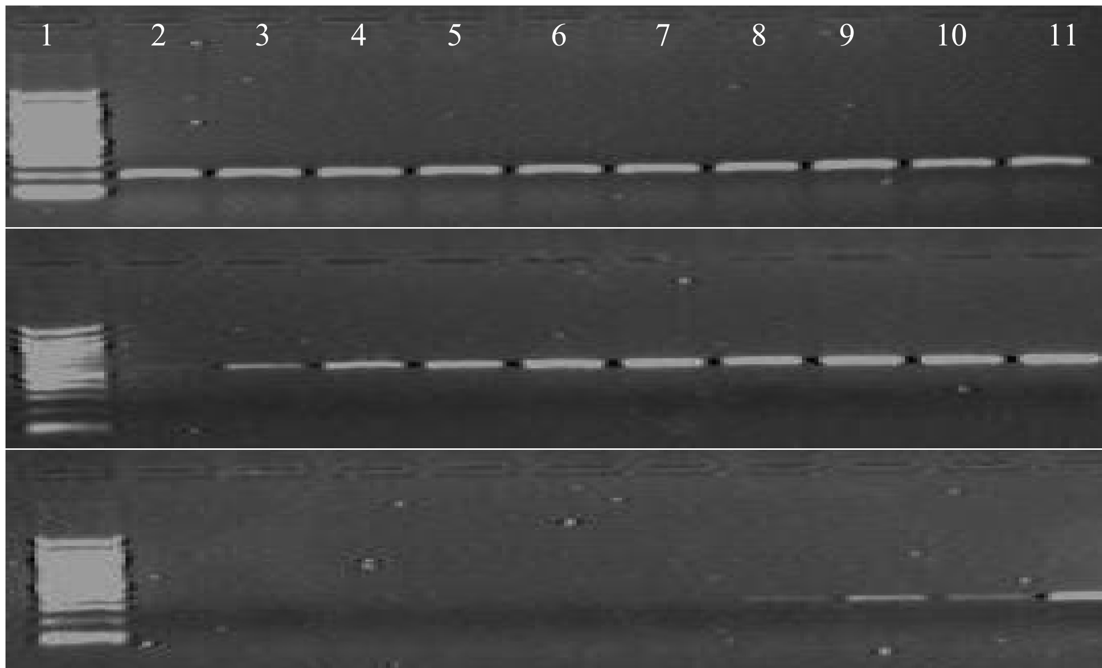


Figure 51. Temperature gradient of simplex PCR reactions for *capA* (top), *pagA* (center), and *gyrB* (bottom) from 62°C– 53°C annealing temperature. DNA from strain 7702 used for *pagA* and *gyrB*, *capA* insert *E. coli* DNA used for *capA*. Lanes- 1: 100 bp ladder, 2: 62°C, 3: 61°C, 4: 60°C, 5: 59°C, 6: 58°C, 7: 57°C, 8: 56°C, 9: 55°C, 10: 54°C, 11: 53°C

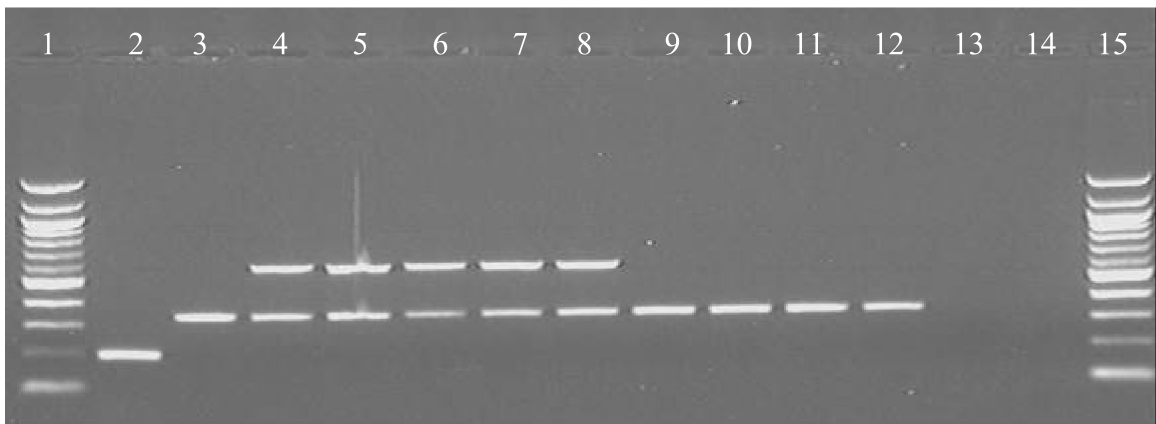


Figure 52. Gel electrophoresis results of *capA, gyrB*, and *pagA* PCR multiplex. Lanes: 1 and 15 100 bp ladder, 2: *E. coli* DH10b with *capA* insert, lanes 3 to 9 *B. anthracis* (3, 9131; 4, 7702; 5, ANR-1; 6, 7702p610; 7, UT258; 8, UM44-1tr203-1; 9, UM23c1-2), 10: *B. cereus* ATCC 14579, 11: *B. mycoides* ATCC 6462, 12: *B. thuringiensis* ATCC 10792, 13: *E. coli* DH10b, 14: H_2O

A.4 Discussion

The current project examined two methodologies for differentiating strains of *Bacillus anthracis* containing the capsule-encoding pXO2 plasmid. Ability to produce

capsule is essential for *B. anthracis* virulence in humans, as it allows the bacteria to escape immune response. Both plasmids pXO1 and pXO2 are critical for virulence however, federal select agent regulations allow for exemptions for researchers using strains devoid of pXO2. Although strains producing capsule can be differentiated on agar media by their shiny, mucoid appearance, this is not conclusive evidence that a particular strain does not have the genetic ability to produce the capsule as expression of capsule may vary between strains and growth conditions. In order to comply with select agent regulations and institution biosafety committee requirements, this project sought to develop a rapid methodology for screening *B. anthracis* strains for the presence of pXO2.

Plasmid isolation was successful in generating small amounts of linearized plasmid. However, the subsequent digestion of the plasmid to ensure its identity as pXO1 was not successful. The most likely reason for failed digestion is the limited amount of plasmid recovered from extraction. This is due to the single copy nature of the plasmid and the tendency for plasmid shearing during preparation. An additional complicating factor may have been the large quantity of chromosomal DNA co-extracted with the plasmid. These issues may have been resolved by using larger culture volumes and separation using a cesium-chloride gradient as described by Kaspar and Robertson (132).

This project successfully used a portion of the *capA* gene as a marker for plasmid pXO2 as a novel PCR positive control based on GenBank accession M24150. This sequence has been previously documented only as part of the *B. anthracis* plasmid pXO2 and *B. cereus* 03BB108, which contains a pXO2 homologue (115). The multiplex PCR designed as part of this project allows for rapid and effective screening for plasmid pXO2

using the surrogate positive control generated in this project. This positive control does not contain the sequence targeted by officially approved diagnostic methods, which may be useful is to differentiate true positives from cases of accidental plasmid transfer. This control can be used as a screening tool for newly isolated strains to meet federal select agent requirements and for large scale prevalence studies as it can be maintained indefinitely.

The multiplex PCR developed permits rapid screening for plasmids pXO1, pXO2 and generic Bacillus DNA. Although no cross reactivity was observed with any of the other bacteria used in this study, the reaction of primers specific for the capA fragment are somewhat prone to mis-priming and require the use Amplitaq Gold. Due to select agent regulations this protocol has not yet been performed on B. anthracis carrying pXO2.

Appendix B. Hydrophobicity of *B. anthracis*

B.1 Mean percent hydrophobicity of *B. anthracis* strains 9131, 7702, and ANR-1 with varying hexadecane to spore suspension ratios with 1 minute vortex mixing

Hexadecane (mL) /spore suspension (mL) ratio	Percent hydrophobicity					
	9131		7702		ANR-1	
0.03	7.47	0.55	2.85	12.55	10.07	19.04
0.07	12.84	2.80	5.24	11.85	19.34	26.26
0.20	17.76	12.80	14.07	17.89	40.12	32.06
0.33	28.71	23.20	18.73	24.57	49.42	45.70

B.2 Mean percent hydrophobicity of *B. anthracis* strains 9131, 7702, and ANR-1 with varying hexadecane to spore suspension ratios with 2 minutes vortex mixing

Hexadecane (mL) /spore suspension (mL) ratio	**Percent hydrophobicity**					
	9131		**7702**		**ANR-1**	
0.03	20.23	4.90	7.75	17.08	10.34	40.77
0.07	27.21	9.45	13.57	12.78	14.57	43.35
0.20	48.93	25.10	22.21	21.95	37.70	57.86
0.33	61.86	44.35	26.34	39.38	52.64	68.01

B.3 Mean percent hydrophobicity of *B. anthracis* strains 9131, 7702, and ANR-1 with varying hexadecane to spore suspension ratios with 4 minutes vortex mixing

Hexadecane (mL) /spore suspension (mL) ratio	Percent hydrophobicity					
	9131		**7702**		**ANR-1**	
0.03	31.05	25.35	11.33	22.58	25.06	41.44
0.07	40.15	40.60	18.92	23.07	44.48	52.19
0.20	78.72	52.05	27.62	39.25	59.64	59.83
0.33	82.63	61.75	53.59	45.10	73.02	66.04

B.4 Percent hydrophobicity of *B. anthracis* 9131, 7702, and ANR-1 with varying hexadecane to spore suspension ratios with 1 minute vortex mixing and pre-incubation at 75°C]

Hexadecane /spore suspension ratio	**Percent hydrophobicity**											
	9131				**7702**				**ANR-1**			
0.03	14.6	35.0	18.2	32.9	25.3	59.2	48.7	45.8	20.4	64.9	43.5	41.6
0.07	15.3	20.6	21.0	39.4	22.3	62.3	51.8	32.3	30.2	72.9	49.6	47.2
0.20	18.6	43.0	26.2	44.8	25.4	78.1	51.9	52.1	30.1	76.6	53.1	41.5
0.33	26.0	49.4	41.5	60.3	27.7	74.4	53.1	68.4	33.9	75.8	56.3	52.0

B.5 Mean percent hydrophobicity of *B. anthracis* 9131, 7702, and ANR-1 over time using a hexadecane: spore suspension ratio of 0.33. Data from single trial in triplicate samples and 95% confidence interval.

Day	Percent hydrophobicity		
	9131	7702	ANR-1
0	38.82 ± 27.93	26.69 ± 13.47	31.22 ± 4.25
2	33.62 ± 6.28	18.35 ± 5.03	43.28 ± 24.40
4	45.66 ± 26.86	23.60 ± 21.89	47.44 ± 20.20
6	28.02 ± 7.68	23.41 ± 6.17	43.63 ± 11.09
8	40.01 ± 5.83	22.96 ± 5.51	34.01 ± 17.31
12	31.50 ± 3.07	23.22 ± 2.92	47.02 ± 4.95

B.6 Percent total *B. anthracis* 9131, 7702, and ANR-1 spores distributing to water and hexadecane fractions at different ratios. Approx. 10^3 spores total initial population.

	Strain	**Hexadecane /spore suspension ratio**			
		0.2		**0.33**	
Percent total spores in hexadecane	9131	0.23	2.43	0.25	0.53
	7702	1.06	3.18	0.00	2.84
	ANR-1	0.00	1.17	0.00	1.33
Percent total spores in water	9131	57.75	113.03	40.88	113.03
	7702	114.71	41.76	62.65	44.03
	ANR-1	65.36	59.84	74.16	67.29

B.7 Percent total *B. anthracis* 9131, 7702, and ANR-1 spores distributing to whole milk and hexadecane fractions at different ratios. Approx. 10^3 spores total initial population.

	Strain	**Hexadecane /spore suspension ratio**			
		0.2		**0.33**	
Percent total spores in hexadecane	9131	15.36	11.13	13.47	17.22
	7702	10.98	13.03	17.27	18.20
	ANR-1	11.69	13.79	11.02	19.51
Percent total spores in whole milk	9131	104.19	92.14	123.95	77.98
	7702	100.52	93.75	75.77	73.68
	ANR-1	79.66	70.88	81.36	92.58

B.8 Percent total *B. anthracis* 9131, 7702, and ANR-1 spores distributing to pulpless orange juice and hexadecane fractions at different ratios. Approx. 10^3 spores total initial population.

	Strain	Hexadecane /spore suspension ratio			
		0.2		0.33	
Percent total spores in hexadecane	9131	11.53	14.02	8.45	11.79
	7702	9.74	12.73	7.73	19.18
	ANR-1	7.76	16.49	6.32	19.03
Percent total spores in orange juice	9131	86.11	89.02	77.08	85.16
	7702	86.60	78.63	105.15	75.45
	ANR-1	83.38	76.01	68.82	67.23

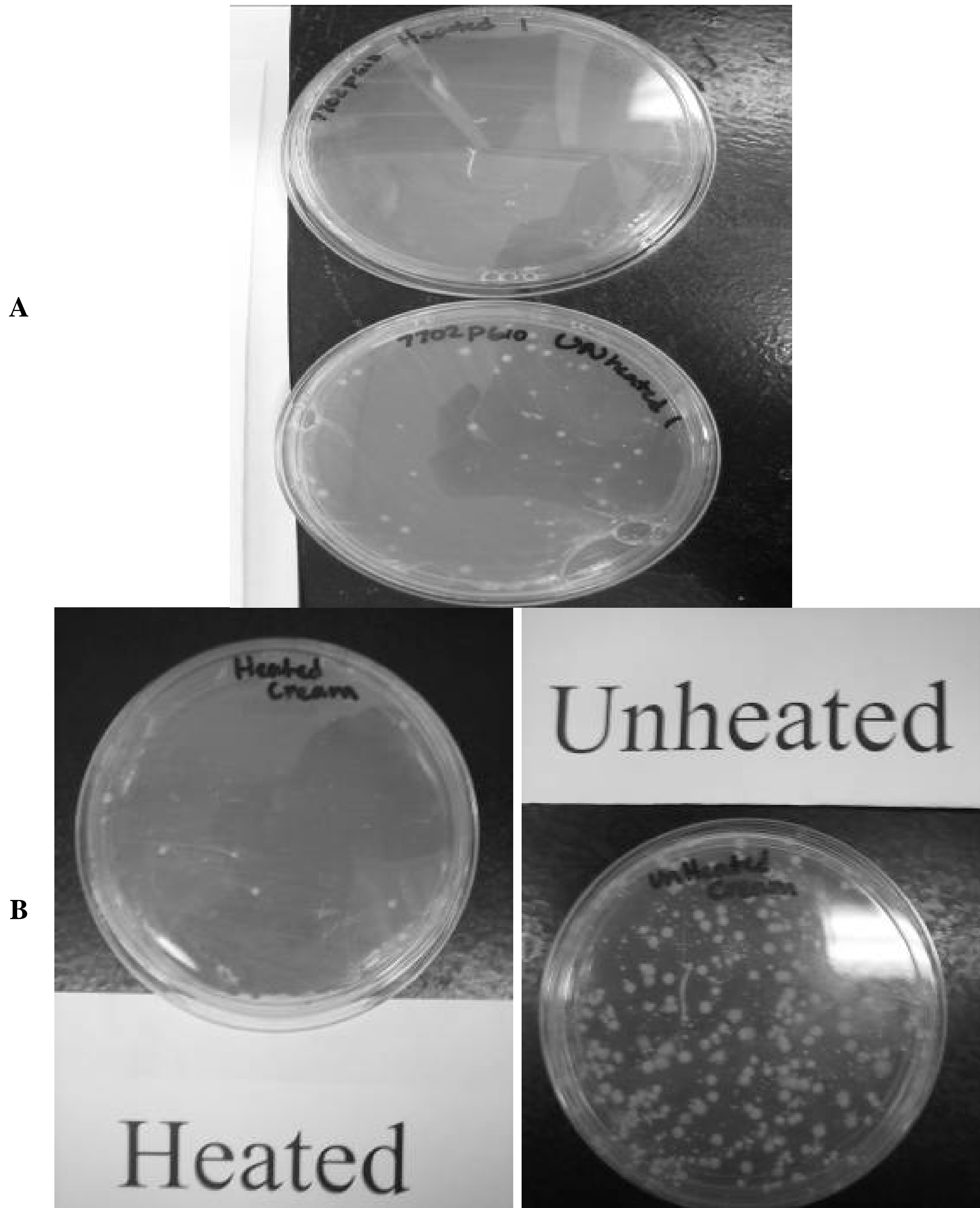
Appendix C. Recovery of *B. anthracis* from various types of milk and distribution following separation

C.1 Percent *B. anthracis* 9131, 7702, and ANR-1 spores recovered from 1 mL milk in silicone oil pellet after centrifugation at 16,250 × *g* for 120 s.

Strain	Commercial whole milk				Raw whole milk		Raw whole milk + 85°C for 45 s		
9131	71.80	112.00	98.27	120	10.83	28.43	89.90	107.30	86.73
7702	80.73	79.70	83.03		38.23	14.00	63.23	84.10	75.83
ANR-1	101.10	97.57			ND*	ND*	69.80	73.23	81.57

* Recovery of ANR-1 was not determined from raw whole milk to the an increase in background flora in the non-inoculated raw milk samples

C. 2 *B. anthracis* spores qualitatively recovered cream from raw whole milk, collected after centrifugation, which was either previously heated to 85°C for 60 s or left untreated. Panel A: strain 7702p610 plated on erythromycin TSA, Panel B: strain 9131 plated on TSA. Spores were added following heat treatment.



C.3 Percent concentration *B. anthracis* 7702p610, UT258, UM44-1tr203-1, and UM23C1-2 spores recovered in skim and cream following separation of raw whole milk

Milk Fraction		**Skim milk**			**Cream**	
7702p610	51.60	80.10	65.48	21.15	11.71	14.19
UT258	88.74	77.88	76.60	6.54	21.24	15.04
UM44-1tr203-1	88.72	67.28	88.36	22.15	6.09	19.93
UM23C1-2	80.31	71.39	77.00	28.37	15.81	18.21

C.4 Percent total *B. anthracis* spores recovered from 1 mL volumes of commercial skim milk after centrifugation at various speeds for 1 minute (starting concentration ~ 10^3 spores/mL).

Strain	Centrifuge speed (× *g*)								
	4,720			**9,630**			**16,250**		
9131	93.7	74.0	98.7	100.6	90.9	100.8	97.6	92.7	95.8
7702		97.4	94.0		93.4	97.3		93.6	90.6
ANR-1	102.8	75.5	95.7	108.3	75.9	93.1	97.9	79.4	97.8
UT258	107.2	66.9	101.2	107.9	96.6	99.4	122.3	97.3	83.9
UM44-1tr203-1	80.6	91.8	89.3	81.5	102.2	102.8	81.5	98.9	94.5
UM23C1-2	96.2	91.1	87.2	97.3	103.9	91.9	99.1	104.8	97.1

C.5 Percent total *B. anthracis* 7702p610 and UT258 spores recovered after centrifugation of 100 mL skim milk (approx. 10^3 spores/mL) at varying centrifuge speeds and time.

Centrifuge speed (× *g*)	***B. anthracis* 7702p610**					***B. anthracis* UT258**				
	5 min			**10 min**		**5 min**			**10 min**	
1500	52.87	56.21	70.13	101.17	90.48	79.39	53.57	73.24	99.18	97.70
2600	88.51	60.33	74.09	101.82	86.62	92.71	56.33	73.90	102.65	94.39
4100	109.77	52.46	80.30	92.97	63.04	93.13	60.15	73.79	98.88	88.69

C.6a Log total initial and recovered *B. anthracis* spores recovered from 100 mL skim milk after centrifugation at 1,500 × *g* for 10 minutes.

Mean log total spores					
***B. anthracis* 9131**		***B. anthracis* 7702**		***B. anthracis* ANR-1**	
Initial	**Recovered**	**Initial**	**Recovered**	**Initial**	**Recovered**
8.02	8.02	7.97	8.02	7.98	7.99
8.01	7.96	8.01	8.02	8.02	8.01
7.02	7.05	6.97	7.03	6.98	7.03
7.01	7.01	7.01	6.99	7.02	7.05
6.02	6.05	5.97	6.03	5.98	6.03
6.01	6.01	6.01	6.00	6.02	6.07
5.02	5.04	4.97	5.06	4.98	5.03
5.01	5.02	5.01	5.07	5.02	5.04
4.02	4.00	3.97	3.96	3.98	3.99
4.01	4.00	4.01	4.01	4.02	4.04
3.02	3.04	2.97	2.89	2.98	3.00
3.01	3.10	3.01	3.01	3.02	3.02

C.6b Log total initial and recovered *B. anthracis* spores recovered from 100 mL skim milk after centrifugation at 1,500 × *g* for 10 minutes.

Mean log total spores			
***B. anthracis* 7702p610**		***B. anthracis* UT258**	
Initial	**Recovered**	**Intial**	**Recovered**
7.84	7.83	7.98	8.00
7.98	7.98	7.44	7.42
6.84	6.83	6.98	7.00
6.98	6.98	6.44	6.44
5.84	5.88	5.98	5.87
5.98	5.98	5.44	5.47
4.84	4.86	4.98	4.95
4.98	4.97	4.44	4.41
3.84	3.84	3.98	4.03
3.98	3.97	3.44	3.36
2.84	2.87	2.98	2.99
2.98	2.97	2.44	2.40

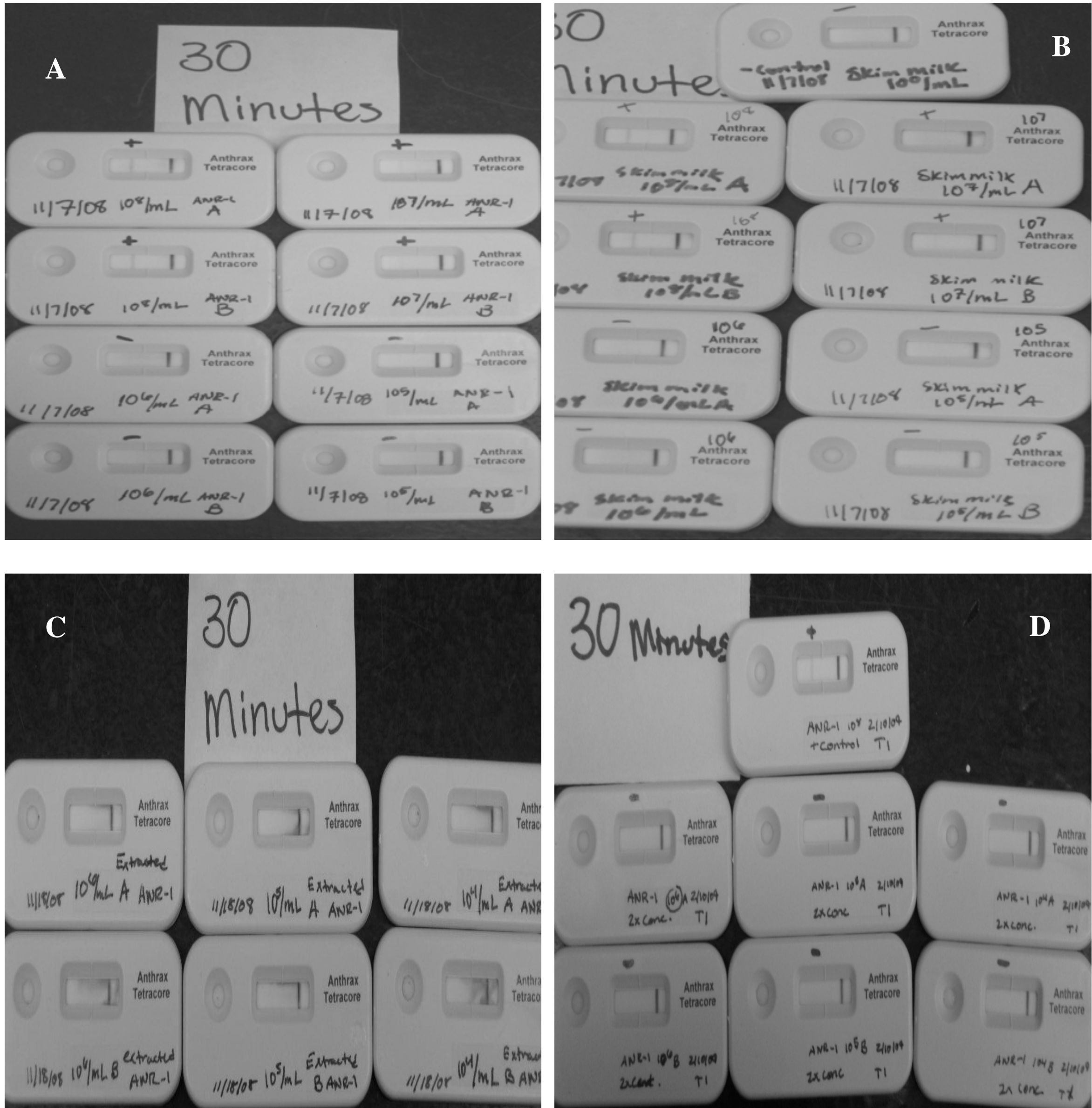
C.7a Log total initial and recovered *B. anthracis* spores recovered from 100 mL whole milk after centrifugation at 1,500 × *g* for 10 minutes.

Mean log total spores					
***B. anthracis* 9131**		***B. anthracis* 7702**		***B. anthracis* ANR-1**	
Initial	**Recovered**	**Initial**	**Recovered**	**Initial**	**Recovered**
7.96	7.93	7.85	7.82	7.94	7.99
7.99	7.99	7.93	8.04	7.97	8.03
6.96	6.94	6.85	6.85	6.94	6.98
6.99	6.99	6.93	7.04	6.97	7.00
5.96	5.92	5.85	5.80	5.94	5.95
5.99	6.00	5.93	6.02	5.97	5.99
4.96	4.94	4.85	4.83	4.94	4.99
4.99	4.98	4.93	5.02	4.97	4.98
3.96	3.93	3.85	3.84	3.94	3.94
3.99	3.98	3.93	3.96	3.97	3.92
2.96	2.88	2.85	2.77	2.94	2.91
2.99	3.00	2.93	2.86	2.97	2.89

C.7b Log total initial and recovered *B. anthracis* spores recovered from 100 mL whole milk after centrifugation at 1,500 × *g* for 10 minutes.

Mean log total spores			
***B. anthracis* 7702p610**		***B. anthracis* UT258**	
Initial	**Recovered**	**Initial**	**Recovered**
7.95	7.95	7.96	7.97
7.85	7.78	7.88	7.95
6.95	6.96	6.96	6.95
6.85	6.87	6.88	6.96
5.95	5.94	5.96	5.98
5.85	5.88	5.88	5.94
4.95	4.94	4.96	4.99
4.85	4.88	4.88	4.97
3.95	3.93	3.96	3.99
3.85	3.85	3.88	3.98
2.95	2.92	2.96	2.96
2.85	2.80	2.88	2.89

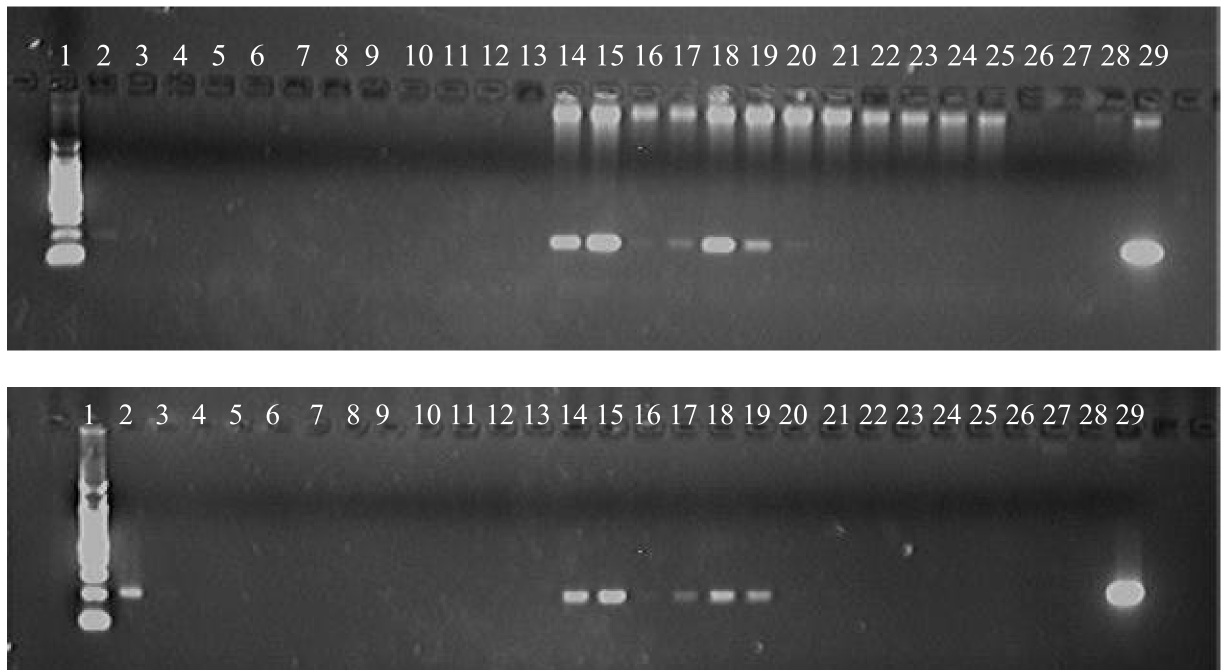
C.8 Sample pictures from lateral flow assay of *B. anthracis* ANR-1 spores suspended in water (A), skim milk (B), milk after concentration (C), and after secondary concentration (D)



C.9 Summary of standard PCR detection for *pagA* results from skim and whole milk inoculated with *B. anthracis* with and without prior concentration

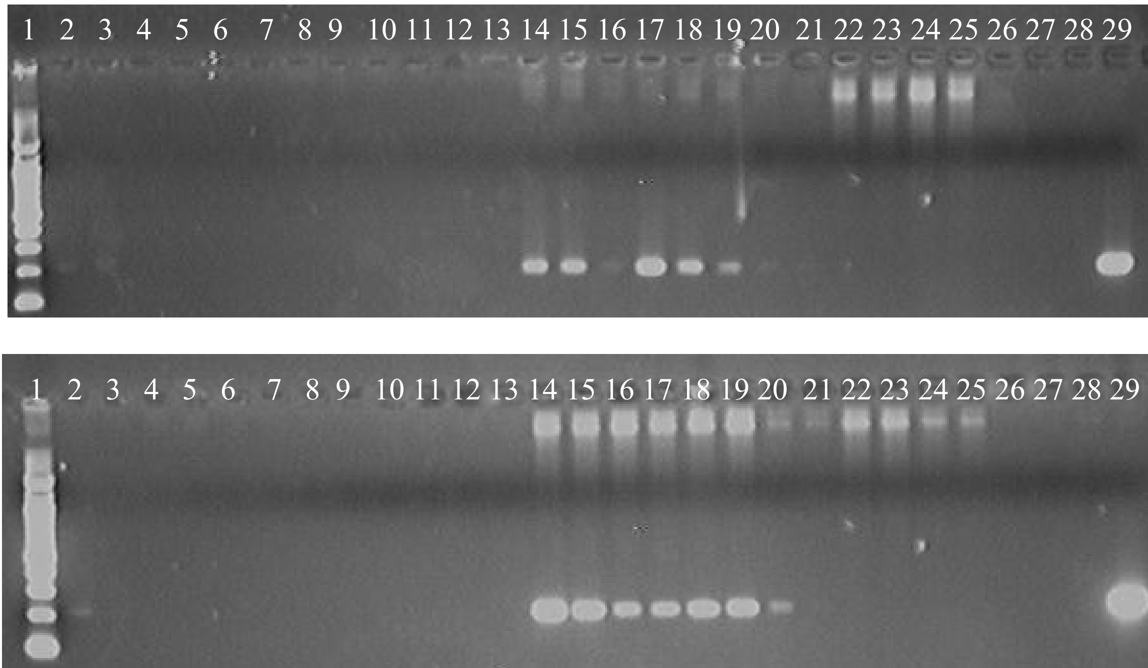
Strain	**Approx. initial concentration *B. anthracis* spores/mL**	**Skim milk**		**Whole milk**	
		Fraction samples positive from milk	**Fraction samples positive from concentrated milk**	**Fraction samples positive from milk**	**Fraction samples positive from concentrated milk**
7702	10^6	1/4	4/4	3/4	4/4
	10^5	0/4	4/4	1/4	4/4
	10^4	0/4	4/4	0/4	4/4
ANR-1	10^6	2/4	4/4	3/4	4/4
	10^5	0/4	4/4	1/4	4/4
	10^4	0/4	4/4	0/4	4/4
7702p610	10^6	3/4	4/4	3/4	4/4
	10^5	0/4	4/4	0/4	4/4
	10^4	0/4	4/4	0/4	4/4
UT258	10^6	3/4	4/4	2/4	4/4
	10^5	0/4	4/4	1/4	4/4
	10^4	0/4	4/4	0/4	4/4

C.10 Example of *pagA* detection from skim milk with and without pre-concentration with *B. anthracis* ANR-1 (top) and UT258 (bottom)



Lanes- Lanes- 1: 100 bp ladder, lanes 2 to13 DNA extraction from milk (2&3, 10^6 spores/mL; 4&5, 10^5 spores/mL; 6&7, 10^4 spores/mL; 8&9, 10^3 spores/mL; 10&11,10^2 spores/mL; 12&13, 10^1 spores/mL), lanes 14 to 25 DNA extraction from concentrated milk (14&15, 10^6 spores/mL; 16&17, 10^5 spores/mL; 18&19, 10^4 spores/mL; 20&21, 10^3 spores/mL; 22&23,10^2 spores/mL; 24&25, 10^1 spores/mL), 26: non-incoluated milk extracted with UltraClean kit, 27: *B. thuringiensis* vegetative cells extracted with UltraClean kit, 28: Sterile distilled H_2O, 29: *B. anthracis* ANR-1 vegetative cells extracted with UltraClean kit.

C.11 Example of *pagA* detection from whole milk with and without pre-concentration with *B. anthracis* 7702 (top) and ANR-1 (bottom)



Lanes- lane 1: 100 bp ladder, lanes 2 to13 DNA extraction from milk (2&3, 10^6 spores/mL; 4&5, 10^5 spores/mL; 6&7, 10^4 spores/mL; 8&9, 10^3 spores/mL; 10&11,10^2 spores/mL; 12&13, 10^1 spores/mL), lanes 14 to 25 DNA extraction from concentrated milk (14&15, 10^6 spores/mL; 16&17, 10^5 spores/mL; 18&19, 10^4 spores/mL; 20&21, 10^3 spores/mL; 22&23,10^2 spores/mL; 24&25, 10^1 spores/mL), 26: non-incoluated milk extracted with UltraClean kit, 27: *B. thuringiensis* vegetative cells extracted with UltraClean kit, 28: Sterile distilled H_2O, 29: *B. anthracis* ANR-1 vegetative cells extracted with UltraClean kit.

C.12 Real-time PCR mean cycle threshold (C*t*) external standard curve of *B. anthracis* ANR-1 in suspended in sterile water extracted with UltraClean kit.

Log total initial ANR-1 spores extracted using UltraClean kit	**Trial 1: 8.98**	**Trial 2: 8.76**
Template dilution	**Mean C*t***	**Mean C*t***
10^{0}	10.01	9.97
10^{-1}	13.26	13.89
10^{-2}	17.16	17.90
10^{-3}	20.92	27.14
10^{-4}	25.04	25.82
10^{-5}	29.01	29.60
10^{-6}	33.15	33.34
10^{-7}	34.50	36.56
10^{-8}	37.24	33.94

C.13a Real-time PCR mean cycle threshold (C*t*) internal standard curve included with each LightCycler run for milk.

***B. anthracis* strain**	**7702**				**ANR-1**			
Milk type	**Skim**		**Whole**		**Skim**		**Whole**	
Dilution of standard	**Mean C*t* value**							
10^{-1}	14.26	11.94	15.07	13.94	13.83	14.66	13.81	14.78
10^{-2}	19.13	16.81	19.77	21.11	18.76	19.97	19.21	20.68
10^{-3}	23.96	26.02	27.51	27.60	23.21	27.07	27.17	28.93
10^{-4}	28.77	29.73	32.09	33.07	27.60	30.85	32.11	34.16
10^{-5}	31.26	32.50	33.12	33.68	31.27	31.54	32.79	34.65
10^{-6}	34.70	32.73	33.20	35.15	36.81	33.30	34.54	34.96

C.13b Real-time PCR mean cycle threshold (C*t*) internal standard curve included with each LightCycler run for milk.

***B. anthracis* strain**	**7702p610**				**UT258**			
Milk type	**Skim**		**Whole**		**Skim**		**Whole**	
Dilution of standard	**Mean C*t* value**							
10^{-1}	13.51	13.88	14.33	16.77	13.86	13.95	14.13	13.54
10^{-2}	18.32	26.82	20.81	22.00	19.25	19.85	19.53	20.97
10^{-3}	24.13	19.49	26.80	28.19	25.62	27.07	27.72	27.21
10^{-4}	26.66	31.68	32.08	32.33	27.86	30.27	33.71	33.55
10^{-5}	29.81	32.80	34.62	31.90	28.06	31.64	31.65	31.72
10^{-6}	34.31	32.16	40.00	33.64	28.06	32.79	32.54	33.16

C.14 Log concentration initial *B. anthracis* 7702 spores in skim milk and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log 7702 concentration (CFU/mL) milk	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	6.01	23.15	16.20
	5.01	27.04	21.32
	4.01	32.30	23.01
	3.01	32.57	27.75
	2.01	33.89*	30.55
	1.01	34.46*	30.12
	Non-inoculated milk (negative control)	33.45	
Trial 2	5.82	22.12	19.01
	4.82	27.65	19.06
	3.82	30.22	23.58
	2.82	31.06	25.03
	1.82	31.18	27.15
	0.82	31.26	29.29
	Non-inoculated milk (negative control)	32.66	

* C*t* values equal or greater to negative control considered negative.

C.15 Log concentration initial *B. anthracis* 7702 spores in whole milk and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log 7702 concentration (CFU/mL) milk	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	5.87	21.21	17.40
	4.87	24.86	21.77
	3.87	27.76	23.80
	2.87	29.84	29.36
	1.87	31.42	28.06
	0.87	31.72*	29.49
	Non-inoculated milk (negative control)	31.70	
Trial 2	5.88	23.47	19.06
	4.88	25.76	24.33
	3.88	29.54	24.93
	2.88	30.55	26.97
	1.88	31.16	28.70
	0.88	31.25	29.61
	Non-inoculated milk (negative control)	32.26	

* C*t* values equal or greater to negative control considered negative.

C.16 Log concentration initial *B. anthracis* ANR-1 spores in skim milk and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log ANR-1 concentration (CFU/mL) milk	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	5.85	24.93	13.72
	4.85	27.30	18.59
	3.85	28.50	21.34
	2.85	30.45	24.71
	1.85	31.93	27.23
	0.85	33.82*	30.26
	Non-inoculated milk (negative control)	33.69	
Trial 2	5.87	22.96	17.05
	4.87	27.22	18.34
	3.87	29.68	22.17
	2.87	30.89	26.22
	1.87	31.47	27.34
	0.87	31.97	28.17
	Non-inoculated milk (negative control)	33.52	

* C*t* values equal or greater to negative control considered negative.

C.17 Log concentration initial *B. anthracis* ANR-1 spores in whole milk and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log ANR-1 concentration (CFU/mL) milk	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	5.86	19.97	14.74
	4.86	22.03	18.34
	3.86	28.33	22.00
	2.86	31.32	26.83
	1.86	30.64	26.87
	0.86	30.76	27.63
	Non-inoculated milk (negative control)	35.31	
Trial 2	5.79	23.27	16.70
	4.79	26.65	20.58
	3.79	28.45	23.62
	2.79	29.20	26.74
	1.79	32.17*	28.93
	0.79	33.35*	29.73
	Non-inoculated milk (negative control)	32.11	

* C*t* values equal or greater to negative control considered negative.

C.18 Log concentration initial *B. anthracis* 7702p610 spores in skim milk and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log 7702p610 concentration (CFU/mL) milk	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	5.67	20.79	15.82
	4.67	23.93	20.63
	3.67	27.34	22.80
	2.67	28.80	26.53
	1.67	29.30	26.95
	0.67	29.71*	27.34
	Non-inoculated milk (negative control)	29.68	
Trial 2	5.72	20.70	14.62
	4.72	25.10	17.68
	3.72	28.12	21.52
	2.72	27.02	24.83
	1.72	29.31	27.36
	0.72	30.47	28.47
	Non-inoculated milk (negative control)	31.78	

* C*t* values equal or greater to negative control considered negative.

C.19 Log concentration initial *B. anthracis* 7702p610 spores in whole milk and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log 7702p610 concentration (CFU/mL) milk	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	5.89	21.25	16.85
	4.89	25.56	20.08
	3.89	28.10	23.49
	2.89	28.47	25.15
	1.89	31.61	28.49
	0.89	34.71*	29.30
	Non-inoculated milk (negative control)	34.34	
Trial 2	5.75	22.42	18.04
	4.75	25.60	20.31
	3.75	27.61	26.62
	2.75	29.37	24.79
	1.75	29.69*	27.60
	0.75	30.20*	28.65
	Non-inoculated milk (negative control)	29.86	

* C*t* values equal or greater to negative control considered negative.

C.20 Log concentration initial *B. anthracis* UT258 spores in skim milk and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log UT258 concentration (CFU/mL) milk	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	6.37	20.04	21.81
	5.37	23.48	22.68
	4.37	26.53	23.50
	3.37	26.69	26.12
	2.37	27.63*	26.13
	1.37	27.25*	26.20
	Non-inoculated milk (negative control)	26.71	
Trial 2	5.98	18.90	14.27
	4.98	24.84	16.40
	3.98	28.98	19.49
	2.98	31.27	27.85
	1.98	31.40	26.66
	0.98	32.55	27.81
	Non-inoculated milk (negative control)	34.16	

* C*t* values equal or greater to negative control considered negative.

C.21 Log concentration initial *B. anthracis* UT258 spores in whole milk and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log UT258 concentration (CFU/mL) milk	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	5.78	20.84	13.58
	4.78	23.77	17.84
	3.78	29.04	21.04
	2.78	28.39	25.66
	1.78	30.22*	26.43
	0.78	32.99*	29.14
	Non-inoculated milk (negative control)	30.12	
Trial 2	5.43	20.57	15.87
	4.43	24.31	19.86
	3.43	24.97	21.45
	2.43	27.72	24.45
	1.43	28.80	26.10
	0.43	32.87	29.54
	Non-inoculated milk (negative control)	34.79	

* C*t* values equal or greater to negative control considered negative.

<u>Appendix D. *B. anthracis* spore survival, concentration, extraction, and detection in fruit juices</u>

D.1a Mean log CFU/mL *B. anthracis* 7702p610 total and spore population in fruit juices and wine over 30 days

Days after inoculation	Orange juice (CFU/mL)				Apple juice (CFU/mL)			
	Trial 1		Trial 2		Trial 1		Trial 2	
	Total	Spore	Total	Spore	Total	Spore	Total	Spore
0	5.51	5.41	5.76	5.83	6.02	5.95	5.22	5.18
1	5.47	5.39	5.83	5.79	6.06	5.99	5.19	5.11
2	5.56	5.35	5.87	5.85	6.07	6.01	5.19	5.16
3	5.55	5.39	5.85	5.87	6.11	6.11	5.18	5.14
4	5.60	5.40	5.89	5.88	6.06	6.06	5.12	5.12
5	5.65	5.53	5.79	5.76	6.06	6.07	5.11	5.10
10	5.57	5.52	5.72	5.63	6.08	6.07	5.05	4.99
15	5.54	5.44	5.76	5.73	6.03	6.00	5.05	5.07
20	5.53	5.54	5.75	5.69	5.86	5.95	4.99	5.02
25	5.36	5.49	5.82	5.81	5.59	5.58	4.93	4.93
30	5.51	5.54	5.76	5.78	4.96	4.99	4.91	4.89

D.1b Mean log CFU/mL *B. anthracis* 7702p610 total and spore population in fruit juices and wine over 30 days

Days after inoculation	Wine (mean log CFU/mL)				Grape juice (mean log CFU/mL)			
	Trial 1		Trial 2		Trial 1		Trial 2	
	Total	Spore	Total	Spore	Total	Spore	Total	Spore
0	5.07	5.06	5.07	5.06	5.07	5.06	5.07	5.06
1	5.24	5.15	5.24	5.15	5.24	5.15	5.24	5.15
2	5.15	5.12	5.15	5.12	5.15	5.12	5.15	5.12
3	5.07	5.12	5.07	5.12	5.07	5.12	5.07	5.12
4	5.13	5.11	5.13	5.11	5.13	5.11	5.13	5.11
5	5.06	5.04	5.06	5.04	5.06	5.04	5.06	5.04
10	4.86	4.81	4.86	4.81	4.86	4.81	4.86	4.81
15	4.46	4.58	4.46	4.58	4.46	4.58	4.46	4.58
20	4.64	4.65	4.64	4.65	4.64	4.65	4.64	4.65
25	4.17	4.20	4.17	4.20	4.17	4.20	4.17	4.20
30	4.04	4.09	4.04	4.09	4.04	4.09	4.04	4.09

D.2a Mean log CFU/mL *B. anthracis* UT258 total and spore population in fruit juices and wine over 30 days

Days after inoculation	Orange juice (mean log CFU/mL)				Apple juice (mean log CFU/mL)			
	Trial 1		Trial 2		Trial 1		Trial 2	
	Total	Spore	Total	Spore	Total	Spore	Total	Spore
0	5.23	5.09	5.67	5.52	5.89	5.75	5.25	5.18
1	5.10	4.95	5.65	5.61	5.97	5.93	5.20	5.05
2	5.15	5.00	5.62	5.60	5.92	5.82	5.21	5.13
3	5.10	4.99	5.61	5.65	6.00	5.93	5.15	5.09
4	5.14	5.11	5.60	5.59	5.91	5.94	5.09	5.02
5	5.07	5.07	5.54	5.53	5.94	5.93	5.03	4.99
10	5.07	5.08	5.46	5.55	5.94	5.91	5.03	5.00
15	5.05	5.06	5.45	5.47	5.67	5.71	4.88	4.91
20	5.00	5.02	5.54	5.59	5.36	5.27	4.85	4.86
25	5.05	5.17	5.54	5.57	5.11	5.18	4.97	4.92
30	4.98	5.05	5.51	5.55	4.65	4.70	4.85	4.83

D.2b Mean log CFU/mL *B. anthracis* UT258 total and spore population in fruit juices and wine over 30 days

Days after inoculation	Wine (mean log CFU/mL)				Grape juice (mean log CFU/mL)			
	Trial 1		Trial 2		Trial 1		Trial 2	
	Total	Spore	Total	Spore	Total	Spore	Total	Spore
0	4.99	4.96	4.99	4.96	4.99	4.96	4.99	4.96
1	5.12	5.05	5.12	5.05	5.12	5.05	5.12	5.05
2	5.15	5.12	5.15	5.12	5.15	5.12	5.15	5.12
3	5.12	5.05	5.12	5.05	5.12	5.05	5.12	5.05
4	5.12	5.05	5.12	5.05	5.12	5.05	5.12	5.05
5	5.06	5.05	5.06	5.05	5.06	5.05	5.06	5.05
10	4.88	4.86	4.88	4.86	4.88	4.86	4.88	4.86
15	4.72	4.76	4.72	4.76	4.72	4.76	4.72	4.76
20	4.82	4.90	4.82	4.90	4.82	4.90	4.82	4.90
25	4.67	4.71	4.67	4.71	4.67	4.71	4.67	4.71
30	4.70	4.71	4.70	4.71	4.70	4.71	4.70	4.71

D.3 Log total initial and recovered *B. anthracis* 7702p610 and UT258 spores recovered from 100 mL grape or apple juice with 0.1% (w/v) cornstarch added after centrifugation at 1500 × *g* for 10 minutes

Apple Juice				**Grape Juice**			
***B. anthracis* 7702p610 (log total spores)**		***B. anthracis* UT258 (log total spores)**		***B. anthracis* 7702p610 (log total spores)**		***B. anthracis* UT258 (log total spores)**	
Initial	**Recovered**	**Initial**	**Recovered**	**Initial**	**Recovered**	**Initial**	**Recovered**
7.92	7.51	7.54	7.43	7.51	7.30	7.59	7.40
7.70	7.64	7.77	7.59	7.62	7.49	7.74	7.54
6.92	6.59	6.54	6.14	6.51	6.23	6.59	6.32
6.70	6.52	6.77	6.62	6.62	6.51	6.74	6.34
5.92	5.51	5.54	5.31	5.51	5.37	5.59	5.51
5.70	5.53	5.77	5.65	5.62	5.51	5.74	5.52
4.92	4.71	4.54	4.11	4.51	4.33	4.59	4.22
4.70	4.51	4.77	4.55	4.62	4.42	4.74	4.45
3.92	3.64	3.54	3.06	3.51	3.30	3.59	3.25
3.70	3.45	3.77	3.74	3.62	3.32	3.74	3.34
2.92	2.39	2.54	1.99	2.51	2.07	2.59	2.07
2.70	2.57	2.77	2.64	2.62	2.44	2.74	2.40

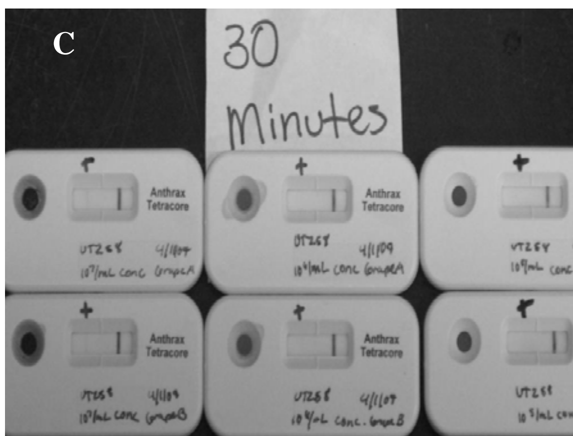
D.4a Mean log initial and final *B. anthracis* 7702p610 (CFU/mL) recovered in orange juice supernatant after centrifugation at varying speed and time.

Centifuge speed (× *g*)	**121**				**655**			
Time (min)	**5**		**10**		**5**		**10**	
Mean log CFU/mL	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**
	3.10	3.01	3.10	2.97	2.98	2.64	2.98	2.44
	3.11	3.02	3.11	2.97	2.87	2.64	2.87	2.25

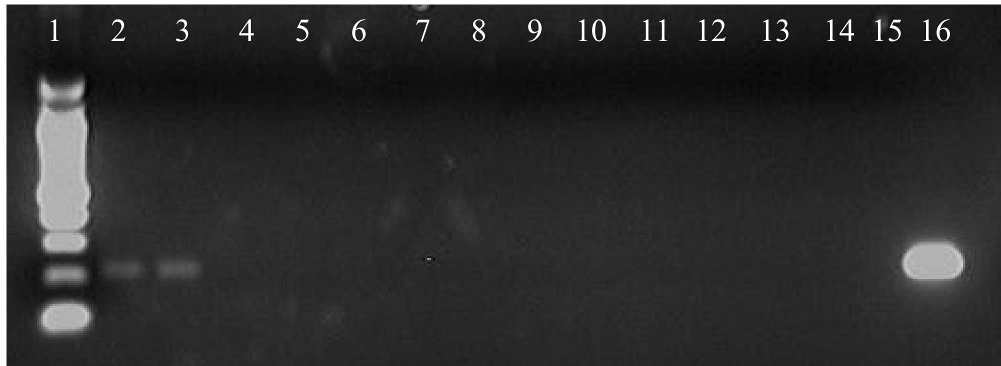
D.4b Mean log initial and final *B. anthracis* UT258 (CFU/mL) recovered in orange juice supernatant after centrifugation at varying speed and time.

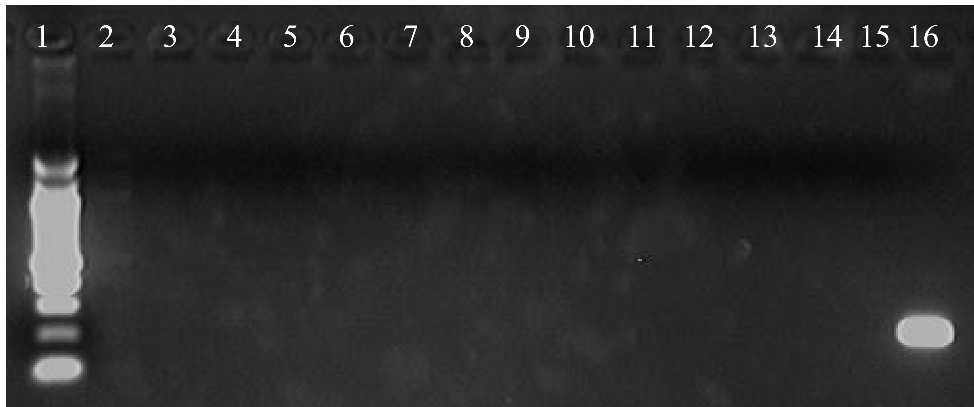
Centifuge speed (× *g*)	**121**				**655**			
Time (min)	**5**		**10**		**5**		**10**	
Mean log CFU/mL	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**
	2.36	2.36	2.36	2.18	2.32	2.00	2.32	1.78
	2.65	2.55	2.65	2.53	2.50	2.38	2.50	2.33

D.5 Sample lateral flow assay results from *B. anthracis* UT258 spores suspended in water (A), apple juice (B), grape juice (C), orange juice (D), apple juice after concentration (E), and grape juice after concentration (F)



D.6 Results of electrophoresis of standard PCR reactions of *B. anthracis* 7702p610 (top) and ANR-1 (bottom) extracted from orange juice using MoBio UltraClean kit





Lanes: 1- 100 bp ladder, 2&3 – 10^6 *B. anthracis* spores/mL orange juice, 4&5- 10^5 *B. anthracis* spores/mL orange juice, 6&7 – 10^4 *B. anthracis* spores/mL orange juice, 8&9 – 10^3 *B. anthracis* spores/mL orange juice, 10&11 – 10^2 *B. anthracis* spores/mL orange juice, 12&13- 10^1 *B. anthracis* spores/mL orange juice, 14 – *B. thuringiensis*, 15 – water, 16 – ANR-1 vegetative cells extracted with UltraClean kit

D.7a Real-time PCR mean cycle threshold (C*t*) internal standard curve included with each real-time PCR run using orange juice.

B. anthracis **strains**	**ANR-1 and 7702p610**		**7702 and UT258**	
Dilution of standard	**Mean C*t* value**			
10^{-1}	13.67	15.04	14.88	14.80
10^{-2}	20.31	21.84	22.28	21.81
10^{-3}	24.24	24.20	24.34	24.11
10^{-4}	28.50	28.11	28.87	28.91
10^{-5}	31.31	29.54	29.44	29.62
10^{-6}	32.20	29.15	29.52	29.16

D.7b Real-time PCR mean cycle threshold (C*t*) internal standard curve included with each real-time PCR run using apple and grape juice.

Juice media	**Apple Juice**				**Grape Juice**			
***B. anthracis* strain(s)**	**7702p610**		**UT258**		**7702p610**		**UT258**	
Dilution of standard	**Mean C*t* value**							
10^{-1}	18.57	16.14	18.69	13.81	24.48	22.80	17.03	14.61
10^{-2}	27.69	24.61	27.66	21.28	24.27	24.25	25.87	22.11
10^{-3}	29.68	24.67	30.57	22.67	31.34	32.11	28.51	23.21
10^{-4}	*	34.58	*	32.46	31.80	32.42	37.93	31.81
10^{-5}	*	34.85	*	31.86	32.93	33.43	*	31.78
10^{-6}	*	36.16	*	31.09	*	*	*	31.19

* Did not cross fluorescence threshold

D.8a Log concentration initial *B. anthracis* spores in orange juice and corresponding real-time PCR cycle threshold (C*t*) after DNA extraction with UltraClean kit.

	Log 7702 concentration (CFU/mL)	**Mean C*t* value**	**Log ANR-1 concentration (CFU/mL)**	**Mean C*t* value**
Trial 1	5.90	23.53	5.73	25.08
	4.90	26.22	4.73	27.15
	3.90	27.62	3.73	30.29
	2.90	28.68*	2.73	30.37
	1.90	28.67*	1.73	31.62
	0.90	28.68*	0.73	33.55*
	Non-inoculated juice (Negative control)	28.48	Non-inoculated juice (Negative control)	33.37
Trial 2	5.96	22.22	6.00	22.85
	4.96	26.46	5.00	25.36
	3.96	28.25	4.00	27.92
	2.96	28.90	3.00	29.14
	1.96	29.97	2.00	29.72*
	0.96	30.37	1.00	29.45*
	Non-inoculated juice (Negative control)	29.23	Non-inoculated juice (Negative control)	29.41

D.8b Log concentration initial *B. anthracis* spores in orange juice and corresponding real-time PCR cycle threshold (C*t*) after DNA extraction with UltraClean kit.

	Log 7702p610 concentration (CFU/mL)	**Mean C*t* value**	**Log UT258 concentration (CFU/mL)**	**Mean C*t* value**
Trial 1	5.91	22.08	5.96	20.98
	4.91	25.21	4.96	23.52
	3.91	27.18	3.96	27.31
	2.91	29.25	2.48	29.63*
	1.91	30.42	1.96	28.10
	0.91	30.33	0.96	29.10*
	Non-inoculated juice (Negative control)	33.37	Non-inoculated juice (Negative control)	28.48
Trial 2	5.56	21.74	5.96	23.23
	4.56	24.91	4.96	25.97
	3.56	27.09	3.96	28.26
	2.56	28.78	2.96	29.00
	1.56	29.83*	1.96	29.94*
	0.56	29.96*	0.96	29.93*
	Non-inoculated juice (Negative control)	29.41	Non-inoculated juice (Negative control)	29.23

* C*t* values equal or greater to negative control considered negative.

D.9 Log concentration initial *B. anthracis* 7702p610 spores in apple juice and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log 7702p610 concentration (CFU/mL) apple juice	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	5.92	25.07	17.66
	4.92	29.06	21.60
	3.92	33.44	26.65
	2.92	36.91	30.69
	1.92	40.00*	34.55
	0.92	40.00*	39.08
	Non-inoculated juice (Negative control)	40.00	
Trial 2	5.70	22.67	15.66
	4.70	26.51	19.69
	3.70	29.81	24.17
	2.70	28.80	27.53
	1.70	31.44	30.45
	0.70	32.02	30.96
	Non-inoculated juice (Negative control)	35.24	

* C*t* values equal or greater to negative control considered negative.

D.10 Log concentration initial *B. anthracis* 7702p610 spores in grape juice and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log 7702p610 concentration (CFU/mL) grape juice	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	5.50	22.33	17.49
	4.50	25.49	21.21
	3.50	28.63	23.82
	2.50	29.28	27.61
	1.50	32.66*	29.96
	0.50	32.99*	31.83
	Non-inoculated juice (Negative control)	32.65	
Trial 2	5.62	20.05	15.11
	4.62	23.45	19.16
	3.62	26.85	22.80
	2.62	30.74	26.75
	1.62	31.32	29.45
	0.62	33.12*	31.62
	Non-inoculated juice (Negative control)	33.09	

* C*t* values equal or greater to negative control considered negative.

D.11 Log concentration initial *B. anthracis* UT258 spores in apple juice and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log UT258 concentration (CFU/mL) apple juice	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	5.53	26.24	20.67
	4.53	29.06	24.70
	3.53	30.20	28.52
	2.53	31.71	29.55
	1.53	31.12	30.84
	0.53	35.56*	31.17
	Non-inoculated juice (Negative control)	34.97	
Trial 2	5.77	22.83	21.73
	4.77	27.01	25.05
	3.77	28.58	28.07
	2.77	29.96	28.34
	1.77	32.11*	28.20
	0.77	34.70*	29.77
	Non-inoculated juice (Negative control)	31.44	

* C*t* values equal or greater to negative control considered negative.

D.12 Log concentration initial *B. anthracis* UT258 spores in grape juice and corresponding real-time PCR cycle threshold (C*t*) with and without centrifugal concentration extracted with UltraClean kit.

	Log UT258 concentration (CFU/mL) grape juice	**Mean C*t* value without pre-concentration**	**Mean C*t* value with pre-concentration**
Trial 1	5.59	30.65	24.77
	4.59	35.82	27.75
	3.59	37.71	33.11
	2.59	39.93*	38.71
	1.59	39.62*	38.91
	0.59	39.58*	35.80
	Non-inoculated juice (Negative control)	39.53	
Trial 2	5.74	25.35	22.73
	4.74	29.28	26.68
	3.74	30.56	28.67
	2.74	30.85	30.65
	1.74	31.34	30.21
	0.74	32.83*	30.79
	Non-inoculated juice (Negative control)	32.68	

* C*t* values equal or greater to negative control considered negative.

Appendix E: Ricin

E.1 Ricin standard curve generated using in-well dilution with +/- Tetracore ELISA assay between 20 µg/mL and 1.98 pg/mL read at A_{405} nm.

Ricin concentration	Mean A_{405} nm			
	Sample Set A	Sample Set B	Sample Set A	Sample Set B
	Trial 1		Trial 2	
20.00 µg/mL	2.89	2.83	2.28	2.45
10.00 µg/mL	2.72	2.75	2.43	2.81
5.00 µg/mL	2.69	2.73	3.02	2.59
2.50 µg/mL	2.55	2.85	2.98	2.43
1.25 µg/mL	2.68	2.76	2.78	2.89
0.63 µg/mL	2.85	2.79	3.10	2.90
0.31 µg/mL	2.80	2.76	2.79	2.67
0.16 µg/mL	2.85	3.09	3.12	2.60
78.13 ng/mL	2.60	2.76	2.42	2.79
39.06 ng/mL	2.60	2.63	2.20	2.17
19.53 ng/mL	2.62	2.37	2.06	1.80
9.77 ng/mL	2.24	2.02	1.75	1.61
4.88 ng/mL	1.35	1.11	0.89	0.66
2.44 ng/mL	2.55	0.78	0.82	0.79
1.22 ng/mL	0.43	0.44	1.08	0.80
61.00 pg/mL	0.33	0.19	0.38	0.37
31.50 pg/mL	0.75	-0.14	0.25	0.32
15.75 pg/mL	0.30	0.05	0.09	0.05
7.89 pg/mL	0.12	0.06	0.06	-0.07
3.95 pg/mL	0.99	0.10	0.05	0.08
1.98 pg/mL	0.04	-0.13	0.11	-0.10

E.2 Ricin standard curve generated using pre-diluted ricin standards with +/- Tetracore ELISA assay between 100 ng/mL and 6.25 ng/mL read at A_{405} nm.

Ricin concentration	Mean A_{405} nm			
	Trial 1	Trial 2	Trial 3	Trial 4
100 ng/mL	2.37	1.60	3.46	1.01
50 ng/mL	1.74	1.53	3.47	0.62
25 ng/mL	1.09	1.17	2.78	0.38
12.5 ng/mL	0.56	1.03	1.78	0.36
6.25 ng/mL	0.17	0.94	0.90	0.07

E.3 Ricin detected via +/- ELISA in samples of ricin in PBS with and without magnetic beads added, the magnetic beads directly, and the resulting bead-free supernatant after 30 minutes of incubation at 37°C with mixing.

Target initial ricin concentration	Mean ricin (ng/mL) detected via ELISA			
	Standard	**Standard with 50 µg Pathatrix magnetic beads**	**Extracted Pathatrix magnetic beads**	**Supernatant**
100 ng/mL	120.05	33.83	37.04	18.77
	94.38	96.49	27.99	52.57
50 ng/mL	23.13	11.91	41.33	3.19
	58.57	56.88	13.17	10.16
25 ng/mL	15.50	3.96	13.96	0*
	33.84	30.49	0.34	0*
12.5 ng/mL	20.88	0.63	21.40	1.59
	12.03	15.10	0*	0*
6.25 ng/mL	32.04	0*	5.24	0*
	0*	5.83	0*	0*

* Below linear range of detection

E.4 Ricin detected via +/- ELISA in samples of ricin in PBS with and without magnetic beads added, the magnetic beads directly, and the resulting bead-free supernatant after 60 minutes of incubation at 37°C with mixing.

Target initial ricin concentration	Mean ricin (ng/mL) detected via ELISA			
	Standard	Standard with 50 µg Pathatrix magnetic beads	Extracted Pathatrix magnetic beads	Supernatant
100 ng/mL	92.02	25.09	15.58	0*
	64.56	83.89	57.70	23.28
50 ng/mL	63.45	4.57	4.80	0*
	59.36	70.41	33.14	0*
25 ng/mL	34.55	2.15	0*	0*
	35.27	29.22	0*	0*
12.5 ng/mL	10.74	0*	0*	0*
	25.64	0*	0*	0*
6.25 ng/mL	0*	0*	10.94	0*
	19.73	0*	0*	0*

* Below linear range of detection

E.5 Ricin (ng/mL) detected in supernatant of 100 ng/mL ricin in PBS after 60 minutes mixing from the original sample and from the magnetic beads after bead beating with 0.1 mm glass beads.

Bead beating time	**Mean ricin detected via ELISA (ng/mL)**					
	Trial 1			**Trial 2**		
	Initial sample	**After bead beating**	**Supernatant**	**Initial sample**	**After bead beating**	**Supernatant**
1 minute	92.25	2.86	9.37	88.62	45.33	7.74
2 minutes	92.25	0.00	1.80	88.62	9.97	0.88
5 minutes	117.70	18.50	0*	90.26	23.22	8.13
10 minutes	117.70	0*	0*	90.26	11.40	0*
15 minutes	111.30	10.21	3.97	123.80	1.86	0*
30 minutes	111.30	0*	0*	123.80	0*	0*

* Below range of linear detection

E.6 Ricin detected in intial sample and supernatant via +/- ELISA after 60 minutes of mixing in standard IMS system with 50 μL of anti-ricin Pathatrix beads

Target initial ricin concentration (ng/mL)	Mean ricin concentration in initial sample detected via ELISA (ng/mL)	Mean ricin concentration detected via ELISA in supernatant (ng/mL)
	264.40	0 *
100	95.57	0 *
	186.87	30.49
	1354.71	0 *
500	554.38	0 *
	852.79	57.17
	2186.76	0 *
1000	1088.41	0 *
	1152.07	156.26
	11262.50	148.57
10000	9102.17	372.70
	10026.62	418.04
	23483.33	4031.00
20000	18715.22	1323.22
	20819.48	816.17

E.7 Ricin detected via +/- ELISA after 60 minutes of mixing using standard IMS system with 50 µL of either anti-ricin Pathatrix beads or unconjugated Pathatrix beads.

Target ricin concentration in initial sample (ng/mL)	Mean ricin detected via ELISA (ng/mL)			
	Anti-ricin beads		Unconjugated beads	
	Intitial sample	Supernatant	Intitial sample	Supernatant
100	264.40	0.00*	120.10	92.64
	95.57	0.00*	120.10	102.81
	146.87	30.49	120.10	104.88
	82.86	3.56	108.00	56.26
	82.86	0.00*	108.00	0.00*
	82.86	15.75	108.00	80.76
500	1354.71	0.00	374.08	155.49
	554.38	0.00	374.08	116.76
	852.76	57.17	374.08	116.47
	615.96	24.83	386.93	194.03
	615.96	40.90	386.93	155.30
	615.96	36.34	386.93	501.83

*Below linear range of detection

E.8 Ricin detected via +/- ELISA after 60 minutes of mixing using Pathatrix IMS system with 50 µL of either anti-ricin Pathatrix beads, unconjugated Pathatrix beads, or no beads added to the system.

Target ricin concentration in initial sample (ng/mL)	Mean ricin detected via ELISA (ng/mL)					
	Anti-ricin beads		Unconjugated beads		No beads added	
	Intitial sample	Pathatrix effluent	Intitial sample	Pathatrix effluent	Intitial sample	Pathatrix effluent
100	77.18	0.00*	96.41	9.214	80.82	32.42
	77.18	12.71	96.41	17.94	80.82	48.02
	77.18	12.71	96.41	0.00*	80.82	10.52
	100.40	1.10	54.38	0.00*	141.20	0.00*
	100.40	0.53	54.38	0.00*	141.20	45.76
	100.40	3.49	54.38	0.00*	141.20	76.5
500	433.10	29.17	636.60	0.00*	357.60	64.62
	433.10	39.50	636.60	28.70	357.60	51.86
	433.10	24.42	636.60	37.53	357.60	23.66
	591.60	1.51	396.20	9.14	572.20	127.3
	591.60	30.68	396.20	63.39	572.20	277.5
	591.60	34.51	396.20	47.27	572.20	296.4

* Below linear range of detection

Appendix F. Additional Protocols

F.1 Protocol for DNA extraction via the MoBio UltraClean kit as provided by the manufacturer

1. Add 1.8 ml of microbial (bacteria, yeast) culture to a 2 ml Collection Tube (provided) and centrifuge at 10,000 x *g* for 30 seconds at room temperature. Decant the supernatant and spin the tubes at 10,000 x *g* for 30 seconds at room temperature and completely remove the media supernatant with a pipet tip. NOTE: Based on the type of microbial culture, it may be necessary to centrifuge longer than 30 seconds.
2. Resuspend the cell pellet in 300 μl of MicroBead Solution and gently vortex to mix. Transfer resuspended cells to MicroBead Tube.
3. Add 50 μl of Solution MD1 to the MicroBead Tube.
4. Optional: To increase yields, to minimize DNA shearing, or for difficult cells, see Alternative lysis methods in the “Additional Information” section before continuing.
5. Secure bead tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (Catalog No. 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes. (See “Alternative lysis method” for less DNA shearing).
6. Make sure the 2 ml MicroBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge the tubes at 10,000 x *g* for 30 seconds at room temperature. CAUTION: Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean 2 ml Collection Tube (provided).
8. NOTE: Expect 300 to 350 μl of supernatant.

9. Add 100 µl of Solution MD2, to the supernatant. Vortex 5 seconds. Then incubate at 4°C for 5 minutes.

10. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

11. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 ml Collection Tube (provided). Expect approximately 450 µl in volume.

12. Add 900 µl of Solution MD3 to the supernatant and vortex 5 seconds.

13. Load about 700 µl into the Spin Filter and centrifuge at 10,000 x *g* for 30 seconds at room temperature. Discard the flow through, add the remaining supernatant to the Spin Filter, and centrifuge at 10,000 x *g* for 30 seconds at room temperature. NOTE: A total of 2 to 3 loads for each sample processed are required. Discard all flow through liquid.

14. Add 300 µl of Solution MD4 and centrifuge at room temperature for 30 seconds at 10,000 x *g*.

15. Discard the flow through.

16. Centrifuge at room temperature for 1 minute at 10,000 x *g*.

17. Being careful not to splash liquid on the spin filter basket, place Spin Filter in a new 2 ml Collection Tube (provided).

18. Add 50 µl of Solution MD5 to the center of the white filter membrane.

19. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.

20. Discard Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

F.2 Protocol for coupling of anti-bodies on to magnetic particle as provided by Matrix MicroScience

1. Separate the beads by pulling them to the side of the vial, using the strong magnet provided, for approximately one minute and discard the supernatant. Remove the magnet and fully re-suspend the beads in 1 ml of Buffer A. magnetically capture and drain the beads fully after washing.

2. Perform a second wash with Buffer A, use magnet and then drain beads fully. (Prepare Buffer E and Proceed to step 4)

3. Add 1,000 µl (1 ml) of Buffer A to the amber glass vial containing Chemical 2 – ensure the powder is completely dissolved. Add a further 1,500 µl of Buffer A to the amber glass vial containing Chemical 1 – ensure the powder is completely dissolved. Transfer 1,500 µl (1.5 ml) of Chemical 1 solution to the amber glass vial containing the Chemical 2 solution. Secure lid and mix to ensure both chemicals are fully dissolved. This creates Buffer E.

4. Pipette 2.5 ml of Buffer E into the vial containing the washed and drained beads. Resuspend the particles fully, secure the universal lid and agitate in either an orbital shaker or end over end rotator for 15 minutes at room temperature.

5. Separate the beads by pulling them to the side of the vial using the strong magnet for approximately one minute and discard the supernatant. Remove the magnet and fully resuspend the beads in 1 ml of Buffer B. Magnetically capture and drain the beads.

6. Resuspend the pellet of particles in 1,480 µl (1.48 ml) of Buffer C, immediately add 1 mg of antibodies (refer to Table 1) and mix.

7. Secure the vial lid and agitate the bead/antibody suspension for 2 hours at room temperature by orbital shaking or by end over end rotation at 30-50 rpm.

8. Separate the beads by pulling them to the side of the vial using the strong magnet for approximately one minute and discard the supernatant. Remove the magnet and fully re-suspend the beads in 1 ml of Buffer C. Magnetically capture and drain the beads fully.

9. Wash beads twice more with buffer C and drain fully.

10. Add 1,000 µl (1 ml) of Buffer D into the vial containing the drained antibody-coated beads and resuspend the antibody-coated beads fully.

11. Transfer all the beads into an appropriately labeled sterile 50 ml blue capped tube and add 4,200 ml of Buffer D to the beads. Secure lid and mix well with minimal frothing to ensure the beads are fully resuspended.

12. Transfer 2,600 µl (2.6 ml) of bead suspension into each bead vial (sterile opaque white plastic vials provided). Secure lid tightly and label appropriately. Store in a fridge at 2-8 ºC. Do NOT freeze.

Performance testing of coated particles

Previous experience indicates that a 50µl dose of beads, coated at [1mg/ml], should be optimal. However, if satisfactory recovery isn't achieved then other bead doses above and below this level should be investigated.

Stock Antibody concentration mg/ml	**Volume (µl) to be added to beads in Buffer C**
2	500

3	333
3.5	286
5	200