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FATE AND DETECTION OF *BACILLUS ANTHRACIS*
SPORES IN PASTEURIZED MILK, JUICE AND EGGS

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FATE AND DETECTION OF *BACILLUS ANTHRACIS* SPORES IN PASTEURIZED MILK, JUICE AND EGGS

By

Sandip H. Shah

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ABSTRACT

FATE AND DETECTION OF *BACILLUS ANTHRACIS* SPORES IN PASTEURIZED MILK, JUICE AND EGGS

By

Sandip H. Shah

Bacillus anthracis is a highly virulent, rapidly growing gram-positive bacterial pathogen that may cause inhalational, gastrointestinal or the cutaneous forms of anthrax in humans. The inhalational form of anthrax is the most lethal and feared form of the disease particularly if spores from a virulent strain are deliberately used as a source of human exposure. However, recently, gastrointestinal anthrax resulting from the consumption of meat from animals that die of the disease has been reported in humans from several parts of the world. Deliberate contamination of common foods such as milk, powdered milk, other milk products, fruit juices, liquid eggs and other foods with spores of this organism could potentially result in a massive epidemic and panic in unsuspecting populations. This could be a real threat to homeland biosecurity. In this study, survival of *B. anthracis* was evaluated by using freshly prepared spore suspensions of *Bacillus anthracis* Sterne (BAS) and Pasteur (BAP) of known concentration to spike different food matrices, followed by subjection of the matrixes to four different pasteurization temperatures and time protocols common to the dairy industry in the United States. These were: i) the traditional – low temperature/longer time: 63^0 C for 30 minutes, ii) high temperature/short

time: 72^0 C for 16 seconds, iii) higher temperature/short time: 78^0C for 16 seconds and iv) the most recent method of subjecting the milk to ultra high temperature/very short time: 100^0C for 3 seconds. The detection and/or enumeration of spores was conducted using microscopic spore count, viable colony count, a real time PCR test (based on a widely used proprietary protocol run on Roche's LightCycler® platform) and an electrochemiluminescence assay (BioVerify Anthrax Test manufactured by BioVeris, run on BioVeris M1M platform). Viability of spores following pasteurization depended mostly upon the type of pasteurization, type of food matrix, spore concentration in the suspension and the *B. anthracis* spore strain used. All pasteurization processes were completely lethal to all vegetative cells. *B. anthracis* Pasteur strain spores were more susceptible to heat inactivation compared to the Sterne strain. Pasteurization had little or no effect on Sterne strain spores. Among the four common pasteurization methods selected, the ultra high temperature/short time pasteurization (100^0C. for 3 sec.) was more lethal to spores, especially the Pasteur strain, resulting in a one to two log reduction in viability. These studies suggest that current pasteurization techniques, as practiced by the dairy industry for various products, would have little to no effect on viability of spores. Using 100^0C for 3 seconds may be helpful in reducing the spore load, and at light contamination levels may be preferred in management of crisis related to milk contamination with *B. anthracis* spores. Technology for rapid detection of spores in raw or processed dairy products is not yet perfected and bacteriological culture remains the "gold standard" (standard method of diagnosis).

Humbly dedicated to my family…

To my wife Parul, who sacrificed immensely to support my academic quest, and especially to my children, Neil, Aastha and Sureel, who painstakingly did not often complain when daddy "went to school or library" spending long hours there missing many family occasions. I am confident that they grew up viewing the hunger, craving and process of education as I did: A challenging and demanding, yet satisfying and fulfilling adventure by which knowledge and understanding of the world we live in is gained.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Some images in this dissertation are presented in color.

KEY TO SYMBOLS

+	positive reaction or detection
-	negative reaction or no detection
>	more than
<	less than
α	alpha
^	10 raised to the power of $^{(x)}$
β	beta
®	registered trade mark
µl	micro-liter
µm	micro-meter
µg	micro-gram

KEY TO ABBREVIATIONS

AMA	American Medical Association
AOAC	Association Of Analytical Communities (International)
APHA	American Public Health Association
APHIS	Animal and Plant Health Inspection Service
APHL	Association of Public Health Laboratories
ASM	American Society for Microbiologists (Washington, D.C.)
ATCC	American Type Culture Collection (Rockville, MD)
BA	*Bacillus anthracis*
BAM	Bacteriology Analytic Manual
BAP	*Bacillus anthracis* Pasteur strain
BAS	*Bacillus anthracis* Sterne strain
BC	*Bacillus cereus*
BSL	biosafety level
BT	bioterrorism
C	Celsius
CCP	critical control point
CDC	Centers for Disease Control & Prevention (Atlanta, GA)
CFU	colony forming units
CLSI	Clinical and Laboratory Standards Institute (Wayne, PA)
DFA	direct fluorescent antibody
dH_2O	distilled water
DHS	Department of Homeland Security (Washington, D.C.)

DMC	direct microscopic count
DNA	deoxyribonucleic acid
ECL	electrochemiluminescence
EDTA	Ethylenediamine tetra acetic acid
EF	edema factor
ELISA	enzyme-linked immunosorbant assay
EPA	Environmental Protection Agency (Washington, D.C.)
FASEB	The Federation of American Societies for Experimental Biology
FDA	Food and Drug Administration (Washington, D.C.)
FDD	flow diversion device
GI	gastro-intestinal
Gm	gram
HACCP	hazard analysis critical control point
HHAs	hand held assays
HHS	Department of Health and Human Services (Washington, D.C.)
HTST	high temperature short time
IgG	immunoglobulin G
IHC	immunohistochemical
LD_{50}	lethal dose
LF	lethal factor
LRN	Laboratory Response Network (Atlanta, GA)
M1M	BioVeris® instrument for electrochemiluminescence assay
MDCH	Michigan Department of Community Health

MIC	minimal inhibitory concentration
Ml	milli-liter
MLVA	multi-locus variable-number tandem repeat analysis
MMWR	Morbidity and Mortality Weekly Report (Atlanta, GA)
MPN	most probable number
MSU	Michigan State University (East Lansing, MI)
NASA	National Aeronautics and Space Administration (Houston, TX)
NSM	new sporulation medium
P1	low temperature/long time pasteurization (63^0 C for 30 minutes)
P2	high temperature/short time pasteurization (72^0 C for 16 seconds)
P3	high temperature/short time pasteurization (78^0C for 16 seconds)
P4	ultra-high temp. /short time pasteurization (100^0C. for 3 seconds)
PA	protective antigen
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PLET	polymyxin-lysozyme EDTA-thallous acetate agar
TSA	Trypticase soy agar
TSB	Trypticase soy broth
USAMRIID	US Army Medical Research Institute of Infectious Diseases
USDA	US Department of Agriculture (Washington, D.C.)
USSR	United Soviet Socialist Republic (former Soviet Union)
WHO	World Health Organization (Geneva)

INTRODUCTION

As a result of the anthrax attack on the postal system in October 2001 and a growing concern for terrorism, the potential for a bioterrorist attack on the US food or water system has increased. The terrorist attack using anthrax spores in the mail resulted in 23 cases with many more exposures of inhalational anthrax and 5 deaths that proved the use of biological weapons in the public environment is a very real threat. Food has been targeted by intentional contamination with microorganisms and chemicals in recent years (Jackson, L. 2001). The fact that microorganisms or their toxins may be inexpensive and easy to conceal, but can produce very high morbidity and mortality, heavy economic loss, social panic or shock, makes it an ideal bioterrorist weapon. *Bacillus anthracis* fits this description and thus is a potential weapon of choice for bioterrorists. The milk and dairy products supply system may be particularly vulnerable because milk, eggs and fruit juices are popular food items used in virtually every household. The increasing concentration of commercial dairy production into large scale operations creates a possible target for a bioterrorist. A single large overland tanker can carry as much as 8,500 gallons of a dairy product that could eventually be distributed to thousands of people (Perdue, Karns Jeff et al. 2003). In the weeks following the fall 2001 anthrax spores attack using the US mail system, questions arose regarding the fate of *B. anthracis* spores if purposely introduced to into food products and in particular milk and dairy products. This project was undertaken to answer some of their questions. The specific objectives of the study are as follows:

1. To measure the effects of various pasteurization methods on the viability and detection of *Bacillus anthracis* spores experimentally used to taint batches of milk, fruit juice and liquid eggs. Four of the most widely practiced pasteurization methods were used: i) the traditional 63^0 C for 30 minutes, ii) 72^0 C for 16 seconds, iii) 78^0C for 16 seconds and vi) the most recent method of subjecting the milk to 100^0C for 3 seconds.
2. To compare the utility and effectiveness of the conventional gold standard plate count method with newer rapid methods such as fluorescence-based real-time PCR (Holland, Abramson et al. 1992; Livak, Flood et al. 1995) and electrochemiluminescence (ECL) spore tests for detection of anthrax spores in milk pasteurized by different methods.
3. Application of the same protocols listed under 2 on liquid eggs and fruit juices.

Background:

Bacillus anthracis is capable of causing acute and lethal disease in humans via two major routes, respiratory (pulmonary) and oral or gastro-intestinal. Until recently, the mortality rate due to ingested *B. anthracis* spores was much lower than that reported for inhaled spores (Dixon; Meselson et al. 1999). Recent outbreaks and mortality related to contaminated meat consumption in parts of Africa, Asia and the former Soviet Union suggests a renewed concern (ProMED

mail post anthrax archives at - http://www.isid.org). Cutaneous anthrax is rarely lethal, which is caused via a third route – skin. There are numerous strains of *B. anthracis,* both highly virulent and avirulent, which differ in the presence of the two virulence plasmids in the *vrrA* region (Andersen, Simchock et al. 1996; Jackson, Walthers et al. 1997). The Sterne strain of *B. anthracis* was reportedly developed in 1937 (Turnbull 1999). This strain and it's variants have been widely used as a vaccine strain, and is commonly used as a surrogate for the virulent strains of *B. anthracis* (Dixon, Meselson et al. 1999; Henderson, Duggleby et al. 1994; Turnbull 2000). It lacks the pX02 plasmid that carries the capsular protein gene that is important for virulence of the bacterium (Uchida, Sekizaki et al. 1985) and has a *vrrA* region indistinguishable from the virulent 'Ames' strain (Jackson, Walthers et al. 1997). Its spores and spore-forming machinery are presumably indistinguishable from those for the virulent strains and it carries the pX01 plasmid coding for three of the other major virulence factors, protective antigen (PA), lethal factor (LF) and edema factor (EF). Conversely the Pasteur strain lacks the pX01 plasmid but otherwise is indistinguishable from the virulent 'Ames" strain (Jackson, Walthers et al. 1997).

There is limited information on the fate of *B. anthracis* (BA) in various foodstuffs and, in particular, on its survival and stability in various foods of commercial interest. It has been shown that BA spores can be recovered from milk of anthrax-infected cows, and a few studies looked at the survival of vegetative and spore forms of BA in unpasteurized and pasteurized milk (Bowen

and Turnbull 1992; Montville, Densgrove et al. 2005; Hanson, Wendorff et al. 2005; Perdue, Karns Jeff et al. 2003). There is no available literature, however, on the stability of BA spores after pasteurization, storage or processing of pasteurized milk, other milk products, fruit juices, liquid eggs, and other food products. Consequently the potential hazard of spores added directly to bulk milk, juice, liquid eggs or other foods has not been assessed. This fact prompted us to evaluate the fate of various concentrations of BA spores if they were added directly to milk, juice and liquid eggs from the bulk tank, and to study their viability over a longer period of time reflective of normal shelf life, and to examine methods for their rapid detection.

There are many so called "rapid", on-site tests for anthrax detection and identification, either in trial phase or on the market. Hand-held assays (HHAs) are also sometimes referred to as "Smart Tickets". Utility, reliability and validity of HHA's sold commercially for rapid detection of BA is unknown. While most of these devices work using similar principles, the most widely known amongst these is the hand held BTA Test Strips, by Tetracore LLC, which is a lateral flow immunochromatographic device that uses two antibodies in combination to specifically detect anthrax in solution. One of the specific antibodies is labeled with a colloidal gold derivative. When sufficient anthrax bacteria are present, the colloidal gold label provides a reddish-brown colored line that is visualized after accumulating in the test sample region on the device. When sample is added to the Anthrax BTA Test Strip, the sample begins to mix with the colloidal gold-

labeled antibody and simultaneously moves along the strip membrane by capillary action. In the sample region of the test strip, if anthrax is present, the second specific antibody captures the colloidal gold-labeled antibody and bound anthrax, forming a colored line or a band in the "S" window of the Test Strip. As an internal control, a second band visualized in the Control ("C") window of the Test Strip is an indication that the Test Strip functioned properly. Two bands or colored lines (in the "S" and "C" windows) are required for a positive result determination.

These assays are intended only for the screening of environmental samples. First responder and law enforcement communities in some jurisdictions are reportedly using these as instant screening devices and are required to forward any positive samples to authorities for more sensitive and specialized confirmatory testing at a sentinel laboratory or a reference laboratory. CDC cautions that the results of these assays should not be used to make decisions about patient management or prophylaxis due to the fact that the utility and validity of these assays are largely unknown. At this time, CDC does not have enough scientific data to even recommend the use of these assays (http://emergency.cdc.gov/agent/anthrax/faq/labtesting.asp). The analytical sensitivity of these assays is limited by the technology, and data provided by manufacturers indicate that a minimum of 10,000 spores is required to generate a positive signal. This number of spores would suggest a heavy contamination of the area (sample). Therefore a negative result does not rule out a lower level of

contamination. Data collected from field use also indicate specificity problems with some of these assays. Some positive results have been obtained with spores of the non-anthrax *Bacillus* bacteria that may be found in the environment.

For these reasons, CDC has been asked to evaluate the sensitivity and specificity of the commercially available rapid, hand-held assays for BA. When this study is completed, results will be made available, but conclusions from this study are not expected in the near future (http://emergency.cdc.gov/agent/anthrax/faq/labtesting.asp).

The recent development of an electrochemiluminescence assay (ECL assay by BioVeris Corp. using M1M instrument, described in later chapters) and a widely used 5' nuclease fluorogenic PCR assay (a proprietary assay, described in later chapters), along with advances in real-time fluorescence-based detection of pathogens, is rapidly bringing this technology to the marketplace. In the future, first-responders may be expected to provide detection results using these new technologies or other more versatile technologies. Thus, it is critical to validate new technologies and reagents by comparing them to existing diagnostic methods. The electrochemiluminescence or ECL assay using M1M instrument is a novel technology which is economical, easy to use, portable, sensitive, and rapid. An expected outcome of this study may result in validation of this technology if our data indicates a good correlation with other methods, which will benefit emergency responders in the field as well as laboratories involved in

bioterrorism testing. Results are available in 16 minutes using this assay, compared to 5 hours for real-time PCR or 24-48 hours by culture.

HYPOTHESES TO BE TESTED

Bacillus anthracis is a highly virulent, rapidly growing gram-positive bacterial agent and a proven bioterror weapon that may cause inhalational, gastrointestinal or cutaneous form(s) of disease in humans. Deliberate contamination of milk, powdered milk, other milk products, fruit juices, liquid eggs and foods with spores of this organism could potentially result in a massive epidemic causing panic in unsuspecting populations and threatening homeland biosecurity.

This study describes the simulation of the fate and detectability of *B. anthracis* in three types of milk products, apple juice and liquid eggs following different pasteurization processes. These food matrices were inoculated with different concentrations of pure spore suspensions of two avirulent laboratory strains of *B. anthracis* and evaluated by two rapid detection methods and the traditional "gold standard" bacteriological culture and colony counts. The objectives of the study were accomplished by testing the following hypotheses:

(1) Hypothesis: A high percentage (75% or more) of spores of *Bacillus anthracis* will easily survive the current common pasteurization methods for milk, milk products and fruit juices. Following pasteurization, these spores will also remain viable for at least 4 weeks, sufficient to pose significant hazard to consumers given the normal shelf life of these products.

(2) Hypothesis: All vegetative cells of *Bacillus anthracis* will be killed following the pasteurization process.

(3) Hypothesis: The electrochemiluminescence (ECL) technique can be used effectively to rapidly detect spores of *Bacillus anthracis* in dairy products and fruit juices in the field.

(4) Hypothesis: The ECL technique used to rapidly detect spores of *Bacillus anthracis* in dairy products and fruit juices in the field will be comparable to the PCR and the "gold standard" culture and colony count techniques.

CHAPTER 1

Literature Review

Based on recent concerns and events, it has become increasingly clear that it is necessary to evaluate the risk associated with various microbiological forms of terrorist attack. One such attack may come in the form of the intentional contamination of food supplies. The dairy industry in the United States is at a high risk for bioterrorist types of attack. This risk is an indirect result of the nature of dairy foods, increasing long distance distribution capabilities, and increasing trends of large scale production farms. *Bacillus anthracis* is recognized as a likely tool of bioterrorism in the milk, juice and commercially pasteurized foods industry because this pathogen has many characteristics that make it a fairly dangerous biological agent, and it is suspected to have been weaponized by several nations.

Bacillus species and their clinical & environmental significance.

The overwhelming majority of *Bacillus* species, defined as aerobic endospore-forming, gram-positive, catalase producing rods, appear to have little or no pathogenic potential and are rarely associated with disease. The principal exceptions to this are *Bacillus anthracis*, the agent of anthrax – a zoonotic disease, and *Bacillus cereus*, an agent of food poisoning. A number of other species, most notably *Bacillus licheniformis*, have been implicated in food poisoning and other human and animal infections. The resistance of the spores

to heat, radiation, disinfectants, and desiccation also results in aerobic endospore formers being troublesome contaminants in the operating room, on surgical dressings, in pharmaceutical products, and in foods (Logan 2007; www.fda.gov).

Apart from *Bacillus anthracis*, the majority of species are common environmental contaminants, and isolation from a single clinical specimen is generally not a sufficient basis for incriminating one of these organisms as the etiological agent. However, moderate or heavy growth of aerobic endospore formers from wounds is usually significant. *Bacillus cereus* infections of the eye are emergencies which should always be taken seriously and evaluated by a physician immediately (Logan 2007).

In the clinical laboratory, the most important questions to ask about an aerobic spore-forming isolate are as follows: 1) Was it isolated in pure culture or at least as the predominant organism? 2) Was it isolated in large numbers? 3) Was it isolated more than once? Low-level contamination of foodstuffs by aerobic endospore formers is common, as is asymptomatic transient fecal carriage. Therefore, in foodborne-illness investigations, qualitative isolation tests are insufficient. The ideal criteria for establishing that an aerobic endospore former is the etiological agent are (i) isolation of significant numbers (>10 CFU/g) of the organism from the epidemiologically incriminated food (and, in the case of suspected *Bacillus cereus* food poisoning, detection of emetic toxin and/or

enterotoxin), and (ii) recovery of the same strain (biovar, serovar, phage type, plasmid type, etc.) in significant numbers from acute phase specimens (feces or vomitus) from the patients, but not from healthy controls (Logan 2007).

Bacillus anthracis continues to be generally regarded as an obligate pathogen; its continued existence in the ecosystem appears to depend on a periodic multiplication phase within an animal host, and its environmental presence reflects contamination from an animal source at some time rather than self-maintenance within the environment. In human and animal specimens, it is usually sought only when the case history suggests that it is reasonable to suspect anthrax. Demonstration of encapsulated or capsule producing *Bacillus anthracis*, even in low numbers, confirms the clinical suspicion of anthrax (Logan 2007).

The Organism: Bacillus anthracis.

Bacillus anthracis is a highly pathogenic, rod-shaped, gram-positive bacterium. It exists in two forms in nature: spores and vegetative cells. Spores are highly resistant to environmental factors, such as temperature, humidity and radiation. Spores germinate into vegetative cells when growth conditions are optimal. It is the growth of the vegetative cells that produces anthrax toxin. B. anthracis grows between 12 and 45 °C. Germination can take place outside of an animal host, if the temperature is between 8 and 45 °C and adequate nutrients are available. Milk provides an optimal environment, and thus it is

required by law to be stored at or below 7.2 °C which is a hostile condition for BA. In practice milk is often stored at 4 °C, therefore growth and germination in milk should not occur (McNamara 2005). Also, vegetative cells of BA are considered more fragile than those of other *Bacillus* species, as they lose viability more readily in environments such as milk. Vegetative cells form spores through the process of sporulation. BA requires temperatures between 15 and 42 °C and free oxygen for sporulation. Vegetative cells do not produce spores in anaerobic environments. While milk may not be a strictly anaerobic environment, the amount of free oxygen is very small. Milk contains an average of 0.47 percent oxygen by volume with a minimum of 0.3% to a maximum of 0.59%. It is not known what amount of free oxygen is necessary for BA sporulation. Because of the low storage temperature and low amounts of oxygen in milk, it is presumed that sporulation will not occur.

B. anthracis has two principal virulence factors, a polyglutamyl capsule and a three component exotoxin. These factors are encoded in plasmids pXO2 and pXO1 respectively (Dixon, Meselson et al. 1999). The genes capA, capB, and capC involved in the synthesis of the capsule are encoded in pXO2. The capsule is thought to inhibit phagocytosis of vegetative anthrax bacilli (Dixon, Meselson et al. 1999). The genes that encode the exotoxins are located on pXO1. These exotoxins inhibit the host immune response to infection. Together these two factors protect the bacteria from the immune response. The exotoxin is produced by combining three basic components: protective antigen, lethal factor, and edema factor. Alone these components have no biological effect, but they

combine to form two binary toxins (Dixon, Meselson et al. 1999). Edema toxin is formed from the protective antigen and edema factor. Lethal toxin is formed from the protective antigen and the lethal factor. The protective antigen in both toxins serves to carry the toxin into the host cell. Edema toxin disrupts water homeostasis causing edema, whereas lethal toxin stimulates the macrophages to release tumor necrosis factor K and interleukin-1 L, which are partly responsible for sudden death in systemic anthrax (Dixon, Meselson et al. 1999). Both plasmids pXO1 and pXO2 are necessary for full virulence and the resulting infection and disease of Anthrax. Any strain lacking one or the other is considered attenuated (Dixon, Meselson et al. 1999; Humes and and Snyder 2007).

Anthrax: The disease

Current reports indicate that anthrax remains the most widely recognized clinical disease caused by a *Bacillus* species (Logan 2007). It is primarily a disease of domestic or wild animals, and prior to the availability of an effective veterinary vaccine in the late 1930s, anthrax was one of the foremost causes worldwide of mortality in cattle, sheep, goats, and horses. Humans almost invariably contract anthrax directly or indirectly from animals. The use of veterinary and human vaccines together with improvements in industrial hygiene and sterilization procedures for imported animal products, and the increased use of man-made alternatives to animal hides or hair, have resulted in a marked decline in the incidence of anthrax in both animals and humans over the past half-century. Nevertheless, the disease continues to be endemic in many

countries, particularly those that lack efficient vaccination policies. Because anthrax spores remain viable in soil for many years and their persistence does not depend on animal reservoirs, *Bacillus anthracis* is exceedingly difficult to eradicate from an area where it is endemic; regions of nonendemicity must be constantly on the alert for the arrival of *Bacillus anthracis* in imported animal products. Anthrax is not contagious, and transmission to humans is usually restricted to direct contact with infected animals or contaminated fomites, including such oddities as communal loofahs and contaminated syringes. Direct animal- to-animal transmission within a species (i.e., excluding scavengers feeding on anthrax carcasses) is also very rare. (Oncu and Sakarya 2003; Humes and Snyder 2007; Logan 2007).

Available literature provides circumstantial evidence that humans are moderately resistant to anthrax compared with strict or obligate herbivores. Consumption of mostly heat cooked food by humans may have contributed to this apparent resistance. Human anthrax infection has traditionally been classified as either "nonindustrial", resulting from close contact with infected animals or their carcasses after death from the disease, or "industrial", indicating disease acquired by those employed in processing wool, hair, hides, bones, or other animal products. A very few reports of laboratory-acquired infections exist. Depending upon the route of infection, there are three major clinical forms of anthrax: cutaneous, inhalation, and oropharyngeal or gastrointestinal. Meningitis as a result of anthrax can develop as a complication of any of these forms.

For the US military and armed forces worldwide, *Bacilus anthracis* has been an organism of special interest. Substantial research has been conducted for anthrax development and deployment as a biowarfare agent in several countries over many years. A major outbreak of anthrax occurred in April 1979 in the city of Sverdlovsk, former USSR (now Yekaterinburg, Russia), in the Urals as a result of the accidental release of spores from a military production facility (Humes and and Snyder 2007). It is speculated that, following attacks on livestock during the First World War, it remains high on the list of agents that could be used currently in biological warfare or bioterrorism. Natural disease is readily controllable, but the October 2001 bioterrorism related anthrax outbreak in the United States increased both governmental and public concerns about this disease immensely (www.cdc.gov/anthrax; Humes and Snyder 2007).

Types of clinical anthrax:

Three types of the disease are known exist.

1. **Cutaneous anthrax**

Cutaneous anthrax refers to a *B. anthracis* infection of the skin, and is usually treatable. Cutaneous anthrax accounts for about 99% of naturally acquired human anthrax worldwide—an estimated 2,000 cases are reported annually. Infection occurs through a break in the skin. Following the incubation period of 2 to 3 days, a small papule appears, progressing over the next 24 h to a ring of

vesicles, with subsequent ulceration and formation of a characteristic blackened eschar. Subsequent eschar formation may become thick and surrounded by extensive edema. Fever and suppuration as well as pain at the site are normally absent; their presence probably indicates a secondary bacterial infection (Chraibi, Haouach et al. 2009; Dixon, Meselson et al. 1999; Bravata, Holty et al. 2007). Before the availability of antimicrobial agents and vaccines, 10 to 20% of untreated cases of cutaneous anthrax were fatal. Less than 1% of cases are fatal today, and they are due mainly to obstruction of the airways by the edema that accompanies lesions on the face or neck, or to the progression of the cutaneous disease into a systemic infection (Chraibi, Haouach et al. 2009; Dixon, Meselson et al. 1999; MMWR 1994).

2. **Inhalation anthrax**

Inhalation anthrax is an infection of the respiratory system as well as the lymph nodes. Inhalation anthrax is fatal in greater than 40 percent of cases with treatment and 97% of cases without treatment (Humes and Snyder 2007). Prior to the bioterrorist attack of 2001, 18 cases of naturally acquired inhalation anthrax had been recorded in the United States since 1900, with 16 (88.9%) of them being fatal (Brachman and Kaufmann 1998); figures from the United Kingdom show a similar picture. Among the 23 cases of the bioterrorism-related outbreak in the United States in late 2001, in which spores were delivered in mailed letters and packages, early recognition and treatment of 12 patients with confirmed inhalation anthrax resulted in 60% survival (Bell, Kozarsky et al. 2002;

Jernigan, Raghunathan et al. 2002; Logan 2007). In inhalation anthrax the inhaled spores are ingested by macrophages and carried from the lungs to the lymphatic system, where the infection progresses. During transit to lymph nodes, spores germinate into vegetative cells, begin to replicate, and produce the capsule and toxins that lead to bacteremia and associated hemorrhage and necrosis. The replacement of the older name for this form of the disease, "pulmonary anthrax," with the newer name, "inhalation anthrax," is a reflection of the fact that active infection occurs in the lymph nodes rather than the lung itself. Analysis of 10 of the cases associated with the bioterrorist events of 2001 (Jernigan, Raghunathan et al. 2002) revealed a median incubation period of 4 days (range, 4 to 6 days). All 12 patients with inhalation anthrax had severe illness and were hospitalized. Their clinical presentation included fever or chills (n=12), fatigue or malaise (n=12), minimal or nonproductive cough (n=10), dyspnea (n=9), nausea or vomiting (n=9), chest pain (n=7), and sweats (n=7). All patients had abnormal chest X-ray images with pleural effusion (n=8), infiltrates (n=7), and mediastinal widening (n=7) (Baggett, Rhodes et al. 2005).

Little dose response information for humans is known; however some data for inhalation anthrax have been obtained through case studies. In one such case study, it was determined that inhalation of 600-2150 anthrax spores results in infection and eventually high mortality (97%) if untreated. In order to enter the alveoli in the lungs, the spores must be less than 5 μm in diameter. Of the 600-

2150 spores inhaled, the calculated dose of spores less than 5 μm in diameter was 150-700 (Watson and Keir 1994).

3. Oropharyngeal and gastrointestinal anthrax

Oropharyngeal and gastrointestinal anthrax, resulting from ingestion of the organism, is also very serious and fatal in 25 to 60% of untreated cases. With treatment, gastrointestinal anthrax has a mortality rate of less than 40% (Sirisanthana and Brown 2002). Gastrointestinal anthrax causes abdominal pain, fever, vomiting, bloody diarrhea, shock and hemorrhagic inflammation of the small intestine. Infection manifests after an incubation period of 1 to 6 days (Beatty, Ashford et al. 2003). Ingestion of infected food or water is likely to cause gastrointestinal anthrax. Determining the minimum number of spores needed to cause infection in humans is difficult and not understood well. Host response to infection is highly variable and is dependant on the interrelationship of many host, agent and environmental factors. Also, necessary data for determining a dose response model are unavailable therefore little dose response information for humans is known for gastrointestinal infection with this agent.

For gastrointestinal anthrax, most infections in humans are known to be caused by ingestion of putrid meats (Sirisanthana and Brown 2002; ProMED mail post anthrax archives at - http://www.isid.org; Ray, Hutin et al. 2009). In case studies it is rarely possible to determine the number of spores ingested. One experiment showed that orally administering 10^8 spores did not cause infection in

guinea-pigs. However, when compared with a different route of inoculation, LD_{50} (the lethal dose for 50% of the population) by subcutaneous injection was estimated to be only 30 spores (Young, Zelle et al. 1946). It is generally thought that both gastrointestinal and orophyngeal anthrax are caused by germination of ingested spores. One source, however, states that ingestion of large numbers of vegetative cells may in fact be the cause of these diseases (Inglesby 2002). Another source states that vegetative cells may have an "effect" at the primary infection site (Beatty, Ashford et al. 2003). Nonetheless, currently it is not clear as to how many spores and/or vegetative cells, if artificially introduced in milk, juice or other food stuffs, would cause infection in humans. At the same time, laboratory prepared concentrations can easily reach extremely high concentration of 10^{10} spores or more in one milliliter of such fluids or food stuffs (Perdue, Karns Jeff et al. 2003). A syringe with a few milliliters of such a preparation in a terrorist's hand would make a very effective tool for intentional or deliberate contamination of large volumes of food stuffs or bulk produced liquid food items.

Regardless of the form of the disease, the initial generalized symptoms that are usually mild (fatigue, malaise, fever, and/or gastrointestinal symptoms) can *rapi*dly develop into the fulminant state, characterized by dyspnea, cyanosis, severe pyrexia, and disorientation followed by circulatory failure, shock, coma, and death. Depending on the host, there is a rapid buildup of the bacteria in the blood stream to catastrophic levels of 10^7 to 10^9 organisms/ml over the last few

hours in the most susceptible species (Logan 2007). Blood fails to clot at death, indicating sepsis – a clue in diagnosis. Enhancing clinical and laboratory expertise and conducting prospective surveillance are critical components of rapid anthrax diagnosis and bioterrorism preparedness.

Environmental and Clinical Specimens

Clinical or environmental specimens for isolation of *Bacillus anthracis* should be handled with special precautions. *Bacillus* species will normally survive transport in freshly collected specimens or in a standard transport medium. Local transport of specimens (over a few hours) can be done at room temperature or at 2 to 8°C for most specimens, including serum (Logan 2007). Generally, if specimens such as swabs, stool, sputum, pleural fluid, and blood are to be shipped overnight or longer they should be sent at 2 to 8°C. Fresh tissue and serum samples should be shipped frozen, whereas formalin fixed tissues can be sent at room temperature primarily for detection using immunohistochemistry and (much less suitable) for PCR. For blood specimens in which PCR will be used to detect *Bacillus anthracis* DNA, collection tubes containing EDTA or citrate as anticoagulants are preferable to those containing heparin (Logan 2007).

Safety considerations in working with Anthrax

As mentioned earlier, the infectious dose in all forms of human anthrax are

largely unknown but considered to be high, with the 50% lethal dose as high as 2,500 to 55,000 spores, according to some publications (Fennelly, Davidow et al. 2004; Inglesby 2002), but as low as 1 to 3 spores (Peters and Hartley 2002) or 2 to 9 spores (Fennelly, Davidow et al. 2004). In specimen handling generally, precautions need to be sensible, not extreme. When collecting specimens for suspected anthrax, personnel should wear disposable gloves, disposable gown or overalls, and boots which can be disinfected after use. For dusty samples that might contain many spores, the use of personal protective equipment such as a face shield and/or a respirator should be considered. It should be noted that hand washing with soap and water, with chlorhexidine gluconate or the use of hypochlorite-releasing towels may all reduce endospore contamination of the skin. Waterless rubs containing ethanol are ineffective at removing spores as indicated by one study with the model organism *B. atrophaeus*, a close relative of *B. subtilis* (Weber, Sickbert-Bennett et al. 2003). Biosafety level 2 practices (BSL 2), containment equipment, and facilities are recommended for activities using clinical materials and diagnostic quantities of infectious cultures (BMBL-CDC 1999). Biosafety level 3 practices (BSL 3), containment equipment, and facilities are recommended for work involving production quantities or concentrations of cultures, and for activities likely to produce aerosols (BMBL-CDC 1999). Disposable items should be discarded into suitable containers for autoclaving. Items that are not autoclavable should be immersed overnight in 10% formalin (5% formaldehyde solution), glutaraldehyde (5%), a pH 7- adjusted 1:10 dilution of household bleach, or an aqueous solution of chlorine dioxide (500 mg/liter)

(Logan 2007). Items that cannot be immersed should be bagged and sent for formaldehyde fumigation. Ethylene oxide and hydrogen peroxide vapor are also effective fumigants, but the latter is inappropriate if organic matter is being treated. The best disinfectant for specimen spills is formalin. In cases where this is considered impractical, a 1:10 dilution of household bleach (6,000-mg/liter hypochlorite solution) can be used, although its limitations should be appreciated; it is rapidly neutralized by organic matter, and it corrodes metals (http://www.epa.gov/pesticides/factsheets/chemicals/bleachfactsheet.htm). Other strong oxidizing agents, such as hydrogen peroxide (5%) and peracetic acid (1%), are also effective but likewise inactivated by organic matter (www.epa.gov).

It is vital to know that when working with pure cultures of *Bacillus anthracis*, direct and indirect contact of broken skin with cultures and contaminated laboratory surfaces, accidental parenteral inoculation, and rarely, exposure to infectious aerosols are the primary hazards to laboratory personnel. Isolation and presumptive identification of *Bacillus anthracis* can be performed safely in the routine clinical microbiology laboratory, provided that the usual good laboratory practice is observed (BSL 2); vaccination is not required for minimal handling of the organism (CDC 1999). All of the other species of aerobic endospore-forming bacteria that may be isolated from clinical specimens can be handled safely on the open bench. Efforts should be made to avoid methods that produce aerosols (Logan 2007). Any procedures that have the potential to generate aerosols should be done in a biological safety cabinet. In addition, all

centrifuging should be done using an aerosol-tight rotor and rotors should be opened within the biological safety cabinet. Centrifuges are the most frequently contaminated pieces of laboratory equipment. Laboratories that frequently centrifuge *Bacillus anthracis* suspensions should use an aerosol-tight rotor that can be repeatedly autoclaved; the rotor and rotor lid should be swabbed regularly to monitor for contamination, and contaminated rotors can be autoclaved before reuse (CDC 1999; Logan 2007).

Clinical Specimens from Suspected Anthrax Patients

In all cases, besides the clinical specimens from the patients, samples from possible sources of exposure that likely resulted in the infection should be sought in addition to patient specimens. For example, possible sources of exposure may involve a dead animal or carcass, hides, hair, bones, etc. in addition to many other environmental sources that may be suspected.

1. Cutaneous Anthrax

It is important to collect sufficient vesicular fluid with swabs to allow both culture and a smear for visualizing the capsule for diagnosis of cutaneous anthrax. For immunohistochemical analysis of cutaneous lesions, a full-thickness punch biopsy fixed in 10% buffered formalin from a papule or vesicle lesion and adjacent skin should be taken. Biopsy specimens should also be taken from both vesicle and eschar if present (Shieh, Guarner et al. 2003).

2. Oropharyngeal or Gastrontestinal Anthrax

Oropharyngeal or gastrointestinal anthrax may be suspected if adequate history of the patient is known. In the developed countries, a case is not considered part of a bioterrorism event until bioterrorism is discovered. If the patient is not severely ill, a fecal specimen may be collected, but isolation may not be successful. If the patient is severely ill, blood should also be cultured, although isolation may not be possible after antimicrobial treatment; treatment should not await laboratory results. A blood smear may reveal the capsulated rods or, if treatment has started, capsule "ghosts" May be seen (Logan 2007).

Postmortem blood collected by venipuncture (a characteristic of anthrax is nonclotting blood at death [Turnbull 1998]) should be examined by smear (for capsule) and culture. Any hemorrhagic fluid from the nose, mouth, or anus should be cultured. If these are positive, no further specimens are needed. If they are negative, specimens of peritoneal fluid, spleen, and/or mesenteric lymph nodes, aspirated by techniques avoiding spillage of fluids, may be collected for smear and culture (Turnbull 2005).

3. Inhalation or Pulmonary Anthrax

As mentioned previously with the gastrointestinal form, inhalational anthrax will be suspected only if the patient's history suggests it. It can also be suspected in pneumonia patients with widened mediastinum on x-rays. In the

developed nations, a case is immediately suspected as part of a bioterrorism event in absence of naturally occurring disease. If the patient is severely ill, blood smear and culture should be done. Following the bioterrorist attacks of 2001 in the United States, PCR on pleural fluid specimens was very useful along with x-ray or CT scan, even when the specimens were collected after antimicrobial therapy had begun; specimens from three patients were negative by culture but still had positive PCR results even when taken =>24 hr. after treatment had begun, but results depend somewhat on previous treatment (Hoffmaster, Meyer et al. 2002). Serology is also useful for the diagnosis of cases when culture fails due to previous treatment. Sera should be taken <7 days after symptoms appear (or after exposure, if known) and again at >14 days. If the patient is not severely ill, immediate specimen collection is likely to be unfruitful and the person should be treated and simply observed; paired sera (when first seen and >10 days later) may be useful for confirmation of diagnosis (Logan 2007). Postmortem, the approach given above for intestinal anthrax should be followed.

Veterinary specimens from Animals Suspected of Anthrax

Anthrax should be considered as the possible cause of death in herbivorous or omnivorous animals that have died suddenly and unexpectedly, particularly if hemorrhage from the nose, mouth, or anus has occurred and if death has taken place at a site with a history of anthrax (even several decades previously) (Logan 2007).

1. 1 to 2 Days Old carcasses

Due to the nonclotting nature of blood in anthrax victims, in the case of 1- or 2-day-old carcasses it is usually possible to aspirate a few drops of blood from a vein for (i) M'Fadyean stained smear and (ii) direct plate culture on blood agar. Pigs frequently do not develop the enormous terminal bacteremia seen in herbivores, and the capsulated rods may not be visible in blood smears (Logan 2007). When cervical edema is present, smears and cultures should be made of fluid aspirated from the enlarged mandibular and suprapharyngeal lymph nodes. In porcine intestinal anthrax, possibly obvious only at necropsy, rods are usually visible in stained smears made from mesenteric lymph nodes (Logan 2007)

2. Putrefying Older Carcasses

Bacillus anthracis competes poorly with putrefactive organisms and may not be seen in smears after 2 to 3 days, so culture is necessary for diagnostic confirmation for older putrefying carcasses. Sections of tissue or any blood-stained material should be collected. If the animal has been opened, spleen or lymph node specimens should be taken. With putrefied and very old carcasses, swabs of the nostrils and eye sockets are likely to yield *Bacillus anthracis*, but the best specimens may be samples of contaminated soil beneath the nose and anus.

Miscellaneous and Other Specimens

Tests for the presence of *Bacillus anthracis* may be requested on a variety of other specimens, such as animal products (e.g., wool, hides, hair, or bonemeal) from regions of endemicity, soil or other materials from old burial sites or tannery or laboratory sites due for redevelopment, or other environmental materials associated with outbreaks (e.g., sewage sludge) (Logan 2007). At present, culture by the selective agar techniques described below is the only confirmatory approach. Suitably equipped laboratories are beginning to use PCR techniques for rapid detection of *Bacillus anthracis* in such samples, but at present it is advisable to confirm positives by conventional methods.

Suspected or potential Bioterrorism-Related Specimens

In 1999, a Laboratory Response Network (LRN) was established in the United States by the Centers for Disease Control and Prevention (CDC) in partnership with the Association of Public Health Laboratories (APHL), the Federal Bureau of Investigation (FBI), and the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) to provide the public health laboratory response to acts of bioterrorism (Morse 2003). This network links local sentinel laboratories to laboratories with more specialized testing and increased biosafety levels at the state and federal level. There are reference level laboratories in all 50 states able to rapidly detect agents such as *Bacillus anthracis*.. State public health laboratories are part of the LRN and will be able to provide guidance, or the LRN can be accessed using the Internet (http://www.bt.cdc.gov/lrn/). State

and territorial public health laboratory contact information and sentinel laboratory guidelines are also available on the American Society for Microbiology website at http://www.asm.org. Emergency response guidelines for state, local, and tribal public health personnel can be found at http://www.bt.cdc.gov/ under "Additional Topics and Resources" and a 24-hr hotline number for urgent advice is 770-488-7100. Although initially limited to the United States, there are now 152 national and international laboratories within the LRN, including Canada, Great Britain, Australia, Germany (U.S. military base), and South Korea (U.S. military base), which are capable of providing a rapid response to acts of biological terrorism, emerging infectious diseases, and other public health threats and emergencies.

Isolation procedures for *Bacillus anthracis*

Specimens with Mixed Microbial Flora:

In specimens submitted for food-poisoning investigations or for isolation of *B. anthracis* from old carcasses, animal products, or environmental specimens, the organisms will be present mostly as spores. Heating specimen preparations (at 62.5 to 80°C for 10 to 30 min.) will both heat shock the spores and effectively destroy non-spore-forming contaminants. A variety of approaches are used to process solid samples prior to heat treatment. Direct plate cultures are made on blood, nutrient, or, selective agars, as appropriate, by spreading up to 0.1 ml volumes from undiluted and 10- and 100-fold dilutions of the treated sample (BAM manual, www.fda.gov).

Enrichment procedures are generally inappropriate for isolations from clinical specimens, and there is no effective enrichment method for *Bacillus anthracis* in old animal specimens or environmental samples; isolation from these is best done with polymyxin-lysozyme EDTA-thallous acetate (PLET) agar (Knisely 1966). A differential/selective chromogenic medium has recently been introduced by R&F Laboratories, Downers Grove, IL. (R&F Anthracis Chromogenic Medium), but it has yet to be thoroughly evaluated. Aliquots (100 µl) of the undiluted and 1:10 and 1:100 dilutions of heat-treated suspension of the specimen are spread across PLET plates, which are read after incubation for 36 to 40 hr at 37°C. Roughly circular, creamy white colonies, 1 to 3 mm in diameter, with a ground-glass texture are subcultured on (i) blood agar plates to test for gamma phage lysis testing and determination of hemolysis and (ii) directly or subsequently in blood to look for capsule production by using M'Fadyean's stain or, less reliably, India ink negative stain (the ink coagulates the blood and makes interpretation difficult); 2.5 ml of horse blood (defibrinated horse blood seems best; horse or fetal calf serum is quite good) or Heart Infusion Broth with 0.8% Sodium Bicarbonate (Trypticase Soy Broth with 0.8% Sodium Bicarbonate is also acceptable) is inoculated with a pinhead quantity of growth from the suspect colony, incubated statically for 6 to 18 hr at 37°C, and then stained (Logan 2007). PCR-based methods are being increasingly used for the direct detection of *Bacillus anthracis* in clinical specimens and environmental samples.

Identification procedures for *Bacillus anthracis*

Gram Stain: Inevitably the first examination of specimens and cultures will be with the gram-stain. In the past, it has been regarded as being of limited value in anthrax diagnosis because it does not reveal the capsule. In recent bioterrorism cases such preparations were clearly considered to be of great value, but caution is still necessary. In a well-developed country, it is unlikely that large numbers of gram-positive bacteria in the blood at death are going to be anything but *Bacillus anthracis*, particularly when supported by the clinical, forensic and laboratory work with fatal cases during the recent bioterrorism events of 2001 in the US. In other circumstances and in animals in particular, the blood or other specimen may not be collected soon enough after death and before putrefactive organisms appear; *Bacillus anthracis* may then be indistinguishable without the use of the proper capsule stain.

The M'Fadyean polychrome methylene blue staining test dates from 1903 and has proved to be a remarkably successful rapid diagnostic test over the decades. However, reliable stain and adequate quality control of its performance are becoming hard to guarantee (Logan 2007) due to commercially available reagent quality and technical skiils issues. A rapid immunochromatographic on-site test has been developed (Burans 1996) but is not commercially available.

It is generally easy to distinguish virulent *Bacillus anthracis* from other members of the *Bacillus cereus* group. *Bacillus anthracis* isolates are

characterized by a typical microscopic appearance and colony morphology: colonies are white or gray, nonhemolytic or only weakly hemolytic, susceptible to the diagnostic "gamma phage" (inquiries about gamma phage should be addressed to the Diagnostics Systems Division, USAMRIID, Fort Detrick, Frederick, MD 21702-5011), generally susceptible to penicillin, nonmotile, and able to produce the characteristic capsule, as shown by M'Fadyean staining or India ink staining. As an alternative to culture in blood, the capsule of virulent *Bacillus anthracis* can be demonstrated on nutrient agar containing 0.7% sodium bicarbonate, incubated overnight under 5 to 7% CO_2 (candle jars perform well). Colonies of the capsulated organism appear mucoid, and the capsule can be visualized by M'Fadyean or India ink staining of smears or by direct fluorescent antibody (DFA) staining or indirect antibody staining (inquiries about indirect fluorescent antibody capsule staining should be addressed as above to the Diagnostics Systems Division, USAMRIID).

In addition to phenotypic analysis, molecular and antigenic detection assays are available for the rapid identification of *Bacillus anthracis*. The LRN PCR (restricted to LRN laboratories; see "Potential Bioterrorism-Related Specimens" above) targets three distinct loci on the *Bacillus anthracis chromosome*, pXO1 virulence plasmid, and pXO2 virulence plasmid. Using several loci increases specificity and allows for the detection of avirulent strains (lacking pXOI or pXO2). The anthrax toxin genes (*pag*A, *lef*, and *cya*) are located on pXO 1, whereas the genes required for capsule biosynthesis (*cap*BCA) are located on

pXO2. Isolates lacking pXO2 or both plasmids are found mostly in the environment and are frequently mistaken for *Bacillus cereus*, due to the lack of a capsule, and discarded (Turnbull, Hutson et al. 1992). A commercial kit is also available for the detection of the *Bacillus anthracis* toxin gene, *pag*A, and the capsule gene, *cap*B (Roche, Mannheim, Germany). These genes have been widely used as *Bacillus anthracis* specific gene targets; however, there have been recent reports of these genes being found in species other than *Bacillus anthracis* (Logan 2007). Recently, several laboratories have developed specific PCR assays for *Bacillus anthracis* that target chromosomal genes such as *rpo*B, *gyr*A, and *plc*R. (Easterday, Van Ert et al. 2005; Hurtle, Bode et al. 2004; Qi, Patra et al. 2001).

A two-component Direct Fluorescent Antibody (DFA) assay has been used to identify encapsulated vegetative cells of *B. anthracis*. This assay uses two different monoclonal antibodies specific for a *B. anthracis* cell wall antigen and the *B. anthracis* capsule. Neither antigen is 100% specific for *B. anthracis*; however, only *B. anthracis* has been found to be positive for both antigens, and thus the assay is 100% specific when both cell wall and capsule components are used together; it was heavily used at the CDC during the 2001 bioterrorism-associated outbreak for the rapid (<4-hr) identification of isolates (De, Bragg et al. 2002; Ezzell, Abshire et al. 1990).

Tetracore, Inc. (Gaithersburg, Md.) has produced a rapid (within 15 min.) immunochromatographic test (RedLine Alert, described in the earlier chapter)

utilizing an antibody specific for one of the *Bacillus anthracis* S-layer proteins. This assay has been approved by the Food and Drug Administration (FDA) for use on nonhemolytic *Bacillus* species colonies cultured on sheep blood agar plates. Manufacturer's data suggest that the test was 98.6% sensitive when tested on 145 *Bacillus anthracis* isolates and 45 nonhemolytic, non- *Bacillus anthracis* isolates; however, such identification of *Bacillus anthracis* is considered presumptive and it should not be used as a stand-alone test.

Molecular Typing and Characterization of *Bacillus anthracis*

Although *Bacillus anthracis* is a genetically monomorphic species, the development of a multiple-locus variable-number tandem repeat analysis (MLVA) has allowed for effective strain differentiation (Keim, Price et al. 2000). This method was relied on during the 2001 bioterrorism-associated outbreak in the United States, and MLVA of the attack strain implicated the Ames laboratory strain (Hoffmaster, Fitzgerald et al. 2002). Recently, Keim et al. (2004) proposed adding canonical single nucleotide polymorphisms, expanding MLVA from 8 to 15 loci, and analyzing four single nucleotide repeats to increase genotyping accuracy and resolution. In addition, although not in widespread use, the availability of genome data has led to the use of microarray technology to detect differences between strains of *Bacillus anthracis* and other *Bacillus* species (Logan 2007). Pulsed-field gel electrophoresis applied to differentiate between different strains of BA and other *Bacillus* species.

Methods for direct Detection of BA in Clinical Specimens

In addition to culturing *Bacillus anthracis*, there are molecular and antigen-based detection methods available for direct detection in clinical and environmental samples. These are essential when cultures fail, as they particularly do after the initiation of antimicrobial therapy. Several methods, including a *Bacillus anthracis* -specific LRN PCR assay, immunohistochemical (IHC) assays, and serology, were useful for confirmation of cases during the 2001 bioterrorism-associated outbreak, particularly when culture failed. At least two such tests need to be positive for a case to be considered laboratory confirmed (Guarner, Jernigan et al. 2003; Hoffmaster, Meyer et al. 2002; Quinn, Dull et al. 2004; Quinn, Semenova et al. 2002; Shieh, Guarner et al. 2003).

The most widely used and available detection method in the U.S. public health system is the LRN PCR as previously described. Following the 2001 anthrax attacks in the United States, this assay was negative on all specimens (n=142) from patients in whom anthrax was excluded (100% specific) (Hoffmaster, Meyer et al. 2002). In addition, testing of specimens from inhalation anthrax patients produced a positive result in 33% of the specimens which were culture negative, with pleural fluid appearing to be the best specimen even after the initiation of antimicrobial therapy. The IHC assay, as performed at the CDC, uses the same antibodies as the DFA assay (specific to cell wall antigen or the capsule) to detect *Bacillus anthracis* in formalin-fixed, paraffin-embedded tissues.

This method was particularly useful in the diagnosis of cutaneous cases during the 2001 bioterrorism-associated outbreak. Skin biopsy specimens from cutaneous lesions from 8 of 10 patients were positive for both the capsule and cell wall antigens (Shieh, Guarner et al. 2003).

The many reports on the use of different PCR assays to detect or identify *Bacillus anthracis* cannot be fully summarized here. Although PCR is currently the most widely used detection technology (Logan 2007), there are increasing reports on novel technologies, or improvements to existing ones, such as mass spectrometry, flow cytometry, time -resolved fluorescent assays, high-performance liquid chromatography, and even the use of engineered B-cells to detect *Bacillus anthracis*. With further improvements and validations, these assays may increasingly be used to detect *Bacillus anthracis* in the future.

Detection of Anthrax by serological assays

Serological assays for the detection of antibody response against the anthrax toxin protein, protective antigen (PA), were used in combination with PCR or IHC assay results to confirm anthrax cases when culture failed (CDC.gov/LRN). A quantitative human anti-PA immunoglobulin G (IgG) enzyme-linked immunosorbent assay was performed at the CDC during the 2001 outbreak and was positive only on sera from individuals with anthrax or vaccinated with Anthrax Vaccine Adsorbed (BioThrax; Emergent Biosolutions, Lansing, Mich.). More recently, a commercially available, FDA-approved, qualitative kit

(QuickELISA Anthrax-PA Kit) from Immunetics (Boston, Mass.) has become available for the detection of anti-PA IgG and IgM antibodies in human serum. Serological assays aided in the effort to confirm cases in the 2001 attack, particularly cutaneous ones; however, the time to seroconversion after infection limits its usefulness in terms of the rapid diagnosis necessary for treatment and a public health response.

In countries of the former USSR, a skin test utilizing Anthraxin®, a heat-stable extract from a non- capsulate strain of *Bacillus anthracis*, which has been licensed for human and animal use since 1962, is widely acclaimed for the retrospective diagnosis of anthrax. The delayed-type hypersensitivity is interpreted as indicating cell-mediated immunity to anthrax and can be used to diagnose anthrax retrospectively or to evaluate the vaccine-induced immune status after periods of several years (Logan 2007). Anthraxin does not contain highly specific anthrax antigens and depends on the nature (type) of anthrax rather than the specificity of the antigens involved. Thus, sensitivity varies with the type of anthrax. It showed highest sensitivity with the diagnosis of cutaneous anthrax (Shlyakhov 1994). This is also true of the Ascoli test, which, dating from 1911, may be one of the oldest antigen detection tests in microbiology. It is a precipitin test using hyperimmune serum raised to *Bacillus anthracis* whole-cell antigen to provide rapid retrospective evidence of anthrax infection in an animal from which the material being tested was derived. The test is still in use in Eastern Europe and Central Asia.

Laboratory maintenance of BA strains

All the clinically significant isolates reported to date are of species that grow, and often sporulate, on routine laboratory media at 37°C. It seems unlikely that many clinically important, but more fastidious strains are being missed for the want of special media or growth conditions. Maintenance is simple if spores can be obtained, but it is a mistake to assume that a primary culture or subculture on blood agar will automatically yield spores if stored on the bench or in the incubator (Logan 2007). It is best to grow the organism on nutrient agar containing 5 mg of manganese sulfate per liter for a few days and refrigerate the culture when microscopy shows that most cells have sporulated (Logan 2007). For most species, sporulated cultures on slants of this medium, sealed after incubation, can survive in a refrigerator for years. Alternatively, cultures (preferably sporulated) can be frozen (-20 to -80^{0}C) or lyophilized.

Bacillus anthracis is defined as a select agent and an "overlap" agent on the U. S. Department of Health and Human Services (HHS) - HHS/CDC and U.S. Department of Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) - USDA/APHIS select agent lists, thus, possession of the agent in the United States requires registration of the laboratory with either CDC or APHIS. When *Bacillus anthracis* is identified in an unregistered clinical or diagnostic laboratory, the identification of this agent must be reported to CDC or APHIS within 7 days and to other authorities as required by federal, state, or local laws.

When *Bacillus anthracis* is isolated in an unregistered laboratory, the organism must either be destroyed on-site by a recognized sterilization or inactivation process or be transferred to a registered laboratory within 7 days. Shipping of this agent requires completion of the APHIS/CDC Form 2 and prior approval from either CDC or APHIS, as required under the "Select Agent Rule" (http://www.selectagents.gov/).

Antimicrobial susceptibility patterns of BA strains

Bacillus anthracis: Most strains of *Bacillus anthracis* are susceptible to penicillin. Of 25 genetically diverse isolates from around the world, 3 strains were resistant to penicillin but were negative for β-lactamase production (Coker, Smith et al. 2002; Mohammed, Marston et al. 2002). Most strains give variable susceptibility results for cephalosporins. In vitro results, even if susceptible, may not predict clinical efficacy, particularly for expanded- and broad-spectrum cephalosporins (Coker, Smith et al. 2002). In a study of 50 historical isolates from humans and animals and 15 clinical isolates from the 2001 bioterrorist attack in the United States, the majority of strains could be regarded as not susceptible to the broad- spectrum cephalosporin - ceftriaxone, and three strains were resistant to penicillin (Mohammed, Marston et al. 2002). Tetracyclines, fluoroquinolones, and chloramphenicol are suitable for the treatment of patients allergic to penicillin (but except for fluoroquinolones, these are not good choices clinically, regardless of in vitro results); most strains in the previously mentioned study showed only intermediate susceptibility to erythromycin (Mohammed,

Marston et al. 2002). Ciprofloxacin and the newer quinolone, gatifloxacin had good in vitro activities against 40 Turkish isolates, but for another new quinolone, levofloxacin, it was observed that MICs were high for 10 strains (Esel, Doganay et al. 2003). Other in vitro studies have shown novel fluoroquinolones and a ketolide to be of potential therapeutic value (Frean, Klugman et al. 2003). Standards for antimicrobial susceptibility testing of *Bacillus anthracis* have been recently adopted (CLSI 2005).

Post-exposure prophylaxis is needed for the prevention of inhalation anthrax following a bioterrorist attack; the recommended regimen is 60 days of antibiotic therapy and three doses of anthrax vaccine, and recommended antimicrobial agents include ciprofloxacin, doxycycline, and levofloxacin (CDC 2004). Amoxicillin is recommended as an option in cases where the *Bacillus anthracis* strain has been demonstrated to be susceptible to penicillins and when other antimicrobial agents are not considered safe, as in the treatment of children and pregnant or lactating women (CDC 2001). The use of penicillins for postexposure prophylaxis or for treatment of inhalation anthrax following the use of *Bacillus anthracis* as a bioweapon gives cause for concern, owing to the potential presence of β-lactamases in *Bacillus anthracis* isolates, and the poor penetration of β-lactams into macrophages, the site of spore germination (Bell, Uhl et al. 2002).

Combination intravenous antibiotic therapy with two or more antibiotics, begun early, such as with ciprofloxacin and one or more other antibiotics to which

the organism is sensitive, appeared to improve survival during the treatment of cases during the 2001 outbreak in the United States (Jernigan, Raghunathan et al. 2002). Following that outbreak, the recommendations for initial treatment of inhalation anthrax is ciprofloxacin or doxycycline along with one or more agents to which the organism is normally susceptible (CDC 2001).

Anthrax as a Bioweapon

During the past two decades, the potential use of biological weapons by terrorist groups has received a great deal of attention, particularly in the United States. The existence of an anthrax bioweapon development campaign by the government of Iraq was revealed during the Persian Gulf War from 1990 to 1991 (Gerberding, Hughes et al. 2002; Alibek 1999). Then, in the aftermath of the September 11, 2001 terrorist attacks on the World Trade Center buildings in New York City and the Pentagon in Washington, DC, letters containing a powdered form of *B. anthracis*, were mailed to government representatives, members of the news media, and others in the United States (Dworkin, Ma et al. 2003; UCLA 2002). The anthrax-laced powder inside the letters was aerosolized (i.e., the spores became airborne) when the letters were opened, and in a few cases were inhaled. The death of a Florida man was the first case of an inhalational anthrax death in the United States since 1978 and as of June 2002, more than 23 cases and five deaths were attributed to the terrorist attacks.

Although anthrax is a relatively new weapon in the hands of modern potential bioterrorists, the threat of death from the inhalation of spores has been part of human history since antiquity. Some scholars argue that anthrax is the sooty "murrain" in the Bible's Book of Exodus, and is likely the "burning wind of plague" that begins Homer's *Iliad*. (Brachman and Kaufmann 1998).

As well, the use of microorganisms such as the anthrax bacteria as weapons is not new. In ancient military campaigns, diseased bodies (including those who died of anthrax) were used to poison wells and were catapulted into cities under siege. Research into the military use of anthrax was carried out during World War I by combatants on all sides of the conflict, and by World War II, anthrax research was actively underway (Heyman 2002). For example, Allied efforts in Canada, the United States, and Britain to develop anthrax-based weapons included the production of five million anthrax "cakes," designed to be dropped on Germany to infect wells and contaminate the food chain. The weapons were never used (Heyman 2002).

Only within the past several decades, however, have biological weapons, including anthrax, been added to the arsenal of terrorists. For example, the Japanese cult Aum Shinrikyo (which released Sarin gas into the Tokyo subway system in 1995, killing 12 people and hospitalizing 5,000) was developing anthrax-based weapons (Inglesby 2001; UCLA 2002). Indeed, the group had released crude anthrax preparations in Tokyo on at least eight separate occasions in 1993 (Inglesby 2001; UCLA 2002). These incidents constituted the first use of anthrax as a weapon against a civilian population. In addition, state-

sanctioned terrorism by the government of Iraq has also involved the production of anthrax bioweapons, and Western intelligence sources openly insist that Iraq—and or terrorist groups operating with Iraq's assistance—continued to develop biological weapons, including anthrax based weapons. Finally, during the terrorist attacks of the United States in the latter part of 2001, the use of anthrax by a terrorist or terrorists (as of July 2009, yet to be identified and not completely proven) pointed out how easily the lethal agent could be delivered. (Heyman 2002; UCLA 2002)

This ease of delivery of anthrax is one feature that has made the bacterium an attractive weapon for terrorists. Scenarios developed by United States government agencies have shown that even a small crop dusting plane carrying only a hundred kilograms of anthrax spores flying over a city could deliver a potentially fatal dose to up to three million people in only a few hours (Heyman 2002). Although variations in weather patterns and concentration variables would substantially reduce the number of expected actual deaths, such an attack could still result in the deaths of thousands of victims and result in a devastating attack on the medical and economic infrastructure of the city attacked. In a less sophisticated effort, spores could simply be released into air intake vents or left in places like a subway tunnel, to be dispersed over a much smaller area (Inglesby 2001).

Another feature of anthrax that has led to its exploitation by terrorists is the physiology of the bacterium. *Bacillus anthracis* can live as a "vegetative cell," growing and dividing in a rapid and cyclical fashion. The bacterium can also form

a metabolically near-dormant form known as a spore. An individual spore is much smaller and lighter than the growing bacterium. The spores can drift on air currents, to be inhaled into the lungs (Heyman 2002; Koehler 2002, UCLA 2002). Once in the lungs, the spores can resuscitate into an actively growing and dividing bacterium. The infections that are collectively termed anthrax can result. Although millions of spores can be released from a few grams (fractions of an ounce) of *Bacillus anthracis*, only about 5,000 to 8,000 spores are sufficient to cause the lung infection when they are inhaled (Inglesby 2001; UCLA 2002). If left untreated or not promptly treated with the proper antibiotics (such as Ciprofloxacin), the lung infection is almost always fatal. Non-inhalation contact with *Bacillus anthracis* can result in cutaneous anthrax—a condition more treatable with conventional antibiotic therapy.

An often-overlooked aspect of the use of anthrax as a terrorist weapon is the economic hardship that the dispersal of a small amount of the spores would exact. A report from the Centers for Disease Control and Prevention, entitled *The Economic Impact of a Bioterrorist Attack*, (Kaufmann 1997) estimated the costs of dealing with an anthrax incident at a minimum of U.S. $26 billion per 100,000 people (UCLA 2002). In just a few months in 2001 alone, a flurry of anthrax incidents, most of which turned out to be hoaxes, cost the United States government millions of dollars (Hughes (2002).

Biotechnology and anthrax

The choice of anthrax as a weapon used by terrorists reflects the growing awareness of biological research and biotechnology among the general community. The ability to grow and disperse infectious microorganisms was once restricted to specialists. However, the explosion of biotechnology in the 1980s and 1990s demonstrated that the many basic microbiological techniques are fairly simple and attainable (Heyman 2002; Koehler 2002). Experts in microbiology testifying before the U.S. Congress estimated that crude weapons could be developed with approximately $10,000 worth of equipment (Hughes (2002, Polyac 2002) . A laboratory sufficient to grow and harvest the bacteria and to dry down the material to powdered form could fit into the average sized household basement. The more highly trained the terrorist, the more effective weapons could be expected to be produced.

Even though *Bacillus anthracis* could be grown in such a makeshift laboratory, the preparation of the spores and the drying of the spores into a powder is not a trivial task. For example, even after a decade of dedicated effort, United Nations inspectors who toured Iraq bioweapons facilities after the Gulf War found that Iraq had only managed to develop crude anthrax preparations. Still, the Iraqi bioweapons program managed to produce 8,500 liters of concentrated anthrax (UCLA 2002).

Despite the technical challenges, the production of anthrax spores in quantities great enough to cause a huge loss of life is not beyond the capability

of a small group of equipped and funded terrorists. The small size and nondescript nature of a bioweapons facility could make detection of such a lab very difficult. Accordingly, the terrorist potential of anthrax will remain a threat for the foreseeable future.

The farm and dairy industry

The farm is the starting point where production of dairy and juice products begins. After milking, the milk is immediately stored in a bulk milk cooling tank where the temperature of the milk is required by law to be less than 7.2 °C within two hours of milking. Many bottling companies, however, prefer the milk to be stored at or below 4.4 °C to minimize the bacterial growth (MSU Dairy, personal interview). Any additional milk added to the tank from subsequent milking(s) must not raise the temperature of the tank to more than 10 °C, and the temperature must return to below 7.2 °C within two hours. Juice is treated the same way. Milk is stored at the farm for up to two days until being transported to the plant for pasteurization and processing. During this time the milk may not always be under constant supervision, and therefore, it may be vulnerable to deliberate contamination.

Once reaching the factory site, the milk or juice is tested for the presence of antibiotics and bacteria. Processing plants perform tests to check the quality of the milk and juice. One of these tests is a direct microscopic bacteria count. Here a stain is used to allow bacteria to be counted by direct observation in a sample of milk or juice. Levowitz/Weber stain, a modification of the Methylene

blue stain, is commonly used in the dairy industry in the United States, which does stain *B. anthracis* vegetative cells along with other bacterial species, but not spores as spores are stain resistant (Logan 2007). The spores are highly refractile and resist staining. The legal limit for this direct count is 100,000 cfu/ml, but fluid milk plants often desire milk to be under 10,000 cfu/ml, and 5,000 cfu/ml is common. If the milk or juice passes these tests, it is accepted and pumped into holding silos where it will remain for up to 72 hours at 4.5 °C. When the plant is ready to process the milk, it is pumped from the silo into a separator, where the raw milk is separated in to cream and skim milk. Depending on the desired fat content of the processed milk, a portion of the cream is added back into the skim milk, and then pasteurized after homogenization. Leftover cream is then used for making other dairy products such as ice cream, butter and yogurt. Pasteurization is designed to kill even the most heat resistant vegetative bacterial cells. In most cases, however, the bacterial spores are not destroyed. Published studies indicate that in the case of anthrax spores, pasteurization will activate spore germination immediately. The most common form of pasteurization in the United States is High Temperature Short Time (HTST) pasteurization, which raises the temperature of milk to 72 °C for no less than 15 seconds. Following pasteurization, milk is cooled and then bottled (MSU dairy; www.idfa.org).

Windows of vulnerabilities.

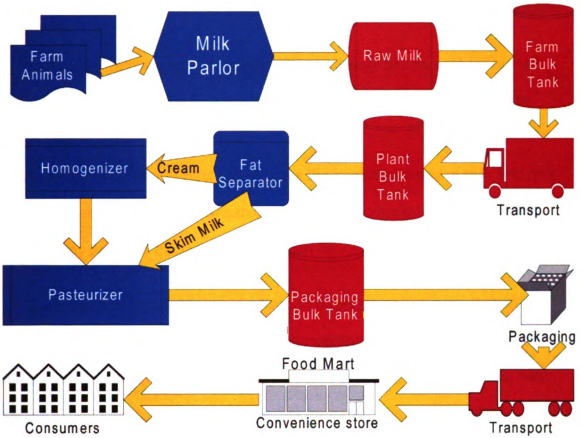
There are four main windows of vulnerabilities from the farm to the consumer's dining table, by which a possible deliberate contamination may occur: the farm bulk tank, the transport tanker, the factory holding silo, and the packaging bulk tank (Figure 1). These vulnerabilities could be chosen as insertion points for initial spore contamination. Each of these tanks has an access port that could allow for injection of contaminated fluid. Critical points in the processing of milk are the quality check and pasteurization. The farm bulk tank and transport tanker insertion points occur before both quality check and pasteurization. Milk or juice enters the factory holding silo after quality checks, before pasteurization. Finally milk or juice in the packaging bulk tank has already passed through both critical points.

Following bottling at the processing plant, the milk is then distributed to establishments such as grocery stores, schools, convenience stores and gas stations. For example, one Michigan dairy distributes over 85,000 gallons of milk daily. There are over 2,300 sites that receive milk, some daily and others that may wait up to a week between shipments. The shipment times range from 30 minutes to 6 hours, with the farthest site 200 miles away. The milk remains there until purchased by a customer.

Pasteurization is designed to kill the vegetative cells of even the most heat resistant bacteria. In the case of *B. anthracis*, pasteurization is likely to kill all vegetative cells. Although pasteurization does not destroy the spores, they may be affected by the heating. Pasteurization causes 99% of the spores to

germinate (Hanson, Wendorff et al. 2005; Perdue, Karns Jeff et al. 2003). This is called "heat shock". The temperature and duration of heat treatment necessary to destroy all *B. anthracis* spores is 121 °C for 15 minutes.

Figure 1. Four points of vulnerabilities marked in red color.



Pasteurization

The process of pasteurization was named after Louis Pasteur who discovered that spoilage organisms could be inactivated in wine by applying heat at

temperatures below its boiling point. The process was later applied to milk and remains the most important operation in the processing of milk (Goff 2006).

Definition:

The heating of every particle of milk or milk product to a specific temperature for a specified period of time without allowing recontamination of that milk or milk product during the heat treatment process.

Purpose: There are two distinct purposes for the process of milk pasteurization.

1. Public Health Aspect - to make milk and milk products safe for human consumption by destroying all bacteria that may be harmful to health (pathogens)
2. Keeping Quality Aspect - to improve the keeping quality of milk and milk products. Pasteurization can destroy some undesirable enzymes and many spoilage bacteria. Shelf life can be 7, 10, 14 or up to 16 days.

The extent of microorganism inactivation depends on the combination of temperature and holding time. Minimum temperature and time requirements for milk pasteurization are based on thermal death time studies for the most heat resistant pathogen found in milk, *Coxelliae burnettii*. Thermal lethality determinations require the applications of microbiology (survival studies) to appropriate processing determinations.

To ensure destruction of all pathogenic microorganisms, time and temperature combinations of the pasteurization process are highly regulated:

Widely used Pasteurization Regulations -

(http://www.idfa.org/facts/milk/pasteur.cfm)

Milk:

63° C for not less than 30 min., 72° C for not less than 16 sec., or equivalent destruction of pathogens and the enzyme phosphatase as permitted by local, state or federal Government authorities. Milk is deemed pasteurized if it tests negative for alkaline phosphatase.

Frozen dairy dessert mix (ice cream or ice milk, egg-nog):

at least 69° C for not less than 30 min; at least 80° C for not less than 25 sec; other time temperature combinations must be approved (e.g. 83° C/16 sec).

Milk based products- with 10% milk fat or higher or added sugar (cream, chocolate milk, etc):

66° C/30 min, 75° C/16 sec. There has also been some progress with low temperature pasteurization methods using membrane processing technology.

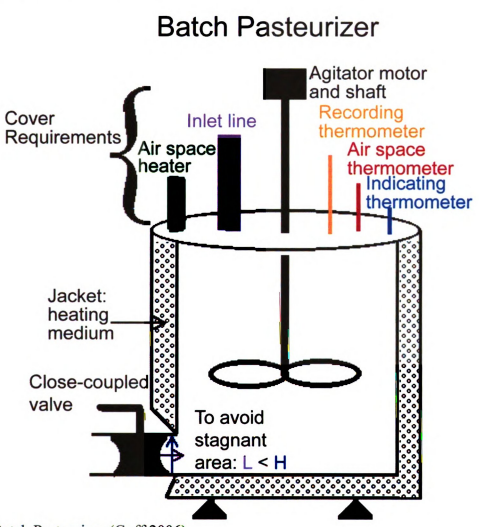
Methods of Pasteurization

There are two basic methods, batch or continuous.

Batch method

The batch method uses a vat pasteurizer which consists of a jacketed vat surrounded by either circulating water, steam or heating coils of water or steam (Figure 2).

Figure 2. Batch Pasteurizer:



Batch Pasteurizer (Goff 2006)

In the vat, the milk is heated and held throughout the holding period while being agitated. The milk may be cooled in the vat or removed hot after the holding time is completed for every particle. As a modification, the milk may be partially heated in a tubular or plate heater before entering the vat. This method has very little use for milk but some use for milk by-products (e.g. creams, chocolate) and

special batches. The vat is used extensively in the ice cream industry for mix quality reasons rather than microbial reasons.

Continuous Method

Continuous process method has several advantages over the vat method, the most important being time and energy saving. For most continuous processing, a high temperature short time (HTST) pasteurizer is used. The heat treatment is accomplished using a plate heat exchanger. This piece of equipment consists of a stack of corrugated stainless steel plates clamped together in a frame. There are several flow patterns that can be used. Gaskets are used to define the boundaries of the channels and to prevent leakage. The heating medium can be vacuum steam or hot water.

HTST Milk Flow Overview

This overview is meant as an introduction and a summary. Each piece of HTST equipment will be discussed in further detail later. Cold raw milk at 4° C in a constant level tank is drawn into the regenerator section of pasteurizer. Here it is warmed to approximately 57° C - 68° C by heat given up by hot pasteurized milk flowing in a counter current direction on the opposite side of thin, stainless steel plates. The raw milk, still under suction, passes through a positive displacement timing pump which delivers it under positive pressure through the rest of the HTST system.

The raw milk is forced through the heater section where hot water on opposite sides of the plates heat milk to a temperature of at least 72° C. The milk, at pasteurization temperature and under pressure, flows through the holding tube where it is held for at least 16 sec. The maximum velocity is governed by the speed of the timing pump, diameter and length of the holding tube, and surface friction. After passing temperature sensors of an indicating thermometer and a recorder-controller at the end of the holding tube, milk passes into the flow diversion device (FDD). The FDD assumes a forward-flow position if the milk passes the recorder-controller at the preset cut-in temperature (>72° C). The FDD remains in normal position which is in diverted-flow if milk has not achieved preset cut-in temperature. The improperly heated milk flows through the diverted flow line of the FDD back to the raw milk constant level tank. Properly heated milk flows through the forward flow part of the FDD to the pasteurized milk regenerator section where it gives up heat to the raw product and in turn is cooled to approximately 32° C - 9° C.

The warm milk passes through the cooling section where it is cooled to 4° C or below by coolant on the opposite sides of the thin, stainless steel plates. The cold, pasteurized milk passes through a vacuum breaker at least 12 inches above the highest raw milk in the HTST system then on to storage tank filler for packaging.

Holding Time

When fluids move through a pipe, either of two distinct types of flow can be observed. The first is known as turbulent flow which occurs at high velocity and in

which eddies are present moving in all directions and at all angles to the normal line of flow. The second type is streamline, or laminar flow which occurs at low velocities and shows no eddy currents. The Reynolds number, is used to predict whether laminar or turbulent flow will exist in a pipe:

Re < 2100 laminar

Re > 4000 fully developed turbulent flow

There is an impact of these flow patterns on holding time calculations and the assessment of proper holding tube lengths.

Dairy Microbiology:

Microorganisms in Milk

Milk is sterile at secretion in the udder but is contaminated by bacteria as it leaves the udder. The usual sources of contamination are manure, bedding, mastitis, and feed. Except in the case of mastitis, the bacteria at this point are harmless and few in number. Further infection of the milk by microorganisms can take place during milking, handling, storage, and other pre-processing activities.

Lactic acid bacteria: this group of bacteria is able to ferment lactose to lactic acid and they are normally present in the milk. These are Lactococci like *L. delbrueckii* subsp. *lactis* and *L. lactis* subsp. *cremoris*; Lactobacilli like *Lactobacillus casei, L.delbrueckii* subsp. *Lactis, L. delbrueckii* subsp. *bulgaricus* and *Leuconostoc* species

Coliforms: coliforms are facultative anaerobes with an optimum growth at 37°C. Coliforms are indicator organisms; they are closely associated with the

presence of pathogens but not necessarily pathogenic themselves. They also can cause rapid spoilage of milk because they are able to ferment lactose with the production of acid and gas, and are able to degrade milk proteins. They are killed by HTST treatment; therefore, their presence after treatment is indicative of contamination and/or failed pasteurization process. *Escherichia coli* is an example belonging to this group.

Spoilage Microorganisms in Milk

The microbial quality of raw milk is crucial for the production of quality dairy foods. Spoilage is a term used to describe the deterioration of a foods' texture, color, odor or flavor to the point where it is unappetizing or unsuitable for human consumption. Microbial spoilage of food often involves the degradation of protein, carbohydrates, and fats by the microorganisms or their enzymes.

Some species and strains of *Bacillus, Clostridium, Corynebacterium, Arthrobacter, Lactobacillus, Microbacterium, Micrococcus* , and *Streptococcus* can survive milk pasteurization and grow at refrigeration temperatures which can cause spoilage problems.

Pathogenic Microorganisms in Milk

Hygienic milk production practices, proper handling and storage of milk and mandatory pasteurization has decreased the threat of milk borne diseases such as tuberculosis, brucellosis, and typhoid fever. There has been a number of food borne illnesses resulting from the ingestion of raw milk, or dairy products made with milk that was not properly pasteurized or that was poorly handled causing

post-processing contamination. The following bacterial pathogens are still of concern today in raw milk and other dairy products (Goff 2006):

- *Bacillus cereus*
- *Listeria monocytogenes*
- *Yersinia enterocolitica*
- *Salmonella* spp.
- *Escherichia coli* O157:H7
- *Campylobacter jejuni*

It should also be noted that moulds, mainly of species of *Aspergillus* , *Fusarium* , and *Penicillium* can grow in milk and dairy products. If the conditions permit, these moulds may produce mycotoxins which can be a health hazard.

Microbial Growth

There are a number of factors that affect the survival and growth of microorganisms in food. The parameters that are inherent to the food, or intrinsic factors, include the following:

- nutrient content
- moisture content
- pH
- available oxygen

- biological structures
- antimicrobial constituents

Each factor, either alone or in combination with each other and/or with additional external forces, affects the duration of survival or growth of the microorganisms. For example, milk with higher fat content protects the microbial survival in larger numbers following pasteurization compared to milk with lower fat content.

Nutrient Requirements: While the nutrient requirements are quite organism specific, the microorganisms of importance in foods require the following:

- water
- energy source
- carbon/nitrogen source
- vitamins
- minerals

Milk and dairy products are generally very rich in nutrients which provide an ideal growth environment for many microorganisms.

Moisture Content: All microorganisms require water but the amount necessary for growth varies between species. The amount of water that is available in food is expressed in terms of water activity (a_w), where the a_w of pure water is 1.0. Each microorganism has a maximum, optimum, and minimum a_w for growth and

survival. Generally bacteria dominate in foods with high a_w (minimum approximately 0.90 a_w) while yeasts and moulds, which require less moisture, dominate in low a_w foods (minimum 0.70 a_w). The water activity of fluid milk is approximately 0.98 a_w.

pH: Most microorganisms have approximately a neutral pH optimum (pH 6-7.5). Yeasts are able to grow in a more acid environment compared to bacteria. Moulds can grow over a wide pH range but prefer only slightly acid conditions. Milk has a pH of 6.6 which is ideal for the growth of many microorganisms.

Available Oxygen: Microorganisms can be classified according to their oxygen requirements necessary for growth and survival:

- Obligate Aerobes: oxygen required
- Facultative: grow in the presence or absence of oxygen
- Microaerophilic: grow best at very low levels of oxygen
- Aerotolerant Anaerobes: oxygen not required for growth but not harmful if present
- Obligate Anaerobes: grow only in complete absence of oxygen; if present it can be lethal

Biological Structures: Physical barriers such as skin, rinds, feathers, etc. have provided protection to plants and animals against the invasion of microorganisms. Milk, however, is a fluid product with no barriers to the spreading of microorganisms throughout the product.

Antimicrobial Constituents: As part of the natural protection against microorganisms, many foods have antimicrobial factors. Milk has several nonimmunological proteins which inhibit the growth and metabolism of many microorganisms including the following most common:

1. lactoperoxidase
2. lactoferrin
3. lysozyme
4. xanthine

More information on these antimicrobials can be found in a chapter on dairy microbiology and safety written by Vasavada and Cousin (Vasavada 1993). Where the intrinsic factors are related to the food properties, the extrinsic factors are related to the storage environment. These would include temperature, relative humidity, and gases that surround the food.

Temperature: As a group, microorganisms are capable of growth over an extremely wide temperature range. However, in any particular environment, the types and numbers of microorganisms will depend greatly on the temperature. According to temperature, microorganisms can be placed into one of three broad groups:

- **Psychrotrophs:** optimum growth temperatures 20 to 30°, and capable of growth at temperatures less than 7° C. Psychrotrophic organisms are specifically important in the spoilage of refrigerated dairy products.

- **Mesophiles:** optimum growth temperatures 30 to 40° C; do not grow at refrigeration temperatures
- **Thermophiles:** optimum growth between 55 and 65° C

It is important to note that for each group, the growth rate increases as the temperature increases to an optimum level, after which it rapidly declines.

Detection and Enumeration of Microorganisms:

There are several methods for detection and enumeration of microorganisms in food. The method that is used depends on the purpose of the testing.

Direct Enumeration:

Using direct microscopic counts (DMC), Coulter counter and other similar instruments allow a rapid estimation of all viable and nonviable cells. Identification through staining and observation of morphology is also possible with DMC.

Viable Enumeration:

The use of standard plate counts, most probable number (MPN), membrane filtration, plate loop methods, spiral plating and other similar methods allow the estimation of only viable cells. As with direct enumeration, these methods can be used in the food industry to enumerate fermentation, spoilage, pathogenic, and indicator organisms.

Metabolic Activity Measurement:

An estimation of metabolic activity of the total cell population is possible using dye reduction tests such as resazurin or methylene blue reduction, acid production, electrical impedance etc. The level of bacterial activity can be used to assess the keeping quality and freshness of milk. Toxin levels can also be measured, indicating the presence of toxin producing pathogens.

Cellular Constituents Measurement:

Using the luciferase test to measure ATP is one example of the rapid and sensitive tests available that will indicate the presence of even one pathogenic bacterial cell.

Isolation of microorganisms is an important preliminary step in the identification of most food spoilage and pathogenic organisms. This can be done using a simple streak plate method.

HACCP

Raw and end-products may be tested for the presence, level, or absence of microorganisms. Traditionally these practices were used to reduce manufacturing defects in dairy products and ensure compliance with specifications and regulations; however, they have many drawbacks:

1. destructive and time consuming
2. slow response

3. small sample size
4. delays in the release of the food

In the 1960's, the Pillsbury Company, the U.S. Army, and NASA introduced a system for assuring pathogen-free foods for the space program (Goff 2006). This system, called Hazard Analysis and Critical Control Points (HACCP), is a focus on critical food safety areas as part of total quality programs. It involves a critical examination of the entire food manufacturing process to determine every step where there is a possibility of physical, chemical, or microbiological contamination of the food which would render it unsafe or unacceptable for human consumption. These identified points are the critical control points (CCP). There are seven principles to HACCP:

1. analyze hazards
2. determine CCPs
3. establish critical limits
4. establish monitoring procedures
5. establish deviation procedures
6. establish verification procedures
7. establish record keeping procedures

Before these principles can be put into place, a prerequisite program and preliminary setup is necessary.

Prerequisite Program:

1) premise control
2) receiving and storage control
3) equipment performance and maintenance control
4) personnel training
5) sanitation
6) recall procedure

Preliminary Setup:

- assemble team
- describe the product
- identify intended use
- construct flow diagram and plant schematic
- verify the diagram on-site

There is extensive information on the Web regarding HACCP initiative, including implementation manuals, HACCP curriculum guidelines, and generic models.

Biosecurity and safety for our nation's dairy and food supply.

With these vulnerabilities and nature of this organism in mind, this proposed project aims to bring the personal and institutional intellectual capital to bear for the long term goal of helping to ensure the biosecurity and safety of the nation's dairy and food supply. This project aligns with national goals to mount a

sustained and innovative research and education effort in specific areas of agricultural security, food security and dairy security, with an emphasis on threats resulting from the intentional introduction of contaminants to the milk or juice supply and associated agricultural systems. The Department of Homeland Security (DHS) is particularly interested in receiving proposals related to on this issue involving deliberate contamination of food and dairy products.

CHAPTER 2

Research Design and Methods

Materials and Methods

Vegetative cell and Spore Preparation:

Bacillus anthracis Sterne strain was obtained from Colorado Serum Company, Denver, CO. *Bacillus anthracis* Pasteur strain was obtained from Centers for Disease Control, Atlanta, GA, *Bacillus cereus* was obtained from Michigan Department of Community Health (MDCH) culture collection, Lansing, MI and *E. coli* (25922) was obtained from American Type Culture Collection (ATCC), Rockville, MD. Vegetative cells were obtained by scraping young colonies from trypticase soy agar plates (TSA, [Remel, Lenexa, KS]), diluting in buffered peptone water (made in-house) for immediate use and then re-plating on TSA for enumeration. Spores from *Bacillus* sp. were prepared using New Sporulation Medium (NSM) which contained, per liter of distilled water (Perdue 2003): tryptone [3g]; yeast extract [3g]; Bacto-Agar [2g], Lab-Lemco Agar (Oxoid) [23g] and $MgSO_4$ $4H_2O$[0.01g]. NSM was autoclaved (121^{0}C, 15 min) and poured into standard 100mm petri dishes. Eight colonies of *Bacillus* strains grown on 5% sheep red blood cell Columbia agar plates (Remel, Lenexa, KS) were suspended in 2 ml phosphate buffered saline (PBS), pH 7.2 (made in-house) and 125ul was spread onto each of 8 NSM plates. The plates were incubated at 37^{0}C for 48 h, then at room temperature (~22^{0}C) for an additional 96 h. Biomass from each plate was suspended in 2 ml of distilled water (dH_2O)

using a plastic spreader and each plate was washed with an additional 1 ml dH_2O. All suspensions and washes were pooled in a 50 ml centrifuge tube and the volume was brought to 40 ml with dH_2O. The suspension was incubated for 3 days at room temperature to allow lysis of vegetative cells. Spores were recovered by centrifugation (8,000xg, 10 minutes) and washed with 40 ml dH_2O, pelleted by centrifugation and suspended in 50 ml dH_2O. The spore suspension was observed under phase contrast microscopy, enumerated by hemocytometer and found to contain less than 0.1% vegetative cells. Highly refractive (viable) spores were counted in the hemocytometer under phase contrast microscopy and the suspension was found to contain between 2.6-4.5 x 10^9 fully refractive spores/ml. This count was confirmed by plating serial dilutions of the suspensions on TSA plates by using either manual plating or a Spiral Plater – Autoplate 4000 (Spiral Biotech, Inc., Norwood, MA) followed by incubation for 12 to18 hours at 35^0C in ambient air. Colonies were enumerated either manually or using the Q-count system (Spiral Biotech, Inc., Norwood, MA).

Real Time PCR (RT-PCR):

PCR samples were prepared (DNA extractions) for each matrix by a boil/lysis method. Briefly, 1 ml samples of respective matrix homogenate were collected into a sterile, screw-capped and O-ringed 2 ml conical tube using a 1.0 ml pipette, and centrifuged at 500 rpm for 5 minutes to pellet food particles. Supernatant was removed to a clean screw-capped and O-ringed 2 ml conical tube using a 1.0 ml pipette and centrifuged at 14000 rpm for 5 minutes.

Supernatant was removed with 1.0 ml pipettor and discarded. The pellet was suspended in 100 µl of dH_2O and heated at 100^0C for 10 minutes followed by PCR analysis. The DNA isolated by this procedure is of sufficient quality and purity that it may be tested by fluorogenic 5' nuclease assay (i.e., TaqMan) for the presence of *B. anthracis* genetic markers. PCR was performed using "proprietary" probes and primers on a Roche LightCycler 2.0 (Roche Diagnostic Corp., Indianapolis, IN 46250), software version 4.0 using LightCycler Fast Start DNA Master Hybridization Probes. Limits of detection studies in dH_2O (distilled water) have been conducted but the data remains undisclosed due to sensitive nature of this information to national security. But, theoretically it can be proposed that one spore per reaction mixture should yield sufficient target DNA that can be adequately amplified and detected by the Real Time PCR. In this case, our template volume was 5 µl. Therefore, one spore in 5 µl or 200 spores/ml is our theoretical limit of detection by PCR.

ECL immunoassay (BioVerify Anthrax Test):

The M-Series M1M Analyzer, 96 well Anthrax test kit, BioVerify test reagents and controls were purchased from BioVeris Corporation, Gaithersburg, MD. The BioVerify Anthrax Test uses a sandwich immunoassay format, utilizing two antibodies which recognize *B. anthracis*, one immobilized on paramagnetic microparticles and the other labeled with BioVeris' BV-TAG™ label. The sample is mixed with these reagents: when *B. anthracis* is present in the sample, both antibodies bind to the antigen, effectively linking the microparticle, the antigen

and the BV-TAG label together. The instrument transports this assay mixture into a measurement cell and collects the microparticles on an electrode. The electrode stimulates the BV-TAG labels bound (via the antibodies and the antigen) to the microparticles and the emitted light reported as the Electrochemiluminescence (ECL) value is measured. If *B. anthracis* is not present in the sample, the microparticle and the label are not linked together, and no signal is generated. Two negative and two positive controls were run with each run. Samples with ECL signals less than 2.0 times the mean ECL signal of the two negative controls were considered negative. Samples with an ECL signal greater than or equal to 2.0 times the mean ECL signal of the negative controls were considered positive. Limits of detection studies (lower limits) in dH_2O indicated that 2000 or more spores/ml of Pasteur strain and $2X10^7$ or more spores/ml of Sterne strain were detectable by this technique. Suspensions with lower concentrations were all negative when tested repeatedly. Spore suspensions prepared in other diluents like phosphate buffered saline (PBS), Peptone water as well as all these diluents supplemented with 0.3% tween20 (a polysorbate surfactant, for dispersion of spore clumps) also revealed the same results and did not increase or change the detection sensitivity.

Matrix-spore suspensions, Pasteurization and culture work:

To assess survival of *B. anthracis* spores through the pasteurization process, we inoculated, in triplicate, six different matrices obtained from a local grocery store (Meijer, Lansing, MI): 1) homogenized whole milk, 2) 2% low fat milk, 3) fat

free skim milk, 4) apple juice, 5) apple cider; and 6) liquid eggs with pure spore suspensions, to achieve final *B. anthracis* Pasteur and Sterne concentrations of a) high - 10^8 spores per ml, b) medium – 10^6 spores per ml and c) low 10^4 spores per ml in the sample matrices. High concentration matrix samples of *Bacillus cereus* (10^8 spores/ml) and *E. coli* (10^8 vegetative cells/ml) were used as positive and negative controls respectively, for the pasteurization process with each run.

For each experiment, a stock of 10^8 spores per ml in each of the matrices was prepared. Further serial dilutions of the stock matrix-spore preparation were made in same matrix. The diluted samples were mixed by vortexing for 15 seconds.

Next, thermal treatment studies were performed to simulate pasteurization. The samples were heated in 0.2 milliliter aliquots in MicroAmp plastic tubes (Applied Biosystems, Foster City, CA) at pasteurization temperatures (63^0 C for 30 minutes [P1], 72^0 C for 16 seconds [P2], 78^0C for 16 seconds [P3] and 100^0C. for 3 seconds [P4]) in a programmed GeneAmp® PCR Systems 9700 thermocycler (Applied Biosystems, Foster City, CA) followed by cooling to 4.4^0C. Stored samples of each matrix suspension, if saved for further work or repeats, were mixed routinely and periodically to prevent the cream or other solids from separating while standing in the refrigerated (4.4^0C) storage.

After subjecting samples to the pasteurization process, each sample was examined for viable (surviving) organisms by the standard microbiological "plate

count" method (FDA) and enumerated either manually or by using the Q-count 510. Uninoculated matrix controls were used as negative controls. All platings of treated and untreated spore and vegetative cell as well as control preparations were performed in triplicate using either a manual plate spreader or the Auto plate 4000 Spiral Plater. Similarly, we also ran real-time PCR testing and the BioVeris electrochemiluminescence assay simultaneously in triplicate to detect the spores and tabulate the comparative data. Organisms were identified using American Society for Microbiology's (ASM) protocols to differentiate *B. anthracis* from other sporulating organisms (Snyder 2007; Logan 2007) known to exist in these matrices, especially milk. These criteria are as follows:

a) Preliminary criteria:

   i) Gram stain – large gram-positive rod (1-1.5 X 3-5 μm) in chains of 2-4 cells, resembling "box car" appearance. They may or may not be encapsulated, depending upon the strain, which may be seen on the Gram stain as clear zones around the bacilli. Spores are seen on rare occasion when grown in air (exposed to low CO_2 levels) as oval, central-to-subterminal (1 X 1.5 μm) that do not cause significant swelling.

   ii) India ink – for capsule demonstration (Pasteur strain).

   iii) Catalase production - detected by hydrogen peroxide.

   iv) Motility - negative demonstrated by wet mount or motility media.

v) Growth - on Trypticase soy agar media after 12-18 hours of incubation at 35-37^{o}C, under ambient conditions, show typical large well isolated colonies 2-5 mm in diameter. *B. anthracis* grows rapidly. The heavily inoculated areas may show growth within 6-8 hours and individual colonies may be well detected within 12-15 hours. This trait is used to recognize or detect and isolate *B. anthracis* from mixed cultures containing slower-growing organisms in all the matrices in this study. The flat or slightly convex colonies are irregularly round, with edges that are slightly undulate (irregular, wavy border), and have a ground-glass appearance. There may be often comma-shaped projections from the colony edge, producing the "Medusa-head" colony. The colonies show tenacious consistency and when teased with a needle the growth will stand up like a beaten egg white.

b) Additional criteria: two of the above preliminary tests rule in *B. anthracis* in the matrices used in this study for counting colony forming units (CFUs). If needed, two of the three additional criteria were used on rare occasions for confirmation. The algorithm or details on reagents we used for obtaining confirmatory results for BA is considered sensitive or privileged information and can not be published at this time in this dissertation. But, commercial reagents are available for the following tests, as suggested by currently available published literature, which could be used as confirmatory criteria.

i) Phage lysis – positive lysis zone (Abshire 2005, Logan 2007)

ii) Direct fluorescent antibody – positive for cell wall or capsule (Logan 2007)

iii) PCR – positive for strain specific markers (either pX01 for Sterne or pX02 for Pasteur strains) (Snyder 2007; Logan 2007; Roche Applied Science Cat#03303411001 – LightCycler® *Bacillus anthracis* Detection Kit).

After the first colony counts (CFU) post pasteurization, all samples were stored at 4.4^{0}C. for long term spore viability study of the product by performing weekly colony counts until the expiration date of the product. Milk products were held for 4 weeks and other products were held for 12 weeks, simulating the farm to consumer dining table time frame including storage conditions. The storage time (manufacturer suggested shelf life) information was obtained from product labels.

All results were obtained in triplicate were tabulated and averages were used for final analysis. Statistical analysis was conducted by using one-way ANOVA repeated measurement design model/ two-way ANOVA, Duncan's multiple range test, Univariate Procedure and Mixed Procedure outputs. SAS ver 9.0 computer program (SAS Corporation, Cary, NC) was used to run the analysis.

CHAPTER 3

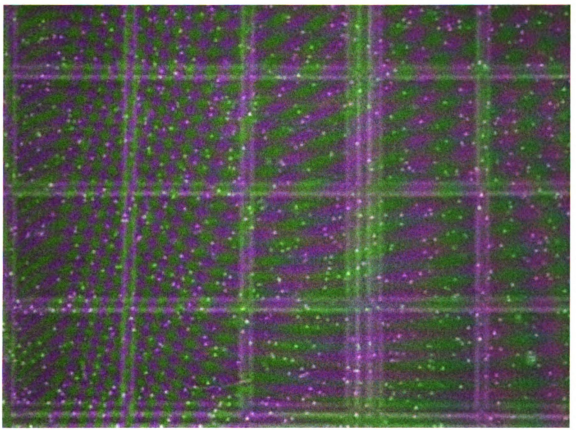
RESULTS

All *Bacillus* spore stock preparations in sterile dH_2O were adjusted to contain 2.0×10^9 spores per ml with a vegetative cell contamination level of less than 0.1% (Fig. 3). Stocks were very stable (with respect to viability over a period of six months) when stored at 4.4^0C. These stock suspensions were used to inoculate the matrix samples which were subjected to pasteurization and enumeration procedures. Pasteurization treatment resulted in complete death of all vegetative cells as shown in Tables 3,6,9,12,15 and 18. *Bacillus cereus* (MDCH culture collection) spore suspension was used as positive control and *E. coli* (25922) was used as negative control (vegetative cells).

Application of pasteurization treatments showed that three of the commonly used treatments (P1-P3) had minimal lethal effect (none to less than 1 log reduction) on the viability of the spore preparations (Tables 1-18). The ultra high temperature/short time pasteurization (100^0C. for 3 sec. [P4]) had the most lethal effect on Pasteur strain of *B. anthracis* spores, which showed a 1 to 2-log reduction in viable spores after the treatment. This reduction was particularly evident at the low and middle spore concentrations. After the pasteurization treatment, the Sterne strain spores at high spike level showed no significant drop in viable cell counts when tested for viability over the duration of storage period, enumerated once each week. Viable cell counts were obtained after 7, 14, 21

and 30 days of storage for milk products and up to 12 weeks for other matrices at refrigeration temperatures (4-8^{0}C).

Figure 3. Highly refractile (viable) *B. anthracis* spores observed in a hemocytometer using phase contrast microscopy at 400x magnification. Non-viable spores would appear as dark objects.



WHOLE MILK STUDIES

Whole milk (homogenized) studies (Table 1-3) showed that the *B. anthracis* Sterne strain spores and *B. cereus* spores did not undergo a significant loss of viability after the pasteurization treatment. The Pasteur strain had a one-log

reduction in viable spores during all pasteurization types selected. These pasteurizations were 100% lethal for *E. coli,* which was used as the pasteurization efficacy process control. PCR failed to detect spores or vegetative cells at any concentration, while the M1M BioVerify test detected high concentrations of Pasteur spores only (10^6 and above). The M1M did not detect spores subjected to ultra high temperature/short time pasteurization (100°C for 3 seconds) at any concentration presumably due to changes in the structure of the surface moieties, which form the base for the immunological properties of the spores. The M1M did not detect any vegetative cells at any level (Table 3). M1M sensitivity did not increase when the samples were diluted (1:10) with buffered peptone water with 0.3% tween20 and retested.

Efficacy of all four pasteurization methods at high, medium and low spike levels of both strains were compared by measuring CFU log differences before and after pasteurizations in whole milk (Fig. 4), which clearly indicated higher susceptibility of the Pasteur strain at most spike levels. Ultra high temperature/short time pasteurization (100°C for 3 seconds) was a relatively more effective method of pasteurization for whole milk compared to the others, as evident from almost a one-log reduction in viable spores of Pasteur strain. Sterne strain showed less than a half-log reduction indicating stronger heat resistant characteristic of the organism. Subsequently, the spore survival study using high spike whole milk suspension of Sterne strain over the product shelf life of 4 weeks showed relatively stable viable spore counts with very little decline (Fig. 5 and 6).

Table 1. STERNE STRAIN: spores detection in WHOLE MILK using plate count (CFU), PCR test (LightCycler) and ECL test (M1M)

Sample Processing ↓	High Spore Spike			Medium Spore Spike			Low Spore Spike		
	CFU	PCR	ECL	CFU	PCR	ECL	CFU	PCR	ECL
Untreated	$2.6x10^8$	-	-	$2.7x10^6$	-	-	$1.9x10^4$	-	-
63°C/30 minutes	$2.2x10^8$	-	-	$1.2x10^6$	-	-	$1.9x10^4$	-	-
72°C/16 seconds	$2.2x10^8$	-	-	$2.2x10^6$	-	-	$3x10^4$	-	-
78°C/16 seconds	$3.1x10^8$	-	-	$1.9x10^6$	-	-	$2.8x10^4$	-	-
100°C/3 seconds	$1.1x10^8$	-	-	$7.1x10^5$	-	-	$9x10^3$	-	-

CFU, colony forming units (spore count); PCR, polymerase chain reaction; ECL, M1M BioVerify assay

Table 2. PASTEUR STRAIN: spores detection in WHOLE MILK using plate count (CFU), PCR test (LightCycler) and ECL test (M1M)

Sample Processing ↓	High Spore Spike			Medium Spore Spike			Low Spore Spike		
	CFU	PCR	ECL	CFU	PCR	ECL	CFU	PCR	ECL
Untreated	$2.1x10^8$	-	+	$1.8x10^6$	-	+	$2.5x10^4$	-	-
63°C/30 minutes	$2.8x10^8$	-	+	$2.1x10^6$	-	+	$7x10^3$	-	-
72°C/16 seconds	$5.2x10^7$	-	+	$7.4x10^5$	-	+	$7.2x10^3$	-	-
78°C/16 seconds	$3.3x10^7$	-	+	$6.3x10^5$	-	+	$5.2x10^3$	-	-
100°C/3 seconds	$1.7x10^7$	-	-	$1.8x10^5$	-	-	$1x10^3$	-	-

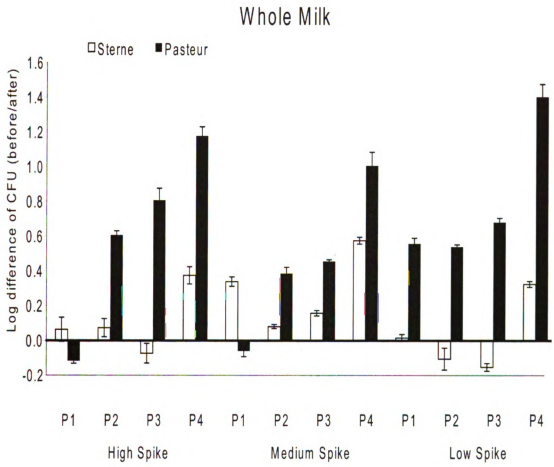
CFU, colony forming units (spore count); PCR, polymerase chain reaction; ECL, M1M BioVerify assay

Table 3. Combined tabulation – WHOLE MILK: Sterne & Pasteur spores, vegetative cells and process control organisms (*Bacillus cereus* spores and *E. coli*), post-pasteurization, immediate readings.

MATRIX: Homogenized Whole Milk, pH = 6.2															
Org.	Colony Counts, PCR (LightCycler®) and ECL (M1M) detection (+/-)														
	Untreated product			P-1 (63ºC/30 min)			P-2 (72ºC/16 sec)			P-3 (78ºC/16 sec)			P-4 (100ºC/3 sec)		
	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL
BAS															
Spores	$2.6x10^8$	-	-	$2.2x10^8$	-	-	$2.2x10^8$	-	-	$3.1x10^8$	-	-	$1.1x10^8$	-	-
	$2.7x10^6$	-	-	$1.2x10^6$	-	-	$2.2x10^6$	-	-	$1.9x10^6$	-	-	$7.1x10^5$	-	-
	$1.9x10^4$	-	-	$1.9x10^4$	-	-	$3x10^4$	-	-	$2.8x10^4$	-	-	$9x10^3$	-	-
Cells	$2.2x10^8$	-	-	<10	-	-	<10	-	-	<10	-	-	<10	-	-
BAP															
Spores	$2.1x10^8$	-	+	$2.8x10^8$	-	+	$5.2x10^7$	-	+	$3.3x10^7$	-	+	$1.4x10^7$	-	-
	$1.8x10^6$	-	+	$2.1x10^6$	-	+	$7.4x10^5$	-	+	$6.3x10^5$	-	+	$1.8x10^5$	-	-
	$2.5x10^4$	-	-	$7x10^3$	-	-	$7.2x10^3$	-	-	$5.2x10^3$	-	-	$1x10^3$	-	-
Cells	$2x10^8$	-	+	<10	-	-	<10	-	-	<10	-	-	<10	-	-
BC															
Spores	$2.6x10^8$	-	-	$3.4x10^8$	-	-	$2.6x10^8$	-	-	$1.4x10^8$	-	-	$6x10^7$	-	-
E.coli															
Cells	$1.8x10^8$	-	-	<10	-	-	<10	-	-	<10	-	-	<10	-	-

Abbreviations: BAS, *Bacillus anthracis* Sterne; BAP, *Bacillus anthracis* Pasteur; BC, *Bacillus cereus*.
+, detected by test; -, not detected by test. Each reading is an average of 3 readings.

Figure 4 – Efficacy of pasteurization methods and strain response differences by comparing BA strains (spores), measuring CFU log differences before and after pasteurizations in whole milk.



P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec.

Figure 5 - Whole Milk: Post Pasteurization Product Shelf Life (4 weeks) Spore Survival Study using High Spike (10^8) Samples *of Bacillus anthracis* Sterne strain (pH on Y2 axis)

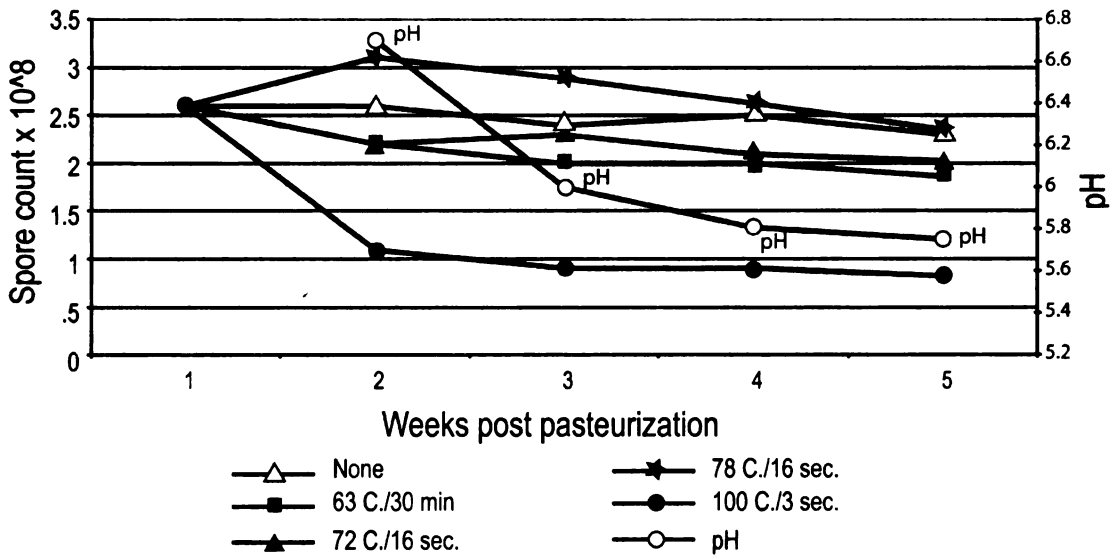


Figure 6 - Whole Milk: Post Pasteurization Product Shelf Life (4 weeks) Spore Survival Study using High Spike (10^8) Samples of *Bacillus anthracis* Sterne strain

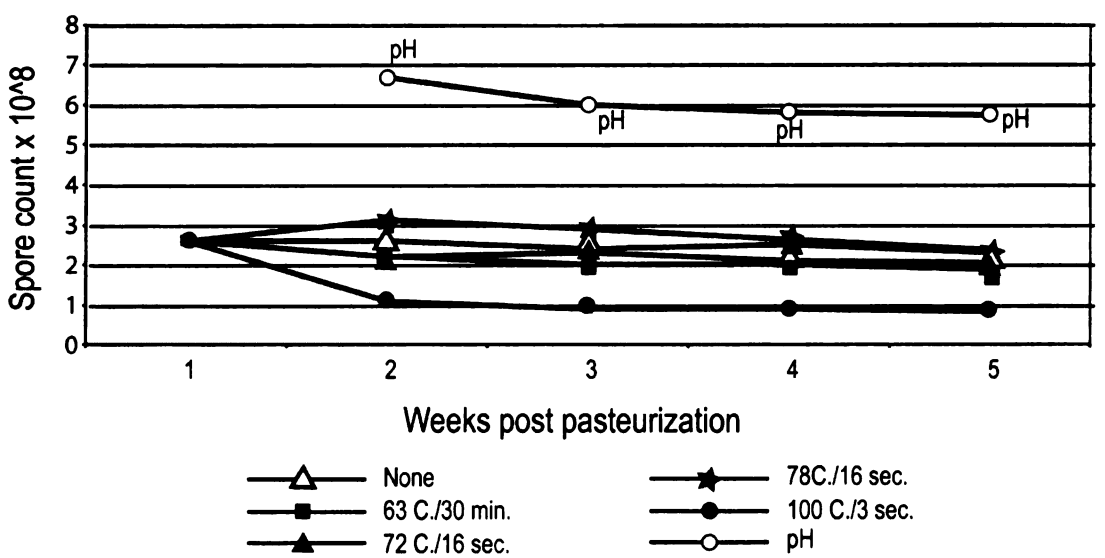


Figure 7 shows log difference in CFU between before and after pasteurization during 4 weeks of storage of whole milk suspensions, measured weekly. Log differences were measured by subtracting log CFU count after pasteurization from log CFU count before pasteurization, for all 3 different spike levels of the suspensions passed through 4 different pasteurization methods. (The spike levels used were High Spike= 10^8/ml, Medium Spike= 10^6/ml, Low Spike = 10^4/ml and the pasteurization methods were: P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec.). Log reductions represent exponential numbers, for example, 0.5 log difference (reduction) means 3.16 times reduction after pasteurization.

Pasteurization method P4 was more effective in reducing spore counts than all other methods - P1, P2, and P3 at all 3 different spike levels as seen in following statistical analysis.

1) At high spike condition (whole milk): pasteurization method was significantly different on the effect of reducing organisms at high spike condition during 4 weeks in both one-way ANOVA repeated measurement design model (when "week" used as repeated measurement, $p<0.0001$) and two-way ANOVA ($p< 0.0001$). [There was no interaction between pasteurization methods and weeks ($p=0.659$) in two-way ANOVA model.] See appendix.

a) Pasteurization method P4 was significantly more effective in reducing organisms than either method P1, P2, and P3

b) Pasteurization method P1 and P2 was significantly more effective in reducing organisms than method P3

c) Pasteurization effect: **method P4 > method P1 and P2 > method P3** (one way ANOVA repeated measure design / two way ANOVA, Duncan's multiple range test, $p < 0.05$) See appendix.

d) Week effect: week 4 > week 3 and week2 > week 1 (two way ANOVA, Duncan's multiple range test, $p < 0.05$) See appendix.

2) At medium spike condition (whole milk): Pasteurization methods were again significantly different in terms of effectiveness in reducing viable spore count (CFU) at medium spike level during 4 weeks in both one-way ANOVA repeated measurement design model (when "week" used as repeated measurement, $p<0.0001$) and two-way ANOVA ($p < 0.0001$). [There was no interaction between pasteurization methods and weeks ($p=0.435$) in two-way ANOVA model.] See appendix.

a) Again, pasteurization method P4 was significantly more effective in reducing organisms than methods P1, P2, and P3 at medium spike conditions.

b) Pasteurization effect (significantly different): **method P4 > method P1 > method P3 > method P2** (one way ANOVA repeated measure design / two way ANOVA, Duncan's multiple range test, $p < 0.05$) See appendix.

c) Week effect: week 4 > week 3 and week2 > week 1 (two way ANOVA, Duncan's multiple range test, $p < 0.05$). See appendix.

3) At Low spike condition (whole milk): Pasteurization methods were similarly significantly different in the effect of reducing organisms at low spike levels during 4 weeks in both one-way ANOVA repeated measurement design model (when "week" used as repeated measurement, $p<0.0001$) and two-way ANOVA with week and pasteurization method ($p< 0.0001$). [There was no interaction between pasteurization methods and weeks ($p=0.693$) in two-way ANOVA model.] See appendix.

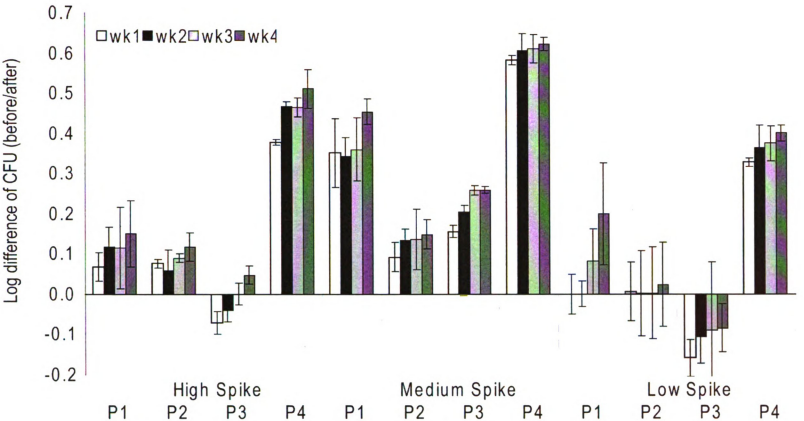
a) Pasteurization method P4 was significantly more effective in reducing organisms than either method P1, P2, and P3

b) Pasteurization method P1 and P2 was significantly more effective in reducing organisms than method P3

c) Pasteurization effect: **method P4 > method P1 and P2 > method P3** (one way ANOVA repeated measure design / two way ANOVA, Duncan's multiple range test, $p< 0.05$) See appendix.

d) Week effect: week 4 > week 1 (two way ANOVA, Duncan's multiple range test, $p< 0.05$) See appendix.

Figure 7 – Whole Milk: Comparative spore Survival Study using High (10^8), Medium (10^6) and Low (10^4) Spike samples of *Bacillus anthracis* Sterne strain in whole milk using log difference in CFU between before and after pasteurizations, during 4 weeks (product shelf life), measured weekly.



P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec.

2% and SKIM MILK STUDIES

The M1M showed better ability to detect Pasteur spores in 2% milk and skim milk in low-level concentration (10^4) as evident from Tables 4-6 and 7-9 respectively, but failed to detect spores in the high temperature/short time samples. The Sterne strain was not detected at any level by the M1M. Vegetative cells of both *B. anthracis* strains were undetectable by the M1M at any concentration. The sensitivity of the M1M did not increase when samples were diluted (1:10) with buffered peptone water containing 0.3% tween20 and retested. On the other hand, PCR did detect both Sterne and Pasteur spores as well as vegetative cells at high and medium concentrations (10^6 and above) but the sensitivity was reduced in samples subjected to high temperature/short time pasteurization.

Efficacy of all four pasteurization methods at high, medium and low spike levels of both strains were compared by measuring CFU log differences before and after pasteurizations in 2% milk (Fig. 8) and skim milk (Fig. 11), which clearly indicated higher susceptibility of the Pasteur strain at all spike levels. Ultra high temperature/short time pasteurization (100°C for 3 seconds) was the most effective method of pasteurization for 2% milk compared to the others, as evident from almost a one half to two-log reduction in viable spores of Pasteur strain. Sterne strain showed very little (less than a half-log) reduction indicating stronger heat resistant characteristic of the organism in this matrix. Subsequently, the spore survival study using the high spike 2% milk suspension and skim milk suspension of Sterne strain over the product shelf life of 4 weeks showed

relatively very stable viable spore counts with very little decline in both matrices (Fig. 9, 10, 12 and 13).

Table 4. STERNE STRAIN: spores detection in 2% MILK using plate count (CFU), PCR test (LightCycler) and ECL test (M1M)

Sample Processing ↓	High Spore Spike			Medium Spore Spike			Low Spore Spike		
	CFU	PCR	ECL	CFU	PCR	ECL	CFU	PCR	ECL
Untreated	$2.4x10^8$	+	-	$2.2x10^6$	+	-	$3x10^4$	-	-
63°C/30 minutes	$2.6x10^8$	+	-	$2.1x10^6$	+	-	$2.7x10^4$	-	-
72°C/16 seconds	$2.2x10^8$	+	-	$3x10^6$	+	-	$2.1x10^4$	-	-
78°C/16 seconds	$2x10^8$	+	-	$2.2x10^6$	+	-	$2x10^4$	-	-
100°C/3 seconds	$2.3x10^8$	+	-	$2.6x10^6$	-	-	$2.2x10^4$	-	-

CFU, colony forming units (spore count); PCR, polymerase chain reaction; ECL, M1M BioVerify assay

Table 5. PASTEUR STRAIN: spores detection in 2% MILK using plate count (CFU), PCR test (LightCycler) and ECL test (M1M)

Sample Processing ↓	High Spore Spike			Medium Spore Spike			Low Spore Spike		
	CFU	PCR	ECL	CFU	PCR	ECL	CFU	PCR	ECL
Untreated	$2.4x10^8$	+	+	$2.6x10^6$	+	+	$1.3x10^4$	-	+
63°C/30 minutes	$2.3x10^8$	+	+	$3.2x10^6$	+	+	$7.9x10^3$	-	+
72°C/16 seconds	$3.1x10^8$	+	+	$1.7x10^6$	+	+	$8.5x10^3$	-	+
78°C/16 seconds	$1.9x10^8$	+	+	$2.1x10^6$	+	+	$6.6x10^3$	-	-
100°C/3 seconds	$2.3x10^6$	+	-	$7.3x10^4$	-	-	$5x10^2$	-	-

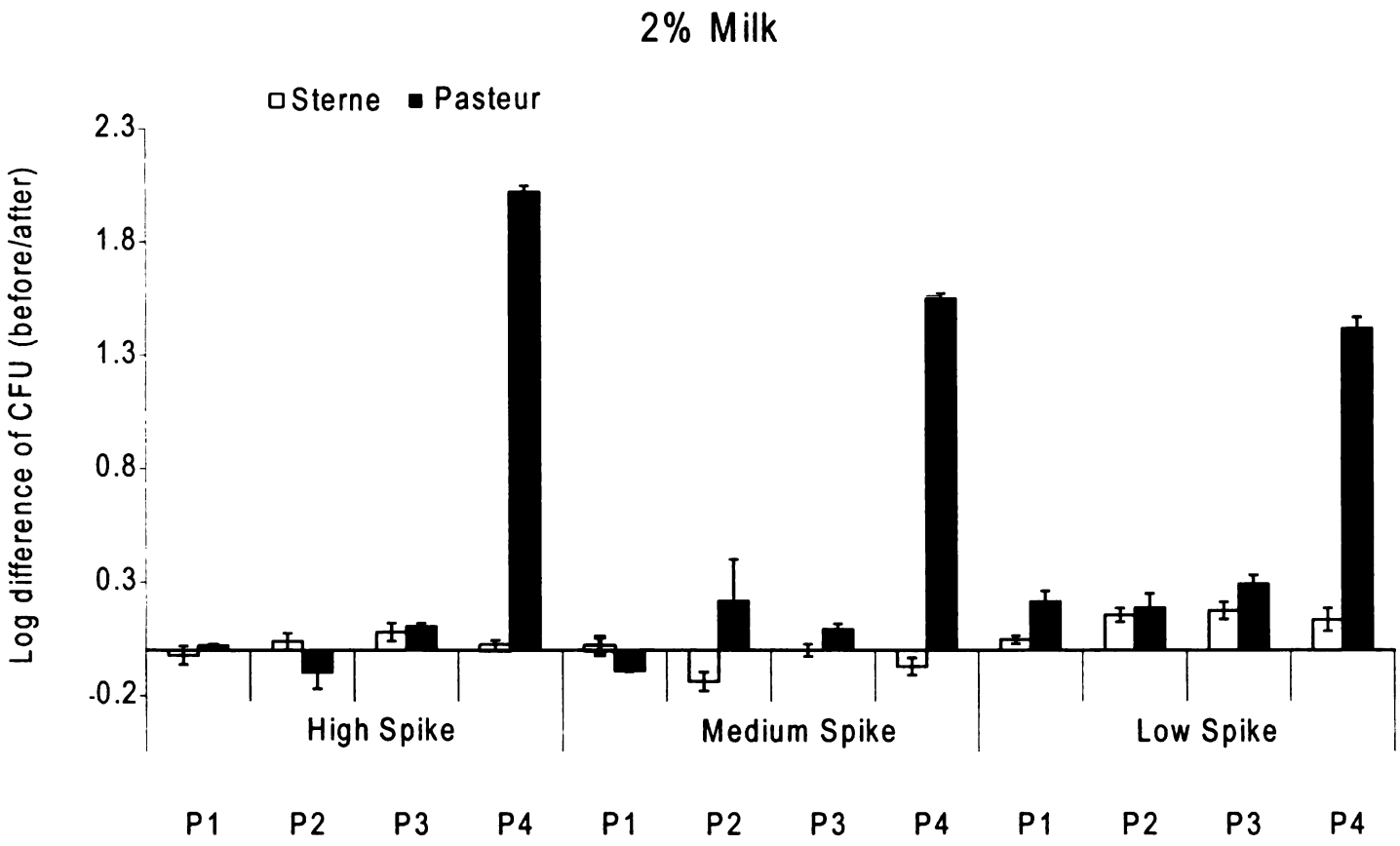
CFU, colony forming units (spore count); PCR, polymerase chain reaction; ECL, M1M BioVerify assay

Table 6. Combined tabulation – 2% MILK: Sterne & Pasteur spores, vegetative cells and process control organisms (*Bacillus cereus* spores and *E. coli*), post-pasteurization, immediate readings.

MATRIX: 2% fat Milk, pH = 6.1															
Org.	Colony Counts, PCR (LightCycler®) and ECL (M1M) detection (+/-)														
	Untreated product			P-1 (63ºC/30 min)			P-2 (72ºC/16 sec)			P-3 (78ºC/16 sec)			P-4 (100ºC/3 sec)		
	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL
BAS															
Spores	$2.4x10^8$	+	-	$2.6x10^8$	+	-	$2.2x10^8$	+	-	$2x10^8$	+	-	$2.3x10^8$	+	-
	$2.2x10^6$	+	-	$2.1x10^6$	+	-	$3x10^6$	+	-	$2.2x10^6$	+	-	$2.6x10^6$	-	-
	$3x10^4$	-	-	$2.7x10^4$	-	-	$2.1x10^4$	-	-	$2x10^4$	-	-	$2.2x10^4$	-	-
Cells	$2.5x10^8$	+	-	<10	+	-	<10	+	-	<10	+	-	<10	+	-
BAP															
Spores	$2.4x10^8$	+	+	$2.3x10^8$	+	+	$3.1x10^8$	+	+	$1.9x10^8$	+	+	$2.3x10^6$	+	-
	$2.6x10^6$	+	+	$3.2x10^6$	+	+	$1.7x10^6$	+	+	$2.1x10^6$	+	+	$7.3x10^4$	-	-
	$1.3x10^4$	-	+	$7.9x10^3$	-	+	$8.5x10^3$	-	+	$6.6x10^3$	-	-	$5x10^2$	-	-
Cells	$3.4x10^8$	+	+	<10	+	-	<10	+	-	<10	+	-	<10	+	-
BC															
Spores	$2.8x10^8$	-	-	$3.4x10^8$	-	-	$2.8x10^8$	-	-	$1.4x10^8$	-	-	$1.8x10^8$	-	-
E.coli															
Cells	$2.4x10^8$	-	-	<10	-	-	<10	-	-	<10	-	-	<10	-	-

Abbreviations: BAS, *Bacillus anthracis* Sterne; BAP, *Bacillus anthracis* Pasteur; BC, *Bacillus cereus.*
+, detected by test; -, not detected by test. Each reading is an average of 3 readings.

Figure 8 – Efficacy of pasteurization methods and strain response differences by comparing BA strains, measuring CFU log differences before and after pasteurizations in 2% milk.



P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec.

Figure 9 - 2% Milk: Post Pasteurization Product Shelf Life (4 weeks) Spore Survival Study using High Spike (10^8) Samples of *Bacillus anthracis* Sterne strain (pH on Y2 axis)

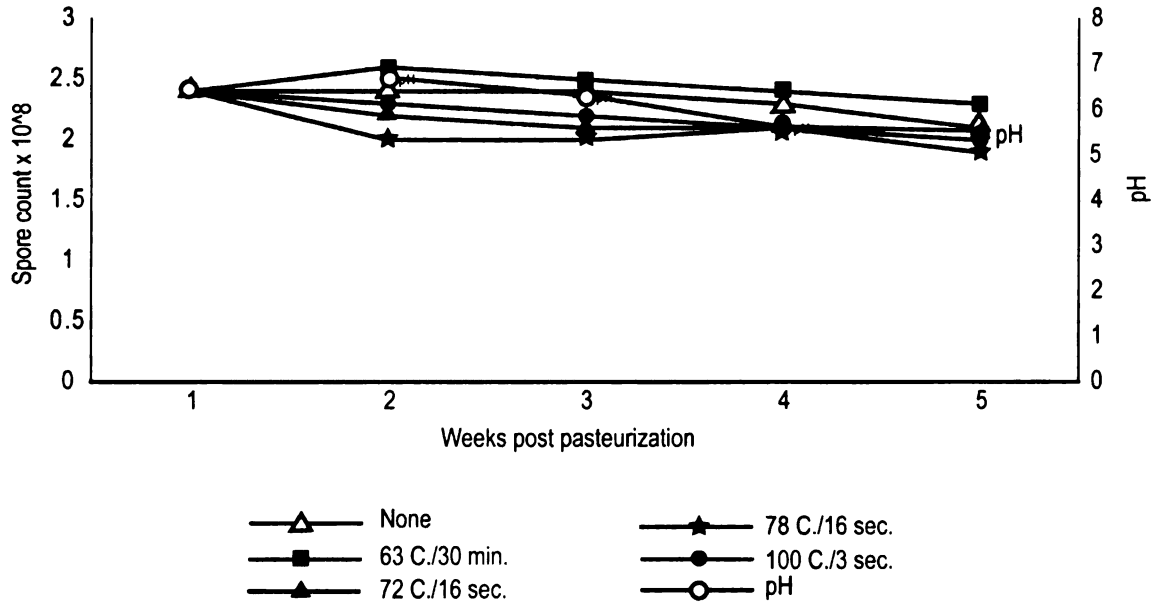


Figure 10 - 2% Milk: Post Pasteurization Product Shelf Life (4 weeks) Spore Survival Study using High Spike (10^8) Samples of *Bacillus anthracis* Sterne strain

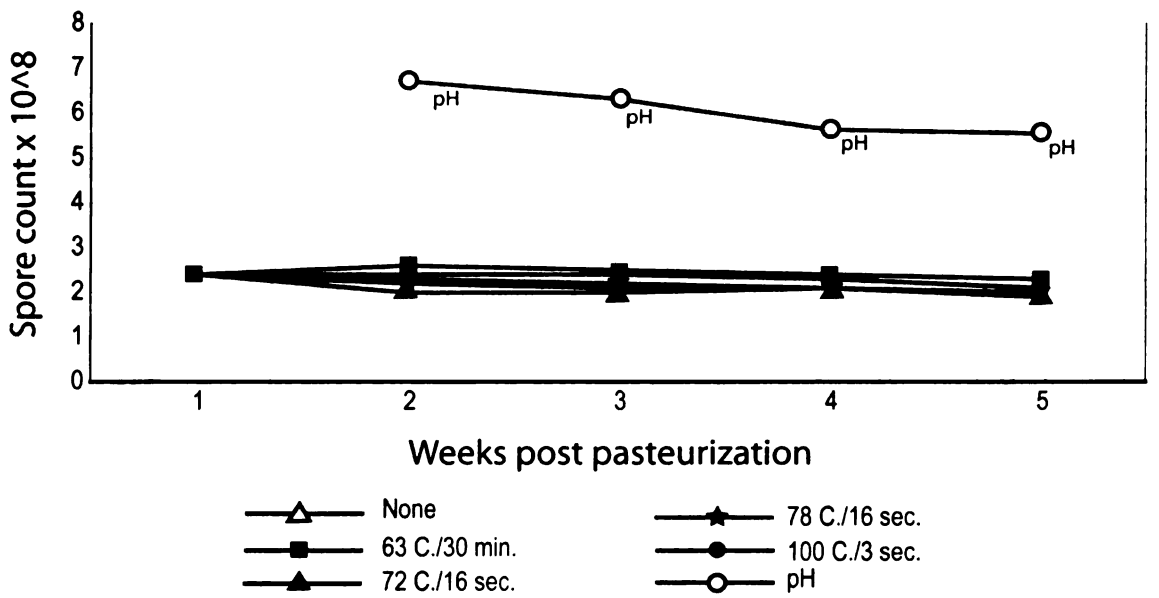


Table 7. STERNE STRAIN: spores detection in SKIM MILK using plate count (CFU), PCR test (LightCycler) and ECL test (M1M)

Sample Processing ↓	High Spore Spike			Medium Spore Spike			Low Spore Spike		
	CFU	PCR	ECL	CFU	PCR	ECL	CFU	PCR	ECL
Untreated	$3.2x10^8$	+	-	$2.6x10^6$	+	-	$3.1x10^4$	-	-
63°C/30 minutes	$1.6x10^8$	+	-	$4.1x10^6$	+	-	$2.3x10^4$	-	-
72°C/16 seconds	$2.3x10^8$	+	-	$3.3x10^6$	+	-	$3.1x10^4$	-	-
78°C/16 seconds	$4.1x10^8$	+	-	$2.1x10^6$	+	-	$2.3x10^4$	-	-
100°C/3 seconds	$2.1x10^8$	+	-	$3.3x10^6$	-	-	$1.4x10^4$	-	-

CFU, colony forming units (spore count); PCR, polymerase chain reaction; ECL, M1M BioVerify assay

Table 8. PASTEUR STRAIN: spores detection in SKIM MILK using plate count (CFU), PCR test (LightCycler) and ECL test (M1M)

Sample Processing ↓	High Spore Spike			Medium Spore Spike			Low Spore Spike		
	CFU	PCR	ECL	CFU	PCR	ECL	CFU	PCR	ECL
Untreated	$1.7x10^8$	+	+	$2.3x10^6$	+	+	$1.2x10^4$	-	+
63°C/30 minutes	$3.6x10^8$	+	+	$7.7x10^5$	+	+	$8x10^3$	-	-
72°C/16 seconds	$3.1x10^7$	+	+	$4.3x10^5$	-	+	$8.4x10^3$	-	-
78°C/16 seconds	$4.8x10^7$	+	+	$6.6x10^5$	-	-	$6.4x10^3$	-	-
100°C/3 seconds	$6.8x10^6$	+	-	$9.5x10^3$	-	-	$3x10^2$	-	-

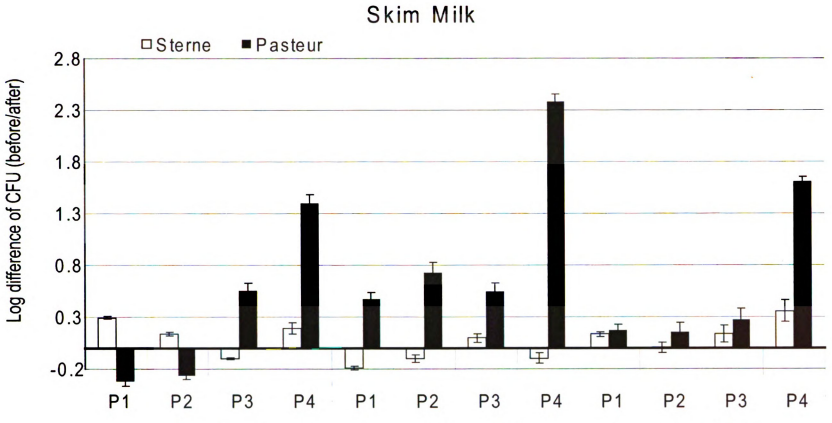
CFU, colony forming units (spore count); PCR, polymerase chain reaction; ECL, M1M BioVerify assay

Table 9. Combined tabulation – SKIM MILK: Sterne & Pasteur spores, vegetative cells and process control organisms (*Bacillus cereus* spores and *E. coli*), post-pasteurization, immediate readings.

MATRIX: Skim Milk, pH = 6.7															
Org.	Colony Counts, PCR (LightCycler®) and ECL (M1M) detection (+/-)														
	Untreated product			P-1 (63^{0}C/30 min)			P-2 (72^{0}C/16 sec)			P-3 (78^{0}C/16 sec)			P-4 (100^{0}C/3 sec)		
	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL
BAS															
Spores	$3.2x10^8$	+	-	$1.6x10^8$	+	-	$2.3x10^8$	+	-	$4.1x10^8$	+	-	$2.1x10^8$	+	-
	$2.6x10^6$	+	-	$4.1x10^6$	+	-	$3.3x10^6$	+	-	$2.1x10^6$	+	-	$3.3x10^6$	-	-
	$3.1x10^4$	-	-	$2.3x10^4$	-	-	$3.1x10^4$	-	-	$2.3x10^4$	-	-	$1.4x10^4$	-	-
Cells	$3.5x10^8$	+	-	<10	+	-	<10	+	-	<10	+	-	<10	+	-
BAP															
Spores	$1.7x10^8$	+	+	$3.6x10^8$	+	+	$3.1x10^7$	+	+	$4.8x10^7$	+	+	$6.8x10^6$	+	-
	$2.3x10^6$	+	+	$7.7x10^5$	+	+	$4.3x10^5$	-	+	$6.6x10^5$	-	-	$9.5x10^3$	-	-
	$1.2x10^4$	-	+	$8x10^3$	-	-	$8.4x10^3$	-	-	$6.4x10^3$	-	-	$3x10^2$	-	-
Cells	$2.1x10^8$	+	+	<10	+	-	<10	+	-	<10	+	-	<10	+	-
BC															
Spores	$2.8x10^8$	-	-	$3.4x10^8$	-	-	$2.6x10^8$	-	-	$1.8x10^8$	-	-	$3x10^8$	-	-
E.coli															
Cells	$2x10^8$	-	-	<10	-	-	<10	-	-	<10	-	-	<10	-	-

Abbreviations: BAS, *Bacillus anthracis* Sterne; BAP, *Bacillus anthracis* Pasteur; BC, *Bacillus cereus.*
+, detected by test; -, not detected by test. Each reading is an average of 3 readings.

Figure 11 - Efficacy of pasteurization methods and strain response differences by comparing BA strains, measuring CFU log differences before and after pasteurizations in skim milk.



P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec.

Figure 16 - Apple Juice: Post Pasteurization Product Shelf Life (12 weeks) Spore Survival Study using High Spike (10^8) Samples of *Bacillus anthracis* Sterne strain (pH on Y2 axis)

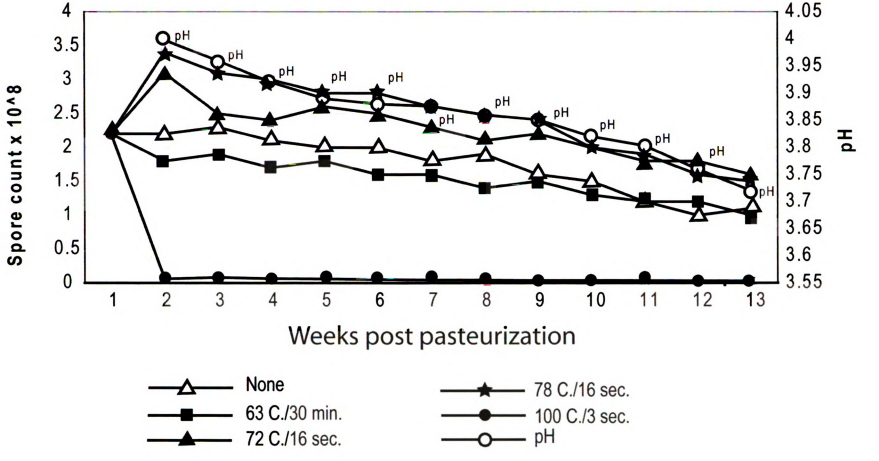


Figure 13 - Skim Milk: Post Pasteurization Product Shelf Life (4 weeks) Spore Survival Study using High Spike (10^8) Samples of *Bacillus anthracis* Sterne strain

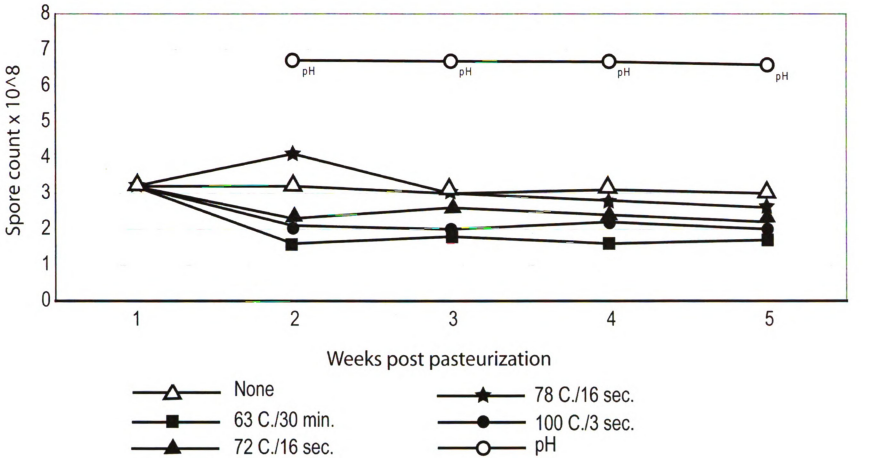
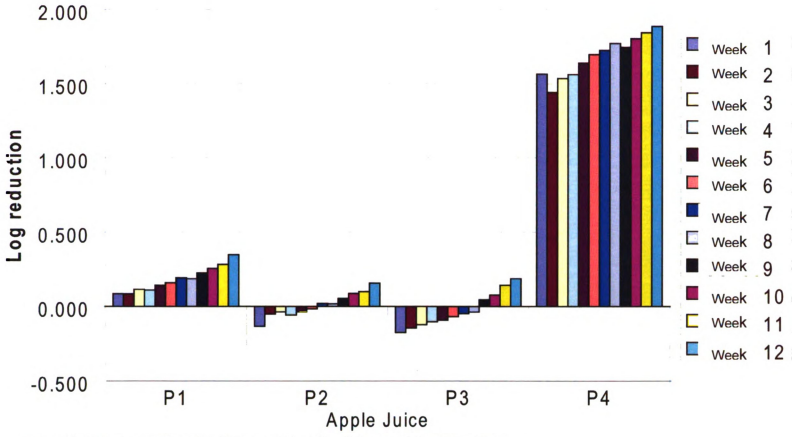


Figure 18 – Apple Juice: Comparative spore Survival Study using High (10^8) spike samples of *Bacillus anthracis* Sterne strain in apple juice using log difference in CFU between before and after pasteurizations, during 12 weeks (product shelf life), measured weekly.



P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec.

Figure 14 shows comparison of log differences in CFU between before and after pasteurization during 4 weeks of storage of all 3 milk matrices - whole milk, 2% milk and Skim milk suspensions, measured weekly.

Log differences were measured by subtracting log CFU after pasteurization from log CFU before pasteurization at all 3 different spike levels passing through 4 different pasteurization methods. Again, for example, 0.5 log difference (reduction) means 3.16 times reduction after pasteurization.

- Pasteurization methods: P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec.
- High Spike= 10^8/ml, Medium Spike= 10^6/ml, Low Spike = 10^4/ml

Statistical analysis summary of milk matrices:

Whole Milk: At high spike level, pasteurization methods showed significantly different effectiveness in reducing viable spore counts during 4 weeks in both one-way ANOVA repeated measurement design model (when "week" used as repeated measurement, $p<0.0001$) and two-way ANOVA ($p< 0.0001$). [There was no interaction between pasteurization methods and weeks ($p=0.659$) in two-way ANOVA model.] See appendix.

a. Pasteurization method P4 was significantly more effective in reducing spore counts than either method P1, P2, and P3

b. Pasteurization method P1 and P2 were significantly more effective in reducing spore counts than method P3

c. Pasteurization effect: **method P4 > method P1 and P2 > method P3** (one way ANOVA repeated measure design / two way ANOVA, Duncan's multiple range test, $p < 0.05$) See appendix.

d. Week effect: week 4 > week 3 and week2 > week 1 (two way ANOVA, Duncan's multiple range test, $p < 0.05$) See appendix.

2) 2% milk: No significant pasteurization or week effects were observed.

3) Skim milk: pasteurization methods were significantly different with respect to the effectiveness in reducing spore counts at high spike level during the 4 weeks storage in both one-way ANOVA repeated measurement design model (when "week" used as repeated measurement, $p<0.0001$) and two-way ANOVA ($p < 0.0001$). [There was an interaction between pasteurization methods and weeks ($p=0.002$) in two-way ANOVA model.] See appendix.

Pasteurization effect: **method P1 > method P4 > P2 > P3** (one way ANOVA repeated measure design / two way ANOVA, Duncan's multiple range test, $p < 0.05$) See appendix.

LIQUID EGG STUDIES

PCR worked well with liquid egg samples (Table 16-18). Both Sterne and Pasteur strain spores and vegetative cells were detected from most spike concentrations before and after pasteurization. The M1M only detected Pasteur spores at high and medium concentrations (10^6 and above) in unpasteurized samples. M1M failed to detect spores and vegetative cells after all pasteurization steps. All the medium and low spore concentration samples tested on the M1M were diluted (1:10) in buffered peptone water with 0.3% tween20 and retested in an effort to increase sensitivity but the results remained unchanged. Dilution in buffered peptone water decreased the liquid eggs matrix pH from 8.7 to 7.4.

Efficacy of all four pasteurization methods at high, medium and low spike levels of both strains were compared by measuring CFU log differences before and after pasteurizations in liquid eggs (Fig. 23), which clearly indicated higher susceptibility of the Pasteur strain at all spike levels. Ultra high temperature/short time pasteurization (100°C for 3 seconds) was a relatively more effective method of pasteurization for liquid eggs compared to the others, as evident from almost a one to nearly two-log reduction in viable spores of Pasteur strain. Sterne strain showed less than one-log reduction indicating a stronger heat resistant characteristic of the organism. Subsequently, the spore survival study using high spike liquid eggs suspension of Sterne strain over the product shelf life of 12 weeks showed relatively stable viable spore counts with very little decline over the period (Fig. 24, 25 and 26).

as evident from almost a one to two-log reduction in viable spores of both Sterne and Pasteur strains. Sterne strain showed generally less reduction, indicating stronger heat resistant characteristic of the organism. Subsequently, the spore survival study using high spike apple juice suspension of Sterne strain over the product shelf life of 12 weeks showed declining viable spore counts with a one-log or less decline during the time period (Fig. 16, 17 and 18) with highest decline seen at ultra high temperature/short time pasteurization.

product shelf life of 12 weeks showed progressively declining viable spore counts up to nearly two-log reductions over the period (Fig. 20, 21 and 22).

Table 13. STERNE STRAIN: spores detection in APPLE CIDER using plate count (CFU), PCR test (LightCycler) and ECL test (M1M)

Sample Processing ↓	High Spore Spike			Medium Spore Spike			Low Spore Spike		
	CFU	PCR	ECL	CFU	PCR	ECL	CFU	PCR	ECL
Untreated	$3.5x10^8$	-	-	$3.2x10^6$	-	-	$2.7x10^4$	-	-
63°C/30 minutes	$2.3x10^8$	-	-	$5x10^6$	-	-	$3.6x10^4$	-	-
72°C/16 seconds	$7.2x10^7$	-	-	$5x10^5$	-	-	$4.2x10^4$	-	-
78°C/16 seconds	$8.2x10^6$	-	-	$7.6x10^5$	-	-	$3.4x10^4$	-	-
100°C/3 seconds	$4.2x10^6$	-	-	$1.5x10^4$	-	-	$6x10^2$	-	-

CFU, colony forming units (spore count); PCR, polymerase chain reaction; ECL, M1M BioVerify assay

Table 14. PASTEUR STRAIN: spores detection in APPLE CIDER using plate count (CFU), PCR test (LightCycler) and ECL test (M1M)

Sample Processing ↓	High Spore Spike			Medium Spore Spike			Low Spore Spike		
	CFU	PCR	ECL	CFU	PCR	ECL	CFU	PCR	ECL
Untreated	$3.6x10^8$	-	+	$2.2x10^6$	-	-	$4.3x10^4$	-	-
63°C/30 minutes	$3.8x10^8$	-	+	$1.8x10^6$	-	-	$1.3x10^4$	-	-
72°C/16 seconds	$1.6x10^8$	-	+	$2.1x10^6$	-	-	$2.6x10^4$	-	-
78°C/16 seconds	$3.1x10^8$	-	+	$3.3x10^6$	-	-	$3.2x10^4$	-	-
100°C/3 seconds	$3.8x10^5$	-	-	$8.8x10^3$	-	-	$3.5x10^2$	-	-

CFU, colony forming units (spore count); PCR, polymerase chain reaction; ECL, M1M BioVerify assay

Table 12. Combined tabulation – APPLE JUICE: Sterne & Pasteur spores, vegetative cells and process control organisms (*Bacillus cereus* spores and *E. coli*), post-pasteurization, immediate readings.

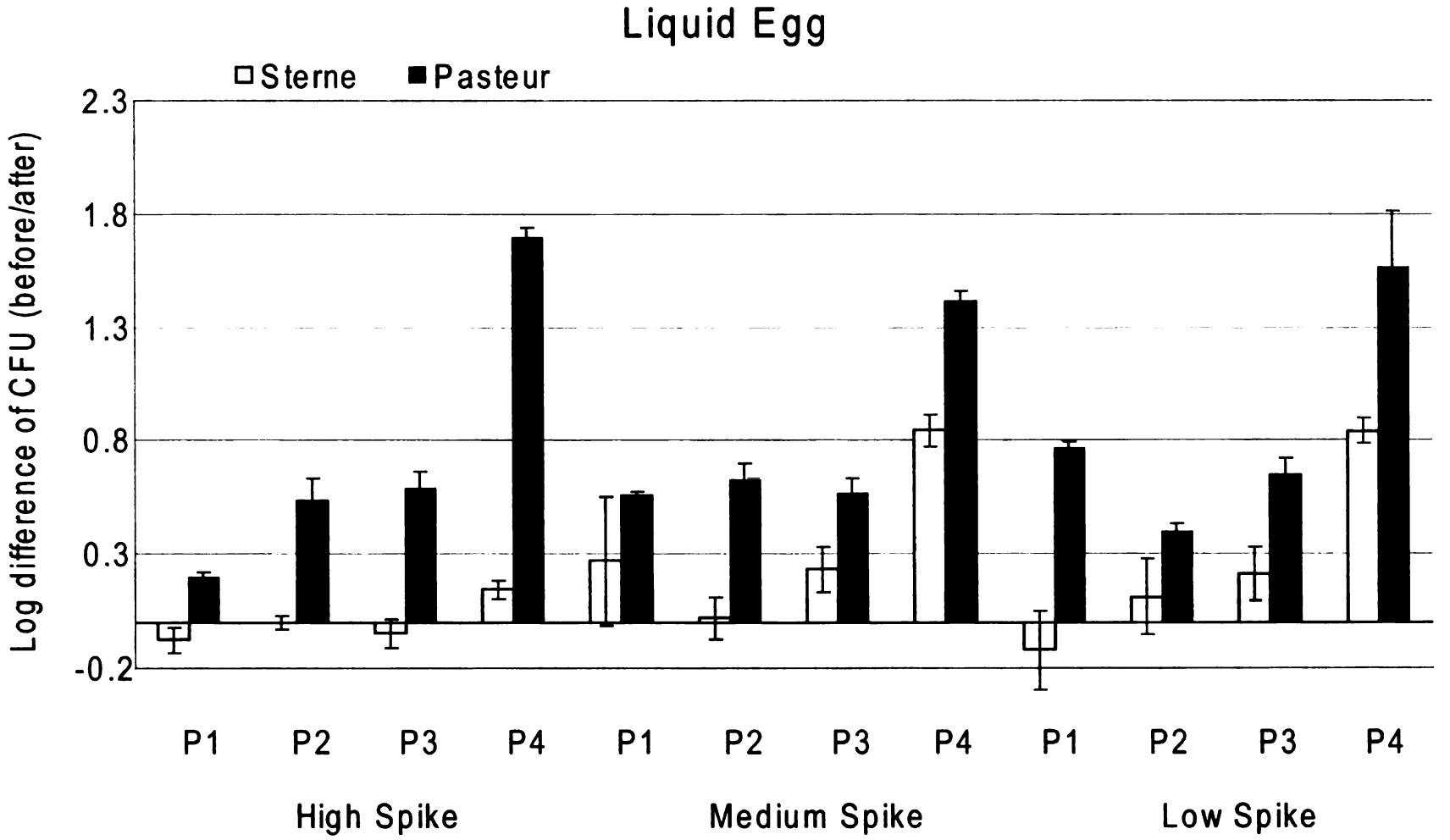
MATRIX: Apple Juice,, pH=3.8. For M1M, diluted with buffered peptone water with 0.3% tween20, pH=7.0, higher sensitivity.															
Org.	Colony Counts, PCR (LightCycler®) and ECL (M1M) detection (+/-)														
	Untreated product			P-1 (63ºC/30 min)			P-2 (72ºC/16 sec)			P-3 (78ºC/16 sec)			P-4 (100ºC/3 sec)		
	CFU/ml	PCR	ECL*	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL	CFU/ml	PCR	ECL
BAS															
Spores	$2.2x10^8$	+	+	$1.8x10^8$	+	-	$3.1x10^8$	+	-	$3.4x10^8$	+	-	$6.1x10^6$	+	-
	$3.3x10^6$	+	-	$2.8x10^6$	+	-	$4.1x10^6$	+	-	$3.2x10^6$	+	-	$8.2x10^4$	-	-
	$1.6x10^4$	-	-	$8.4x10^3$	-	-	$1.2x10^4$	-	-	$1.3x10^4$	-	-	$1.8x10^3$	-	-
Cells	$3.3x10^8$	+	-	<10	+	-	<10	+	-	<10	+	-	<10	+	-
BAP															
Spores	$2.5x10^8$	+	+	$8.6x10^7$	+	+	$1.6x10^8$	+	+	$2.1x10^8$	+	+	$2.9x10^7$	+	+
	$1.8x10^6$	+	+	$2x10^6$	+	-	$9.2x10^5$	+	+	$1.9x10^6$	+	+	$5.6x10^4$	-	-
	$1.4x10^4$	-	-	$6.4x10^3$	-	-	$1.1x10^4$	-	-	$9.3x10^3$	-	-	$1x10^2$	-	-
Cells	$2.4x10^8$	+	+	<10	+	-	<10	+	+	<10	+	+	<10	+	-
BC															
Spores	$2.3x10^8$	-	-	$2.5x10^8$	-	-	$3.2x10^8$	-	-	$2.9x10^8$	-	-	$2.1x10^8$	-	-
E.coli															
Cells	$2x10^8$	-	-	<10	-	-	<10	-	-	<10	-	-	<10	-	-

Abbreviations: BAS, *Bacillus anthracis* Sterne; BAP, *Bacillus anthracis* Pasteur; BC, *Bacillus cereus.*

+, detected by test; -, not detected by test

***** For ECL (M1M) testing, the high concentration samples were prepared in apple juice and then diluted in buffered peptone water to prepare the medium and low concentration samples. Data for testing the medium and low concentrations in apple juice are not shown. Each reading is an average of 3 readings.

Figure 23 - Efficacy of pasteurization methods and strain response differences by comparing BA strains, measuring CFU log differences before and after pasteurizations in liquid eggs.



P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec.

Figure 24 – Liquid Eggs: Post Pasteurization Long Term (12 weeks) Spore Viability Study using *Bacillus anthracis* Sterne strain (pH on Y2 axis)

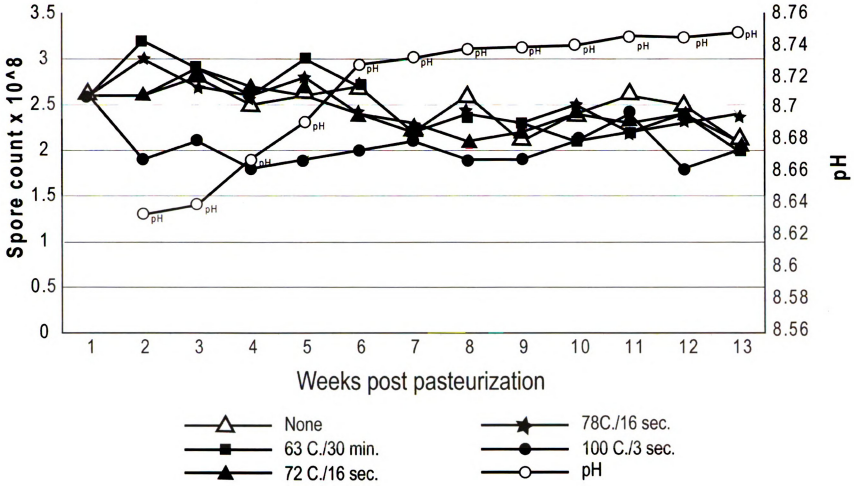


Figure 25 – Liquid Eggs: Post Pasteurization Long Term (12 weeks) Spore Viability Study using *Bacillus anthracis* Sterne strain

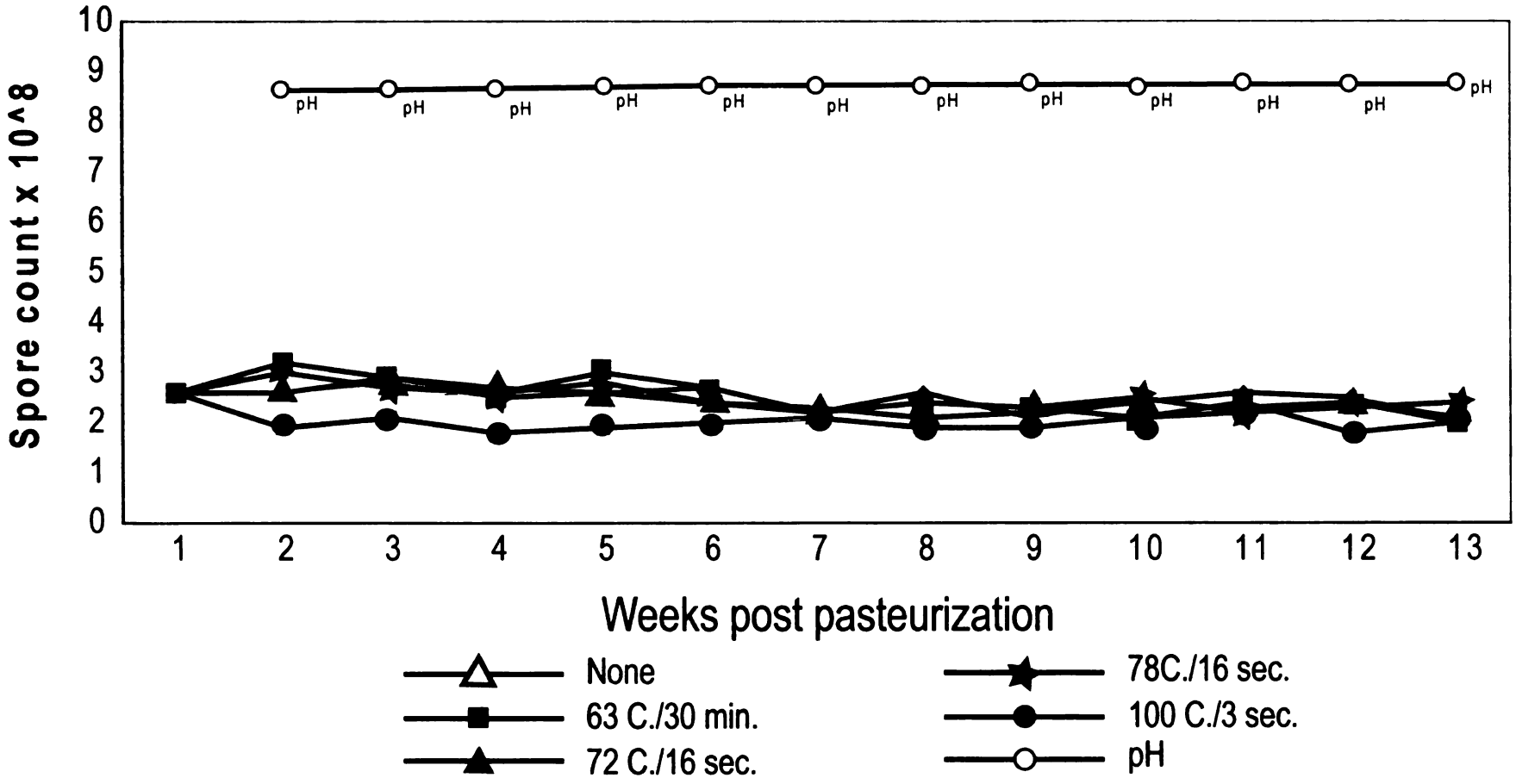
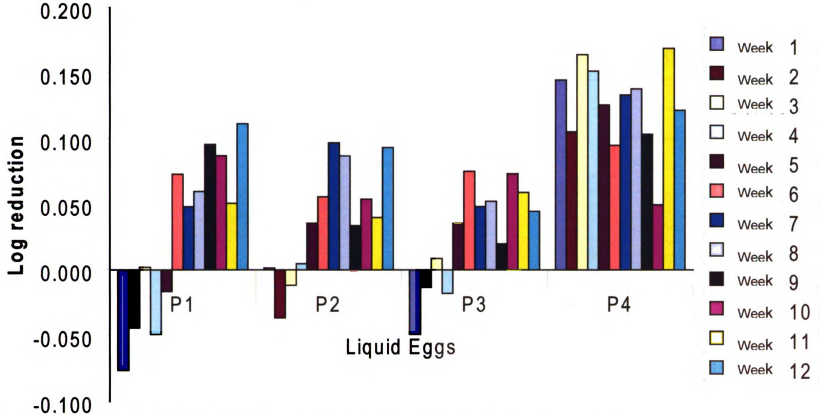
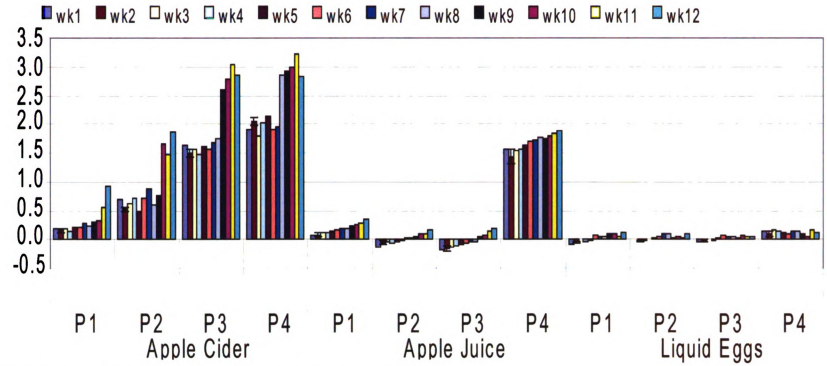


Figure 26 – Liquid Eggs: Comparative spore Survival Study using High (10^8) spike samples of *Bacillus anthracis* Sterne strain in liquid eggs using log difference in CFU between before and after pasteurizations, during 12 weeks (product shelf life), measured weekly.



P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec., Series=weeks

Figure 27 - Other matrices: Comparative spore Survival Study using High Spike (10^8) samples of *Bacillus anthracis* Sterne strain in apple juice, apple cider and liquid eggs using log difference in CFU between before and after pasteurizations, during 12 weeks (product shelf life), measured weekly.



P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec.

Figure 27 compares log difference in CFU between before and after pasteurization during 12 weeks of refrigerated storage of apple cider, apple juice and liquid eggs suspensions (simulating product shelf life and storage conditions), measured weekly.

Log differences were measured by subtracting log CFU after pasteurization from log CFU before pasteurization at all 3 different spike levels passing through 4 different pasteurization methods. Again, 0.5 log difference (reduction) means 3.16 times reduction after pasteurization.

- Pasteurization methods: P1= 63c/30mins., P2= 72c./16sec., P3= 78c./16sec., P4= 100c./3sec.
- High Spike= 10^8/ml, Medium Spike= 10^6/ml, Low Spike = 10^4/ml

Statistical analysis summary of apple juice, apple cider and liquid eggs:

1) Apple juice: pasteurization methods were significantly different in reducing the viability of spores at high spike level during 12 weeks in both one-way ANOVA repeated measurement design model, when "week" used as repeated measurement, $p<0.0001$ and two-way ANOVA $p< 0.0001$. [There was no interaction between pasteurization methods and weeks ($P=0.4918$) in two-way ANOVA model.] See appendix.

*Pasteurization effect: **method P4 > method P1 > Method P2 > Method P3** (One way ANOVA repeated measure design / two way ANOVA, Duncan's multiple range test, $p < 0.05$) See appendix.

2) Apple cider: pasteurization methods were significantly different in reducing viability of spores at high spike condition during 12 weeks in both one-way ANOVA repeated measurement design model, when "week" used as repeated measurement, $p<0.0001$ and two-way ANOVA $p < 0.0001$. [There was an interaction between pasteurization methods and weeks ($p=<0.0001$) in two-way ANOVA model.] See appendix.

*Pasteurization effect: **method P4 > method P3 > method P2 > method P1** (one way ANOVA repeated measure design / two way ANOVA, Duncan's multiple range test, $p < 0.05$) See appendix.

3) Liquid eggs: differences in pasteurization methods were insignificant in reducing viability of spores at high spike condition during 12 weeks in both one-way ANOVA repeated measurement design model, when "week" used as repeated measurement, and two-way ANOVA, $p > 0.05$. See appendix.

Global Statistical Analysis Summary of All Matrices, Pasteurization Methods and Survival (weeks) in a mixed procedure:

(See appendix for the SAS Univariate Procedure and Mixed Procedure outputs as well as the SAS codes used).

1. Based upon quartiles analysis (univariate procedure) of low to high CFU counts against test positivity (detection), BioVeris M1M showed direct proportionality and as expected, detection was progressively better with higher CFU counts (Table 19). See appendix.

2. Based upon quartiles analysis (univariate procedure) of low to high CFU counts against test positivity (detection), PCR showed direct proportionality and as expected, detection was progressively better with higher CFU counts, and was better than BioVeris M1M (Table 20). See appendix.

3. In comparing both detection methods, PCR and M1M, the percent agreement is 0.60 (60%), p=0.000066, risk ratio (relative risk) of 1.866, indicating rate of being positive for one is only about twice as great if the other one is positive`(Table 21). See the 2x2 table with details in the appendix.

4. The mixed procedure indicated that pasteurization method 4 was significantly better than all other methods, "matrix" and "pasteurization" both had significant effect on spore survival (p=0.0001) but "week" had no significant effect on spore survival (p=0.8511) (Table 22-23). See appendix.

Table 19. Progressively better detection by M1M, directly proportional to CFU count, by quartile analysis and by frequency of the listed variables:

Quartile (low to high CFU)		Negative	Positive	Total
1	Frequency	123	12	135
	Percent	22.78	2.22	25.00
	Row Percent	91.11	8.89	
	Column Percent	29.71	9.52	
2	Frequency	112	27	139
	Percent	20.74	5.00	25.74
	Row Percent	80.58	19.42	
	Column Percent	27.05	21.43	
3	Frequency	100	31	131
	Percent	18.52	5.74	24.26
	Row Percent	76.34	23.66	
	Column Percent	24.15	24.60	
4	Frequency	79	56	135
	Percent	14.63	10.37	25.00
	Row Percent	58.52	41.48	
	Column Percent	19.08	44.44	
Total		414	126	540
Total Percent		76.67	23.33	100.00

Table 20. Progressively better detection by PCR, directly proportional to CFU count, by quartile analysis and by frequency of listed variables:

Quartile (low to high CFU)		Negative	Positive	Total
1	Frequency	116	19	135
	Percent	21.48	3.52	25.00
	Row Percent	85.93	14.07	
	Column Percent	38.41	7.98	
2	Frequency	97	42	139
	Percent	17.96	7.78	25.74
	Row Percent	69.78	30.22	
	Column Percent	32.12	17.65	
3	Frequency	51	80	131
	Percent	9.44	14.81	24.26
	Row Percent	38.93	61.07	
	Column Percent	16.89	33.61	
4	Frequency	38	97	135
	Percent	7.04	17.96	25.00
	Row Percent	28.15	71.85	
	Column Percent	12.58	40.76	
Total		302	238	540
Total Percent		55.93	44.07	100.00

Table 21. Statistical Comparison of PCR and M1M test technologies:

2 x 2 Table: Single Table Analysis

		BioVerify Kit - M1M (+)	(-)	Total
PCR (LRN)	(+)	75	163	238
	(-)	51	251	302
Total		126	414	540

Uncorrected chi square = 15.92, p=0.000066
Yates corrected chi square = 15.11, p=0.0001015
Mantel-Haenszel chi square = 15.89, p=0.00006729.
Risk ratio (relative risk) = 1.866 (CI = 1.365, 2.551)
Percent agreement between two tests = 75+251/540 = 60%.

Table 22. Type 3 Tests of Fixed Effects on colony forming unit (CFU) counts, overall mixed procedure statistic:

Effect	Num DF	Den DF	F Value	Pr > F
Matrix	5	347	52.78	< 0.0001
Pasteurization	4	347	48.45	< 0.0001
Week	3	347	0.26	0.8511

Table 23. Least Squares Means Table, overall mixed procedure statistic:

Effect	Matrix	Pasteurization	Week	Estimate	Pr > | t |
Pasteurization		0		0.01872	0.6386
Pasteurization		1		0.1055	0.0084
Pasteurization		2		0.1419	0.0004
Pasteurization		3		0.2473	<0.0001
Pasteurization		4		0.7205	<0.0001
Matrix	1			0.1334	0.0024
Matrix	2			0.04632	0.2891
Matrix	3			0.1352	0.0021
Matrix	4			0.2866	<0.0001
Matrix	5			0.8644	<0.0001
Matrix	6			0.01478	0.7351
Week			1	0.2296	<0.0001
Week			2	0.2359	<0.0001
Week			3	0.2510	<0.0001
Week			4	0.2707	<0.0001

The matrices tested in this study appeared to pose some obstacle to extraction and real-time PCR procedures as well as to the BioVerify test protocol presumably due to test interference from the matrix itself. This interference may be due to matrix factors like fat content, other matrix constituents, low pH or other yet unidentified factors. As discussed earlier in chapter 2, lower limit of detection (LOD) in dH_2O was lower than what was seen in these matrices, which shows variable and matrix dependent detection capability (sensitivity).

We found no solution to these problems for *B. anthracis* strains particularly when samples were spiked at low (10^4) and middle (10^6) concentrations. Detection of low spore concentrations ($<10^4$) is beyond the capacity of the procedures without using concentration or enrichment techniques. Some matrices such as skim milk, fat free milk, apple juice, and liquid eggs are suited for rapid evaluation using RT-PCR or the BioVerify test without concentration, but only at high spore concentrations ($>10^6$).

BENEFITS:

Electrochemiluminescence assay is a novel technology. It is economical, easy to use, portable, sensitive, and rapid. This study may result in validation of this technology if our data indicates a good correlation with other methods. Such a validation will benefit emergency responders in the field as well as laboratories involved in bioterrorism testing. Results will be available in 16 minutes by this assay compared to 5 hours for real-time PCR or 24-48 hours by culture.

CHAPTER 4

DISCUSSION

Bacillus anthracis is a highly virulent, rapidly growing gram-positive bacterial pathogen that may cause inhalational, gastrointestinal or the cutaneous forms of anthrax in humans. The inhalational form of anthrax is the most lethal and a highly feared form of the disease particularly if spores from a virulent strain are deliberately used as a source of human exposure. Events' following the September 2001 terrorist attacks, targeting an unsuspecting population using our nation's postal system as an effective delivery mechanism for spores in the form of powder in envelopes is a testament to it. However, recently, gastrointestinal anthrax resulting from the consumption of meat from animals that die of the disease has been reported in humans from several parts of the world, making it a disease of very serious epidemiological implications and a public health priority. Dozens of human fatalities have been reported from part of Africa, independent nations once part of the Soviet Union and several regions of Asia, especially India as reported on an almost daily basis by the ProMED-mail post (http://www.promedmail.org), a program of the International Society for Infectious Diseases (http://www.isid.org). Dose-response data or models for human infection with *B. anthracis* are not found in the scientific literature. There is very little published empirical data to predict the dose of consumed *B. anthracis* spores that would cause disease in humans. Subject experts at USAMRIID have suggested a number between 8,000 to 50,000 spores

consumed, coupled with the condition of the GI tract and immunological status of the individual at the time of the consumption, may cause the gastrointestinal or oropharyngeal form of the disease. While gastrointestinal anthrax has not been reported in the US, reports from heavily endemic countries indicate symptoms generally appear within 2-5 days after ingestion of spore-contaminated meat from diseased animals. In the case of intentional tainting of food with *Bacillus anthracis* spores, it can be assumed that spore preparations could easily reach concentrations of 10^{10} per milliliter of liquid or 10^{12} per gram of dried preparations. This would mean that contamination levels of 10^{6} or greater spores per milliliter of bulk dairy product could be attained even in a large tanker. While morbidity and mortality from outbreaks of contaminated meat has certainly been documented and can be high, (http://www.who.int/emc-documents/docs/zoonosis/whoemczdi986.html), doses of cells ingested have not been measured, so little is actually known about what to expect in the case of an intentional contamination of various matrices of food. There is some suggestion that a lesion in the GI tract would be required to initiate the lethal form of the disease (Beatty et al, AIM, 2003).

Deliberate contamination of common foods such as milk, powdered milk, other milk products, fruit juices, liquid eggs and other foods with spores of this organism could potentially result in a massive epidemic and panic in unsuspecting populations and would be a threat to homeland biosecurity. In this study, survival of *B. anthracis* was evaluated by using freshly prepared spore suspensions of *Bacillus anthracis* Sterne (BAS) and Pasteur (BAP) of known

concentration to spike the six different food matrices followed by subjection of the matrixes to four different pasteurization temperatures common to the dairy industry in the United States. The matrices selected are the most common, nutritive and wholesome pasteurized items that virtually all the households and all age groups consume on daily basis. These items are whole milk, 2% milk, skim milk, apple juice, apple cider and liquid eggs. The detection and enumeration of spores was conducted using microscopic spore count, viable colony count, a widely used real time PCR test based on proprietary protocols and an electrochemiluminescence assay called M1M BioVerify Anthrax Test Protocol manufactured by BioVeris Corporation for rapid field detection. Viability of spores following pasteurization depended mostly upon the type of pasteurization, type of matrix, spore concentration in the suspension and the *B. anthracis* spore strain used. *B. anthracis* Pasteur strain was more susceptible to heat inactivation compared to the Sterne strain. All pasteurization processes were completely lethal to vegetative cells. Ultra high temperature/short time pasteurization (100^0C. for 3 sec.) was more lethal to spores, especially the Pasteur strain, resulting in a one to two log reduction in viability. Pasteurization had little or no effect on Sterne strain spores. These studies suggest that current pasteurization techniques, as practiced by the dairy industry for various products, would have little to no effect on viability of spores, except ultra high temperature/short time pasteurization treatment. Using 100^0C for 3 seconds (ultra high temperature/short time) may be helpful in reducing the spore load, especially at low contamination levels it may be preferred in management of a BT

event/crisis related to milk contamination with *B. anthracis* spores. Other widely used methods of pasteurization largely failed, which may be an indication of what could happen if there was an incidence of an intentional contamination by an act of a bioterrorist. For this very reason, our findings suggest that the preferred method of pasteurization in our dairy system should be the ultra high temperature/short time method, and it should be widely adopted by the dairy industry.

Rapid detection of spores in raw or processed dairy products is not yet perfected. Most rapid methods available on the market have been tested by various study groups including the Centers for Disease Control (CDC) and have been found to be less reliable. In this study, we selected a widely used real time PCR test based on proprietary protocols, and an electrochemiluminescence assay, M1M BioVerify Anthrax Test Protocol, manufactured by BioVeris Corporation. Both of these methods are intended for the rapid detection of *B. anthracis* in human, animal or environmental specimens, including food items. Both can be used by first responders in the field. Successful detection by both of these methods at various concentration (spike) levels of *B. anthracis* spores in selected matrices following pasteurization were compared to standard bacteriological culture colony counts (colony forming units or CFUs) and tabulated for analysis. This study demonstrates that portable real-time PCR and the BioVerify electrochemiluminescence assay can provide a rapid assay for detecting high levels of BA spores in some raw or pasteurized products; but, these assays are inhibited by some matrices due to nature of the matrix, matrix

constituents, pH, heat effect on spore surface antigen and other unknown factors, and thus are unreliable at this time for a widespread use in all types of conditions. They may be useful in selective scenarios only, with respect to specific matrices and level of contamination both prevalidated by users. For example, PCR test worked well with 2% and skim milk but not with whole milk. ECL testing by M1M also showed similar patterns of sensitivity. Bacteriological culture, though time consuming, remains the gold standard, for detection and enumeration of *B. anthracis.*

Previous studies on the stability of spores and vegetative cells indicated that both were relatively stable when added to pasteurized and unpasteurized milk at 5^0C. Incubation at higher temperatures resulted in loss of viability of vegetative cells but stability was only measured over a 48 h period and the effect of pasteurization was not measured (Bowen and Turnbull 1992). These studies also supported the general notion that contamination of milk from an infected animal in a milking herd would not pose a significant health risk, due to dilution into larger quantities. However, no quantitative data was presented. Another study (Anonymous 1966; Perdue, Karns Jeff et al. 2003) demonstrated the likelihood of 'natural' contamination of milk from an infected herd would be quite small. It is fortunate that *B. anthracis* spores have not been frequently-encountered contaminants of the milk supply system since from our studies it is clear that spores of the *Bacillus* species are quite refractory to pasteurization, and remain viable even after prolonged storage, particularly at higher spore concentrations. Whether these data would be consistent with results for virulent

strains of *B. anthracis* is unknown. Since the only differences between Sterne, Pasteur and virulent anthrax strains are minor, it is conceivable that spore formation and germination along with stability of the organism would be similar among the strains. Many stabilizing additives are added to commercially pasteurized products which prevent bacterial growth. Effect of such stabilizing additives on the viability of spores in pasteurized products is unknown, though research on, *B. cereus,* indicated no significant effect on spores' stability with three common stabilizers (Mazas, Lopez et al. 1999).

STUDY LIMITATIONS:

Two limitations are evident.

1) Simulated pasteurization.

In absence of other options, we have to make assumptions that our study will mimic a large processing plant.

2) Working with avirulent strains of *Bacillus anthracis*.

We will have to make assumptions that these laboratory strains will behave very similarly in regard to thermal sensitivity and detectability by PCR and M1M detection methods, to the wild type strains of the organism, which may be used, in a terrorist activity.

CONCLUSIONS

1) The concern over the potential for use of *B. anthracis* as a biological weapon in food and dairy products is valid.
2) Many foods are considered safe following pasteurization and under circumstances in which *B. anthracis* vegetative cells might be introduced.
3) The thermal lethal effects associated with cooking or pasteurization should prevent infection from normal microbial contaminants. However, following the intentional introduction of large number of spores, the risk of infection may be high, and it seems prudent to have rapid assays to evaluate the levels of contamination in questionable food or dairy products.
4) This study demonstrates that real-time PCR as well as an electrochemiluminescence assay (M1M BioVerify anthrax spore test) could provide a rapid assay for detecting high levels of BA spores in some raw or pasteurized products, but these assays are inhibited by some matrices and thus are unreliable at this time. Test inhibition may be due to nature of matrix, matrix constituents, pH, heat effect on spore surface antigen or other unknown factors.
5) Strain differences in test sensitivity and detectable limits remain, making it difficult to extrapolate the predictability of test results for wild type pathogenic strains.

6) Further work to improve the reliability and sensitivity of these methods is necessary. We conclude that certain pasteurization methods, particularly one which uses 100^0C. for 3 seconds may lead to a more significant reduction in the number of viable spores in milk and other food matrices.

7) When considering the minimal effective spore dose of *Bacillus anthracis* that may pose significant risk to human health and the level of contamination in foods, it may be prudent to suggest the high temperature short time pasteurization method as an additional precaution to reduce the risk from consumption of food suspected of being tainted with *Bacillus anthracis* spores.

8) Bacteriological culture remains the gold standard for detection of BA in food matrices.

CHAPTER 5

SUMMARY

Bacillus anthracis is a highly virulent, rapidly growing gram-positive bacterial pathogen that may cause inhalational, gastrointestinal or the cutaneous forms of anthrax in humans. The inhalational form of anthrax is the most lethal and a highly feared form of the disease particularly if spores from a virulent strain are deliberately used as a source of human exposure. Events' following the September 2001 terrorist attacks, targeting an unsuspecting population using our nation's postal system as an effective delivery mechanism for spores in the form of powder in envelopes is a testament to it. However, recently, gastrointestinal anthrax resulting from the consumption of meat from animals that die of the disease has been reported in humans from several parts of the world, making it a disease of very serious epidemiological implications and a public health priority. Dozens of human fatalities have been reported from part of Africa, independent nations once part of the Soviet Union and several regions of Asia, especially India as reported on almost daily basis by the ProMED-mail post (http://www.promedmail.org), a program of the International Society for Infectious Diseases (http://www.isid.org). Deliberate contamination of common foods such as milk, powdered milk, other milk products, fruit juices, liquid eggs and other foods with spores of this organism could potentially result in a massive epidemic and panic in unsuspecting populations in a wide area can also be a

threat to homeland biosecurity. In this study, survival of *B. anthracis* was evaluated by using freshly prepared spore suspensions of *Bacillus anthracis* Sterne (BAS) and Pasteur (BAP) of known concentration to spike the six different food matrices followed by subjecting the matrixes to four different pasteurization temperatures common to the dairy industry in the United States. The matrices selected are the most common, nutritive and wholesome pasteurized items that virtually all the households and all the age groups consume on daily basis. These items are whole milk, 2% milk, skim milk, apple juice, apple cider and liquid eggs. The detection and enumeration of spores was conducted using microscopic spore count, viable colony count, a widely used real time PCR test based on proprietary protocols and an electrochemiluminescence assay called M1M BioVerify Anthrax Test Protocol manufactured by BioVeris Corporation for rapid field detection. Viability of spores following pasteurization depended mostly upon the type of pasteurization, type of matrix, spore concentration in the suspension and the *B. anthracis* spore strain used. *B. anthracis* Pasteur strain was more susceptible to heat inactivation compared to the Sterne strain. All pasteurization processes were completely lethal to vegetative cells. Ultra high temperature/short time pasteurization (100^0C. for 3 sec.) was more lethal to spores, especially the Pasteur strain, resulting in a one to two log reduction in viability. Pasteurization had little or no effect on Sterne strain spores. These studies suggest that current pasteurization techniques, as practiced by the dairy industry for various products, would have little to no effect on viability of spores, while using 100^0C for 3 seconds may be helpful in reducing the spore load and at

low contamination levels may be preferred in management of a crisis related to milk contamination with *B. anthracis* spores. Other widely used methods of pasteurization largely failed, which may be an indication of what could happen if there was an incidence of an intentional contamination by an act of a bioterrorist. For this very reason, this study suggests that the preferred method of pasteurization in our dairy system should be the ultra high temperature/short time method and it should be widely adopted by the dairy industry. Rapid detection of spores in raw or processed dairy products is not yet perfected. Most rapid methods available on the market have been tested by various study groups including the Centers for Disease Control (CDC) and have been found to be less reliable. In this study, we selected a widely used real time PCR test based proprietary protocols and an electrochemiluminescence assay called M1M BioVerify Anthrax Test Protocol manufactured by BioVeris Corporation. Both of these methods are intended for rapid detection of *B. anthracis* in human, animal or environmental samples including food items. Both can also be used by first responders in the field. Successful detection by both of these methods at various concentration (spike) levels of *B. anthracis* spores in selected matrices following pasteurization were compared to standard bacteriological culture colony counts (colony forming units or CFUs) and tabulated for analysis. This study demonstrates that portable, real-time PCR as well as the BioVerify electrochemiluminescence assay could provide a rapid assay for detecting high levels of BA spores in some raw or pasteurized products, but these assays are inhibited by some matrices (matrix constituents, pH, heat effect on spore surface

antigen etc.) and thus are unreliable at this time for detection, in general, for widespread use in all types of conditions. They may be useful in selective scenarios only, with respect to specific matrices and level of contamination. Bacteriological culture, though time consuming, remains the gold standard, for detection and enumeration of *B. anthracis*.

CHAPTER 6

OVERVIEW OF LITERATURE CITED

Cases of anthrax are rare in the United States, but there is a substantial amount of literature published on the subject as a result of events during and after the October 2001 of the anthrax attack on the postal system and a growing concern for biological terrorism in the United States. A large number of sources of information on anthrax provide brief descriptions of the organism *B. anthracis*, its natural environment, and brief descriptions of the disease anthrax. The following is a review of most of the work followed by the list of references.

The American Society for Microbioloty (asm.org), The Centers for Disease Control (cdc.gov), The Federation of American Societies for Experimental Biology (faseb.org), American Medical Association (ama-assn.org) and many other professional groups have held numerous conferences on anthrax over the last decade. The collection of papers from these conferences contains a large amount of information, case studies, epidemiological data, field data, and other work on anthrax. In particular, "Value of field data for extrapolation in anthrax" (Lincoln, J.S. Walker et al. 1967), "Anthrax of the Gastro-intestinal Tract" (Sirisanthana and Brown 2002) and "Gastro-intestinal Anthrax: Review of the literature" (Beatty, Ashford et al. 2003) discusses in detail dose response for various animals, humans, anthrax toxin, and the effectiveness of treatment in all three types of anthrax, especially the gastro-intestinal type infection.

The American Society for Microbiology, Centers for Disease Control and The Infectious Diseases Society of America have a large number of resources posted on their web pages also. These sites contain an enormous list of publications, articles, and other various resources covering nearly every aspect of *B. anthracis*. As of this submission the list was last updated in 2009. The sites also contain a medical summary, last updated in 2009. This summary is an extremely thorough compilation of information concerning both *B. anthracis* and the disease it causes - anthrax. Similarly, Pathport the pathogen portal project at Virginia Bioinformatics Institute also has an in depth online compilation of information. This compilation was also last updated in 2009.

A discussion that details the life cycle of *B. anthracis* can be found in Eiko and Masayuki's 2003 paper. Growth characteristics, both aerobic and anaerobic, are discussed in this paper, including verification of common assumptions about the growth of *B. anthracis*. A detailed description of the requirements for spore germination can be found in Ireland and Hanna's 2002 paper, which focuses discussion on genetic issues.

A plethora of information concerning the disease anthrax is prevalent in the literature. Virtually all publications found contain some description of the disease. Lethal dose (LD_{50}), the critical number of anthrax spores necessary to cause illness or death in 50% population, however, remains uncertain and largely

speculative for all three forms of the disease. The probability of infection due to contact (exposure) with a given level of infectious agent (number of spores) is called the dose response. A comprehensive discussion source of dose response is found in Watson and Keir's 1994 publication – "Information on which to base assessments of risk from environments contaminated with anthrax spores". This paper contains a review of the organism and its virulence factors, as well as a large amount of animal dose response data. Human dose response is also briefly discussed. Out of all the different types of infections possible, oropharyngeal and gastrointestinal anthrax are the most important types for this study.

Highly detailed descriptions of the gastrointestinal or oropharyngeal disease are found in "Anthrax of the Gastro-intestinal Tract" (Sirisanthana and Brown 2002) and "Gastro-intestinal Anthrax: Review of the literature" (Beatty, Ashford et al. 2003). These publications contain descriptions of the disease's characteristics, treatment, disease management as well as case studies. Information concerning the behavior of *Bacillus anthracis* in milk can be found in Perdue and Karns' 2003 publication "Detection and Fate of *Bacillus anthracis* (Sterne) Vegetative Cells and Spores Added to Bulk Tank Milk". This publication describes limited experiments conducted to study the response of *Bacillus anthracis* sterne strain to pasteurization and it's viability in milk. PCR and use of a field deployable system known as R.A.P.I.D. for detection of *B. anthracis* in milk is also discussed.

APPENDIX

Codes for Outputs of SAS Analysis of Data for individual matrices

- Whole Milk, 2% Milk, Skim Milk, Apple Juice, Apple Cider and Liquid Eggs Studies

SAS CODE:

```
proc anova data=work.siz ;
class Pasteurization ;
model Diff = Pasteurization ;
means Pasteurization / duncan ;
title "one way_ANOVA";
run;

proc anova data=work.siz ;
class week ;
model Diff = week ;
means week / duncan ;
title "one way_ANOVA";
run;

proc anova data=work.siz ;
class week Pasteurization ;
model Diff = week Pasteurization ;
means Pasteurization / duncan ;
title "one way repeated measures design (week)";
```

```
 run;

proc GLM data=work.siz ;
class Pasteurization week ;
model Diff = Pasteurization week Pasteurization*week / SS3;
means Pasteurization week Pasteurization*week / LSD ;
title "Two way_GLM";
run;

proc ANOVA data=work.siz ;
class Pasteurization week ;
model Diff = Pasteurization week Pasteurization*week ;
means Pasteurization week Pasteurization*week / DUNCAN ;
title "Two way_ANOVA";
run;
```


Codes for Outputs of SAS Analysis of Data for all Matrices, Pasteurizations and Survival Time Period (weeks) combined:

SAS CODE:

```
Data one;
  set sandip.cfu;

  flask = round(tube,1);
```

```
  If week le 4;

  proc sort; by matrix past week tube;

 proc mixed;

 Class matrix past week flask;
 Model diff = matrix past week / ddfm=satterth outp=yhat;
 *repeated week/ type=un subject=flask;
lsmeans past/diff adj=tukey;
lsmeans matrix/diff adj=tukey;
lsmeans week/diff adj=tukey;

*This model won't run;
*proc mixed;

 *Class matrix past week flask;
 *Model diff = matrix past flask week / ddfm=satterth outp=yhat;
*repeated week/ type=cs subject=flask;
*lsmeans past/diff adj=tukey;
*lsmeans matrix/diff adj=tukey;
*lsmeans week/diff adj=tukey;

  run;
```

Codes for Outputs of The Univariate procedure – Detection comparison of PCR and M1M by quartiles analysis:

SAS CODE:

```
data one;
 set sandip.m1m;

 *proc contents;
grp = 0;

If log_end lt 4.356 then grp = 1;
if log_end ge 4.356 and log_end lt 6.279 then grp = 2;
If log_end ge 6.279 and log_end lt 7.929 then grp = 3;
If log_end ge 7.929 then grp = 4;

 proc univariate;
 var log_end;

 proc sort;
 by grp;

proc freq;

Tables grp;

tables grp*m1M/chisq;
tables grp*pcr/chisq;

 run;
```

Outputs of SAS Analysis of Data

SAS output:

The Univariate procedure – Detection comparison by quartiles analysis:

The SAS System 11:41 Friday, July 10, 2009

The UNIVARIATE Procedure
Variable: LOG_END (LOG_END)

Moments

N	540	Sum Weights	540
Mean	6.01403551	Sum Observations	3247.57918
Std Deviation	1.78476309	Variance	3.1853793
Skewness	-0.053362	Kurtosis	-1.1797619
Uncorrected SS	21247.9759	Corrected SS	1716.91944
Coeff Variation	29.6766304	Std Error Mean	0.07680397

Basic Statistical Measures

Location		Variability	
Mean	6.014036	Std Deviation	1.78476
Median	6.278754	Variance	3.18538
Mode	4.380211	Range	6.71705
		Interquartile Range	3.57331

NOTE: The mode displayed is the smallest of 2 modes with a count of 8.

Tests for Location: Mu0=0

Test		-Statistic-	-----p Value------	
Student's t	t	78.3037	Pr > |t|	<.0001
Sign	M	270	Pr >= |M|	<.0001
Signed Rank	S	73035	Pr >= |S|	<.0001

Quantiles (Definition 5)

Quantile	Estimate
100% Max	8.62014
99%	8.56820
95%	8.50035
90%	8.41497
75% Q3	7.92930
50% Median	6.27875
25% Q1	4.35599
10%	3.86033
5%	3.51851
1%	2.39794
0% Min	1.90309

The UNIVARIATE Procedure
Variable: LOG_END (LOG_END)

Extreme Observations

------Lowest------		-----Highest-----	
Value	Obs	Value	Obs
1.90309	358	8.57864	417
1.95424	360	8.58433	416
2.11394	359	8.58659	209
2.34242	268	8.61490	208
2.36173	450	8.62014	210

The FREQ Procedure

grp	Frequency	Percent	Cumulative Frequency	Cumulative Percent
1	135	25.00	135	25.00
2	139	25.74	274	50.74
3	131	24.26	405	75.00
4	135	25.00	540	100.00

Table of grp by M1M

grp	M1M(M1M)		
Frequency			
Percent			
Row Pct			
Col Pct	0	1	Total
1	123	12	135
	22.78	2.22	25.00
	91.11	8.89	
	29.71	9.52	
2	112	27	139
	20.74	5.00	25.74
	80.58	19.42	
	27.05	21.43	
3	100	31	131
	18.52	5.74	24.26
	76.34	23.66	
	24.15	24.60	
4	79	56	135
	14.63	10.37	25.00
	58.52	41.48	
	19.08	44.44	
Total	414	126	540
	76.67	23.33	100.00

The FREQ Procedure

Statistics for Table of grp by M1M

Statistic	DF	Value	Prob
Chi-Square	3	41.7957	<.0001
Likelihood Ratio Chi-Square	3	42.3082	<.0001
Mantel-Haenszel Chi-Square	1	39.2813	<.0001
Phi Coefficient		0.2782	
Contingency Coefficient		0.2680	
Cramer's V		0.2782	

Sample Size = 540

Comparison of effectiveness of "Matrix", "Pasteurization" and "week" by the Mixed Procedure Modeling Analysis:

The Mixed Procedure

Model Information

Data Set	WORK.ONE
Dependent Variable	Diff
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information

Class	Levels	Values
Matrix	6	1 2 3 4 5 6
Past	5	0 1 2 3 4
Week	4	1 2 3 4
flask	30	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Dimensions

Covariance Parameters	1
Columns in X	16
Columns in Z	0
Subjects	1
Max Obs Per Subject	360

Number of Observations

Number of Observations Read	360
Number of Observations Used	360
Number of Observations Not Used	0

Covariance Parameter Estimates

Cov Parm	Estimate
Residual	0.1142

Fit Statistics

-2 Res Log Likelihood	284.0
AIC (smaller is better)	286.0

The Mixed Procedure

Fit Statistics

AICC (smaller is better)	286.0
BIC (smaller is better)	289.9

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Matrix	5	347	52.78	<.0001
Past	4	347	48.45	<.0001
Week	3	347	0.26	0.8511

Least Squares Means

Effect	Matrix	Past	Week	Estimate	Standard Error	DF	t Value	Pr > |t|
Past		0		0.01872	0.03983	347	0.47	0.6386
Past		1		0.1055	0.03983	347	2.65	0.0084
Past		2		0.1419	0.03983	347	3.56	0.0004
Past		3		0.2473	0.03983	347	6.21	<.0001
Effect	Matrix	Past	Week	Estimate	Standard Error	DF	t Value	Pr > |t|
Past		4		0.7205	0.03983	347	18.09	<.0001
Matrix	1			0.1334	0.04363	347	3.06	0.0024
Matrix	2			0.04632	0.04363	347	1.06	0.2891
Matrix	3			0.1352	0.04363	347	3.10	0.0021
Matrix	4			0.2866	0.04363	347	6.57	<.0001

Matrix	5		0.8644	0.04363	347	19.81	<.0001
Matrix	6		0.01478	0.04363	347	0.34	0.7351
Week		1	0.2296	0.03562	347	6.44	<.0001
Week		2	0.2359	0.03562	347	6.62	<.0001
Week		3	0.2510	0.03562	347	7.05	<.0001
Week		4	0.2707	0.03562	347	7.60	<.0001

Differences of Least Squares Means

Effect	Matrix	Past	Week	Matrix	Past	Week	Estimate	Standard Error	DF	t Value	Pr > |t|
Past		0			1		-0.08677	0.05632	347	-1.54	0.1243
Past		0			2		-0.1231	0.05632	347	-2.19	0.0295
Past		0			3		-0.2286	0.05632	347	-4.06	<.0001
Past		0			4		-0.7018	0.05632	347	-12.46	<.0001
Past		1			2		-0.03637	0.05632	347	-0.65	0.5189
Past		1			3		-0.1418	0.05632	347	-2.52	0.0122
Past		1			4		-0.6150	0.05632	347	-10.92	<.0001
Past		2			3		-0.1055	0.05632	347	-1.87	0.0620

The Mixed Procedure

Differences of Least Squares Means

Effect	Matrix	Past	Week	Matrix	Past	Week	Estimate	Standard Error	DF	t Value	Pr > |t|
Past		2			4		-0.5787	0.05632	347	-10.27	<.0001
Past		3			4		-0.4732	0.05632	347	-8.40	<.0001
Matrix	1			2			0.08710	0.06170	347	1.41	0.1590
Matrix	1			3			-0.00180	0.06170	347	-0.03	0.9768
Matrix	1			4			-0.1532	0.06170	347	-2.48	0.0135
Matrix	1			5			-0.7309	0.06170	347	-11.85	<.0001
Matrix	1			6			0.1186	0.06170	347	1.92	0.0553
Matrix	2			3			-0.08889	0.06170	347	-1.44	0.1506
Matrix	2			4			-0.2403	0.06170	347	-3.89	0.0001
Matrix	2			5			-0.8180	0.06170	347	-13.26	<.0001
Matrix	2			6			0.03155	0.06170	347	0.51	0.6095
Matrix	3			4			-0.1514	0.06170	347	-2.45	0.0146
Matrix	3			5			-0.7291	0.06170	347	-11.82	<.0001
Matrix	3			6			0.1204	0.06170	347	1.95	0.0517
Matrix	4			5			-0.5777	0.06170	347	-9.36	<.0001
Matrix	4			6			0.2719	0.06170	347	4.41	<.0001
Matrix	5			6			0.8496	0.06170	347	13.77	<.0001
Week			1			2	-0.00630	0.05038	347	-0.13	0.9005
Week			1			3	-0.02144	0.05038	347	-0.43	0.6706
Week			1			4	-0.04115	0.05038	347	-0.82	0.4146
Week			2			3	-0.01514	0.05038	347	-0.30	0.7639
Week			2			4	-0.03485	0.05038	347	-0.69	0.4896
Week			3			4	-0.01971	0.05038	347	-0.39	0.6959

Differences of Least Squares Means

Effect	Matrix	Past	Week	Matrix	Past	Week	Adjustment	Adj P
Past		0			1		Tukey	0.5368
Past		0			2		Tukey	0.1874
Past		0			3		Tukey	0.0006
Past		0			4		Tukey	<.0001
Past		1			2		Tukey	0.9674
Past		1			3		Tukey	0.0889
Past		1			4		Tukey	<.0001
Past		2			3		Tukey	0.3341
Past		2			4		Tukey	<.0001
Past		3			4		Tukey	<.0001
Matrix	1			2			Tukey	0.7200
Matrix	1			3			Tukey	1.0000
Matrix	1			4			Tukey	0.1319
Matrix	1			5			Tukey	<.0001
Matrix	1			6			Tukey	0.3899
Matrix	2			3			Tukey	0.7021
Matrix	2			4			Tukey	0.0016
Matrix	2			5			Tukey	<.0001

The Mixed Procedure

Differences of Least Squares Means

Effect	Matrix	Past	Week	Matrix	Past	Week	Adjustment	Adj P
Matrix	2			6			Tukey	0.9957
Matrix	3			4			Tukey	0.1410
Matrix	3			5			Tukey	<.0001
Matrix	3			6			Tukey	0.3724
Matrix	4			5			Tukey	<.0001
Matrix	4			6			Tukey	0.0002
Matrix	5			6			Tukey	<.0001
Week			1			2	Tukey	0.9993
Week			1			3	Tukey	0.9741
Week			1			4	Tukey	0.8465
Week			2			3	Tukey	0.9906
Week			2			4	Tukey	0.9002
Week			3			4	Tukey	0.9797

Statistical Comparison of PCR and BioVerify M1M test technologies:

2 x 2 Table Statistics

Single Table Analysis

		BioVerify kit - M1M (+)	(-)	
	(+)	75	163	238
PCR (LRN)	(-)	51	251	302
		126	414	540

Chi Square and Exact Measures of Association

Test	Value	p-value(1-tail)	p-value(2-tail)
Uncorrected chi square	15.92	0.00003312	0.00006625
Yates corrected chi square	15.11	0.00005076	0.0001015
Mantel-Haenszel chi square	15.89	0.00003364	0.00006729
Fisher exact		0.00005254	0.0001051
Mid-P exact		0.00003735	0.00007470

All expected values (row total*column total/grand total) are >=5
OK to use chi square.

Risk-Based* Estimates and 95% Confidence Intervals
(Not valid for Case-Control studies)

Point Estimates		Confidence Limits	
Type	**Value**	**Lower, Upper**	**Type**
Risk in Exposed	31.51%	25.94, 37.68	Taylor series
Risk in Unexposed	16.89%	13.06, 21.54	Taylor series
Overall Risk	23.33%	19.96, 27.09	Taylor series
Risk Ratio	1.866	1.365, 2.551[1]	Taylor series
Risk Difference	14.63%	7.367, 21.88°	Taylor series
Etiologic fraction in pop.(EFp)	27.63%	14.03, 41.22	
Etiologic fraction in exposed(EFe)	46.41%	26.75, 60.79	

Odds-Based Estimates and Confidence Limits

Point Estimates		Confidence Limits	
Type	**Value**	**Lower, Upper**	**Type**
CMLE Odds Ratio*	2.261	1.507, 3.41[1]	Mid-P Exact
		1.479, 3.478[1]	Fisher Exact
Odds Ratio	2.265	1.508, 3.401[1]	Taylor series
Etiologic fraction in pop.(EFp|OR)	33.24%	18.18, 48.29	
Etiologic fraction in exposed(EFe|OR)	55.84%	33.68, 70.6	

*Conditional maximum likelihood estimate of Odds Ratio
(P)indicates a one-tail P-value for Protective or negative association; otherwise one-tailed exact P-values are for a positive association.
Martin,D; Austin,H (1991) An efficient program for computing conditional maximum likelihood estimates and exact confidence limits for a common odds ratio. Epidemiology 2, 359-362.
° ¹ 95% confidence limits testing exclusion of 0 or 1, as indicated
P-values < 0.05 and confidence limits excluding null values (0,1, or [n]) are highlighted.

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