AN ABSTRACT OF THE THESIS OF

Matthew Applegate for the Master of Science Degree

in Biology Presented on Wednesday, September 8, 1999

Title: Detection of *Listeria monocytogenes* in ground turkey and turkey frankfurters using ELISA, DNAH, and USDA protocols prior to, and following, exposure to ultracold temperatures

Abstract Approved: Bessie L. Johnson

Listeria monocytogenes is one of the most prevalent food-borne pathogens. *Listeria* has lead to several deaths, and has been determined to be the cause of several recent food recalls, including Ball Park, Bil Mar, Mr. Turkey and Sara Lee Deli Meats. What makes *Listeria* important to the food industry, the USDA, and alert consumers, is that *Listeria* can proliferate well within a wide range of temperatures, from 1 to 45 degrees Celsius. Despite its importance, little information is known about how well *Listeria* can survive under extended periods of time in ultracold environments, such as a freezer. Eighty-one ground turkey and eighty-one turkey frankfurter samples were obtained from a local grocer and tested for the presence of *Listeria monocytogenes*, using three diagnostic tests. A total of 150 samples were artificially inoculated with a standard inoculum of 1 X 10^6 cells per milliliter per 100 grams of sample. The remaining twelve samples were used as negative controls. The samples were then exposed to ultracold –70

C for two, four, and six months. At the end of each time period, samples were removed from the freezer and diagnostic tests were performed. Enzyme-linked immunosorbent assay (ELISA), DNA hybridization assay (DNAH), and a modified USDA culture-based method of isolation (USDA protocol) were used as diagnostic tests. Revived cells were then injected into mice, intraperitoneally (IP), at a standard inoculum to determine if virulence was retained. A few of the initial samples from the grocer tested positive for the presence of *Listeria* by at least one diagnostic test. All three diagnostic tests identified the presence of *Listeria* within both substrates throughout all three experimental time periods. Additionally, virulence was retained throughout all time periods.

Detection of *Listeria monocytogenes* in ground turkey and turkey frankfurters using ELISA, DNAH, and USDA protocols prior to, and following, exposure to ultracold temperatures

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A Thesis

Presented to

The Division of Biological Sciences

EMPORIA STATE UNIVERSITY

In Partial Fulfillment

of the Requirement for the Degree

Master of Science

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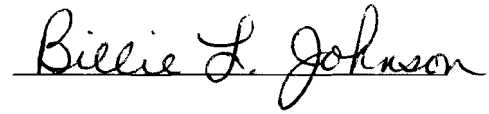
by

Matthew Roger Applegate

October 1999



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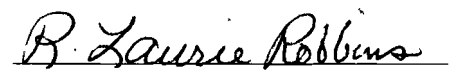

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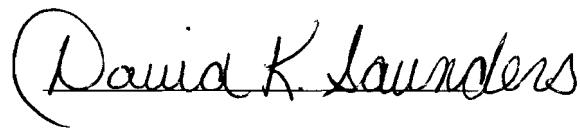

Approved by Committee Member

Approved for Major Department

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Acknowledgments

I would like to thank my mentor, friend, and major advisor, Dr. Billie Johnson for all of her support, encouragement, and advice on my thesis and research. I would also like to thank my committee members, Dr. Laurie Robbins and Dr. David Saunders for their influence, suggestions, and tutelage in my years at Emporia State University. Their help will always be greatly appreciated.

Thanks also go to John Kudla of Gene-Trak Systems for the use of his equipment, and Organon Teknika for donating all of the ELISA test kits free-of-charge. I also want to extend my thanks to Roxana Johnson, Rex Johnson, Linda Johnson, and Brent Lee for encouraging me to enter into graduate school and for the ideas that started the brainstorming for my research topic. Last, I want to give my love and thanks to my wife, mother, father, sister, aunt, and grandma for all of their encouragement and love.

To all that helped me, put up with me, and were there for me--Thank You!

Literature Cited Format

The literature cited was done in the format following the instructions given by the American Society for Microbiology.

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CHAPTER 1

INTRODUCTION

General Background

There are currently seven species of *Listeria,* two of which are believed to be the same species. The genus is made up of *Listeria monocytogenes*, *L. innocua*, *L. welshimeri*, *L. ivanovii*, *L. seeligeri*, *L. murrayi*, and *L. grayi*. Of these species, only two are infectious to man, *L. monocytogenes* and *L. ivanovii* (Low and Donachie, 1997). *Listeria* is a gram-positive, non-spore-forming, aerobic and facultatively-anaerobic bacillus. It is commonly found inhabiting soil, fertilizer, water, vegetation, processed meats, and dairy products (Southwick and Purich, 1996). *L. monocytogenes* is approximately 0.4 to 0.5 micrometers (μm) in diameter and 0.5 to 2 μm long (Schuchat et al., 1991). It is beta-hemolytic on blood agar, developing a narrow zone of hemolysis around the colonies, and is motile between 20 and 25°C.

Listeria monocytogenes proliferates well within a wide range of temperatures from 1 to 45°C; the optimum temperature for growth ranges from 30 to 37°C. It can also grow within a wide pH range from 6.0 to 9.0, giving it the ability to survive within a multitude of foodstuffs (Schuchat et al., 1991). *Listeria monocytogenes* most often leads to infections that are mistaken for common food poisoning, but when a serious case arises, it can be a significant problem. Generally, *L. monocytogenes* infections are not serious and can be treated with the use of general antibiotics. Penicillin G, ampicillin, erythromycin, chloramphenicol, rifampicin, and tetracyclines all work well in fighting *Listeria* infections (Schuchat et al., 1991).

L. monocytogenes is the causative agent of the disease listeriosis. Listeriosis was first identified in animals in New Zealand sheep. The sheep suffered from a form of encephalitis known as "circling disease," which eventually induced death of the infected animal (Gill, 1937). Human listeriosis was first observed in East Germany in a newborn baby that exhibited characteristic granulomatosis infantiseptica (Potel, 1943-54). Healthy humans are most often not in any danger of developing listeriosis from slight food contamination. People at highest risk of getting listeriosis are pregnant women, the elderly, and the immunocompromised.

Outbreaks and Food Recalls

Food-borne pathogens remain a serious problem throughout the world. Food safety has become a widespread concern, not only among the scientific community, but also among the general public. To prevent the spread of food-borne illnesses, food-processing plants are now being regulated. Processing plants must adhere to relatively-strict guide lines set by the United States Department of Agriculture (USDA) and the United States Food and Drug Administration (FDA). These regulations cover everything from the harvesting of the product through its processing. Outbreaks of food-borne illnesses have become "big news." For instance, Hudson Beef had an incident in August 1997 in which *Escherichia coli* was found contaminating processed beef patties. All of the contaminated beef patties, 25 million pounds, had to be recalled, resulting in an enormous monetary loss to the company.

In October 1998, Dixie Packers had to recall 108,000 pounds of frankfurters after they tested positive for the presence of *Listeria monocytogenes* (Anonymous, 1998). Hot

dogs and luncheon meat (about 30 million pounds) were recalled from a Thorn Apple Valley plant in January, 1999. Another recall in December, 1998 involved the Bil Mar company, producer of Ball Park Franks, Mr. Turkey, Sara Lee Deli Meat, and Grillmaster brands. Bil Mar had to recall about 35 million pounds of luncheon meat and hot dogs (Anonymous, 1999a). As a result of the December 1998 incident, twenty-one deaths were reported, six of which were fetal deaths. In addition, about 100 other individuals became ill (Anonymous, 1999b).

One of the first reported outbreaks of listeriosis was in the Maritime Provinces of Canada during the months of March to September in 1981. Listeriosis occurred in approximately 1.3% of births at the Halifax hospital in Nova Scotia during that time. There were also seven adult and 34 perinatal cases, which resulted in a 27% fatality rate among liveborn infants (Schuchat et al., 1991). This outbreak was determined to be caused by coleslaw that was made with cabbage that had been fertilized with infected sheep manure. The second notable outbreak occurred in the summer of 1983 in Massachusetts. This outbreak was caused by tainted milk, which resulted in an overall case fatality rate of 29% (Schuchat et al., 1991).

One of the largest outbreaks in North America occurred in 1985 in Los Angeles, California. There was a fatality rate of 63% for early neonatal and fetal infections. This particular outbreak was traced back to tainted Mexican-style soft cheese that had been made with unpasteurized milk (Schuchat et al., 1991). Another large outbreak occured in France in 1992, in which a total of 279 cases lead to 22 abortions and 63 deaths. Because of poor disinfection techniques, post-processing contamination occurred on deli meats, resulting in the outbreak (Salvat et al., 1995).

L. monocytogenes is not a discriminating organism and does not prefer one sex over the other or any particular age group, as could be seen by examining the case studies from 40 of the infected individuals, after the Bil Mar recall occured. From the Bil Mar incident, 55% of the inflicted patients were women, along with six infected newborns. Infected adults ranged in age from 18 to 88 (MMWR, 1998). These data show that practically everyone is at risk of becoming infected by food-borne bacteria, such as *L. monocytogenes*.

Surveillance

Over the past several years, listeriosis cases have been on the rise. This information is critical, especially when considering that in 1989, there had been a decrease of about 44% in the number of serious cases of listeriosis. This decrease was attributed to the implementation of USDA guidelines. Food-borne illnesses presently result in an estimated expense of $5.6 to $22 billion annually for medical treatment. Although many precautions have been taken to prevent food-borne illnesses, people are still getting sick. The majority of these illnesses result from post-processing contamination, or contamination from improper food preparation, most of which occur within the home. Food-borne infections account for more than 81 million cases annually, with approximately 9,000 deaths worldwide. These numbers are clear evidence that more effective precautions and identifying tests are required to prevent contamination and to identify individuals with food-borne illnesses.

In the 1950s, Canadian officials recorded approximately fifteen cases of listeriosis per year (Farber et al., 1996). Currently in North America, listeriosis accounts for about

1,700 to 2,000 cases annually, according to the Centers for Disease Control. This results in about 450 adult and 100 fetal and postnatal deaths per year. Perinatal listeriosis occurs in about 12.7 cases per 100,000 live births (Schuchat et al., 1991).

Pathogenesis and Virulence

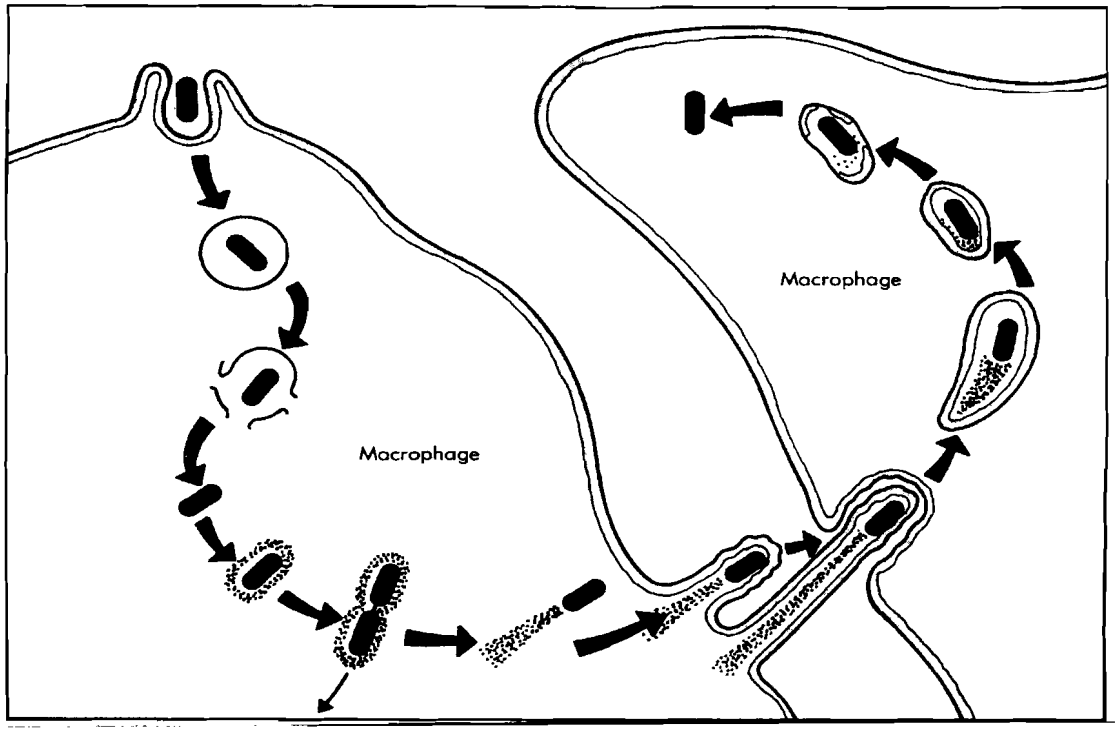
Listeria monocytogenes is an intracellular pathogen that survives and replicates within many different kinds of phagocytic and non-phagocytic cells (Southwick and Purich, 1996). *L. monocytogenes* gains entrance into the host organism through the gastrointestinal tract by means of contaminated foods (El-Kest et al., 1991). While in the gastrointestinal tract, *Listeria* must fend off and out-compete the normal microflora of the gut. Once *Listeria* attaches to the epithelial cells of the intestine, it then uses a family of cell wall surface proteins known as internalins (Czuprynski, 1994). Internalins work by facilitating attachment and promoting entry of materials into host cells such as epithelial cells, hepatocytes, and macrophages (Southwick and Purich, 1996).

Internalins can actually initiate the phagocytosis or uptake of *Listeria* by non-phagocytic cells. Once in the cell, the bacterium is surrounded by a phagosomal membrane, which is usually an inhospitable environment for most bacteria. However, the pH within the phagolysosome stimulates *Listeria* to produce an exotoxin known as lysteriolysin O (Southwick and Purich, 1996). Listeriolysin O lyses the phagolysosome, releasing the bacterium back into the cytoplasm. Once freed from the phagolysosome, the bacterium then surrounds itself with globular actin, one of the most abundant proteins in mammalian cytoplasm. The bacterium begins to proliferate at the same time that it is being coated by actin.

At some point, the *Listeria* is transferred into a new cell. *Listeria monocytogenes* does this by polymerizing the globular actin that surrounds it. A surface protein known as ActA is the vector by which this polymerization occurs. ActA polymerizes the globular actin into filamentous actin, which is used to attach to the host cell cytoskeleton. Once attached, the actin tail builds at its junction with the *Listeria* and pushes the bacterium to the cell membrane where filopodia are formed. With the elongation of actin tails, the bacterium is pushed through the cell membrane and into the adjacent cell. In formation of the filamentous actin, an initial trimer that is thermodynamically unstable is formed. When the unstable trimer is formed, monomeric actin molecules quickly attach and elongate the polymer. This is what actually pushes the bacterium through the cell membrane. When passing into the next cell, the filamentous actin tail breaks off and dissociates (Southwick and Purich, 1996).

Once in the adjacent cell, the bacterium is surrounded by a double membrane, which is lysed using two bacterial phospholipases and listeriolysin O (Moors et al., 1999; Southwick and Purich, 1996). This type of transfer from one cell to another prevents the bacteria from coming in contact with the extracellular environment, thus preventing the destruction of the bacteria by activated macrophages within the extracellular environment. The whole infection process then starts over (Southwick and Purich, 1996). This process is illustrated in Figure 1. Without the production of internalin, listeriolysin O, and ActA, *Listeria monocytogenes* is avirulent. These three virulence factors are *L. monocytogenes*' "aces in the hole." According to Moors et al. (1999), *L. monocytogenes* that contained mutations to the *ActA* gene or the *hly* gene, the gene that encodes for listeriolysin O, were unable to cause infection *in vivo*. To what degree *Listeria* can cause

Figure 1. Phagocytosis of *Listeria.* After phagocytosis, the bacterium is surrounded by a phagosome. Within a short time, the phagosome fuses with the lysosome, thereby exposing its contents to caustic lysozymes. *L. monocytogenes* prevents this fusion by lysing the phagosome with listeriolysin O before the fusion can occur. Once freed into the cytoplasm, the bacterium is surrounded with globular actin. This globular actin is then polymerized with the use of ActA into filamentous actin. With the use of the polymerized filamentous actin, the bacterium pushes its way into an adjacent cell. This picture was adapted from Murray et al. (1994).


Macrophage
Macrophage

infection is dependent upon many different factors, such as the amount of organism ingested, stability of the host's immune system, and the condition of the host's current intestinal microflora (Schuchat et al., 1991).

Host Defenses

Although *Listeria monocytogenes* has well-adapted virulence mechanisms, it is not an extremely common infectious agent. There are many factors that affect whether or not *L. monocytogenes* can cause disease. One of the main preventative factors is that the bacteria must fight off normal intestinal microflora (Czuprynski, 1994). Once out of the intestine, *Listeria* can be destroyed by neutrophils, monocytes, and most often, activated macrophages. It has been found that the production of cytokines during *Listeria* infection activates an antibody-production mechanism that targets *Listeria* (Czuprynski, 1994). Natural killer cells, one of the body's first defenses, are thought to invade infection sites, where they release interfeuron-gamma (Czuprynski, 1994). The interferon-gamma then activates CD4+ and CD8+ T cells to initiate a cytotoxic immune response. CD4+ and CD8+ T cells play a large role in the body's defense against *Listeria*. It is speculated that the CD4+ and CD8+ T cells recognize certain epitopes located on the hemolysin, which is produced by all virulent strains of *L. monocytogenes*. Once targeted, activated macrophages can inactivate the listeriolysin O and then destroy the bacteria (Czuprynski, 1994).

Another host defense is the lysis of infected hepatocytes by neutrophils. According to Czuprynski and Balish (1981), cell-mediated immunity is effective within the intestine of *Listeria* monoassociated rats. Also, levels of serum IgA, along with

secretory IgA, were elevated. These elevated levels of IgA are suspected to inhibit the adherence of *L. monocytogenes* to the intestinal epithelial tissue, making it difficult for entry into the epithelial cells (Czuprynski and Balish, 1981). Unfortunately, some 1,700 to 2,000 individuals annually, these defenses do not work and listeriosis becomes a serious illness.

Diseases and Symptomology

Listeria infections occur mainly in pregnant women, the elderly, and the immunocompromised, namely people suffering from HIV infections (Schlech, 1991). *Listeria monocytogenes* presents several different disease characteristics. The most frequent is the septic disease that inflicts pregnant women. These women develop 'flu-like' symptoms with a pronounced fever, headache, and malaise (Schlech, 1991). The infection can then spread, most likely becoming bacteremia, along with an intrauterine infection, resulting in pre-term labor, amnionitis, stillbirth, spontaneous abortion, or early-onset neonatal disease (Schuchat et al., 1991). Early-onset neonatal disease, or early-onset listeriosis, causes illness to the neonate at birth or shortly after. This disease results in sepsis, or sometimes granulomatosis infantiseptica, characterized by granulomas in the liver and other organs. The second disease type in infants is late-onset listeriosis, which can arise within a few days to a few weeks after birth. This disease type develops in infants that were presumably healthy at birth. It leads to central nervous system infections, or meningitis, in approximately 93% of infected infants (Schuchat et al., 1991).

Fatality rates run lower in late-onset than in early-onset disease, 26% and 38%, respectively. Listeriosis can also present itself as an invasive disease within immunocompromised individuals. These individuals can develop meningitis, meningoencephalitis, or sepsis. This can then lead to central nervous system damage, such as abscesses on the brain and spinal cord, or altered mental status (Schuchat et al., 1991).

Listeria in the Food Processing Environment

Because of the danger of *L. monocytogenes*, the FDA and the USDA have adopted a "zero tolerance" policy against all species of *Listeria*. That is, if any species of *Listeria* is found contaminating food products that are being sold within the United States, those products must be recalled. This zero tolerance policy has been accepted because of *L. monocytogenes*' ability to grow well in a very broad range of temperatures. This enables the organism to contaminate a food stuff with a low initial inoculum, and by the time the product reaches the consumer, a substantial population can be achieved (Schuchat et al., 1991).

The resiliency of *L. monocytogenes* presents problems for food processing facilities, because it can grow on almost any surface, and in almost any environment throughout the processing plant. Common colonization sites are in cool, damp areas that might or might not come in contact with food, such as conveyors, drains, and floors (Slade, 1992). *L. monocytogenes* is able to attach to many different surfaces that are used throughout a food processing plant, including stainless steel, glass, polypropylene, and rubber (Mafu et al., 1990). In the process of attaching, *L. monocytogenes* produces a

primary acidic polysaccharide that serves to initially attach and anchor the bacterium to a surface. Once attached, a secondary fibrous acidic polysaccharide is produced. This secondary fibrous polysaccharide appears to add strength to the attachment by increasing the surface area with which the bacterium comes in contact (Mafu et al., 1990). As with all microorganisms, their attachment to a material is subject to the surrounding menstruum (the material to which it is attached) and the characteristics of the particular organism (Helke et al., 1993).

In food processing environments, it is extremely important to know to what objects, and with what tenacity, an organism attaches. This information is particularly important when it comes to properly sanitizing food-processing equipment. Many different cleaners, including Cygnus-14, a broad-spectrum enzymatic cleaner, and Tuff Stuff XF, an alkaline detergent, are used for disinfection. Also, many different sanitizers, including sodium hypochlorite and iodophor, are used to improve disinfection. These cleaners and sanitizers do not work efficiently by themselves, but when used in combination, they have a much higher rate of effectiveness. Although combinations of these chemicals are used in the food-processing environment, difficulties persist in obtaining complete sanitation, mainly because of an organism's ability to attach to a surface.

Once attached to a surface, the organism becomes extremely difficult to remove and eradicate. When attached, *L. monocytogenes* is especially resistant to a multitude of sanitizers and cleaners. Slade (1992) showed that *Listeria* could remain viable on a surface in a processing plant for several months after thorough cleaning. According to Krysinski et al. (1992), when *L. monocytogenes* is attached to polyester/polyurethane,

none of the sanitizers or cleaners tested were effective in sanitizing the material. Polyester/polyurethane is the material that makes up the rollers used on conveyor belts in food processing plants. If *Listeria* cannot be effectively destroyed on these surfaces, contamination problems become very serious, both for the processing plant and the consumer (Krysinski et al., 1992). Another common antimicrobial technique is to use sodium chloride to slow or prevent bacterial growth on processed salmon. When *Listeria* is exposed to sodium chloride in relatively low concentrations and at low temperatures, it can still grow. Sodium chloride can decrease numbers of viable *Listeria* cells, but exposure to high concentrations for extended periods of time is required for more complete elimination (Hudson, 1992).

Another problem presents itself when *Listeria* is allowed prolonged exposure to a particular environment; the bacterium becomes resistant, or adapts to that particular environment. When stressed, *Listeria* reacts by regulating the production of several proteins. This provides the bacteria with the ability to modify their cellular physiology and adapt to the stress. For instance, *Listeria* does not normally grow well within foods that have a low pH, such as cottage cheese, yogurt, and cheddar cheese. However, when *Listeria* adapts to a low-pH environment, it can then grow in a variety of low pH foods. It is also possible that when the bacteria become resistant to low pH, they can then survive the process of active milk fermentation by a lactic acid culture. This means that *Listeria monocytogenes* can survive fermentation in milk and pass from the processing plant into someone's home (Gahan et al., 1996), a potentially hazardous situation.

Behavior and Injury of *Listeria monocytogenes* Caused by Heating

Listeria monocytogenes has been known to adapt to a variety of growth temperatures, allowing it to grow more efficiently in a multitude of temperatures (Smith et al., 1991). Smith et al. (1991) showed that when microorganisms are cultured within a low-temperature environment, they slowly become more susceptible to high temperature environments. It is thought that adaptation to growth temperatures might be attributed to an organism's ability to regulate the amount of unsaturated fatty acids within the cytoplasmic membrane. When cultured in higher temperatures, the microorganism decreases the number of unsaturated fatty acids, making the cytoplasmic membrane more viscous and less fluid (Smith et al., 1991).

According to Bunduki et al. (1995) *Listeria* injury from exposure at 56°C for 20 minutes ranges from 84-98%. However, the heat-injured cells were able to repair themselves within three to five hours when cultured on *Listeria* repair broth (LRB) (Bunduki et al., 1995). It has been shown that organisms can be recovered from milk heated to between 60 and 66°C. However, no cells can be recultured from milk heated to 69°C for at least 16.2 seconds. It has also been shown that heat resistance of the organism within meat can be considerably increased if curing salts are present (Farber and Peterkin, 1991). It is possible that the curing salts act similarly to cryoprotectants in freezing conditions.

Farber et al. (1992) showed that *L. monocytogenes* is able to withstand minimum pasteurization at 71.7°C for 16 seconds within certain products. These are the parameters of high-temperature, short-time pasteurization (HTST), a technique used in the processing of several products, including cottage cheese and dehydrated milk.

Considering that *Listeria* can survive this type of pasteurization, the concern about whether or not pasteurized products are free of *Listeria* is valid. Most of the damage inflicted on *Listeria* with heating deals with biochemical alterations that affect cell division, metabolism, and bioenergetic mechanisms (Smith et al., 1991). Alterations to these systems are only semilethal to the bacteria, making it possible for the injured bacteria to recover when given enough time. In milk, it is extremely unlikely that *Listeria* would be able to make a recovery before the shelf expiration date arrives. All in all, *L. monocytogenes* is fairly resistant to heating up to about 54°C. There is some variation between strains in the ability to resist heating, but for the most part, *Listeria* can survive rather well in heat-stressed conditions.

Behavior and Injury of *Listeria monocytogenes* Caused by Freezing

The most widely used technique in preventing food spoilage is frozen storage. Freezing prevents bacterial growth and destroys bacteria by forming intracellular and extracellular ice crystals. When frozen, the extracellular water freezes first, then the intracellular water travels externally and freezes, or it freezes within the cell at about –5 to –10°C. Slow freezing rates facilitate extracellular crystallization, and rapid freezing facilitates intracellular crystallization (El-Kest and Marth, 1992a). Formation of ice crystals either can result in dehydration of the cell or can puncture the cytoplasmic membrane, allowing contents of the cell to leak; this presents a severe problem for the bacterium. Repeated freezing and thawing also damages the membrane, leading to leakage of materials from the cell. Slow freezing leads to the formation of large extracellular ice crystals and extensive damage, whereas rapid freezing leads to formation

of small extracellular and intracellular ice crystals, with little damage to the cell (El-Kest et al., 1991). As with heating, *Listeria* can gain some resistance to freezing by altering composition of the cell membrane (El-Kest and Marth, 1992a).

The medium in which *Listeria* is suspended greatly influences how well it can resist freezing. Some foods, such as ice cream, contain cryoprotectants such as glycerol, which coat the bacteria and prevent freeze injury. Cryoprotectants are chemicals that aid in the preservation of a cell's viability. They serve to protect the cell from freezing and thawing, and thus protect it from frozen storage (El-Kest and Marth, 1992b). El-Kest and Marth (1992a) showed that glycerol, casein, and lactose can protect *L. monocytogenes* from death and freeze injury for up to six months in storage. Damage from freezing has been shown to be less severe than that done by heating (Busch and Donnelly, 1992).

El-Kest and Marth (1992a) found that ice cream held at –18°C is able to harbor *L. monocytogenes* Scott A for up to five months with no significant decrease in population size. It is also known that *L. monocytogenes* can sustain a relatively constant viable count when suspended within frozen ground beef, ground turkey, and frankfurters (Palumbo and Williams, 1991). Palumbo and Williams (1991) also found that the ability of *Listeria* to survive freezing in a foodstuff is not only dependent upon the cryoprotectants present but also upon the pH of the foodstuff itself. *Listeria* survives freezing best in foods with a pH of 5.8 or above. Not only can *Listeria* survive freezing, but even if it does become injured, when given the right nutrients, it can repair itself and possibly regain any lost virulence capabilities (Flanders and Donnelly, 1994).

The amount of injury and death to *Listeria* from freezing is dependent upon several factors, including the suspending menstrua, the temperature, and the storage time.

For instance, when stored at –18°C for seven days *L. monocytogenes* Scott A sustained injury to about 71% of the population. However, when held at the same temperature for fourteen days, only 72% of the population sustained injury (Golden et al., 1988). One problem with the freezing process is that possibly up to 90% of the living cells within the product after freezing might be injured and unable to proliferate (Speck and Ray, 1977). This can mean that a negative sample might not necessarily be free of the organism being tested.

Based on the results obtained by Golden et al. (1988), *L. monocytogenes* suffers most freeze injury within the first seven days of exposure to –18°C. The extent of injury caused by freezing is currently unknown. Freezing might affect the functionality of the peptidoglycan layer, teichoic acids, nucleotides, and ribosome (El-Kest and Marth, 1992b). *Listeria* has a unique ability to maintain itself within freezing environments for extended periods of time. With this ability, *Listeria* can contaminate a food product during processing, and by the time the consumer eats the product, infectious doses can still exist. Thus, the threat of food contamination remains a concern, even after freezing.

Selective and Non-selective Media for Culturing *Listeria*

Listeria spp. are rather fastidious organisms, and are difficult to isolate because of the competitive background microflora that outgrow *Listeria* when plated on enrichment media. To isolate *Listeria* with some success, enrichment media had to be designed that could prevent or slow the growth of competitive microflora. These media are collectively known as *Listeria*-selective media. When plated on non-selective media, *Listeria* has a substantially-reduced growth rate compared to that obtained when plated on selective

media (Sheridan et al., 1994). Over the past ten years, several of these agars and broths have been developed. Selective media include Oxford agar (OA), Palcam agar, lithium chloride-phenylethanol-moxalactam agar (LPM), Al-Zoreky-Sandine *Listeria* medium (ASLM), lithium chloride-ceftazidime agar (LCA), and many others.

Of these agars, OA and LPM are the most widely used in the laboratory because of their effectiveness. Heisick et al. (1989) found that LPM was effective in inhibiting background microflora on vegetables; moxalactam inhibits the background microflora but not the *Listeria*. Lee and McClain (1986) found that *L. monocytogenes* was resistant to moxalactam in the amount of 128 μg or more. Moxalactam is a wide-spectrum antibiotic effective against several gram-negative and gram-positive organisms (Lee and McClain, 1986). LPM has also been observed to be as good as Modified Vogel Johnson agar (MVJ) when culturing *Listeria* from food products (Buchanan et al., 1989). To identify *Listeria* from a LPM plate, the colonies must be microscopically examined. Another way to identify *Listeria* organisms from LPM is to isolate the organism in question and run a rapid Sodium hippurate hydrolysis test to identify hydrolysis of Sodium hippurate, which *Listeria* spp. can do. Oxford agar, another selective agar used by the USDA, contains cycloheximide, acriflavin, cefotetan, colistin sulfate, and phosphomycin as selective agents. Oxford agar also contains esculin, a product that when broken down, forms byproducts that combine with iron citrate to form a black product that colors the agar around *Listeria* colonies (Westoo and Peterz, 1992). With the addition of esculin to the OA media, no microscopic examination is needed.

The main inhibitory agents within OA and LPM, cycloheximide and moxalactam, respectively, are similar in their effectiveness (Lachica, 1990). Likewise, both OA and

LPM media are rather evenly matched in overall performance. The only true advantage that OA has over LPM is that OA does not need to be examined microscopically for *Listeria* conformation because of the esculin byproducts. Al-Zoreky and Sandine (1990) formulated a new selective media that combined the best aspects of both the OA and the LPM media. This media, Al-Zoreky-Sandine *Listeria* medium (ASLM), combined the cycloheximide, acriflavin, and esculin from OA with the highly-effective moxalactam from LPM. Al-Zoreky-Sandine listeria medium also has an additional selective agent, ceftazidime pentahydrate, to increase the media's effectiveness. The Al-Zoreky-Sandine *Listeria* medium was found to not inhibit *L. monocytogenes*, but completely inhibited all common background microflora that were tested (Al-Zoreky and Sandine, 1990). The only significant problem with the use of selective agars is that injured *Listeria* cells, especially those that are heated injured, can have some difficulty multiplying with the selective agents in place.

Lithium chloride-ceftazidime agar (LCA) is another *Listeria*-selective agar that is somewhat similar to LPM. LCA is a modified brain/heart infusion agar (BHI) that has lithium chloride, glycine anhydride, and ceftazidime pentahydrate as added components (Lachica, 1990). LCA, like OA, is able to demonstrate *Listeria* presence with the formation of a color reaction. On LCA, *Listeria* colonies present a bluish hue, whereas streptococci form iridescence around the colonies when observed under oblique-transmitted light (Lachica, 1990). A final selective medium that is also used to resuscitate injured *Listeria* is University of Vermont Media (UVM), which is a two-step enrichment procedure (Ralovich, 1989). When considering all of the current selective

media available, those that contain esculin are among the most widely-used because microscopy is unnecessary.

Culture Identification Protocols

Currently, there are two main culture-based identification protocols. The U. S. Department of Agriculture and U. S. food and Drug Administration each have their own protocols. There is no significant difference between the two protocols in overall effectiveness. According to Hayes et al. (1992) the USDA and the FDA protocols have only a 75% selective sensitivity. This means that neither protocol is very sensitive and both more than likely will identify some positive samples as being negative. Although both are similar in effectiveness, both are not always equally advantageous for every situation. The USDA formulated their protocol around the isolation of *Listeria* from meat, poultry, and environmental samples, whereas the FDA formulated their protocol around isolation of *Listeria* from dairy products (Westoo and Perez, 1992). Another difference between the two procedures is that the USDA method provides a much better platform for the recovery of heat-injured cells, whereas the FDA method is not effective at recovering heat-injured cells (Slade, 1992).

The use of different methods with different media is important, because there is no single medium that is effective under all circumstances. The main problem with the devised protocols is that they really are not sensitive enough to identify small amounts of *Listeria* within food products. They also require long enrichment intervals that require 3-28 days to obtain results (Walker et al., 1990).

The USDA protocol used in this study was slightly modified to add the use of Sodium hippurate hydrolysis. This particular protocol is based on the use of two non-selective enrichment periods followed by one selective enrichment period. Non-selective enrichment used a two-step broth media known as UVM-I and UVM-II, in which the selective enrichment stages used two highly-selective plating media known as Oxford agar (OA) and lithium-chloride phenylethanol moxalactam agar (LPM). According to McClain and Lee (1988), the two-step enrichment process increases the effectiveness of recovery of *L. monocytogenes* from meat products. In their particular study, the two-step enrichment protocol was 42% more efficient than a single step enrichment procedure in the recovery of *L. monocytogenes*. Although the protocol is time-consuming and lengthy, the USDA protocol has an isolation efficiency of approximately 96% (Hayes et al., 1991).

To increase isolation efficiency and sensitivity, the selective media Oxford agar and LPM were used. Because the USDA protocol is culture-based, there is an added variable that might alter the results. Competition from other organisms living within the food product could cause interference in the results, thereby producing a false negative. Most of the competitors to *L. monocytogenes* are *Enterococcus* spp., *Staphylococcus* spp., and *Corynebacterium* spp. (Dallas et al., 1991). Use of the two selective media minimizes the effects of competition, also known as competitive inhibition. Even with the use of highly-selective media, test results can still be skewed if the food samples are heavily contaminated (Hayes et al., 1991).

Oxford agar uses bile esculin to identify bile esculin hydrolysis products which is characteristic of *L. monocytogenes*. Fraser and Sperber (1988), found that all

Listeria spp. hydrolyze esculin, making it a universal characteristic for the genus. According to Art and Andre (1991), Oxford agar also works well with food samples, making it ideal for this study. As with all selective media, certain isolates proliferate better than others, depending on what the isolate is and from where it was isolated. For example, when isolating *Listeria* from cold-processed salmon products, OA performs with only marginal efficiency (Paranjpye et al., 1992).

Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay is a technique developed to identify the presence of an antigen or an antibody. There are two types of ELISA, a direct and an indirect ELISA. The indirect ELISA involves the detection of an unknown antibody within the serum of a patient. The procedures involve adding a known antigen to a polystyrene well that anchors the antigen. Then, the serum with the unknown antibody is added to the well. If a known antigen is the complement to the unknown antibody, then the antibody binds to the antigen. An enzyme-linked (or enzyme-labeled) antibody that is specific to the unknown antibody that is being screened for is then added to the well. Finally, with the addition of an enzyme substrate, the enzyme that is linked to the antibody reacts and produces a colorimetric reaction. A figure of the indirect ELISA is not shown because it was not the type of ELISA used in this study.

The second ELISA type is the direct ELISA that tests for an unknown antigen. Direct ELISA involves coating a polystyrene well with a known antibody to a particular antigen. With the addition of an unknown antigen, this antigen binds to the known antibody if they are complements. An enzyme-linked antibody is then added that binds

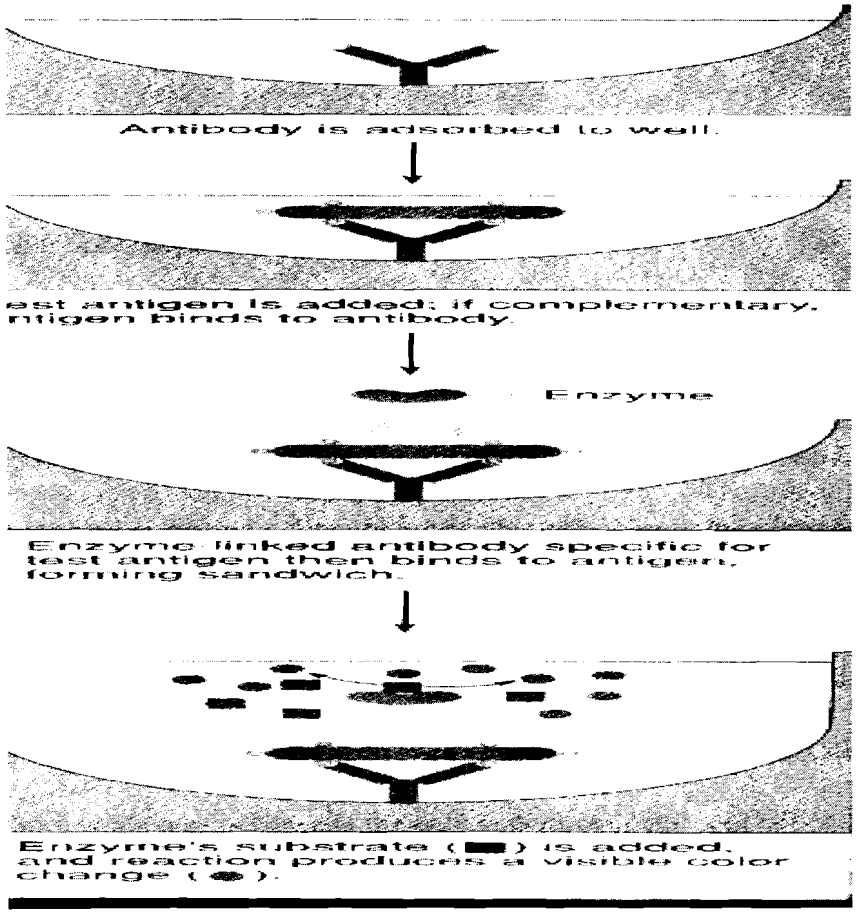
to the antigen being screened for, if it is present. If the antigen is present, the "sandwich," which is a complex of antibody-antigen-antibody, is formed. The last step of the direct ELISA is to add an enzyme substrate that is reacted upon by the linked enzyme, forming a colorimetric reaction. The direct ELISA procedure is illustrated in Figure 2.

Both the direct and indirect ELISA procedures use extremely-specific monoclonal antibodies that are usually produced by hybridomas (Comi et al., 1991). In other words, it is an antibody that is produced by lymphocyte clones that respond to a particular antigen and only produce antibodies specific to that antigen. According to Mattingly et al. (1988), the Organon Teknika *Listera*-Tek ELISA kit identified only *Listeria* as positive in samples among 18 *Listeria* spp. and 21 non-*Listeria* spp. When *Listeria* was present, the ELISA expressed strongly positive readings that were off of the optical density scale (Mattingly et al., 1988). On the other hand, the *Listeria*-Tek ELISA kit has received some unfavorable reviews. Meier and Terplan (1993) reported receiving unsatisfactory results with the *Listeria*-Tek when using only a one-step enrichment process. According to Organon Teknika (1994), the best results are obtained when using a two-step enrichment procedure. Because of the difficulty of isolating organisms from food products, this extended enrichment allows for more time to attain an approximate population of 10^5 to 10^6 organisms. The population of that size is needed to produce a positive reaction with the ELISA (Noah et al., 1991).

Although the one-step enrichment procedure might not be ideal, the Listeria-Tek produced no false negative results and only one false positive out of 36 samples (Meier and Terplan, 1993). Another advantage to the ELISA is that even with the two-step

Figure 2. ELISA procedure. The first step of the direct enzyme-linked immunosorbent assay (ELISA) is to coat a polystyrene well with a monoclonal antibody specific to *Listeria* spp. Second, the unknown antigen is added to the well. If the antigen and the antibody are complements to each other, then the antigen-antibody complex is formed. Step three, the enzyme-linked antibody added to the well. If the initial antigen-antibody complex is formed, then the enzyme-linked antibody binds to the other side of the antigen, forming the "sandwich" complex. The final step involves addition of the enzyme substrate that is acted upon by the enzyme, forming a colorimetric reaction This picture was adapted from Talaro and Talaro (1999).

a) Direct Antibody Sandwich Method



enrichment procedure in place, the whole assay only takes about 50 hours, compared to current USDA culture-based protocol that takes about one week. Given these results and the specificity of the assay, the ELISA is a proficient method for identifying *Listera* spp. in food samples. The enrichment procedure is an important part that makes the assay proficient.

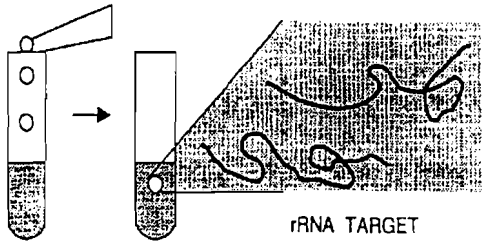
Deoxyribonucleic Acid Hybridization Assay (DNAH)

One of the more widely-adopted assays is the colorimetric DNA hybridization-based assay. The DNAH, like the ELISA, takes about 50 hours for completion (Bottari et al., 1995). This method involves the addition of two DNA fragments, a capture probe and a detector probe, that hybridize to the *Listeria* ribosomal RNA. Both of the probes hybridize to *Listeria*-specific 16S rRNA, including the detector probe, which is a synthetic, P32-labeled, phosphorus radioisotope, DNA probe (Klinger et al., 1988). With the use of a plastic dipstick, which contains a polydeoxythymidylic acid tail, hybridization occurs between the polydeoxythymidylic acid tail on the dipstick and the polydeoxyadenylic acid tail located on the capture probe that is already hybridized to *Listeria* ribosomal RNA. The dipstick is then transferred to an antibody-enzyme conjugate solution that can then recognize the detector probe. Once recognized, an additional chemical substrate is then cleaved and provides a visual color reaction, whose intensity is then quantified to determine positive or negative results (Curaile et al., 1994). The Gene-Trak Systems DNAH procedure is illustrated in Figure 3.

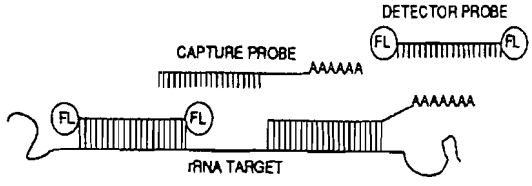
Klinger et al. (1988) tested a total of 139 *Listeria* isolates and 73 non-*Listeria* isolates with the DNAH. It was found that all *Listeria* isolates gave strong positive

Figure 3. DNAH assay procedure, by Gene-Trak Systems. First, the cells are lysed releasing the ribosomal RNA. Two DNA fragments (a capture probe and a detector probe) are then added to the suspension in which they both hybridize to the *Listeria* 16S rRNA. A plastic dipstick that contains a polydeoxythymidylic acid tail (PolyT) is then added, and the PolyT tail then hybridizes to a polydeoxyadenylic acid tail (PolyA). The whole complex is then transferred to an antibody-enzyme conjugate that recognizes the detector probe and forms a colorimetric reaction. Permission was obtained from Gene-Trak Systems Corporation (1999) for the use of this figure.

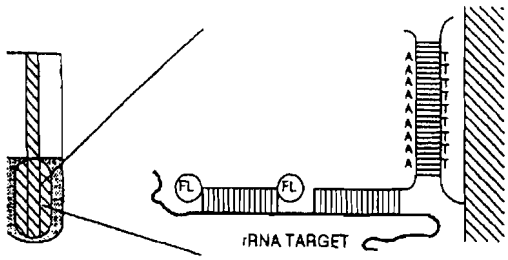
1. SAMPLE LYSIS



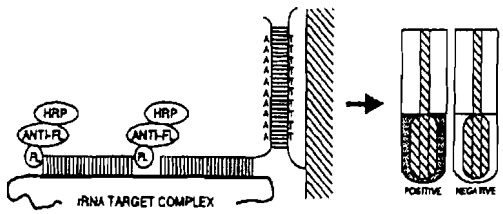
2. HYBRIDIZATION



3. CAPTURE



4. DETECTION



results. Of the non-*Listeria* isolates (which could be found within food samples as background microflora) all presented negative results (Klinger et al., 1988). The DNAH assay by Gene-Trak Systems (1997), has been reported to be more reliable when compared to the FDA culture-based protocol for milk testing (Url et al., 1993). In another study, it was found that DNAH had a sensitivity of approximately 93.9%, with a false negative rate of about 6.1%. It was also shown that the FDA protocol had a sensitivity of approximately 82.6% and a false negative rate of occurrence of about 17.4%. The USDA protocol had a sensitivity of approximately 71.0%, with a false negative rate around 29.0% (Bottari et al., 1995). Clearly, the current USDA and FDA protocols are not as sensitive as the current technology allows.

However, one of the main problems with rapid detection assays (DNAH assay and ELISA) is that there is no current way to quantify the amount of organism present within the sample (Murphy et al., 1996). One must remember that no single assay is 100% accurate (Martin and Katz, 1993). Also, to identify a positive result, at least 1 x 10^6 organisms need to be present, which might be difficult when testing food samples. The results might also misrepresent the entire food product because only a small portion of the sample is tested (Murphy et al., 1996).

Research Objectives

The objectives of this research were four fold: first, to determine survival of *Listeria monocytogenes* in ultracold –70°C temperatures over extended periods of time (two, four, and six months); second, to determine survival under ultracold –70°C within two different substrates (ground turkey and turkey frankfurters); third, to observe the

effectiveness of three commonly-used diagnostic tests (Organon Teknika *Listeria*-Tek ELISA kit, a Gene-Trak Systems *Listeria* assay DNA hybridization test for detection of *Listeria*, and a modified USDA culture based protocol for isolation of *Listeria*); and fourth, to determine if *Listeria monocytogenes* strain Scott A would maintain its virulence throughout the experimental time periods at –70°C.

There were several reasons for choosing the -70°C as the experimental temperature to work with. First, a prior study by Lee (1997), showed that L. monocytogenes strain Scott A could survive exposure to –5° and –10°C for five months in dried dairy products. Also, El-Kest and Marth (1992a) showed that L. monocytogenes could survive exposure to -18°C for five months in ice cream. Based on previous data it was assumed that if L. monocytogenes could survive at -70°C, then it would be able to survive at standard home deep freeze temperatures of approximately -20°C. Another factor that had to be considered, was the availability of freezer space for 162 samples and the reliability of the freezer.

A reliable and large cryofreezer that was already set for -70°C was commissioned for use for this study. The last factor to be considered was that all of the water within the samples needed to be frozen. According to El-Kest and Marth (1992a), water that contains no nucleation agents and is between 1μm and 1 cm in diameter will not freeze until below -39°C. The temperature was also chosen because at -70°C it is impossible for the organisms to proliferate, and most of the challenge is for the *Listeria* to survive. The temperature chosen goes beyond the temperature of household consumer freezers and even deep freezers. This created a higher level of stringency at which the organisms were being measured.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Listeria monocytogenes strain Scott A was obtained from American Type Culture Collections (Rockville, MD). Freeze-dried cells were cultured following directions as outlined by ATCC. The freeze-dried disc was rehydrated with the aseptic addition of 0.3 to 0.4 ml of tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI). The suspension of *L. monocytogenes* was then inoculated onto a tryptic soy agar (TSA) slant and into a tube containing 5 ml of TSB. The cultures were then incubated for 48 hours at 37°C. After the incubation period, a 500 ml flask of TSB was inoculated with the suspension of cells and incubated at 37°C for 48 hours with periodic agitation. At the same time, two Oxford agar (OA) (Difco Laboratories, Detroit, MI) plates were inoculated with a swab of the *L. monocytogenes* suspension and incubated at 37°C for 24 hours to reinforce the presence of *Listeria*.

Standardization of Inoculum

To establish a known number of bacterial cells per ml of suspension, turbidity was measured using a Bausch and Lomb spectronic 20 spectrophotometer (Rochester, NY). Samples were taken from TSB tubes and transferred to sterile cuvettes. The optical density (OD), or absorbance, of the samples was then measured and adjusted, by dilution, to 20% at a wavelength of 550 nm. The optical density corresponds to the amount of light transmitted through the suspension in agreement with the equation:

OD=log100 – 10g%T, where g=growth and T=time. According to Lee (1997), 20% transmittance with *Listeria monocytogenes* corresponds to approximately 1 x 10^7 cells/ml.

Initial Testing and Inoculation of Substrates

Eighty-one ground turkey and 81 turkey frankfurter samples, totaling 162 samples were obtained from local grocers. All samples were then tested with all three diagnostic assays to determine initial presence of *Listeria*. Twelve of the negative samples, six ground turkey and six turkey frankfurters, were set up as negative control samples. Ground turkey samples were separated into 227 g aliquots and placed into Stomacher bags. Upon inoculation, the samples were homogenized and the Stomacher bags were placed into Glad Lock freezer bags.

Turkey frankfurter samples were separated into one frankfurter (45 g) per Stomacher bag. The frankfurters were massaged and then injected with organism using a 1 ml syringe. The Stomacher bags were then placed into Glad Lock freezer bags. A total of 150 samples, 75 ground turkey and 75 turkey frankfurters, were inoculated with a set inoculum of 1 x 10^6 organisms/ ml/ 100 g of sample. The set inoculum was obtained by diluting the spectrophotometrically-measured amount of 1 x 10^7 cells/ml with TSB. To keep track of the samples, all were numbered with a system devised to distinguish the designated experimental time period, the sample number, and the substrate type. The sample designations were as follows: 4-22-F or 4-22-T, 4 as the experimental time period, 22 as sample 22 out of 25 for that particular sample set, and T representing ground turkey or F representing turkey frankfurter.

Freezing of Inoculated Samples

After inoculation, all 162 samples were then placed into the freezer at –70°C for the designated time periods. Twenty-five inoculated samples and two negative samples of each substrate were set up for each time period; two, four, and six months, respectively. Glad Lock freezer bags were used to house the stomacher bags, and prevented possible freezer burn of the products. The prevention, or suppression, of freezer burn was important because the burned appearance on the product is actually degradation of proteins within the meat. If degradation of proteins within the meat occurred, it is possible that the bacteria might lose a cryoprotectant. The freezer bags were also used to help maintain the integrity of the stomacher bags throughout the extended periods within the freezer that were required for the study. Prevention of protein degradation and integrity stabilization was necessary to maintain a low number of possible external variables.

Sample Preparation for *Listeria*-Tek ELISA Test System

The same sample preparation procedure was used for both substrates for the *Listeria*-Tek ELISA test system. The sample preparation for the initial testing of samples involved removing a 25 g portion of substrate and placing it into a stomacher bag containing 225 ml of Fraser broth (Difco Laboratories, Detroit, MI). The samples were homogenized into the broth to expose as much organism as possible. Samples were then incubated at 35°C for 24 ± 2 hours. Upon completion of the incubation period, a 0.1 ml aliquot of the sample was then transferred to 10 ml of buffered *Listeria* enrichment broth (BLEB) (Difco Laboratories, Detroit, MI). The BLEB tubes were then incubated at 30°C

for 26 ± 2 hours. Upon completion of the second incubation period, 1 ml aliquots of the samples were transferred to clean screw-top glass test tubes. The tubes were then autoclaved, with the caps slightly unscrewed, under running steam, slightly above 100°C, for twenty minutes. Immediately after the autoclaved material was cooled to ambient temperature, testing was performed.

Listeria-Tek ELISA Testing Procedure

The Organon Teknika Corporation (Durham, NC; 1994) donated four enzyme-linked immunosorbent assay (ELISA) test kits (#52100 and lot 151000). Included in these test kits were; one strip holder containing 96 polystyrene wells coated with monoclonal antibodies to *Listeria* species, negative control, positive control, 12 ml of peroxidase conjugate antibody to *Listeria* species, 7 ml 3,3',5,5'-tetramethylbenzidine, 7 ml hydrogen peroxide, 25 ml phosphate buffered saline with surfactant, 12 ml 2N sulfuric acid, and 10 sheets of plate sealers. The strip holder was set up with 57 polystyrene wells (50 inoculated samples, 4 negative samples, 2 negative controls, and one positive control) for the testing procedure.

100 µl of pre-treated samples were then transferred to each assigned well. Then, 100 µl of peroxidase conjugate antibody was pipetted into each well, including the control wells. Upon addition of peroxidase conjugate, the wells took on an orange coloration. Plate sealers were used to seal the polystyrene wells to prevent spillage of contents during agitation. The plate was then incubated at 37°C for one hour in a covered water bath. Upon completion of the incubation period, all wells were washed six times with phosphate buffered saline wash solution as follows. All wells were aspirated and

the contents discarded into a waste flask. All wells were then filled with approximately 0.2 to 0.3 ml of wash solution, which was then aspirated into the waste flask with the original contents. This procedure was repeated six times for each well and the wells were not allowed to dry before proceeding to the next step.

During the one-hour incubation period, the TMB substrate was prepared. TMB preparation involved calculating the total volume of substrate needed for the test run. The number of samples, including controls, was multiplied by 0.12 in order to determine this volume. Once determined, equal volumes of TMB peroxidase substrate A (3,3',5,5'-tetramethylbenzidine) and TMB peroxidase substrate B (hydrogen peroxide) were combined into a sterile test tube. After washing was done, 100 µl of prepared TMB substrate was pipetted into each well. The wells were then incubated at ambient temperature, approximately 20°C, for 30 minutes. Wells that contained *Listeria* took on a blue coloration as the incubation time proceeded. The intensity of the blue coloration was directly correlated to the relative amount of *Listeria* antigen present within the samples. Upon completion of the incubation period, 100 µl of stop solution (2N sulfuric acid) was added to each well. This changed the blue coloration into a yellow coloration of corresponding intensity.

The plates were read immediately to prevent any false readings of the absorbance levels of the wells resulting from change in color. Absorbance of the well contents were read with an EL307C Microplate reader (Bio Tek Instruments, Winooski, VT). The reader was blanked on air and then the absorbance was read at 450 nm. To determine control values, the mean negative control (NCX) value was calculated. The NCX value had to be less than 0.300 to qualify as a negative value and the positive control had to be

greater than 0.700. The cut-off value for samples was equal to NCX + 0.150; anything greater than or equal to this value is considered presumptive positive. If absorbance of the sample is less than this value, it is considered negative.

Sample Preparation for Gene-Trak *Listeria* DNAH Assay

The same sample preparation procedure was used for both of the substrates for the Gene-Trak Systems Corporation (Hopkinton, MA) *Listeria* assay DNA hybridization test for detection of *Listeria.* Sample preparation for the testing of samples involved the transfer of a 25 g aliquot of sample to 225 ml of Fraser broth. The samples were homogenized into the broth to expose as much organism as possible. Samples were then incubated at 35°C for 24 ± 2 hours. This primary enrichment procedure was the same as that used for the *Listeria*-Tek ELISA. Upon completion of the initial incubation, a sterile cotton swab was used to transfer material from the Fraser broth culture to an Oxford agar plate (Difco Laboratories, Detroit, MI). The inoculated Oxford plates were then incubated at 35°C for 24 ± 2 hours. After the incubation was concluded, a sterile swab was used to transfer organisms from the Oxford plates to test tubes containing 1 ml of sterile phosphate-buffered saline. The swabs were swirled for 5 seconds to suspend as much organism as possible into the saline solution. As much liquid as could be expressed from the swab was done so before being discarded. Once this was performed, the samples were ready for testing.

Gene-Trak *Listeria* DNAH Assay Testing Procedure

The testing procedure for the DNA hybridization assay as outlined by Gene-Trak Systems was followed. Included in the DNA hybridization test kit was: pre-treatment reagent concentrate (1 vial), pre-treatment reagent buffer (12 ml), lysis reagent concentrate (2 vials), lysis reagent buffer (12 ml), *Listeria* probe solution (10 ml, caution: contains 0.1% sodium azide), wash solution 20X concentrate (2 x 250 ml), enzyme conjugate 100X concentrate (1 ml), substrate-chromogen solution (90 ml), stop solution (25 ml, caution: contains 4.0 N sulfuric acid), dipsticks (2 x 50), positive control (5 ml, caution: contains 0.1% sodium azide), and negative control (5 ml). Three test tube racks, repeating pipette, disposable tips, and Gene-Trak photometer were obtained from Gene-Trak Systems.

Prior to starting the testing procedure, two water baths were adjusted and stabilized to 37 +/- 1°C and 65 +/- 1°C. Both water baths were filled to an approximate level of 1.5 inches (4 centimeters). Next, 12 ml of pre-treatment reagent buffer was added to the pre-treatment reagent concentrate vial. The pre-treatment reagent concentrate was dissolved by gentle swirling and was then placed on ice to prevent degradation. Six ml of lysis reagent buffer was added to one of the vials of lysis reagent concentrate for each 50 test samples that were run. The lysis reagent concentrate was dissolved by shaking thoroughly and then was placed on ice to prevent degradation. The pre-treatment reagent and lysis reagent are stable for 60 days after reconstitution when stored at –20°C. Thawing of frozen reagent requires soaking of the vial in a 37°C water bath for approximately 10 minutes, then is immediately placed on ice until needed.

Borosilicate glass test tubes (12 x 75 mm) were used for the testing procedure. For each sample being tested, a test tube was labeled with the sample designation and placed into the test tube rack. Tubes were also set up for one positive and one negative control. A second set of test tubes was set up (in the same manner as stated above) for the enzyme conjugate step. A third rack was set in the same manner as stated above except one more tube was added and labeled "reagent blank." This third rack was set up for the substrate-chromagen step. First, 1.3 liters (L) of 1X wash solution was prepared for each 25 samples being run. Then, the 20X wash solution was diluted with distilled water (65 ml 20X wash solution plus 1235 ml distilled water). A wash basin containing approximately 300 ml of 1X wash solution was placed into the 65°C water bath for each 25 samples being run. Also, three additional water baths were set up containing approximately 300 ml of 1X wash solution, which were stored at room temperature during the testing procedure. All four wash basins were covered and remained covered except while in use.

Each test tube containing suspended organisms in 1 ml of phosphate-buffered saline was vortexed just before testing began in order to resuspend the organisms. A 0.5 ml aliquot was removed immediately following vortexing and transferred to a properly-labeled 12 x 75 mm borosilicate glass test tube. Next, 0.5 ml of both the positive and negative controls were transferred to the proper tubes. Then, 0.10 ml of reconstituted pre-treatment reagent was added to each test tube, including the controls. The test tube rack was then agitated for 5 seconds to mix the contents. After addition of the pre-treatment reagent, the contents turned purple. The test tubes were then incubated at 37°C for 15 minutes. Then, 0.10 ml of reconstituted lysis reagent was added to each of

the tubes while they remained in the water bath. The rack was then removed just long enough to mix the contents by agitation for 5 seconds and then placed back into the water bath. After mixing was complete, the tubes took on a dark green coloration. Tubes were incubated for another 15 minutes in the 37°C covered water bath.

During the second incubation period, the proper number of dipsticks needed were placed into the dipstick holders. The dipsticks were then rinsed in 1X wash solution at ambient temperature for two to three minutes. Excess wash solution was removed by blotting on absorbent paper. Upon completion of the second incubation period, the rack of tubes were transferred from the 37°C water bath to the 65°C water bath. An aliquot of 0.10 ml of *Listeria* probe solution was then added to each tube. The *Listeria* probe solution turned the tube contents to a dark red color. The washed dipsticks were then inserted into the sample tubes and used to mix the contents thoroughly. The dipsticks were raised and lowered five times and then incubated at 65°C for one hour. Approximately 30 minutes prior to the end of the one-hour incubation period, the proper amount of 1X enzyme conjugate was prepared by diluting the 100X enzyme conjugate concentrate with 1X wash solution. The enzyme conjugate remains stable for one hour after dilution. Then, 0.75 ml of 1X enzyme conjugate was dispensed into each of the empty tubes prepared earlier.

When incubation was complete, the dipsticks were removed from the tubes and washed. They were washed sequentially for one minute each, first in the 65°C wash solution and then in ambient temperature wash solution. Dipsticks were blotted on absorbent paper and placed into the second set of tubes containing 1X enzyme conjugate. The tubes were incubated at ambient temperature for 20 minutes. During the 20-minute

incubation period, 0.75 ml of substrate-chromagen solution was placed into the third set of tubes. The substrate-chromagen was placed into the third tube set at least five minutes prior to use to allow the reagent to reach ambient temperature. On completion of the 20-minute incubation, the dipsticks were removed and washed sequentially in the remaining two wash basins for one minute each. The dipsticks were blotted on absorbent paper and placed into the third set of tubes containing substrate-chromagen, followed by gently shaking the rack to mix the contents. Tubes were then incubated at ambient temperature for 30 minutes.

As the 30-minute incubation period continued, the solution took on a blue coloration where *Listeria* was present. When the incubation period was completed, the dipsticks were removed from the tubes and discarded. Finally, 0.25 ml of stop solution was added to the tubes and the solution was mixed with gentle shaking of the rack. Upon addition of stop solution, a yellow coloration of the solution was produced of the equivalent intensity to that of the blue coloration produced by the substrate-chromagen. Absorbance of the samples was determined as soon as possible after addition of stop solution.

For absorbance of the samples to be read, absorbance values of the positive and negative controls had to be established. The tube labeled "reagent blank" was placed into the left slot of the Gene-Trak photometer set at 450 nm, and the negative control tube was placed into the right side of the photometer. This determined the absorbance value of the negative control. To determine absorbance of the positive control, the "reagent blank" was left in the left side of the photometer and the positive control was placed into the right side. The sample absorbances were then read with the negative control occupying

the left slot and the sample occupying the right slot. To prevent possible misreading due to color changes, all absorbance values were read and recorded as soon after addition of stop solution as possible.

As outlined by Gene-Trak Systems the negative control absorbance value had to be less than 0.15, and the positive control absorbance value had to be greater than 1.00. If the controls did not adhere to the standards, then the assay was considered invalid. The assay would have been invalid if the results did not conform to these standards. The negative criterion required that the samples must produce an absorbance value of 0.10 or less, indicating that the sample did not contain *Listeria* species. The positive criterion required that the samples must produce an absorbance value greater than 0.10, indicating that the sample contained *Listeria* species.

Modified USDA Culture-Based Method of Isolation

A modified USDA culture-based method of isolation was used to compare with the ELISA and DNAH techniques. This method involved the common primary enrichment procedure shared by all three diagnostic tests. Twenty-five grams of sample was transferred to 225 ml of Fraser broth and then incubated at 35°C for 24 ± 2 hours. Then, 0.1 ml of Fraser broth was transferred to properly-labeled test tubes containing 10 ml of University of Vermont Media (UVM-I) broth. The tubes were then incubated at 30°C for 24 hours. After incubation was completed, 0.1 ml of UVM-I broth was transferred to labeled tubes containing 10 ml of UVM-II broth. After inoculation, the tubes were incubated at 30°C for 24 hours.

After the second incubation period at 30°C, the tubes were vortexed to suspend the organism evenly. Sterile cotton swabs were used to transfer the organisms from the UVM-II cultures to appropriately-labeled lithium chloride-phenylethanol-moxalactam (LPM) agar plates and Oxford (OA) agar plates. The LPM plates were incubated at 30°C for 24-48 hours, and the OA plates were incubated at 37°C for 24-48 hours. The use of wide range on incubation periods was because *Listeria monocytogenes* is a fastidious organism. During the incubation period, the OA plates were checked at intervals to identify any color change to the plates. If *Listeria* was present, the plates turned black with bile esculin hydrolysis.

The method recommended for visualizing Listeria growth on LPM plates is known as Henry illumination. This technique did not seem to work for this study, so *Listeria* identification on LPM plates was modified. To determine *Listeria* growth on LPM plates, a technique known as rapid Sodium hippurate hydrolysis was used. Rapid Sodium hippurate hydrolysis involved preparing a 1% solution of Sodium hippurate. This was done with the addition of 1 g of Sodium hippurate salt, or hippuric acid, (Sigma Chemicals, St. Louis, MO) to 100 ml of distilled water. The solution was mixed until the hippuric acid completely dissolved. The solution was then dispensed in 0.4 ml aliquots into 12 x 75 mm borosilicate glass test tubes. The remaining solution was stored frozen and was useable for up to six months.

Ninhydrin reagent was then prepared by dissolving 3.5 g of ninhydrin in a mixture of 50 ml acetone and 50 ml butanol and stored in a glass bottle wrapped in foil. This solution can be kept at ambient temperature in this manner for up to six months. Each tube was labeled corresponding to the sample number and heavily-inoculated using a

sterile loop to tranfer organisms from the respective LPM plates. All tubes were covered with a foil seal and incubated in a covered water bath at 35°C for two hours. Upon completion of the incubation period, 0.2 ml of ninhydrin reagent was added to the Sodium hippurate substrate and organism mixture. The tubes were then reincubated at 35°C for 15 minutes. As the 15 minutes progressed, the tubes took on a deep purple coloration, indicating that the hippurate had been hydrolyzed and that *Listeria* was present (Baron, et al., 1994).

Inoculation and Observation of Mice for Virulence Testing

To determine the virulence retention of *Listeria monocytogenes* throughout the experimental time periods, samples were taken and injected into experimental mice. A set inoculum of $2x10^6$ organisms per ml was prepared by isolating organism from OA plates and transferring organism to 10 ml of TSB. Tubes were then incubated for approximately 24 hours at 35°C. The standard inoculum was determined using a spectrophotometer to determine 20% transmittance followed by dilution to the specified concentration. Then, 1 ml of isolate was taken into a syringe with a 24-gauge needle. Amount of organism injected was estimated based on the amounts suggested by Stephens et al. (1991). Stephens et al. (1991) inoculated mice with *L. monocytogenes* in the doses of 10^4 for intravenous injection and 10^{10} for the oral-gastric infection. This made an infective dose of 2×10^6 organisms intraperitoneally seem adequate for this study.

Mice were injected intraperitoneally (IP) with the set inoculum of organism. Mice were then separately placed into cages of the same dimensions with fresh food, water, and litter. Thereby preventing any fights that could have contributed to the death

of any particular mouse. Four test groups were set up, one for each time period (two, four, and six months), and an additional set was injected with organism that had not been subjected to the experimental conditions at –70°C. For each set of mice, there were three experimental mice that had been injected with organism and three control mice that had been injected in the same manner but with sterile peptone. All mice were observed for a maximum of two weeks after injection to allow sufficient time for *Listeria* to cause an active infection. The mice were observed at regular intervals of approximately five hours and their behaviors recorded. When a mouse died, the time was recorded and the mouse was immediately examined.

Examination of Mice for Virulence Testing

Examination of the mice involved initially recording where in the cage the mouse had died and how stiff the mouse had become. The mouse was then removed from the cage and pinned at all four legs to a disinfected dissection board. The abdomen of the mouse was then moistened with alcohol to mat down the hair, making it easier to dissect. A long incision was made from the base of the chin to the genitals. Four more incisions were made from the centerline incision to the pit of each limb.

The skin was carefully pulled back, the membrane lining the peritoneal cavity was cut open and gently pulled to the same spot as the skin. The skin flaps were then extended to each side and pinned to the dissecting board. Care was taken when making the incisions to prevent the cutting of any organs. Once opened, a sterile swab was used to swab the external surfaces of the organs. The swab was then swabbed onto an OA plate to reculture any *Listeria* present. Observations of the major organs were also taken

as to whether they were discolored, had external lesions, or had any other unusual characteristics. A portion of the liver was removed and blotted onto two clean glass slides to make impression slides. Following blotting of the liver, the spleen was removed, cut in two, and blotted onto two additional clean glass slides. All slides were then heat fixed and stained with Wright's Giemsa stain. Slides were later examined microscopically to determine if *Listeria* had infected the cells within either of the organs. These organs were chosen because they are two of the major infection sites common to *L. monocytogenes*. Positive results were indicated by the presence of *Listeria* within polymorphonuclear neutrophils, monocytes, and macrophages.

Criteria for Positive and Negative Samples

An easily-accessible definition of the criteria for positive and negative samples for each of the diagnostic protocols is as follows. For the ELISA, a positive sample was defined as one with an absorbance value equal to or greater than that of the cutoff value, which is the mean negative control value plus 0.150. A negative sample is one that has an absorbance value less than the cutoff value. Positive samples for the DNAH assays were defined as having an absorbance value greater than 0.10, and a negative reading was one that with an absorbance value less than or equal to 0.10. The positive criterion for the USDA protocols were defined as turning the color of an Oxford agar plate from a greenish hue to an opaque black. Oxford agar from negative samples retained the greenish hue. The sodium hippurate hydrolysis produced a dark purple coloration, indicating a positive result and the absence of color formation indicating a negative result.

Statistical Analyses

The Z statistic was used to compare the proportion of positive ground turkey and turkey frankfurter room temperature samples found prior to inoculation. The Z statistic actually tests the differences among proportions of a contingency table (Zar, 1996). This test was used to test the null hypothesis that there was no difference in the proportion of positive samples found at room temperature from either the ground turkey or the turkey frankfurters.

Chi square analysis was used to determine if any statistical significant difference existed between the ELISA and DNAH assay in identifying *Listeria monocytogenes* in ground turkey and turkey frankfurter products. The Chi square was also used to determine if there was a significant difference in L. monocytogenes's ability to survive freezing within the two food products. Survival over all three-time periods was analyzed. Chi square analysis was calculated using the formula: $\chi^2=\sum(O-E)^2/E$, where O is observed frequency in a given category and E is expected frequency in a given category (Bartz, 1988).

A one-way analysis of variance (ANOVA) was used to identify any statistical significant difference among the lengths of time that it took for the mice to die after being inoculated with organism from all three experimental time periods at -70°C, and from room temperature isolates. A one-way ANOVA was chosen because there was only one factor, experimental time, and one variable, hours till death, that were being tested (Zar, 1996). The ANOVA was done using the number of hours it took, after injection, for death to occur.

CHAPTER 3

RESULTS

Room Temperature Samples

Zero-time, room temperature testing resulted in a wide range of positive and negatives using the three testing protocols. ELISA determined 18 positive and 63 negative ground turkey samples. The DNAH assay and the USDA protocol identified zero positive and 81 negative ground turkey samples. The turkey frankfurter samples tested with the ELISA resulted in two positive and 79 negative samples. The DNAH assay determined six positive and 75 negatives, and the USDA protocol determined 15 positive and 66 negatives. The room temperature test results are presented on Table 1.

A comparison of the total percent positive and negative values for the three protocols from the initial testing can also be found on Table 1. The ELISA determined approximately 22.2% of the total samples of ground turkey at room temperature to be contaminated with *Listeria* spp. On the other hand, both the DNAH assay and the USDA protocol determined 100% of the ground turkey samples to be free of *Listeria* contamination. Turkey frankfurter results are as follows: ELISA determined 2.5% of the total turkey frankfurter samples to be contaminated with *Listeria*; DNAH assay determined 7.4% contamination; and the USDA protocol identified 18.5% positive. Statistical comparison of the results on the number of positive and negative samples found with all three protocols for both food products are shown on Table 2. The Z statistic was performed for each of the testing protocols.

Table 1. Results using ELISA, DNAH assay, and USDA protocol for both food products on all experimental sample sets tested at room temperature prior to inoculation. GT=ground turkey, TF=turkey frankfurters

Product	Samples	Positive(%)	Negative(%)	False (+)	False (-)
ELISA					
GT	81	18(22.2)	63(77.8)	0	0
TF	81	2(2.5)	79(97.5)	0	0
DNAH					
GT	81	0(0)	81(100)	0	0
TF	81	6(7.4)	75(92.6)	0	0
USDA					
GT	81	0(0)	81(100)	0	0
TF	81	15(18.5)	66(81.5)	0	0

Table 2. Comparison of results using ELISA, DNAH assay, and USDA protocol for both food products over all experimental sample sets tested at room Temperature prior to inoculation. GT=ground turkey, TF=turkey frankfurters

	ELISA		DNAH		USDA	
Product	Positive	Negative	Positive	Negative	Positive	Negative
GT	18	63	0	81	0	81
TF	2	79	6	75	15	66

*Z=3.58270 for ELISA, 2.0795 for DNAH, 3.7945 for USDA

$Z_{0.05}(2)=1.9600$

Z is significant for all of the testing protocols

*Z analysis was calculated by using the formula as stated by Zar.

The Z values determined for each testing protocol were; ELISA = 3.5827, DNAH assay = 2.0795, and USDA protocol = 3.7945. With two degrees of freedom at an alpha of 0.05 the critical value is 1.9600. Thus, the Z value for the all three testing protocols was significant. In other words, there was significant difference observed in the number of positive samples found in ground turkey and turkey frankfurters from all of the protocols. Refer to appendices A-F for absorbance values from the ELISA and DNAH.

Listeria Determinations in Two-, Four-, and Six-Month Samples Using the ELISA

Results were as expected for the samples run through the ELISA from all three experimental testing periods. From the two-month, four-month, and six-month sample sets, 25 positive and two negative samples were determined for each food product. There were zero false positive and zero false negative samples identified within all three testing periods and both food products (See Table 3). The two negative samples identified from each experimental time period were the two negative control samples for each experimental sample set. A chi square value comparing all three-sample periods, presented a value of zero. Meaning that there was no difference in survival ranging from two months to six months at -70°C.

Listeria Determinations in Two-, Four-, and Six-Month Samples Using the DNAH Assay

Results were as expected for the samples run through the DNAH assay from all three experimental testing periods. The two-month, four-month, and six-month sample sets produced 25 positive and two negatives for each food product. Results from the two-month, four-month, and six-month experimental sample sets can be found in Table 4.

Table 3. Results using ELISA for both food products after two, four, and six months exposure to -70°C. GT=ground turkey, TF=turkey frankfurters.

Product	Samples	Positive	Negative	False (+)	False (-)
Two-month					
GT	27	25	2	0	0
TF	27	25	2	0	0
Four-month					
GT	27	25	2	0	0
TF	27	25	2	0	0
Six-month					
GT	27	25	2	0	0
TF	27	25	2	0	0

Table 4. Results using DNAH for both food products after two, four, and six months exposure to -70°C. GT=ground turkey, TF=turkey frankfurters.

Product	Samples	Positive	Negative	False (+)	False (-)
Two-month					
GT	27	25	2	0	0
TF	27	25	2	0	0
Four-month					
GT	27	25	2	0	0
TF	27	25	2	0	0
Six-month					
GT	27	25	2	0	0
TF	27	25	2	0	0

As with the ELISA, there were zero false positive and zero false negative samples determined throughout all three experimental sample sets in both food products. A chi square value comparing all three-sample periods, presented a value of zero. Meaning that there was no difference in survival ranging from two months to six months at -70°C.

Listeria Determinations in Two-, Four-, and Six-Month Samples Using the USDA Protocol

Results from the USDA protocol were determined by using the selective agar Oxford agar and rapid Sodium hippurate hydrolysis for the three experimental testing periods. The Sodium hippurate and Oxford agar both produced 25 positive and two negative samples for both food products from the two-month sample set. From the four-month sample set, the Sodium hippurate hydrolysis produced 25 positive and two negative samples for the ground turkey and 26 positive and one negative sample from the turkey frankfurters. Oxford agar identified 25 positive and two negative samples for both of the food products. The six-month sample set demonstrated the expected pattern of 25 positive and two negative samples for both of the food products from both the Oxford agar and the Sodium hippurate hydrolysis. As with the previous ELISA and DNAH assay, the negatives consisted of only the set of negative controls. For both of the food products from the two-month and six-month sample sets using both Oxford agar and Sodium hippurate hydrolysis, zero false positive and false negative samples were identified. Within the four-month sample set, there were zero false positive and false negatives from both food products using the Oxford agar. Sodium hippurate hydrolysis also produced zero false positives and false negatives from the ground turkey, but there

Table 5. Results using Sodium hippurate hydrolysis and Oxford agar from the USDA protocol on both food products after two, four, and six months exposure to -70°C. GT=ground turkey, TF=turkey frankfurters, SH=Sodium hippurate, OA=Oxford agar.

Product	Samples	Positive	Negative	False(+)	False(-)
Two-month					
SH					
GT	27	25	2	0	0
TF	27	25	2	0	0
OA					
GT	27	25	2	0	0
TF	27	25	2	0	0
Four-month					
SH					
GT	27	25	2	0	0
TF	27	26	1	1	0
OA					
GT	27	25	2	0	0
TF	27	25	2	0	0
Six-month					
SH					
GT	27	25	2	0	0
TF	27	25	2	0	0
OA					
GT	27	25	2	0	0
TF	27	25	2	0	0

was one false positive and zero false negatives from the turkey frankfurters. For results from the two-month, four-month, and six-month testing periods, refer to Table 5. A chi square value comparing all three-sample periods, presented a value of 0.04, well below the critical value of 5.99. Meaning that there was no significant difference in survival ranging from two months to six months at -70°C.

Sensitivity and Specificity of Testing Protocols

Sensitivity was determined using the calculation of the number per method positive divided by the total number of positive samples. Sensitivity was extremely high for the ELISA, demonstrating 100% sensitivity for all three experimental testing periods. Specificity was determined using the calculation of the number per method negative divided by the total number of negative samples. Specificity was also extremely high for the ELISA, with 100% specificity over all three testing periods. Likewise, the DNAH exhibited 100% sensitivity and 100% specificity across all three experimental testing periods. The USDA protocol exhibited 100% sensitivity and 100% specificity for both the two-month and six-month sample sets. Sensitivity remained high, at 99% for the four-month sample set. However, the four-month sample set specificity was lower at 87.5%. Refer to Table 6 for sensitivity and specificity of ELISA, DNAH, and USDA protocols, respectively.

When comparing the sensitivity and specificity of the ELISA and the DNAH, the two testing protocols are equally sensitive and specific. This is important to know because of the current debate on whether to use an ELISA or a DNAH assay in certain

Table 6. Sensitivity and specificity of ELISA, DNAH assay, and USDA protocol for the three testing periods.

Time of Sample Testing	Sensitivity %			Specificity %		
	2-month	4-month	6-month	2-month	4-month	6-month
ELISA	100	100	100	100	100	100
DNAH assay	100	100	100	100	100	100
USDA protocol	100	99	100	100	87.5	100

Sensitivity was determined by the number per method positives divided by the total number of positive samples.

Specificity was determined by the number per method negatives divided by the total number of negative samples.

testing circumstances. This demonstrates that the two testing protocols work equally well. Upon comparison of sensitivity from the ELISA, DNAH assay, and USDA protocol, it is evident that the USDA protocol is only slightly less sensitive than the ELISA and DNAH. Upon comparison of specificity from the ELISA, DNAH assay, and USDA protocol, it is evident that the USDA protocol is less specific than the ELISA and DNAH assay.

Virulence Retention

Virulence retention was measured on the basis of Koch's postulates, which require that if a pure culture of a particular organism is injected into an animal subject, and the animal dies, the organism can be reisolated in quantity. The isolates can then be tested using selective media and biochemical tests. Positive virulence retention was defined as the isolation of *Listeria monocytogenes* from the peritoneal cavity of the deceased mouse. The isolates were microscopically examined and plated onto the selective, differential Oxford agar. Presence or absence of substantial bile esculin hydrolysis, within the agar was used to determine if the isolates were positive or negative. All of the mice that died produced positive identification for *Listeria monocytogenes*. The number of hours elapsed between injection with organism and death was also recorded (Table 7). The period of time that it took for the mice that had been infected with room temperature, zero freezer time organism to die was 42, 42, and 72 hours. The two-month exposure mice died in 19, 56.5, and 64.5 hours after injection. Two of the mice injected with the four-month exposure organisms died in 59 hours. The third mouse did not die. This might have resulted if injection of the organism was

Table 7. Time (hrs) elapsed between injection of bacteria and time of death of inoculated mice.

Treatment	Range	Mean	n
Control	N/A	N/A	N/A
Room Temperature	42-72	52	3
Two-month	19-64.5	46.67	3
Four-month	59	59	2
Six-month	30-43.5	38.67	3

directly into the intestine, and the organism would have been mostly excreted. The six-month exposure organisms killed the mice in 30, 42.5, and 43.5 hours after injection. All controls survived and at no time did they show any sign of illness or unusual behavior.

Observations of the organs of all dead mice presented no unusual characteristics as far as external appearance was concerned. All of the dead mice exhibited the presence of *Listeria monocytogenes* within both the spleen and liver. The organism was identified as possibly inhabiting neutrophils, monocytes, eosinophils, and macrophages. Refer to figures 4-11 for impression slides from mice from each time period, showing the presence of *L. monocytogenes* within the spleen and liver. All Oxford agar plates that were swabbed from the peritoneal cavity identified the presence of *L. monocytogenes*. The one-way analysis of variance determined there to be no significant statistical difference in the number of hours after injection between the four sample groups, with a power of 0.589. Refer to Table 8 for ANOVA data.

Table 8. One Way Analysis of Variance Thursday, October 28, 1999, 14:25:24

Normality Test: Passed (P = 0.376)

Equal Variance Test: Passed (P = 0.419)

Group	N	Missing
room	3	0
two	3	0
four	2	0
six	3	0

Group	Mean	Std Dev	SEM
room	52.000	17.321	10.000
two	46.667	24.292	14.025
four	59.000	0.000	0.000
six	38.667	7.522	4.343

Power of performed test with alpha = 0.050: 0.050

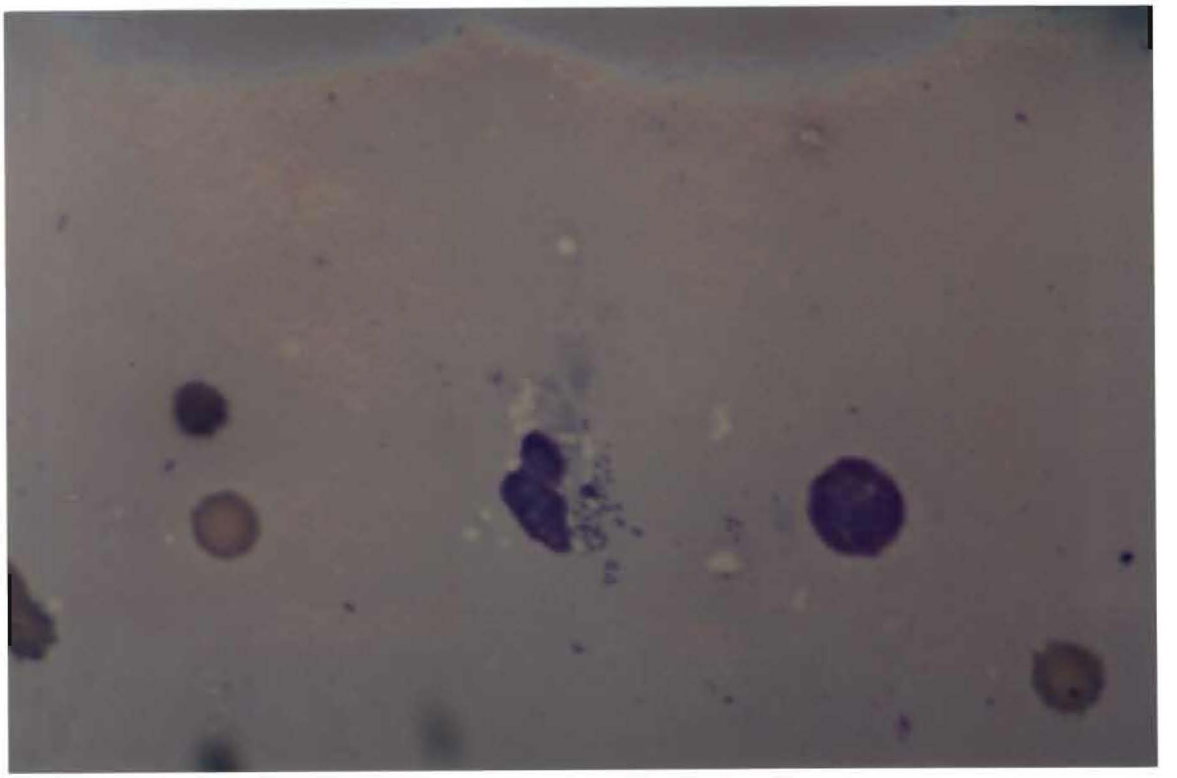
The power of the performed test (0.050) is below the desired power of 0.800.
You should interpret the negative findings cautiously.

Source of Variation	DF	SS	MS	F	P
Between Treatments	3	556.303	185.434	0.686	0.589
Residual	7	1893.333	270.476		
Total	10	2449.636			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.589).

Figure 4. Spleen impression slide from the zero-time room temperature sample set.

Figure 5. Liver impression slide from the zero-time room temperature sample set.



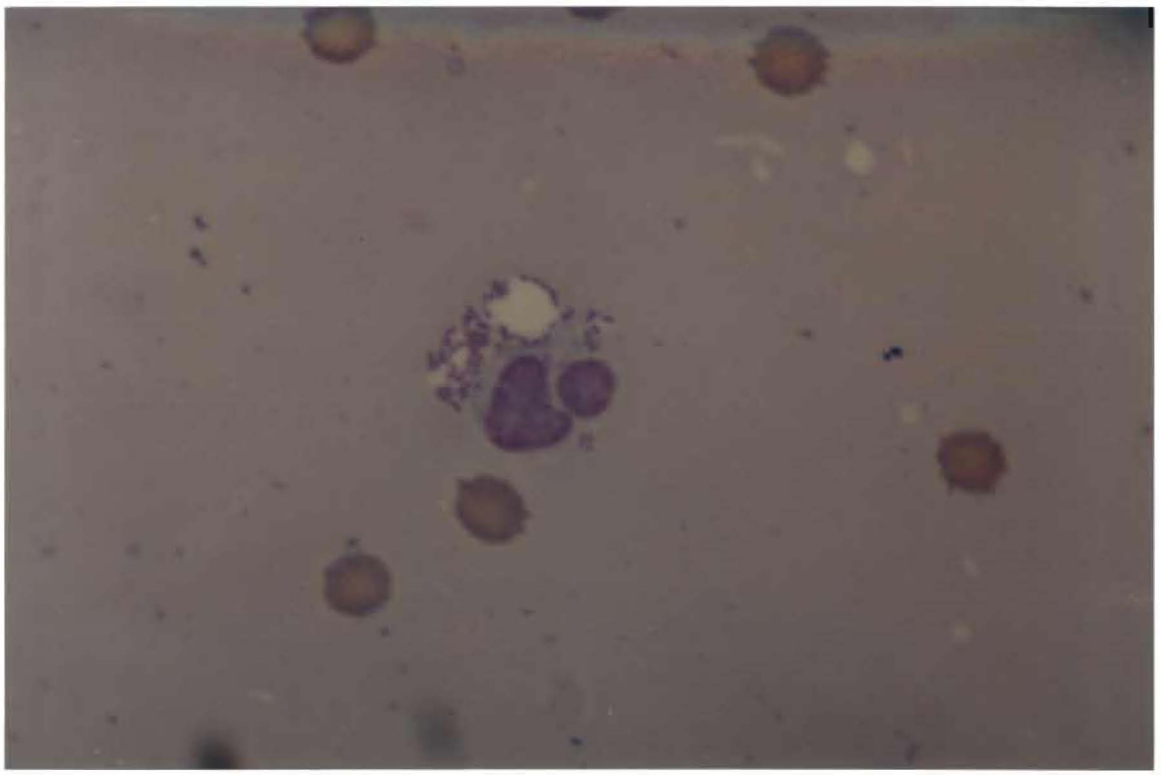
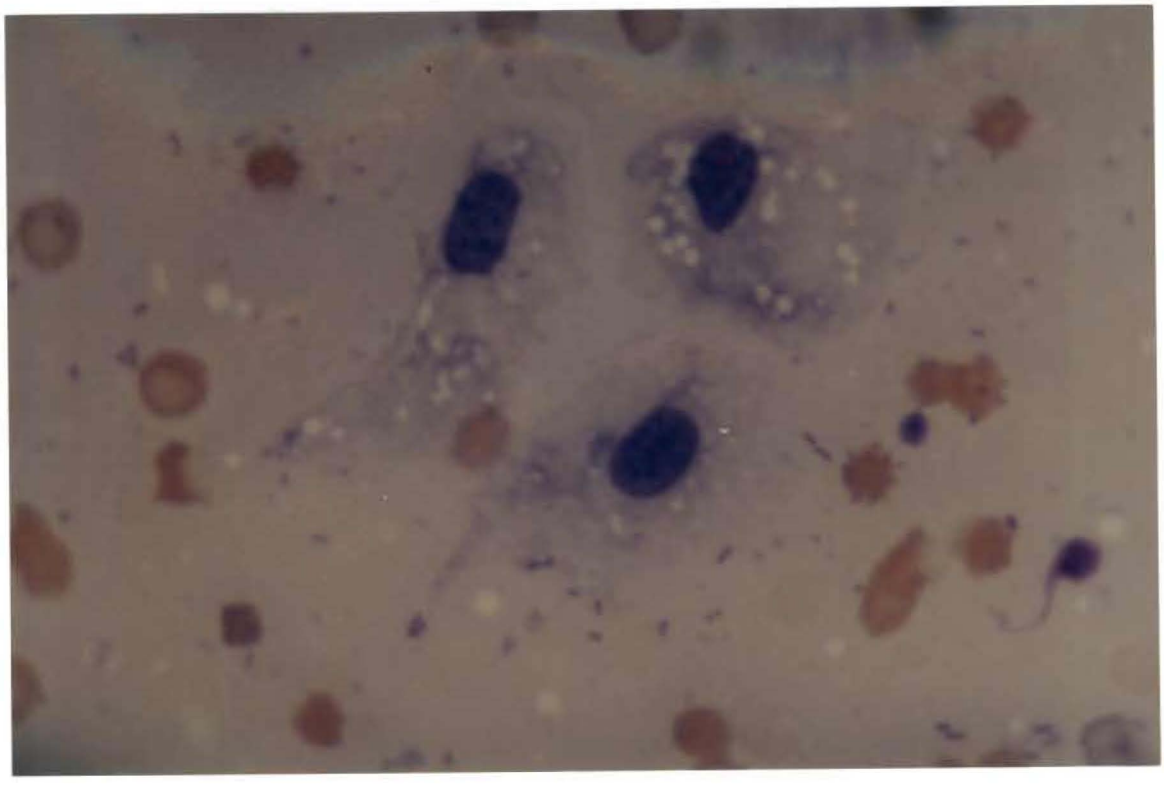


Figure 6. Spleen impression slide from the two-month experimental sample set.

Figure 7. Liver impression slide from the two-month experimental sample set.



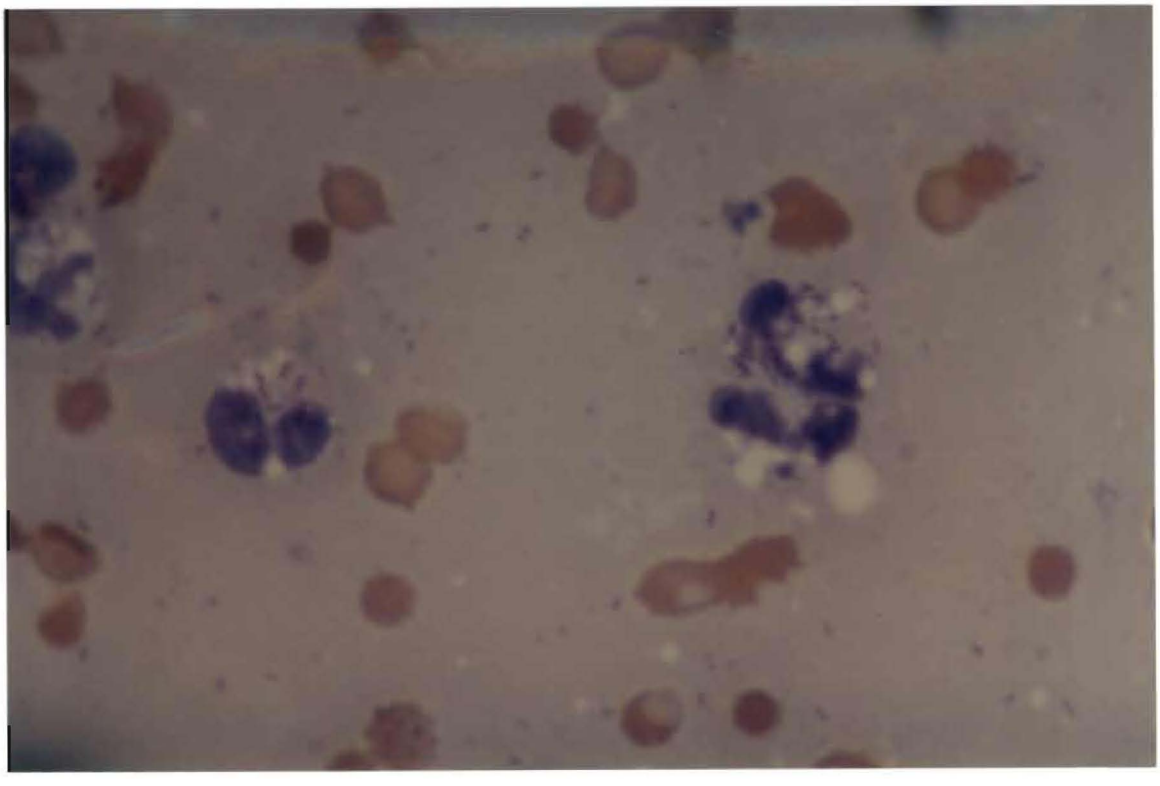
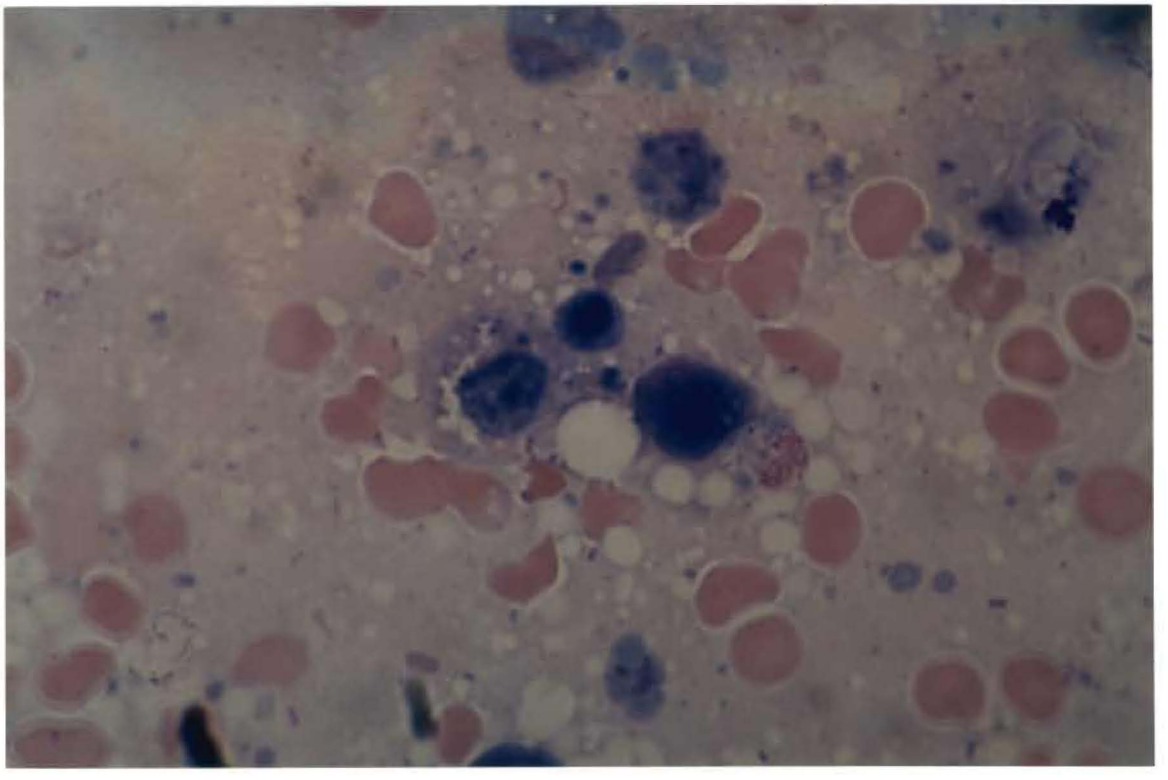


Figure 8. Spleen impression slide from the four-month experimental sample set.

Figure 9. Liver impression slide from the four-month experimental sample set.



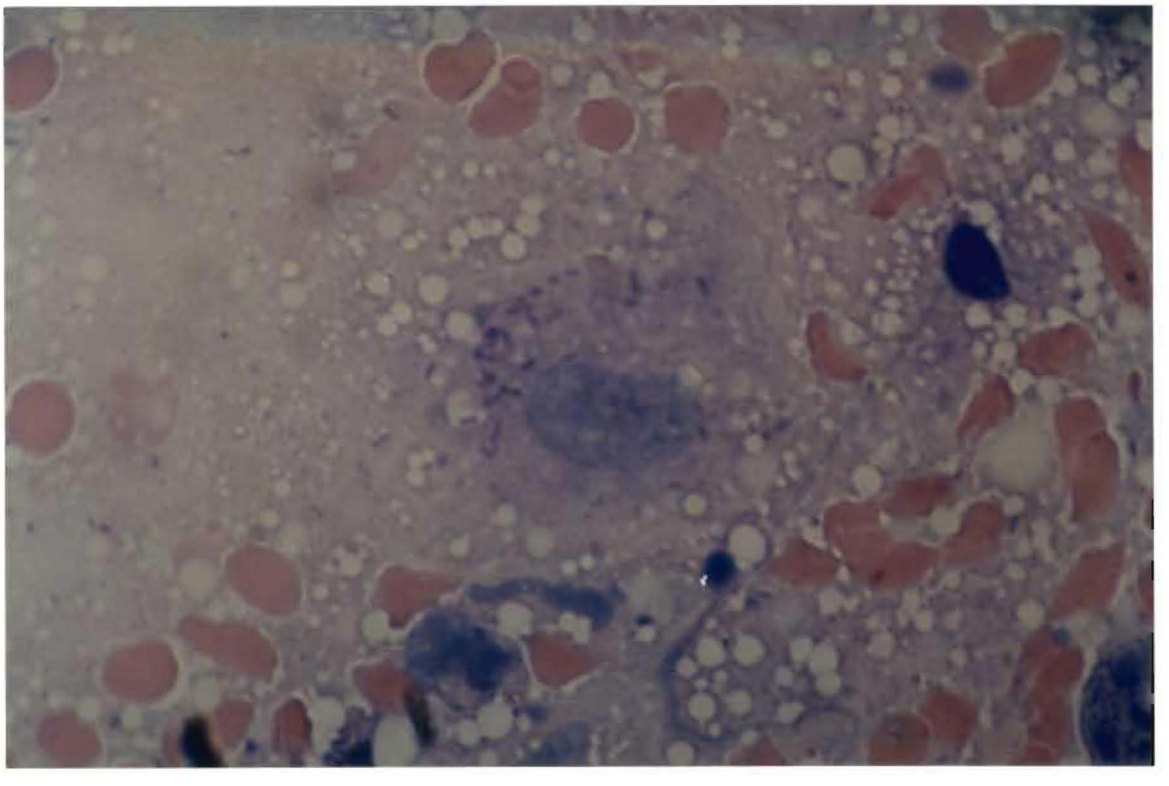
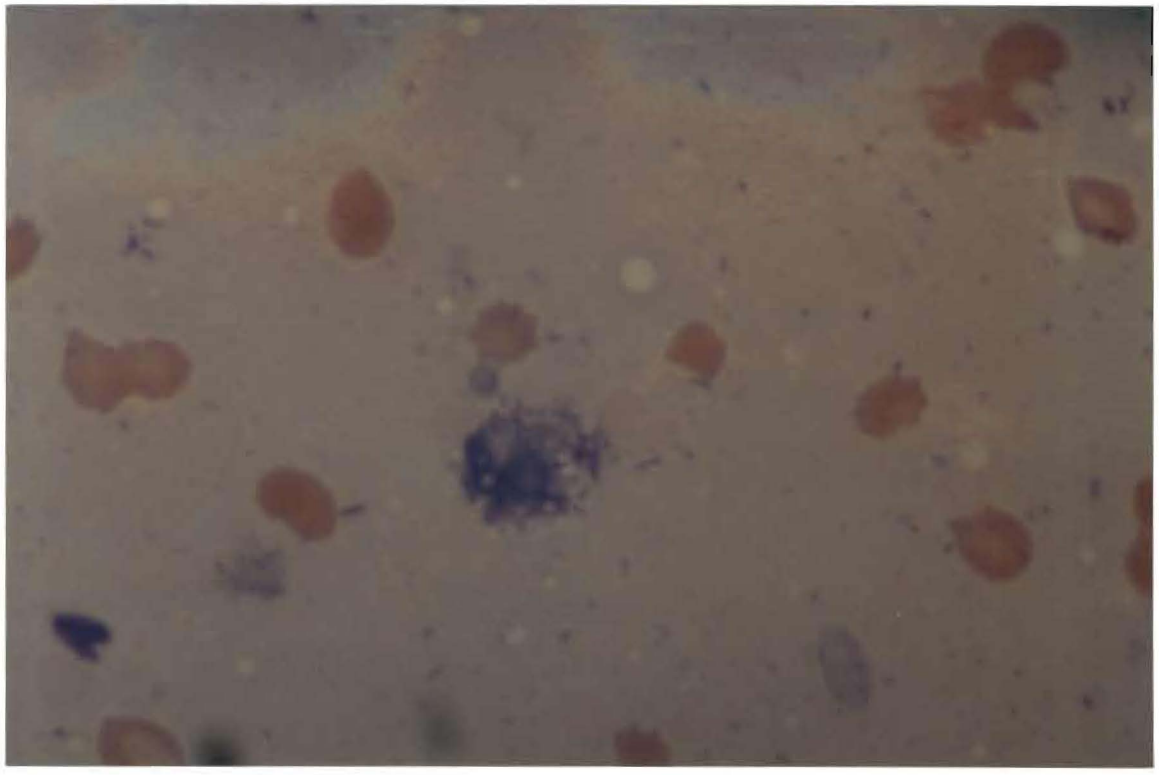
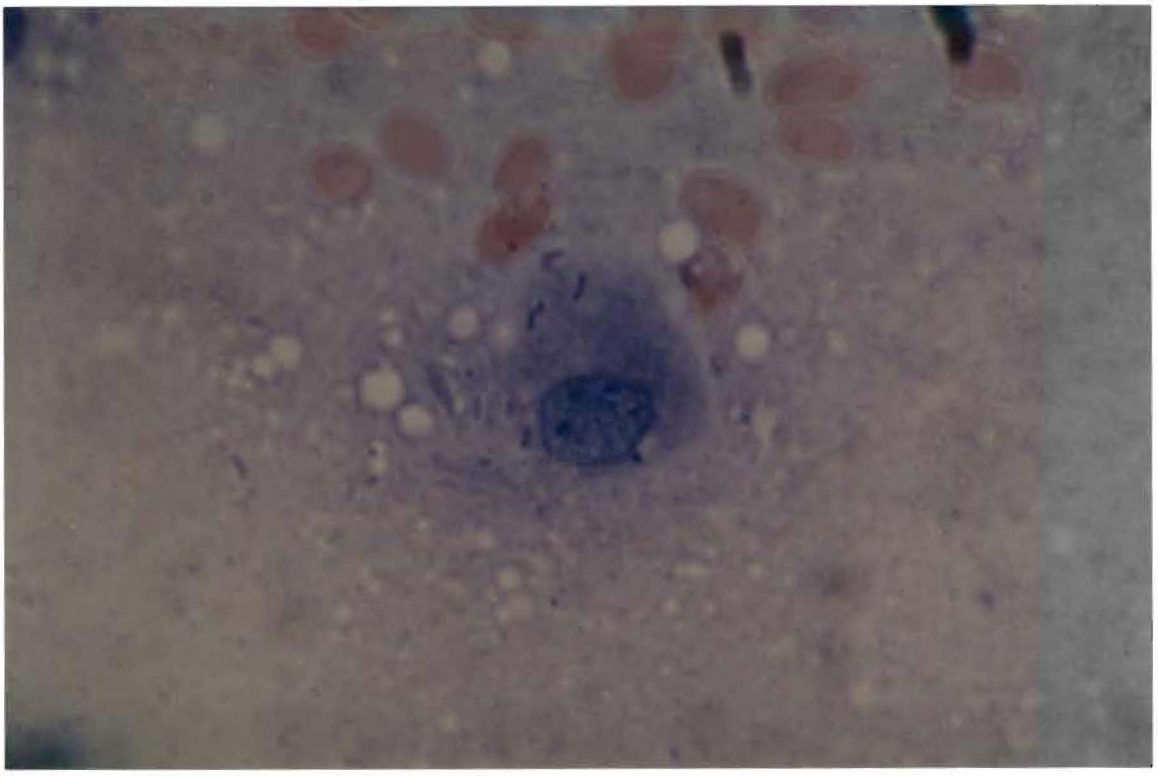


Figure 10. Spleen impression slide from the six-month experimental sample set.

Figure 11. Liver impression slide from the six-month experimental sample set.





CHAPTER 4

DISCUSSION

Presence of *Listeria* Within Food Products

Within the past few years, the number of food related illnesses has increased. There has also been an increase in awareness regarding the pathogens that cause these illnesses. *Listeria monocytogenes,* one of the primary food-borne pathogens, was the main focus of this study. *Listeria monocytogenes* has eluded physicians since its first description in 1926. The difficult isolation and identification of *Listeria,* along with the length of time to manifest symptoms, has been the main reason for its low profile (Low and Donachie, 1997). Both the United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA) have developed their own culture-based protocols for the isolation and identification of *Listeria* species. However, the main problem with these protocols is that they take about two weeks for test completion. The key to detection is the use of rapid detection assays and improved selective plating media. Two of the rapid detection assays that were the main interest to this study were the enzyme-linked immunosorbent assay (ELISA) and the deoxyribonucleic acid hybridization assay (DNAH).

According to the results obtained from this study, it is evident that *Listeria* spp. exist and survive within readily-available consumer products. The ELISA found 20 positive samples out of a total of 162 samples of the two food products straight from the grocer (Table 1), showing 12.35 percent of the total samples contaminated with *Listeria.* The DNA hybridization protocol found only six positive samples, or 3.7 percent of the

total contaminated samples (Table 1), and the USDA protocol identified 15 total positive samples, or 9.26 percent of the total contaminated (Table 1). Thus, a given grocery store's meat department could have as little as 3.7 or as much as 12.35 percent of their total product contaminated with *Listeria* spp. Although the average of 8.44% contamination might seem to be fairly low, if a particular grocer who fell in this average sold 1,000 pounds of meat in a day, 84.4 pounds of that total could be contaminated.

Given enough time, a small population of *Listeria* held at household refrigerator temperatures can proliferate into a substantial colony able to cause a food-borne infection. Once a critical population has been achieved, even cooking the product does not necessarily destroy the organisms. According to Farber (1989), rare-cooked meats might not be free of *Listeria*, even if only small amounts existed in the first place. Glass and Doyle (1989) showed that *L. monocytogenes* grew rather well on both chicken and turkey products stored at 4.4°C, or ordinary refrigerator temperatures. Their results showed an increase in population of 10^3 to 10^5 CFU/g in a four week span.

Detection of *Listeria monocytogenes* Using the ELISA

Results from the ELISA exhibited the expected values. Throughout the three testing periods and from both food products, the ELISA identified 25 positive and two negative samples. The 25 positives were the samples that had been inoculated with *L. monocytogenes* strain Scott A. The two negatives for each food product and time period were composed of the set of negative controls. None of the samples identified by the ELISA were false positive or false negative. Results from the ELISA can be interpreted to show that the enzyme-linked immunosorbent assay was extremely sensitive and

specific. Sensitivity and specificity reached 100% across the three experimental testing periods.

Detection of *Listeria monocytogenes* Using the DNAH Assay

As with the ELISA, the results presented were as expected. The two-month, four-month, and six-month sample sets exhibited 25 positives and two negatives for both food products. No false positive or false negative samples were determined throughout the three sample sets and both food products. In the same manner as the ELISA, the DNAH performed with 100% sensitivity across the three experimental time periods. Specificity was equally impressive with 100% specificity across all time periods for both the ground turkey and the turkey frankfurters.

The debate of the past few years about whether the ELISA or the DNAH is the best assay to use for food testing is definitely not over yet. According to the results of this particular study, neither is better than the other. Upon comparison of the results obtained using the ELISA and DNAH it can be stated that both assays are equally proficient in the detection of *Listeria monocytogenes* strain Scott A from both ground turkey and turkey frankfurters after being exposed to ultracold -70°C for two, four, and six months. A Chi square of the results from both assays run would show a Chi square value of zero, meaning that there was no statistically significant difference observed between the specificity and sensitivity of the two assays.

Detection of *Listeria monocytogenes* Using the USDA Protocol

In this study, Oxford agar identified 25 positive samples and two negative samples from both food products across all three time periods. No false positives or false negatives were identified using the Oxford agar. Sodium hippurate hydrolysis from the LPM agar identified 25 positives and two negatives from the ground turkey, across the three time periods. No false positives or false negatives were identified from the ground turkey samples.

Sodium hippurate hydrolysis on the turkey frankfurter samples, on the other hand, produced 25 positive readings and two negative readings from both the two-month and six-month sample sets. No false positive samples or false negative samples were identified from the two-month and six-month turkey frankfurter sample sets. From the four-month sample set, the Sodium hippurate hydrolysis identified 26 positive samples and one negative sample. One of the 26 positive samples was identified to be a false positive. Overall sensitivity and specificity of the USDA protocol were rather good. Sensitivity and specificity from the two-month and six-month sample sets were all 100%. Sensitivity for the four-month sample set was 99%, and specificity for the four-month sample set was a relatively low 87.5%, when compared to the ELISA and DNAH total specificity of 100% each. In comparison to the ELISA and DNAH assay, the USDA protocol was almost as sensitive but not quite as specific. The USDA is somewhat inferior to the ELISA and DNAH, not only because it is not quite as sensitive and specific but also because it takes more than an extra week for completion.

Virulence Retention and Survival of *Listeria monocytogenes*

One of the objectives of this study was to see if *L. monocytogenes* strain Scott A could survive extended periods of time under ultracold -70°C temperature. *L. monocytogenes* strain Scott A survived for two, four, and six months of exposure to the freezing conditions. Additionally, not only did *L. monocytogenes* survive over the three time periods but it also did so within two different food products that are known to harbor *Listeria* spp., ground turkey and turkey frankfurters. Poultry products tested by McClain and Lee (1988) were shown to be 33% contaminated.

Most of the injury done to organisms during freezing occurs within the first 24 hours of freezing (El-Kest and Marth, 1991), and continues to rise with time. One might predict that at six months, the injury toll would near 100%. However, the results in this study from the DNAH assay and the ELISA demonstrate that at least enough organisms survived the freezing periods to multiply and produce a positive reading after enrichment. Thus, during the enrichment period, the injured cells must repair themselves, aided by the enrichment media. As Morichi and Irie (1973) determined it is extremely difficult to know exactly what a particular organism needs to repair itself.

All of the mice that died were positive for *Listeria* infection. The number of hours after injection to the time of death was recorded. These numbers gave only a slight indication of the retained virulence of the resuscitated organism. *L. monocytogenes* that had not been subjected to ultracold conditions killed mice injected with it in 42, 42, and 72 hours, respectively. Mice injected with organism from two months of exposure died in 19, 56.5, and 64.5 hours, respectively. Mice injected with organism from four months of exposure died in 59 and 59 hours, respectively, and one did not die. The mouse that

did not die might have been injected directly into the intestines. If this had happened then most of the inoculum would have been passed from the body safely. Mice injected with organisms from six months of exposure died in 30, 42.5, and 43.5 hours respectively. All of the control mice survived and exhibited no signs of illness or unusual behavior.

The room temperature mice died in an average of 52 hours, the two-month mice died in 46.7 hours, the four-month mice in 59 hours, and the six-month mice in 38.7 hours. The overall trend shows a decrease in the amount of time that it took for the mice to die after injection. Although it cannot be definitively stated, because of small sample sizes, it appears that the ultracold -70°C might have selected for a more virulent organism. However, *L. monocytogenes* did retain its virulence throughout the experimental time periods in freezing temperatures.

CHAPTER 5

SUMMARY

Listeria monocytogenes has been one of the most detrimental food-borne pathogens within the past few years. It has cost companies such as Bil Mar and Thorn Apple Valley, millions of dollars on food recalls. *Listeria monocytogenes* has also had a human cost by those who have been ill or died from eating contaminated food. The key to controlling and preventing the spread of contaminated food products to the consumer is the use and development of highly-sensitive and specific rapid detection assays. To develop new and improved assays, more information is needed about how well the current assays work and about the limits of the organism itself.

The objectives of this study were four-fold. The primary objective was to see if *Listeria monocytogenes* strain Scott A could survive extended periods of time under an ultracold -70°C. The second objective was to determine if there was a difference in the survival of *L. monocytogenes* within two different substrates. The third objective was to compare the effectiveness of isolation of an enzyme-linked immunosorbent assay (ELISA), a deoxyribonucleic acid hybridization assay (DNAH), and a modified USDA culture-based protocol from the two substrates. The fourth objective was to determine if *L. monocytogenes* strain Scott A subjected to -70°C could retain its virulence.

Results from this study revealed that both of the rapid detection assays, the ELISA and DNAH, were extremely sensitive and specific. The USDA culture-based protocol was still quite sensitive and specific but less so than either of the rapid detection assays. In running both of the rapid detection assays, one can get a feel for which is a

better assays and which is easier to run. I found the ELISA and DNAH were equally efficient, but the DNAH was easier to run, even though it took about one hour longer for completion. All three protocols seemed to work equally well within the two different food products. No difference in effectiveness of the rapid detection assays for a particular food product was shown.

This study also demonstrated that *L. monocytogenes* strain Scott A can survive within ground turkey and turkey frankfurters for extended periods of time under ultracold (-70°C) temperatures. Data of this kind show that freezing does not kill all of the organisms that reside within the product, even after six months of freezing under even more stringent conditions than normal. Of more importance are the results showing that the organism did not lose its virulence throughout the experimental time periods. More studies need to be done to examine the issue of virulence retention in ultracold temperatures.

CHAPTER 6

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APPENDICES

Appendix A. Absorbance values from the two-month experimental sample set Using the DNAH. The absorbance values are from both ground turkey and turkey Frankfurters at room temperature and after exposure to -70°C. Positives and Negatives were determined with the use of cutoff values.

Sample #	02tD	02fD	2tD	2fD
1	0.01(-)	0.01(-)	1.88(+)	1.91(+)
2	0(-)	0.03(-)	1.82(+)	1.94(+)
3	0(-)	0.01(-)	2.04(+)	1.86(+)
4	0.01(-)	0.45(+)	1.83(+)	1.96(+)
5	0.01(-)	0(-)	1.81(+)	1.83(+)
6	0(-)	0.02(-)	1.51(+)	1.85(+)
7	0(-)	0.02(-)	1.46(+)	1.77(+)
8	0(-)	0.01(-)	2.05(+)	1.90(+)
9	0.01(-)	0.01(-)	1.87(+)	1.80(+)
10	0(-)	0.14(+)	1.95(+)	1.78(+)
11	0(-)	0.01(-)	1.98(+)	1.84(+)
12	0(-)	0.01(-)	2.04(+)	1.98(+)
13	0.06(-)	0.02(-)	1.81(+)	1.68(+)
14	0(-)	0.68(+)	1.85(+)	1.87(+)
15	0(-)	0.01(-)	2.01(+)	1.88(+)
16	0(-)	0(-)	1.96(+)	1.71(+)
17	0.01(-)	0.01(-)	2.02(+)	1.85(+)
18	0.01(-)	0.01(-)	1.49(+)	1.84(+)
19	0(-)	0.02(-)	1.78(+)	1.88(+)
20	0(-)	0.01(-)	1.82(+)	1.84(+)
21	0.01(-)	0.01(-)	1.60(+)	1.74(+)
22	0(-)	0.01(-)	1.78(+)	1.66(+)
23	0.06(-)	0.03(-)	1.98(+)	1.87(+)
24	0.08(-)	0.28(+)	1.88(+)	1.82(+)
25	0.01(-)	0.01(-)	1.71(+)	1.81(+)
26	0.01(-)	0(-)	0(-)	0(-)
27	0(-)	0.01(-)	0.01(-)	0.01(-)
Control (+)	3.05(+)	1.37(+)	1.91(+)	1.91(+)
Control (-)	0.04(-)	0.03(-)	0.01(-)	0.01(-)

02tD= room temperature, two-month set, ground turkey, DNAH
02fD= room temperature, two-month set, turkey frankfurters, DNAH
2tD= experimental, two-month set, ground turkey, DNAH
2fD= experimental, two-month set, turkey frankfurters, DNAH

Appendix B. Absorbance values from the four-month experimental sample set Using the DNAH. The absorbance values are from both ground turkey and turkey Frankfurters at room temperature and after exposure to -70°C. Positives and Negatives were determined with the use of cutoff values.

Sample #	04tD	04fD	4tD	4fD
1	0.01(-)	0.01(-)	2.32(+)	2.16(+)
2	0.01(-)	0.01(-)	2.30(+)	1.71(+)
3	0.01(-)	0.01(-)	2.26(+)	2.02(+)
4	0.02(-)	0.03(-)	2.24(+)	1.87(+)
5	0.06(-)	0(-)	2.13(+)	2.00(+)
6	0.01(-)	0.03(-)	2.15(+)	1.89(+)
7	0(-)	0.01(-)	2.23(+)	1.94(+)
8	0.02(-)	0(-)	2.25(+)	2.01(+)
9	0.01(-)	0.01(-)	2.08(+)	1.84(+)
10	0.05(-)	0.02(-)	2.09(+)	1.90(+)
11	0(-)	0.01(-)	2.21(+)	2.06(+)
12	0.02(-)	0.01(-)	2.10(+)	1.97(+)
13	0(-)	0(-)	2.11(+)	1.91(+)
14	0.02(-)	0(-)	2.16(+)	1.86(+)
15	0.02(-)	0.06(-)	2.25(+)	1.95(+)
16	0.02(-)	0.02(-)	2.13(+)	1.91(+)
17	0.01(-)	0.01(-)	2.06(+)	1.86(+)
18	0.01(-)	0.02(-)	2.10(+)	1.80(+)
19	0.01(-)	0.02(-)	2.18(+)	2.02(+)
20	0.03(-)	0(-)	2.14(+)	1.89(+)
21	0.01(-)	0(-)	2.25(+)	2.07(+)
22	0.04(-)	0.02(-)	2.09(+)	2.00(+)
23	0.01(-)	0.03(-)	2.16(+)	2.01(+)
24	0.01(-)	0.02(-)	2.07(+)	1.75(+)
25	0(-)	0.01(-)	2.10(+)	1.93(+)
26	0(-)	0(-)	0(-)	0.02(-)
27	0.02(-)	0(-)	0(-)	0.01(-)
Control (+)	3.66(+)	1.37(+)	2.08(+)	2.08(+)
Control (-)	0.04(-)	0.03(-)	0.02(-)	0.02(-)

04tD= room temperature, four-month set, ground turkey, DNAH
04fD= room temperature, four-month set, turkey frankfurters, DNAH
4tD= experimental, four-month set, ground turkey, DNAH
4fD= experimental, four-month set, turkey frankfurters, DNAH

Appendix C. Absorbance values from the six-month experimental sample set Using the DNAH. The absorbance values are from both ground turkey and turkey Frankfurters at room temperature and after exposure to -70°C. Positives and Negatives were determined with the use of cutoff values.

Sample #	06tD	06fD	6tD	6fD
1	0.02(-)	0.02(-)	1.58(+)	1.55(+)
2	0(-)	0.02(-)	1.40(+)	1.70(+)
3	0.01(-)	0.02(-)	1.38(+)	1.77(+)
4	0(-)	0.02(-)	1.53(+)	1.83(+)
5	0.01(-)	0.33(+)	1.46(+)	1.86(+)
6	0.01(-)	0.01(-)	1.07(+)	1.88(+)
7	0(-)	0.02(-)	1.52(+)	1.68(+)
8	0.01(-)	0.02(-)	1.52(+)	1.84(+)
9	0(-)	0.02(-)	1.34(+)	1.82(+)
10	0.01(-)	0.01(-)	1.61(+)	1.66(+)
11	0.01(-)	0.01(-)	1.42(+)	1.83(+)
12	0(-)	0.02(-)	1.85(+)	1.69(+)
13	0.04(-)	0.03(-)	1.56(+)	1.79(+)
14	0.01(-)	0.03(-)	1.50(+)	1.66(+)
15	0.02(-)	0.02(-)	1.43(+)	1.73(+)
16	0.07(-)	0.01(-)	1.35(+)	1.71(+)
17	0(-)	0.26(+)	1.58(+)	1.65(+)
18	0.03(-)	0.02(-)	1.66(+)	1.75(+)
19	0.02(-)	0.01(-)	1.54(+)	1.73(+)
20	0.03(-)	0.01(-)	1.29(+)	1.78(+)
21	0.03(-)	0.01(-)	1.34(+)	1.71(+)
22	0.02(-)	0.01(-)	1.56(+)	1.78(+)
23	0.01(-)	0.02(-)	1.44(+)	1.79(+)
24	0.01(-)	0.02(-)	1.41(+)	1.82(+)
25	0.02(-)	0.02(-)	1.60(+)	1.20(+)
26	0.01(-)	0(-)	0.01(-)	0.01(-)
27	0.01(-)	0.02(-)	0.01(-)	0.01(-)
Control (+)	1.98(+)	1.37(+)	1.78(+)	1.78(+)
Control (-)	0.04(-)	0.03(-)	0.01(-)	0.01(-)

06tD= room temperature, six-month set, ground turkey, DNAH
06fD= room temperature, six-month set, turkey frankfurters, DNAH
6tD= experimental, six-month set, ground turkey, DNAH
6fD= experimental, six-month set, turkey frankfurters, DNAH

Appendix D. Absorbance values from the two-month experimental sample set Using the ELISA. The absorbance values are from both ground turkey and turkey Frankfurters at room temperature and after exposure to -70°C. Positive and Negatives were determined with the use of cutoff values.

Sample #	02tE	02fE	2tE	2fE
1	*.***(+)	0.017(-)	*.***(+)	*.***(+)
2	1.568(+)	0.108(-)	*.***(+)	*.***(+)
3	0.934(+)	0.084(-)	*.***(+)	*.***(+)
4	0.437(+)	0.102(-)	*.***(+)	*.***(+)
5	0.270(+)	0.076(-)	*.***(+)	*.***(+)
6	0.165(-)	0.109(-)	*.***(+)	*.***(+)
7	0.073(-)	0.072(-)	*.***(+)	*.***(+)
8	0.232(-)	0.201(-)	*.***(+)	*.***(+)
9	0.478(+)	0.074(-)	*.***(+)	*.***(+)
10	0.534(+)	0.067(-)	*.***(+)	*.***(+)
11	0.142(-)	0.138(-)	*.***(+)	*.***(+)
12	0.116(-)	0.136(-)	*.***(+)	*.***(+)
13	0.048(-)	0.086(-)	*.***(+)	*.***(+)
14	0.049(-)	0.091(-)	*.***(+)	*.***(+)
15	0.064(-)	0.111(-)	*.***(+)	*.***(+)
16	0.167(-)	0.080(-)	*.***(+)	*.***(+)
17	0.349(+)	0.090(-)	*.***(+)	*.***(+)
18	0.122(-)	0.093(-)	*.***(+)	*.***(+)
19	0.099(-)	0.093(-)	*.***(+)	*.***(+)
20	0.079(-)	0.213(-)	*.***(+)	*.***(+)
21	0.065(-)	0.069(-)	*.***(+)	*.***(+)
22	0.071(-)	0.064(-)	*.***(+)	*.***(+)
23	0.110(-)	0.140(-)	*.***(+)	*.***(+)
24	1.092(+)	0.174(-)	*.***(+)	*.***(+)
25	0.962(+)	0.095(-)	*.***(+)	*.***(+)
26	0.072(-)	0.072(-)	0.100(-)	0.106(-)
27	0.075(-)	0.074(-)	0.112(-)	0.109(-)
Control (+)	*.***(+)	*.***(+)	*.***(+)	*.***(+)
Control (-)	0.067(-)	0.149(-)	0.128(-)	0.128(-)
Control (-)	0.104(-)	0.103(-)	0.105(-)	0.105(-)
NCX	0.0855	0.126	0.1165	0.1165

Cutoff	0.2355	0.276	0.2665	0.2665

02tE= room temperature, two-month set, ground turkey, ELISA
02fE= room temperature, two-month set, turkey frankfurters, ELISA
2tE= experimental, two-month set, ground turkey, ELISA
2fE= experimental, two-month set, turkey frankfurters, ELISA

*.*** indicates that the absorbance, or optical density, was greater than the Microplate plate reader could indicate with a number value.

NCX= Mean negative control value
Cutoff= NCX + 0.150

Appendix E. Absorbance values from the four-month experimental sample set Using the ELISA. The absorbance values are from both ground turkey and turkey Frankfurters at room temperature and after exposure to –70°C. Positives and Negatives were determined with the use of cutoff values.

Sample #	04tE	04fE	4tE	4fE
1	0.346(+)	0.086(-)	*.***(+)	*.***(+)
2	0.086(-)	0.118(-)	*.***(+)	*.***(+)
3	0.076(-)	0.109(-)	*.***(+)	*.***(+)
4	0.059(-)	0.070(-)	*.***(+)	*.***(+)
5	0.067(-)	0.154(-)	*.***(+)	*.***(+)
6	0.093(-)	0.040(-)	*.***(+)	*.***(+)
7	0.146(-)	0.102(-)	*.***(+)	*.***(+)
8	0.456(+)	0.067(-)	*.***(+)	*.***(+)
9	0.084(-)	0.109(-)	1.333(+)	*.***(+)
10	0.069(-)	0.093(-)	0.750(+)	*.***(+)
11	0.062(-)	0.069(-)	*.***(+)	*.***(+)
12	0.081(-)	0.052(-)	*.***(+)	*.***(+)
13	0.059(-)	0.199(-)	*.***(+)	*.***(+)
14	0.074(-)	0.087(-)	*.***(+)	*.***(+)
15	0.164(-)	0.089(-)	*.***(+)	*.***(+)
16	0.192(-)	0.147(-)	*.***(+)	*.***(+)
17	0.070(-)	0.116(-)	1.836(+)	*.***(+)
18	0.060(-)	0.086(-)	*.***(+)	*.***(+)
19	0.086(-)	0.093(-)	1.231(+)	*.***(+)
20	0.071(-)	0.099(-)	*.***(+)	*.***(+)
21	0.061(-)	0.106(-)	1.096(+)	*.***(+)
22	0.095(-)	0.054(-)	*.***(+)	*.***(+)
23	0.202(-)	0.073(-)	*.***(+)	*.***(+)
24	0.873(+)	0.132(-)	1.687(+)	*.***(+)
25	0.078(-)	0.070(-)	*.***(+)	*.***(+)
26	0.078(-)	0.071(-)	0.279(-)	0.178(-)
27	0.070(-)	0.076(-)	0.286(-)	0.195(-)
Control (+)	*.***(+)	*.***(+)	*.***(+)	*.***(+)
Control (-)	0.067(-)	0.149(-)	0.154(-)	0.154(-)
Control (-)	0.104(-)	0.103(-)	0.153(-)	0.153(-)
NCX	0.0855	0.126	0.1535	0.1535

Cutoff	0.2355	0.276	0.3035	0.3035

04tE= room temperature, four-month set, ground turkey, ELISA
04fE= room temperature, four-month set, turkey frankfurters, ELISA
4tE= experimental, four-month set, ground turkey, ELISA
4fE= experimental, four-month set, turkey frankfurters, ELISA

*.*** indicates that the absorbance, or optical density, was greater than the Microplate plate reader could indicate with a number value.

NCX= Mean negative control value
Cutoff= NCX + 0.150

Appendix F. Absorbance values from the six-month experimental sample set Using the ELISA. The absorbance values are room both ground turkey and turkey Frankfurters at room temperature and after exposure to -70°C. Positives and Negatives were determined with the use of cutoff values.

Sample #	06tE	06fE	6tE	6fE
1	0.078(-)	0.127(-)	*.***(+)	*.***(+)
2	0.079(-)	0.086(-)	*.***(+)	*.***(+)
3	0.075(-)	0.164(-)	*.***(+)	*.***(+)
4	0.088(-)	0.135(-)	*.***(+)	*.***(+)
5	0.168(-)	0.391(+)	*.***(+)	*.***(+)
6	0.251(+)	0.160(-)	*.***(+)	*.***(+)
7	0.375(+)	0.111(-)	*.***(+)	*.***(+)
8	0.110(-)	0.086(-)	*.***(+)	*.***(+)
9	0.086(-)	0.109(-)	1.685(+)	*.***(+)
10	0.110(-)	0.101(-)	*.***(+)	*.***(+)
11	0.087(-)	0.145(-)	*.***(+)	*.***(+)
12	0.083(-)	0.091(-)	*.***(+)	*.***(+)
13	0.082(-)	0.120(-)	*.***(+)	*.***(+)
14	0.097(-)	0.238(-)	*.***(+)	*.***(+)
15	0.514(+)	0.159(-)	*.***(+)	*.***(+)
16	0.224(-)	0.095(-)	*.***(+)	*.***(+)
17	0.116(-)	0.079(-)	*.***(+)	*.***(+)
18	0.086(-)	0.169(-)	*.***(+)	*.***(+)
19	0.067(-)	0.103(-)	*.***(+)	*.***(+)
20	0.087(-)	0.094(-)	*.***(+)	*.***(+)
21	0.103(-)	0.144(-)	*.***(+)	*.***(+)
22	0.196(-)	0.153(-)	*.***(+)	*.***(+)
23	0.684(+)	1.056(+)	*.***(+)	*.***(+)
24	0.446(+)	0.138(-)	*.***(+)	*.***(+)
25	0.162(-)	0.151(-)	*.***(+)	*.***(+)
26	0.082(-)	0.069(-)	0.185(-)	0.143(-)
27	0.076(-)	0.078(-)	0.241(-)	0.175(-)
Control (+)	*.***(+)	*.***(+)	*.***(+)	*.***(+)
Control (-)	0.067(-)	0.149(-)	0.125(-)	0.125(-)
Control (-)	0.104(-)	0.103(-)	0.127(-)	0.127(-)
NCX	0.0855	0.126	0.126	0.126

Cutoff	0.2355	0.276		0.276	0.276

06tE= room temperature, six-month set, ground turkey, ELISA
06fE= room temperature, six-month set, turkey frankfurters, ELISA
6tE= experimental, six-month set, ground turkey, ELISA
6fE= experimental, six-month set, turkey frankfurters, ELISA

*.*** indicates that the absorbance, or optical density, was greater than the Microplate plate reader could indicate with a number value.

NCX= Mean negative control value
Cutoff= NCX + 0.150

Signature of Graduate Student

Billie L. Johnson

Signature of Major Advisor

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Date

Detection of *Listeria monocytogenes* in ground turkey and turkey frankfurters using ELISA, DNAH, and USDA protocols prior to, and following, exposure to ultracold temperatures

Title of Thesis

Signature of Graduate Office Staff

December 9, 1999

Date Received