AN ABSTRACT OF THE THESIS OF

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Title: Acquisition of *Listeria monocytogenes* from Powdered Dairy Products Using ELISA and DNA Gene Probe Assays Prior to, and Following, Freezing of Samples at -10^{0}C and -70^{0}C

Abstract Approved: Billie L Johnson

Listeria monocytogenes is a human pathogen commonly found in soil, water, vegetation, and foods such as meats, poultry, and dairy products. Over the past two decades, there has been an increase in disease caused by *Listeria.* Research has shown that *Listeria monocytogenes* can survive heating, freezing, and other harsh conditions. The injured cells have the capability to repair and multiply under favorable conditions, and thus, regain their ability to cause disease. Therefore, there is a need to identify injured bacterial cells as well as healthy cells. Cultivation based methods developed by the Food and Drug Administration and the United States Department of Agriculture take from 3-28 days to determine if a food product is contaminated with *Listeria monocytogenes.* An enzyme-linked immunosorbent assay (ELISA) and a deoxyribonucleic acid hybridization assay (DNAH) have been developed to analyze food contamination more rapidly than the standard culture methods.

This study compared the ELISA and the DNAH assay by testing dairy products that had been inoculated with *Listeria monocytogenes* and then exposed to freezing conditions. The ELISA identified all positive samples, whereas the DNAH assay identified all but three positives. Both assays mis-identified only one negative sample each. This study also compared four plating agars used in the determination of *Listeria*. Trypticase soy agar-yeast extract (TSA-YE) allowed growth on all plates streaked, but gram staining showed that *Listeria* was not always present because of other competitive flora. Lithium chloride-phenylethanol-moxalactam (LPM) and Modified Oxford medium (MOX) produced the best results. The presence of *Listeria* was more identifiable on MOX plates.

Acquisition of *Listeria monocytogenes* from Powdered Dairy Products Using ELISA and DNA Gene Probe Assays Prior to, and Following, Freezing of Samples at -10^{0}C and -70^{0}C

A Thesis

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by

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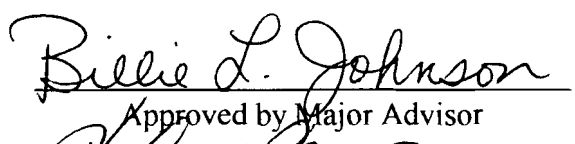

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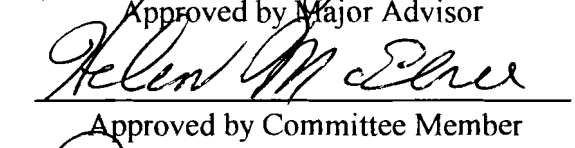

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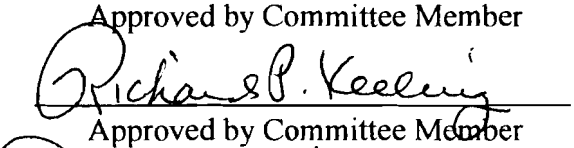

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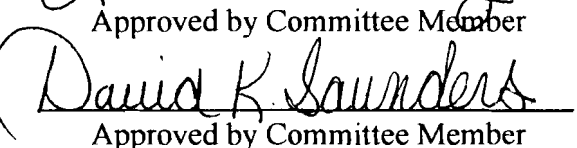

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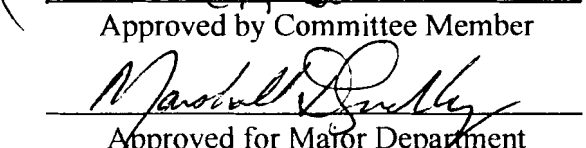

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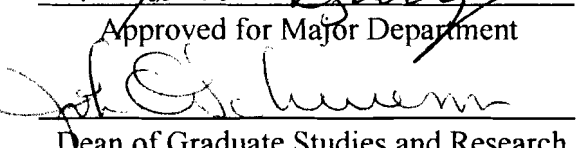

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CHAPTER 1

INTRODUCTION

General Background

There are seven different species of the genus *Listeria,* with the only human pathogen being *Listeria monocytogenes*, which was first described in 1926 (Murray et al., 1994; Butman et al., 1988). *Listeria monocytogenes* are gram-positive, facultative anaerobes, and non-spore forming bacilli approximately 0.4 to 0.5 μm in diameter and 0.5 to 2.0 μm in length (Gray and Killinger, 1966). This organism possesses great motility at temperatures of 20^0C to 25^0C and can grow in a wide pH range from 6.0 to 9.0 (Schuchat et al., 1991). *Listeria* can also multiply in temperatures ranging from 2.5^0C to 37^0C (Murray et al., 1994). The optimum temperature for growth is between 30^0C and 37^0C (Schuchat et al., 1991). *Listeria* species are primarily found in soil, in water, on plants and vegetation, and in sewage (Salyers and Whitt, 1994). However, in the past few decades, *Listeria* has been identified in numerous food products, including various meats, poultry, fish, ice cream, and dairy products (Schuchat et al., 1991). Contamination of food products resulting in public consumption has led to a human disease known as listeriosis, and thus has caused a great concern for health officials (Gellin et al., 1991).

Listeriosis was first identified in New Zealand sheep after causing a characteristic form of encephalitis known as "circling disease." The sheep developed a severe disturbance in their gait, which was soon followed by death (Gill, 1937). Listeriosis was also later found in cattle and other breeds of sheep. The first case of human listeriosis

was identified in East Germany when a newborn baby was described as having granulomatosis infantiseptica (Potel, 1943-54). In the late 1950s, the first adult cases involved meningitis and cerebritis symptomology (Bowie et al., 1983). Other symptomology seen in patients with listeriosis are bacteremia, septicemia, fever, chills, endocarditis, fatigue, nausea, vomiting, diarrhea, miscarriages, stillbirths, pneumonia, and death (Murray et al., 1994; Salyers and Whitt, 1994). Symptoms can occur anywhere from one to six weeks after consumption of contaminated food. Listeriosis can be responsible for epidemic outbreaks or sporadic cases.

Treatment for listeriosis includes penicillin G, ampicillin, aminoglycosides, erythromycin, trimethoprim-sulfamethoxazole, chloramphenicol, rifampin, and tetracyclines (Schuchat et al., 1991). Penicillin and ampicillin are usually recommended. Ampicillin is believed to be superior to penicillin (Lavetter et al., 1971). Prevention methods include washing raw vegetables, avoiding consumption of raw foods of animal origin, not drinking unpasteurized milk, thoroughly cooking all foods and heating leftovers, and not leaving foods at room temperature more than two hours (Anonymous, 1992).

Outbreaks

Since the identification of contaminated food products, *Listeria monocytogenes* has been responsible for several outbreaks worldwide. Reports have indicated that there is a significant upward trend in disease caused by *Listeria* (Schlech, 1991). The first outbreak occurred in Maritime Provinces of Canada where listeriosis occurred in 1.3% of

births between March and September of 1981. There were seven adult and 34 perinatal cases with a fatality rate among newborn infants of 27% (Schlech et al., 1983). The outbreak was caused by coleslaw that had been made from cabbage fertilized by raw sheep manure. It was determined that the manure contained *Listeria monocytogenes* from infected sheep (Schuchat et al., 1991).

A second large outbreak occurred in Massachusetts from the end of June to the end of August in 1983. This outbreak affected immunocompromised nonpregnant adults, and the fatality rate was 29%. The contaminated food product was traced to pasteurized whole and 2% milk. Scientists first questioned the ability of *Listeria* to survive the pasteurization process, but it was later concluded that contamination had occurred after pasteurization in the processing plant. This outbreak was, however, responsible for future experiments in determining the heat resistance of *Listeria* (Fleming et al., 1985).

The largest outbreak in North America occurred in 1985 in Los Angeles, California. The majority of infections were found in pregnant women and their newborn infants. The case fatality rate was 63% for early-onset (newly-born) infected infants and 37% for late-onset (several days to weeks after birth) infected infants. *Listeria monocytogenes* was traced to a Mexican-style soft cheese that had been processed with unpasteurized milk (Linnan et al., 1988).

One of the most recent outbreaks occurred in France in 1992. There were 279 cases, resulting in 22 abortions and 63 deaths. Contamination of cooked products had occurred in the processing plants due to poor disinfection techniques (Salvat et al., 1995).

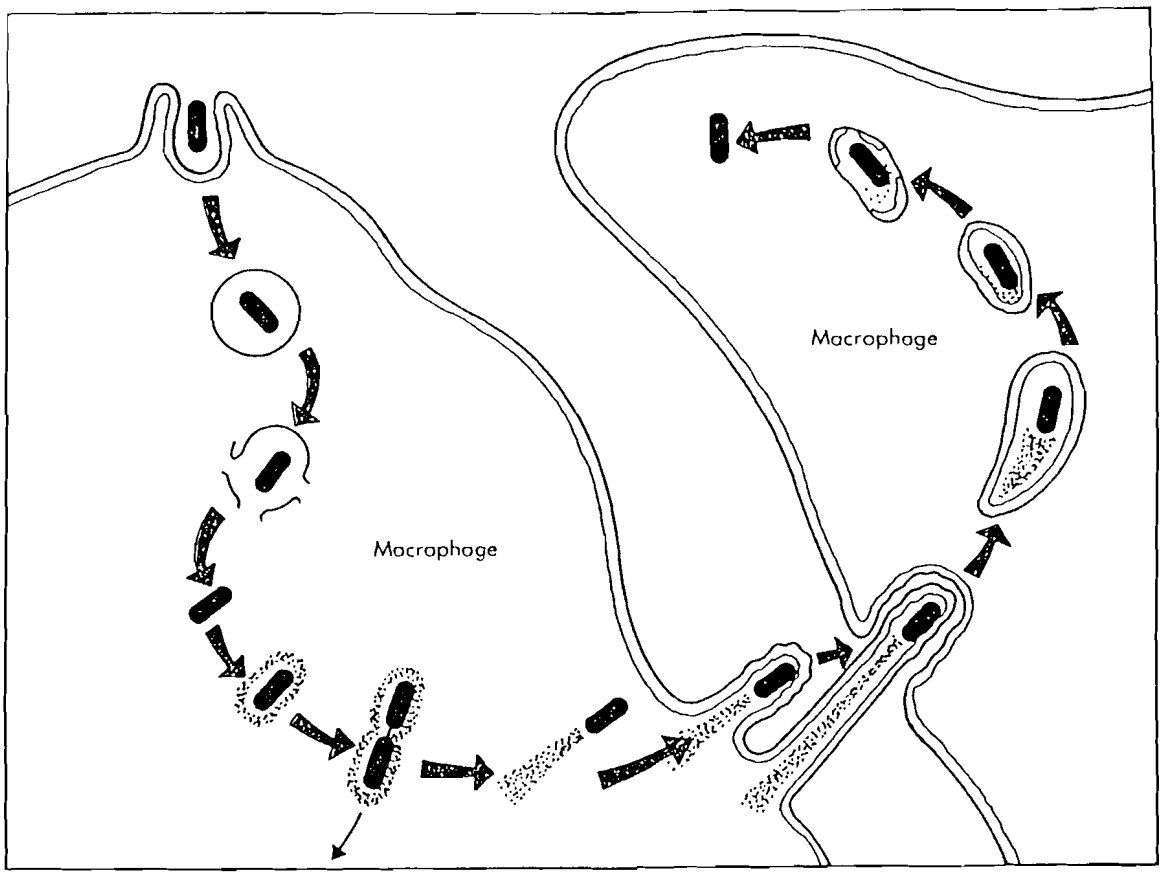
Surveillances

Over the past few decades, there has been an increase in listeriosis cases worldwide (Schlech, 1991). This increase not only means more infected persons, but also more contaminated food products, causing an increase in the total cost created by listeriosis disease. In the 1950s, Canadian officials recorded approximately 15 cases a year. However, surveillances now estimate 48 cases a year with an approximate annual cost of 11.1-12.6 million dollars (Farber et al., 1996). In Scotland, Campbell (1990) recorded an increase of 0.5 cases per million to 7 cases per million between the years of 1967 to 1988. In the United States, a surveillance projected over 1,700 cases and approximately 550 deaths in the year of 1986 from listeriosis (Gellin et al., 1991). This research group found over 40 commercial food products were recalled or not distributed because of contamination with *Listeria monocytogenes*. Gellin et al. (1991) concluded that persons with conditions such as immunosuppressive disorders, diabetes, renal failure, alcoholism, decreased gastric acidity, narcotic addiction, cigarette smoking, pregnancy, and aging were at higher risk than others for developing listeriosis. Gellin et al. (1991) also agreed with MacDonald et al. (1986) that listeriosis is responsible for the highest case fatality rate out of all the foodborne illnesses, including botulism. Even though listeriosis is a fairly uncommon illness, the high mortality rate (30-50%) is a cause of great concern for health officials and indicates a need for continued research in developing new strategies for controlling *Listeria monocytogenes* and listeriosis (Schlech, 1991).

Pathogenesis and Virulence

Human listeriosis is a foodborne disease caused by *Listeria monocytogenes* when contaminated food products are consumed. Once inside the body, *Listeria monocytogenes* penetrates the intestinal lining (Berche et al., 1988) and invades macrophages, epithelial cells, fibroblasts, and hepatocytes (Murray et al., 1994; Gregory et al., 1992). Being an intracellular pathogen is one of the important virulence characteristics of *Listeria monocytogenes.* A surface protein on the bacteria, called internalin, allows the bacteria to adhere to cell membranes and enter the cells, even those that are not phagocytic (Gaillard et al., 1991). Inside the cell, the bacteria elude the immune response by evading the lysosomal enzymes and then multiplies. *Listeria* can multiply in the Peyer's patches (MacDonald and Carter, 1980), in addition to the liver (Gregory et al., 1992) and spleen (Roll and Czuprynski, 1990). *Listeria monocytogenes* produces a β-hemolysin, known as listeriolysin, which dissolves the membrane-bound vesicle surrounding the bacterium following ingestion by phagocytic processes (Portnoy et al., 1992). This allows the bacterium to act freely in the cytoplasm of the macrophage and other cells for multiplication (Portnoy et al., 1992). Another surface protein on the bacteria, known as ActA, aids in actin polymerization (Domann et al., 1992). Actin surrounds the bacterium allowing it to multiply. A tail is then formed to move the bacterium to the cell membrane (Czuprynski, 1994). *Listeria* penetrates the cell membrane and invades another cell where the process repeats (Portnoy et al., 1992). Figure 1 illustrates this mechanism. Onset of clinical disease usually takes three weeks after ingestion of the contaminated food product (Czuprynski, 1994). The virulence

Figure 1. After phagocytosis, the bacterium is surrounded by an endosome that fuses with lysosomes. Listeriolysin O dissolves the phagolysosomal membrane before the bacterium is damaged by the lysosomal enzymes. Actin filaments surround the bacterium and following bacterial replication, a tail moves the bacterium towards the macrophage surface. A pseudopod extends outward and facilitates transfer to another macrophage. This process allows the bacteria not to be exposed to antibodies and external cellular factors. This picture was adapted from Murray et al. (1994).


Macrophage
Macrophage

of the infecting organism, the susceptibility of the host, and the amount of organism ingested are all factors that influence the invasiveness and disease-causing ability of *Listeria monocytogenes* (Schuchat et al, 1991). The minimal infective number it takes to cause listeriosis is unknown at this time (Schuchat et al., 1991).

Neutrophils are thought to play an important role in the fight against *Listeria monocytogenes* (Rosen et al., 1989). Evidence has shown that a large number of inflammatory neutrophils can ingest and kill *Listeria monocytogenes* by engulfing *Listeria*-infected cells, thus stopping multiplication and spreading (Rosen et al., 1989). In the liver, neutrophils are believed to phagocytize and lyse the *Listeria*-infected hepatocytes, thus preventing intracellular transmission to neighboring cells (Conlon and North, 1991).

Behavior of *Listeria* in Food Processing Plants

Listeria monocytogenes is found in various dairy and meat products. There are numerous sources where contamination can occur throughout the handling and processing of food before it reaches the consumer. First, contamination can come directly from animal origin or, secondly, it can come from environmental areas of the processing plant. The Food and Drug Administration (FDA) Dairy Safety Initiatives Program has identified several environmental sources such as floors in coolers, freezers, processing rooms, entrances, cases and case washers, floor mats, and plant personnel where *Listeria monocytogenes* is usually found (Kozak, 1986; Donnelly, 1990). During processing of meats and dairy products, sanitation techniques and pasteurization procedures are used to

eliminate contamination. However, food products can still become contaminated, depending upon the survival of the organism throughout processing.

Listeria monocytogenes has demonstrated the ability to attach to stainless-steel, (Herald and Zottola, 1988; Speers et al., 1984) rubber, and Buna-N gaskets (Czechowski, 1990). However, Helke et al. (1993) observed a reduction in attachment to stainless-steel and Buna-N in the presence of whole, skim, 2%, and chocolate milk. Research performed by Buazzi et al. (1992) showed that *Listeria monocytogenes* did not survive the manufacturing of Mozzarella cheese. They agreed with Bunning et al. (1988) that contamination probably occurs during handling after the cheese has been processed.

Listeria monocytogenes has also exhibited the ability to adapt, survive, and grow in harsh conditions during processing. Hudson (1992) showed that *Listeria* grew in 6% sodium chloride (NaCl) and tryptic soy broth at refrigeration. The results from this research also showed that *Listeria* numbers remained constant at 16% NaCl and declined at 26% NaCl at refrigeration temperatures, thereby demonstrating a high salt tolerance.

Gahan et al. (1996) have shown that *Listeria monocytogenes* has the ability to adapt to acidic environments, a term they called acid tolerance response (ATR). *Listeria* can acquire a resistance to low pH following exposure to acidic conditions (Hill et al., 1995). Gahan et al. (1996) also observed that acid adaption increased the survival of *Listeria* in cottage cheese, yogurt, whole-fat cheddar cheese, orange juice, and salad dressing. Research is underway to determine if *Listeria* acid tolerance occurs in nature.

Injury to *Listeria monocytogenes* Caused by Heating

There have been many experiments performed to determine if *Listeria monocytogenes* is heat resistant and can survive pasteurization. Knable et al. (1990) found that *Listeria* grown at high temperatures (43^0C) had a greater thermotolerance than the organisms grown at lower temperatures. Farber et al. (1992) demonstrated in their study that *Listeria* present in milk can survive minimum high temperature (72^0C), short time (16.2 seconds) pasteurization. However, cell populations did decrease in number upon recovery after pasteurization. Smith et al. (1991) showed that *Listeria* grown at low temperatures are more susceptible to death when introduced to heat. Why cells grown at high temperatures are able to become more thermoresistant than cells grown at lower temperatures is not known (Farber et al., 1992). Some researchers believe this effect is related to the physical condition of a cell. When growth temperature is lowered, the bacterial cells increase the proportion of unsaturated fatty acids, which increases the fluidity of the membrane phospholipids. This causes a decrease in heat resistance (Beuchat, 1978). It is believed that, with decreased unsaturation brought about by high growth temperatures, bacteria membranes aid in heat resistance (Smith et al., 1991).

Factors that cause injury or death to *Listeria monocytogenes* are heating, freezing, dehydration, and exposure to sanitizers (Busta, 1978; Golden et al., 1988a). In some foods, injured cells can repair sub-lethal damage, multiply, and regain their virulence potential (McCarthy, 1991; Meyer and Donnelly, 1992). Failure of identification methods to detect sublethal injured cells could result in adverse public health consequences (Bunduki et al., 1995).

What damage is done to the cell that is sub-lethally injured by high temperatures? Bunduki et al. (1995) showed that the cell wall is not a site of damage because there was no leakage of nucleic acids and cellular proteins when observed. They also showed that sublethal heat-damaged cells undergo repair through protein synthesis and oxidative phosphorylation. They believe knowledge of injury and repair will lead to improvements in identification methods for detecting injured *Listeria.*

Injury to *Listeria monocytogenes* Caused by Freezing

Freezing is used in food processing for control of foodborne pathogens and to minimize food spoilage (El-Kest and Marth, 1991b). Freezing can injure or kill *Listeria* by formation of ice crystals forming both extracellularly and intracellularly, thus causing the cell to expand and burst (El-Kest and Marth, 1991b). However, *Listeria monocytogenes* can survive freezing, repair itself, and regain the capacity to multiply under favorable conditions (Ray, 1979). This ability of *Listeria* has attracted great interest in trying to understand the specific injury to the cell, how the bacterium repairs itself, and what protection the food product provides (Flanders and Donnelly, 1994).

The freeze-injury to *Listeria* is less severe than that of heat induced injury (Busch and Donnelly, 1992); however, there is a strong concern about the identification of injured cells in food products. Survival of the bacterial species depends on several factors such as bacteria species, strains, age, growth conditions, nature of suspending media, conditions of freezing and thawing, temperature of freezing, and the period of freezing (Ray, 1984). Freezing causes damage to the cell wall by crystallization (El-Kest

and Marth, 1992b). As the ice crystals expand, the cell wall is torn. Once thawing occurs, leakage is determined by the presence of nucleic acids and proteins extracellularly (El-Kest and Marth, 1992b). However, it is still undetermined what specific damage is done to the cell wall other than tearing. There are still questions about the effects of freezing on specific components of the cell such as the peptidoglycan layer, teichoic acids, plasma membrane, nucleotides, and ribosomes (El-Kest and Marth, 1992b). There are also unanswered questions relating to the repair mechanisms of freeze-injured *Listeria monocytogenes* cells.

The survival of *Listeria monocytogenes* is influenced by the food product (Meyer and Donnelly, 1992). El-Kest and Marth (1991b) demonstrated that *Listeria monocytogenes* is more resistant to death and injury when suspended in milk (1991b), casein, lactose, and milk fat (1991a) rather than in phosphate buffer solution. They concluded that some dairy products protect the cells during exposure to frozen environments. These results supported the research of Speck and Ray (1977), which reported that foods with proteins, carbohydrates, and triglycerides increase the resistance to freezing. Palumbo and Williams (1991) added that the pH of foods might explain the resistance to freezing. They reported that *Listeria monocytogenes* was not injured by freezing in foods with a pH of 5.8 or above.

Time exposure to freezing temperatures also influences the survival of *Listeria monocytogenes* (Ray, 1984). El-Kest and Marth (1992a) reported that damage to the plasma membrane increased as storage increased at -18^{0}C for up to six weeks. They observed irregularity in the shape of the cells. However, El-Kest and Marth (1992a) also

showed some strains of *Listeria monocytogenes* resisted frozen storage for six weeks, thawing, and treatment with lysozyme. El-Kest et al. (1991) showed that "repeated freezing and thawing caused more death and injury than did a single freeze and thaw cycle" (p.1070).

There have not been many studies conducted to improve efforts for identifying injured *Listeria* cells after exposure to frozen environments (Flanders, 1991). Work done by Busch and Donnelly (1992) showed that using selective media for direct plating was not recommended after exposure to freezing or heating. Research done by Flanders and Donnelly (1994) showed that low numbers of organisms, caused by freezing, might not produce adequate growth on selective media. However, other research has recorded that freezing does not affect the ability of injured *Listeria* to grow on a selective media (Golden et al., 1988b).

Selective and Non-selective Media for Agar Plating

There are several selective and non-selective media used to identify *Listeria monocytogenes* in contaminated food products and clinical specimens. A non-selective medium is one that will allow growth of many different organisms, where as selective medium inhibits the growth of all competitive flora except the selected species (Speck and Ray, 1977). Blood agar is one example of a non-selective agar used to identify *Listeria monocytogenes*, which produces a β-hemolysis (a clear zone around the colonies caused by the destruction of erythrocytes) on blood agar.

Certain chemicals are used to make media selective for *Listeria.* These chemicals include acriflavine, glycine anhydride, lithium chloride, nalidixic acid, nitrofurazone, potassium tellurite, and potassium thiocyanate (Klinger, 1988). The first selective medium of *Listeria* for agar plating was developed by McBride and Girard in 1960. Since then, several selective media for agar plating have been developed. One of the most common is lithium chloride-phenylethanol-moxalactam, known as LPM (Lee and McClain, 1986). Another selective medium, Oxford medium, has a coloration change when *Listeria* is present. The colonies appear black and are surrounded by halos, resulting from the hydrolysis of esculin (Curtis et al., 1989). Research conducted by Westöö and Peterz (1992) showed that Oxford agar allowed for greater recovery than LPM agar. Visualization of colonies and color change make Oxford media better for plating than LPM (Paranijpye et al., 1992). Art and Andre (1991) concluded that Oxford agar, when compared to blood agar had an advantage by reducing competitive flora. However, Pini and Gilbert (1988) summarized that no one particular medium is suitable for recovery of all different food types and methods of injury. Other studies have also supported this conclusion (Lammering and Doyle, 1989; Paranijpye et al., 1992).

Culture Identification Protocols

The two standard cultural protocols for identifying *Listeria monocytogenes* in food products have been developed by the United States Department of Agriculture (USDA) and the Food and Drug Administration (FDA). These methods require long enrichment stages in liquid media, incubation, and plating techniques that can take

anywhere from 3-28 days to determine the results for the samples (Walker et al., 1990). Both methods vary in some degree, depending on which method is used. Research performed by Slade (1992) concluded that the USDA method provided more accurate results in most applications when compared to the FDA method. With the increase of disease caused by *Listeria* in 1986, numerous studies were conducted to improve the accuracy and shorten the time on identification methods (Walker et al., 1990).

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was first described by Mattingly et al. (1988) as a rapid diagnostic test for the detection of *Listeria monocytogenes* in food products. This assay uses murine monoclonal antibodies that are specific for a protein antigen found in all species of *Listeria* (Butman et al., 1988). The monoclonal antibodies were tested against *Staphylococcus, Streptococcus, Citrobacter, Pseudomonas,* and *Salmonella* species and found to be non-reactive. The targeted protein is heat stable with a molecular weight between 30-38,000 depending on the species. The antigen found in *Listeria monocytogenes* has an approximate molecular weight of 30-34,000. One monoclonal antibody is used for capture of the antigen (if present) and another monoclonal antibody is used for enzyme conjugation (Mattingly et al., 1988). This test is considered very sensitive and highly specific.

Before the food samples can be tested by the ELISA, enrichment procedures are performed to enhance the growth of *Listeria*. Samples are collected and mixed with a selective enrichment broth (broths vary depending on which procedure is used and which

food product is tested) and incubated for 24 hours. Another selective enrichment broth is inoculated after the incubation period and also incubated for 24 hours. Samples are collected from the second enrichment stage and pipetted into polystyrene microELISA wells precoated with the monoclonal antibodies. Immune complexes form as the antigen and antibody bind. Enzyme-labeled antibodies (conjugate) are added to the wells and bind to the antigen-antibody immune complex. Incubation for one hour follows and the wells are washed with phosphate-buffered saline solution. The sample is then incubated for 30 minutes with a combined substrate solution containing 3, 3', 5, 5'-Tetramethyl-benzidine and hydrogen peroxide. A blue coloration appears, caused by the reaction of the chromogen binding to the antibody-antigen complex in the wells during incubation . The stop solution (2N sulfuric acid) is added to the wells at the end of incubation to stop the chromogen/antibody complex reaction and a yellow coloration indicates the presence of *Listeria*. The samples are then read with a microELISA reader to determine absorbance or optical density of the chromogen/antibody/antigen complex. The intensity of the color is determined by the absorbance at 450nm using a photometer. Positive samples have an absorbance level over the cutoff value (negative control value plus 0.150). The positive control should have an absorbance over 0.700 OD and the negative control should be less than 0.300 OD (Organon Teknika Corporation, 1994).

Since the ELISA was developed, several studies have compared the ELISA to the cultural methods of the FDA and USDA. Meier and Terplan (1993) were able to determine negative samples within two days which is two days earlier than the cultural methods. Walker et al. (1990) stated that "the ELISA kit was as good as the best cultural

methods" (p. 340). They also concluded that the ELISA procedure was more rapid than the cultural methods. Curiale et al. (1994a) recommended that the ELISA was a good alternative to the cultural methods. It should be noted that the ELISA can only detect the presence of *Listeria*, it can not identify the strains (Comi et al., 1991). It should also be noted that the ELISA requires at least 10^5-10^6 organisms/ml before it can be detected (Martin and Katz, 1993).

Deoxyribonucleic Acid Hybridization Assay (DNAH)

The DNAH assay was first described by Klinger et al. in 1988 as an alternative method of identification of *Listeria* in food products. This assay captured the cells with the use of membrane filters, and the cells are lysed to release ribosomal ribonucleic acid (rRNA). A synthetic DNA probe labeled with a phosphorus radioisotope (P^{32}) is complementary to the rRNA of the *Listeria*. Hybridization of the strands is monitored with a beta particle detector (Klinger et al., 1988).

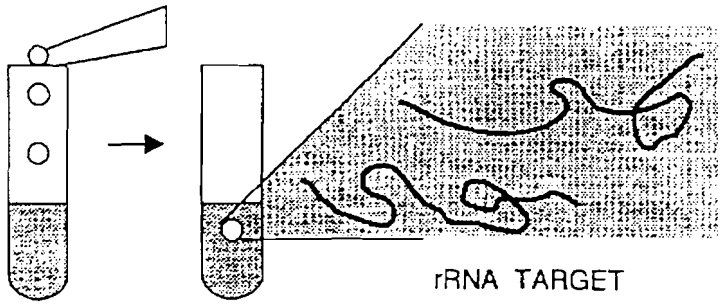
The DNAH method was improved by the modification of a liquid-phase hybridization capture and colormetric detection method (King et al., 1989). The enrichment procedure consists of an incubation period of 24 hours in a selective broth to increase growth of the cell population. Agar plates are then swabbed and incubated again for 24 hours. Cells from the plates are collected and suspended in phosphate buffered saline solution. Pre-treatment reagents and lysis reagents are added and incubated as outlined by Gene-Trak Systems Corporation (1995). The bacterial cells are lysed by the lysis reagents, which causes the release of rRNA. Synthetic DNA probes complementary

in sequence to *Listeria* rRNA are added, and binding occurs during another incubation period. Plastic dipsticks are introduced and the polydeoxyadenylic acid tail on the DNA probe attaches to a deoxythymidylic acid sequence on a plastic dipstick. The dipsticks are then introduced to an anti-fluorescein antibody-enzyme conjugate in which the detector probe is recognized during incubation. The dipstick is again transferred to an substrate-chromogen where the hydrogen peroxide (HRP), in the presence of chromogen, binds to the antibody-rRNA-detector probe complex, thus producing a blue coloration during incubation (Figure 2). A stop solution, sulfuric acid, is added to stop the chromogen/antibody complex reaction and a yellow coloration appears. The intensity of the color is determined by the absorbance at 450nm using a photometer supplied by Gene Trak Corporation. The negative control should have an absorbance less than 0.15 OD and the positive control should be greater than 1.00 OD (Gene-Trak Systems Corporation, 1994; Gene-Trak Systems Corporation, 1995).

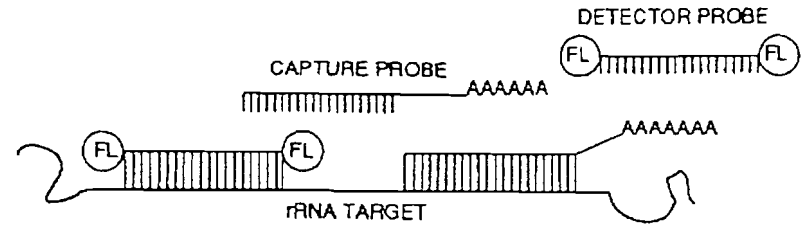
After the DNAH method was developed, many studies compared the assay to the cultural methods of the FDA and the USDA. Curiale et al. (1994b) showed the DNAH method can determine negative results in two days, whereas cultural methods take four days. Research has showed that the DNAH method was statistically equivalent to the FDA and the USDA methods (Bottari et al., 1995). Url et al. (1993) recommended the Gene-Trak DNAH assay as an alternative method to the cultural methods. The DNAH assay requires at least 10^5-10^6 organisms/ml before detection (Martin and Katz, 1993).

Figure 2. In stage one, the cell is lysed and ribosomal ribonucleic acid (rRNA) strands are released. Stage two shows the capture probe with the polydeoxyadenylic acid tail and the detector probe labeled with fluorescein ends attach to the complementary sequences on the rRNA. In stage three, the dipsticks are added and the poly-A tail is attached to the polydeoxythymidylic acid sequence coated on the dipsticks. With the addition of anti-fluorescein antibodies conjugated to the enzyme horseradish peroxidase (HRP), the conjugate binds to the fluorescein molecules on the detector probe. In the presence of the HRP substrate, a blue coloration appears. The intensity of the color depends on the amount of enzyme conjugate bound to the complex and the proportion of target rRNAs captured. The reaction is stopped with sulfuric acid, changing the color to yellow if *Listeria* is present. The intensity of the color is measured by determining the absorbance at 450nm using a photometer. Permission was obtained from Gene-Trak Systems Corporation (1995) for the use of this figure.

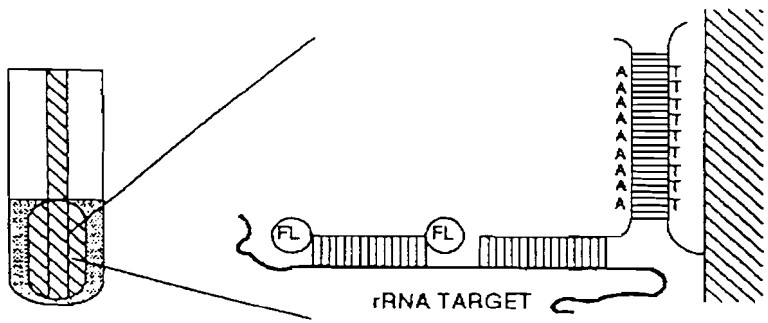
1. SAMPLE LYSIS



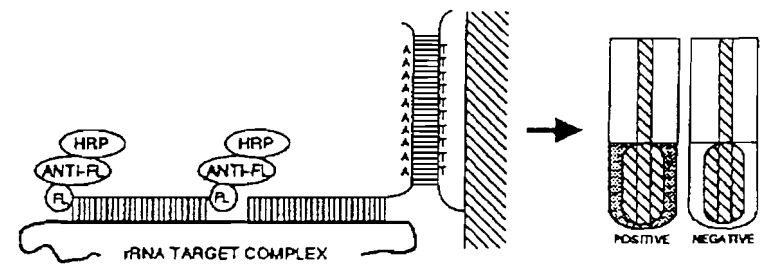
2. HYBRIDIZATION



3. CAPTURE



4. DETECTION



Use of Rapid Detection Methods

The use of rapid detection methods such as the ELISA and DNAH assay can reduce analysis time of determining the contamination of food products with *Listeria monocytogenes*. However, no one method is 100% accurate because of the method of sampling (Martin and Katz, 1993). It is difficult to obtain a true representative sample from processed food products (Murphy et al., 1996). Problems with sampling methods include a non-uniform distribution of the pathogen throughout the food product, and only a small sample is analyzed from a large batch, which can be misleading in the analysis of the entire food product (Murphy et al., 1996).

Research Objectives

The objectives of this research were twofold. First, comparisons of the ELISA and DNAH assay methods in detecting *Listeria monocytogenes* in powdered dairy food products after exposure to frozen environments (-10^{0}C and -70^{0}C) was conducted. Testing was conducted at room temperature, one month after freezing, and four months after freezing. Second, comparison of four different media for plating and analysis in showing adequate growth after freezing were made. The selective media included McBride's agar, Modified Oxford agar (MOX), LPM and one non-selective medium, tryptic soy agar with yeast extract.

CHAPTER 2

MATERIALS AND METHODS

Bacteria and Cell Suspensions

Listeria monocytogenes strain J. Pelzar, Jr. was obtained from Presque Isle Cultures (Presque Isle, Pennsylvania). A test tube containing 10 ml of trypticase soy broth (Difco Laboratories, Detroit, MI) was inoculated and incubated at 35^0C for 48 hours. Cells were inoculation from the test tube to a flask of trypticase soy broth (500 ml) and incubated at 35^0C for 48 hours.

Standardization of Inoculum

To establish a known number of bacterial cells per ml of suspension, turbidity measures were conducted using a Bausch and Lomb spectronic 20 spectrophotometer (Rochester, NY). Samples were transferred from the trypticase soy broth (TSB) flask into a sterile cuvette and 10-fold dilutions were made to measure the percentage of transmission. The absorbance or optical density (OD) of the bacterial suspension is related to the percentage of light transmitted through the suspension according to the formula: OD=log100 - 10g%T where g=growth and T=time. The suspension of *Listeria* cells was then adjusted to equal 20% transmittance at a wavelength of 550nm. It has been determined that at 20% transmittance with the known size of *Listeria* being 2.0 μm, the bacteria present would correlate to be $1x10^7$ cells/ml (Murray et al., 1994). Trypticase soy agar (TSA) plates were used to conduct standard plate counts from each of the

10-fold dilutions. Cell colonies were counted and multiplied by the dilution factor to yield the total number of viable bacteria per ml of the original sample. The original suspension was then confirmed to contain approximately $1x10^7$ cells/ml.

Inoculation of Food Product

Powder dairy products (i.e., cheese, lactose, milk) were obtained from a dairy processing plant in the area (no names are indicated at the request of the plant). The powdered products were poured into 30 stomacher bags and weighed to equal 500 g per bag. Some of these products might have been naturally-contaminated with other food borne pathogens such as *Streptococcus, Escherlia*, and *Salmonella* species (as indicated by the dairy processing plant officials). Samples numbered 1-13 contained lighter-orange/yellowish color, probably a cheddar cheese product. Samples numbered 21-25 were also a cheddar cheese product, with a darker orange appearance. Samples numbered 31-37 consisted of milk powder. Finally, samples numbered 41-45 contained lactose. Twenty-five samples (1-10, 21-25, 31-35, and 41-45) were inoculated with 10 ml of *Listeria monocytogenes* suspension ($1x10^7$ cells/ml) that had been standardized previously. The five remaining samples (11-13 and 36-37) were left uninoculated to serve as a negative control. The product samples were mixed and left at room temperature for 48 hours. Samples weighing 150 g were collected into new stomacher bags from each of the previously-inoculated samples. These samples were labeled as room temperature products and served as the control for this experiment.

Freezing the Contaminated Product

The initial contaminated products (those from which the control samples had been removed) were placed in two different frozen atmospheres. The two temperatures chosen for this experiment were -10^{0}C and -70^{0}C. The samples were separated equally among different products (i.e., cheese, milk, lactose) and placed into the frozen environments. Samples 1-8, 11-13, 31-32, and 43-45 were placed in the -70^{0}C freezer. Samples 9-10, 21-25, 33-37, and 41-42 were placed in the -10^{0}C freezer. After one month exposure, 150 g samples were again collected and placed in sterile stomacher bags. The initial product was placed back into the frozen environment, while the newly- collected samples were allowed to reach room temperature (20^{0}C) for testing. After four months, the initial samples were removed from the frozen environment and allowed to thaw to room temperature.

Sample Preparation for the *Listeria*-Tek ELISA Test System

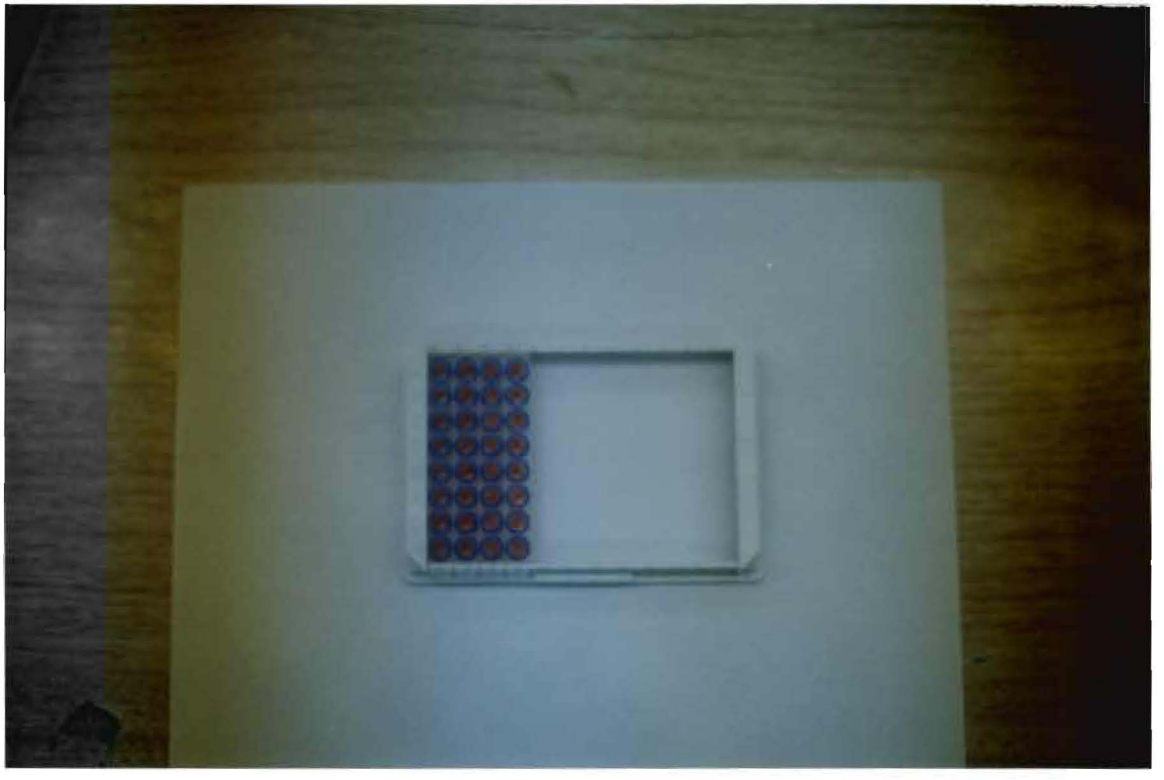
Twenty-five gram samples were collected in stomacher bags from each sample stored at room temperature, one month exposure, and four months exposure to a frozen environment. The pre-enrichment stage involved the addition of 225 ml of modified Fraser broth to each 25 g sample, mixing thoroughly, and incubated at 30^{0}C for 24 hours. Samples were mixed again and 100 µl was pipetted into test tubes containing 10 ml of UVM Modified *Listeria* enrichment broth. The tubes were mixed and incubated at 30^{0}C for 24 hours. Samples were mixed once again before removing 1.0 ml and placing into clean, glass screw-top test tubes. The samples were placed in an autoclave for 10 minutes

and then allowed to cool to room temperature before testing. This procedure is conducted as specified by the test system.

Listeria-Tek ELISA Test Procedure

The enzyme-linked immunosorbent assay (ELISA) test kit (#52100 and lot 150762) was purchased from Organon Teknika Corporation (Durham, North Carolina). Included with the test kit were polystyrene microELISA wells pre-coated with monoclonal antibodies specific for a protein antigen found on all species of *Listeria,* negative and positive controls, a murine-peroxidase conjugated antibody, TMB substrate consisting of 3, 3', 5, 5'-tetramethylbenzidine and hydrogen peroxidase, a buffered wash solution, and a 2N sulfuric acid stop solution. The strip holder was filled with 33 microELISA pre-coated wells (one for each sample, two for negative controls, and one for a positive control). From the pre-treated sample tubes, 100 µl was pipetted into each of the assigned wells. The positive and negative controls were also pipetted into their assigned wells. The murine-peroxidase conjugated monoclonal antibodies to *Listeria* species (100 µl) was added to each of the wells including the controls. An orange coloration was seen in each well from the color of the conjugate (Figure 3). The strip holder was covered with a Mylar sealing tape (Sigma, St. Louis, MO) and the samples were mixed gently by tapping. Samples were incubated at 37^0C for one hour. The contents in the wells were aspirated into a waste area and each well was filled with 200 µl of wash solution (diluted to 1:25 with distilled water). Aspiration and washing of the wells was performed six times. TMB peroxidase solution A (3, 3', 5, 5'- tetramethyl-

Figure 3. Orange coloration is due to the color of the conjugate solution added to the wells.



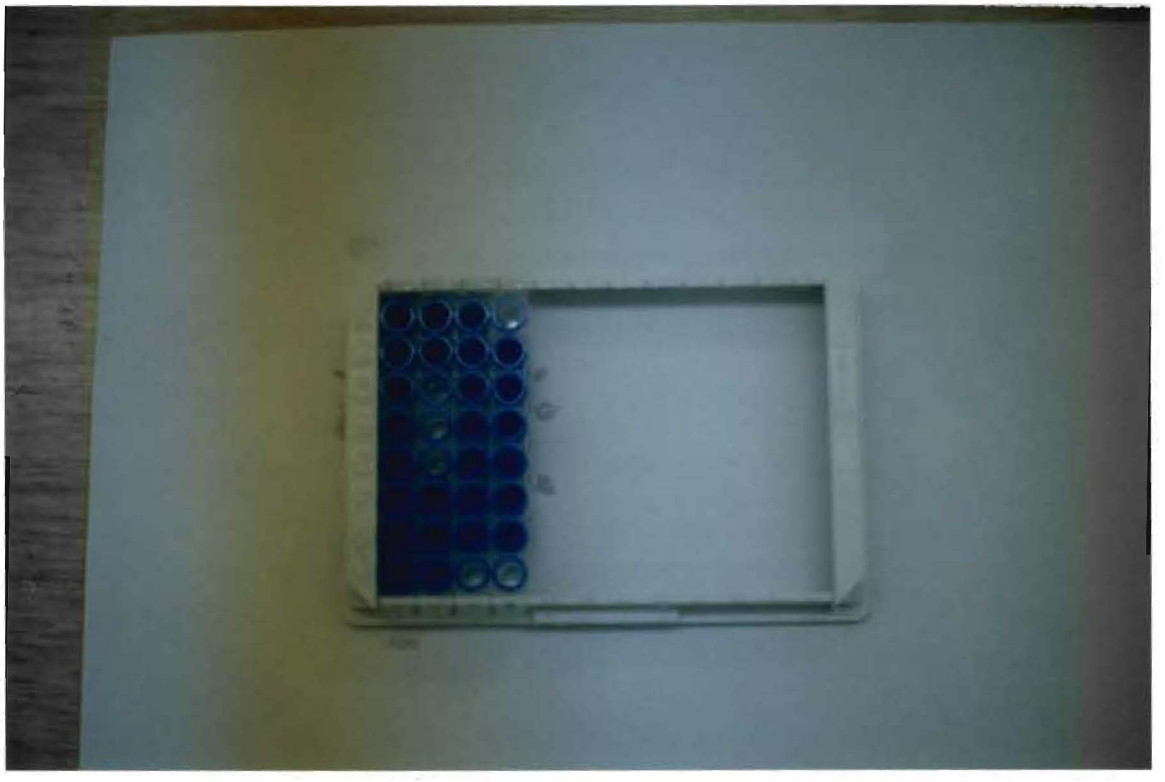
benzidine) and solution B (hydrogen peroxidase) were mixed in equal amounts to form the TMB substrate. Each sample received 100μl of the substrate solution, and was covered and incubated at room temperature (20^0C) for 30 minutes. A blue coloration appeared if *Listeria* was present because of the reaction of the chromogen that had been bound to the antibody-antigen complex (Figure 4). The stop solution (100 μl) was pipetted into the wells, creating a yellow coloration (Figure 5), and absorbance was determined with an EL307C Microplate Reader (Bio Tek Instruments, Vermont). The reader was cleared and calibrated on air before analyzing the samples. The absorbance was read at 450 nm. The mean negative control should have an absorbance less than 0.300 OD and the positive control should be greater than 0.700 OD. If the absorbance level was below 0.300 OD, it was determined as being negative for *Listeria.* If the absorbance level of a sample was greater than 0.300 OD, it was considered positive for *Listeria.* All positive samples were streaked on agar plates for confirmation.

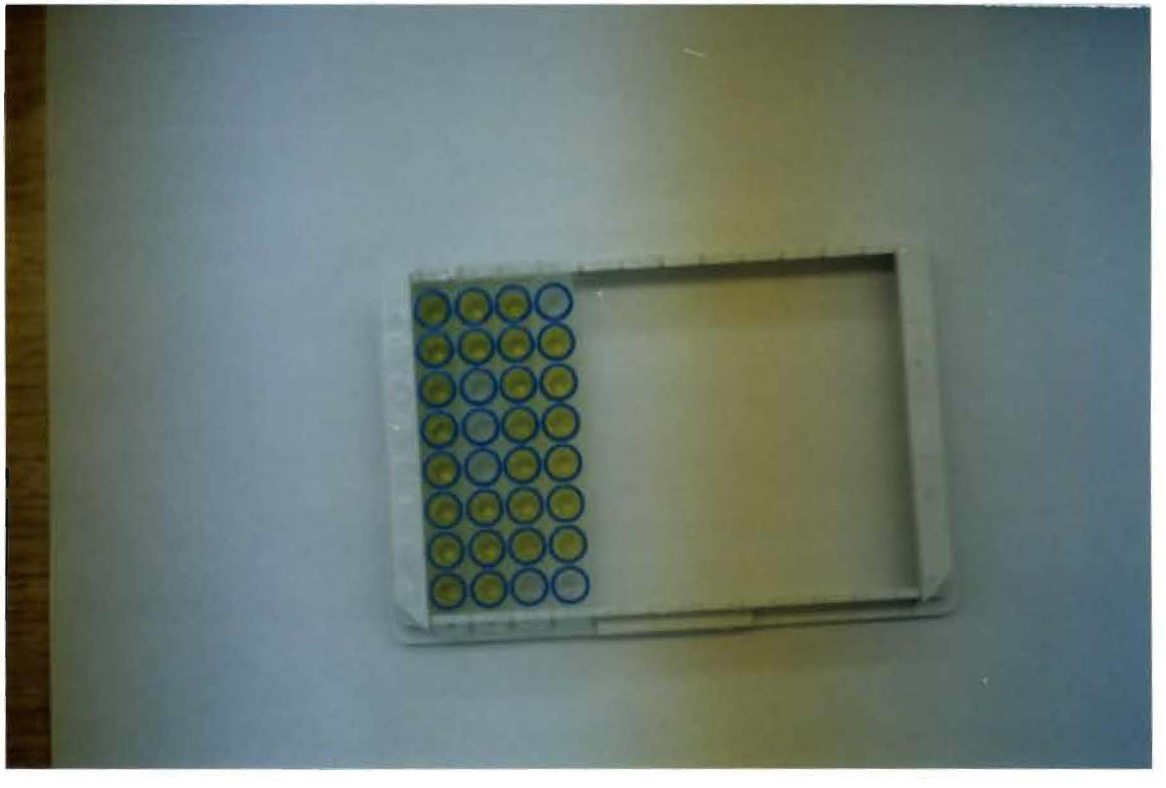
Sample Preparation for the Gene-Trak *Listeria* Assay

Samples were collected exactly like the ELISA sample preparation procedures. Modified Fraser broth (225 ml/25 g of sample) was used as the primary enrichment stage. The samples were incubated at 35^0C for 24 hours. After mixing the samples, cotton swabs were dipped into the culture broth and streaked onto lithium chloride-phenylethanol-moxalactam (LPM) plates which were then incubated at 35^0C for 48 hours.

Figure 4. Blue coloration due to the presence of *Listeria monocytogenes*.

Figure 5. The stop solution (2N sulfuric acid) is used to stop the reaction of the chromogen/antibody complex and in result, a yellow coloration is observed if *Listeria* is present.





DNA Hybridization Test Procedures

The DNA hybridization test kit (#GT0808 and lot 1499) for detection of *Listeria* was obtained from Gene-Trak Systems (Hopkinton, MA). The test kit included pre-treatment reagent concentrate and buffer, lysis reagent concentrate and buffer, *Listeria* probe solution, wash solution (20x concentrate), enzyme conjugate (100x concentrate), substrate-chromogen solution, stop solution, dipsticks, positive and negative controls, four washing containers, six dipstick holders, and a photometer at 450 nm. Using a sterile cotton swab, growth from the LPM plates were collected and suspended in 1 ml of phosphate buffered saline (PBS) in sterile test tubes. Swabs were stirred vigorously in the saline and then discarded.

Prior to starting the assay, two water baths were filled to a level of 5 cm at a water temperature of 37^0C and 65^0C respectively. The lysis reagent concentrate and buffer were combined into one solution, mixed, and placed on ice. The pre-treatment reagent concentrate and buffer were also combined, mixed, and placed on ice. The wash solution (65 ml) was diluted by adding 1235 ml of distilled water. Each wash basin was filled with 300 ml of wash solution, and the metal wash basin was placed in the 65^0C water bath, and the other three were kept at room temperature. Three racks of 12 x 75 mm test tubes were numbered and set aside for future use.

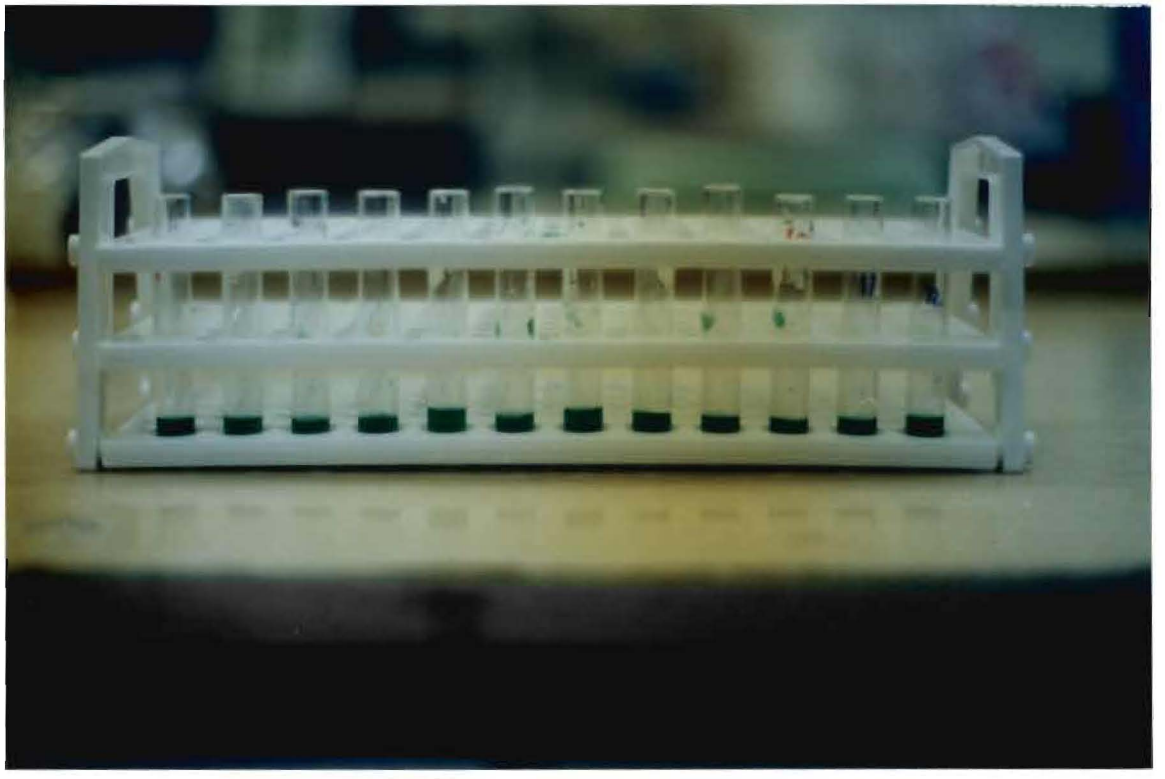
To perform the Gene-Trak assay, 500 µl of the growth suspension (PBS) was pipetted into the appropriate 12 x 75 mm test tubes, along with 500 µl of the positive and negative controls. The pre-treatment reagent (100µl) was added to each test tube resulting in a purple coloration (Figure 6). The samples were mixed and incubated in the

Figure 6. A purple coloration is seen after the addition of the pre-treatment reagent to the PBS solution.

37^0C water bath for 15 minutes. Without removing the rack of tubes, 100 µl of lysis reagent was added resulting in a green coloration due to the mixture of reagents (Figure 7). The tubes were gently mixed and incubated in the water bath for additional 15 minutes. The appropriate number of dipsticks were placed in one of the room temperature wash basins. The *Listeria* probe solution (100 µl) was added to each sample resulting in a red coloration, again because of the mixture of reagents (Figure 8). The dipsticks were removed from the wash basin, blotted on absorbent paper, and placed in the appropriate tubes. The solutions were mixed by raising and lowering the dipstick five times. The rack of tubes was removed from the 37^0C water bath and placed in the 65^0C water bath for one hour. Prior to the end of the one hour incubation period, the enzyme conjugate was mixed with the remaining wash solution in a 1:100 proportion. A dilution chart was supplied with the test kit. Enzyme conjugate (750 µl) was pipetted into empty 12 x 75 mm test tubes that had been prepared earlier. After the one hour incubation period, the dipsticks were removed from the test tubes and washed gently, first in the metal wash basin (65^0C) and then in a room temperature wash basin. The dipsticks were then added to the appropriate test tubes containing the conjugate enzyme (Figure 9). The samples were incubated at room temperature for 20 minutes. During the incubation period, 750 µl of the substrate-chromogen solution was added to a second rack of clean test tubes that had been prepared earlier. One additional tube was added to this group and labeled as a blank reagent. This blank reagent tube served as the control for reading absorbance later in the testing procedures. After the 20 minute incubation period, the dipsticks were removed from the test tubes and washed for one minute each in the

Figure 7. With the addition of the lysis reagent to the test tubes, a green coloration results from the mixing of reagents.

Figure 8. The addition of the *Listeria* probe solution causes a red coloration to appear because of the mixing of reagents. Dipsticks are added to each appropriate test tube and then incubated in a water bath at 65^0C.



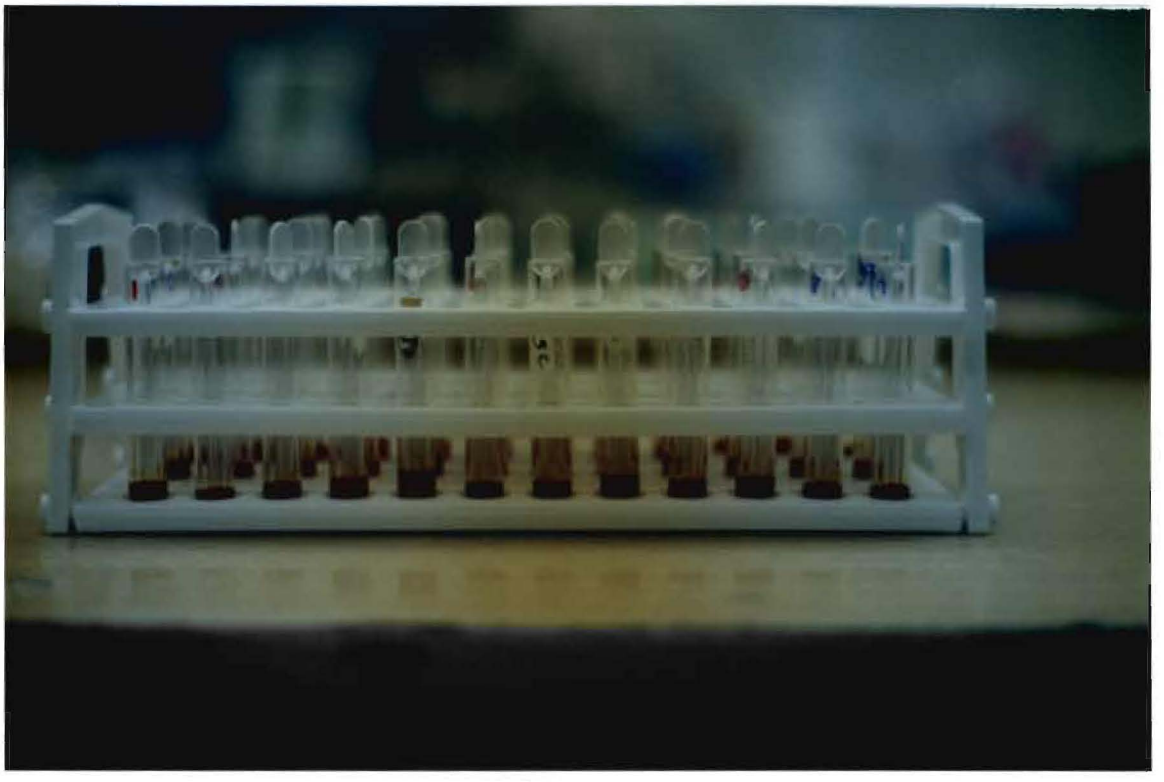
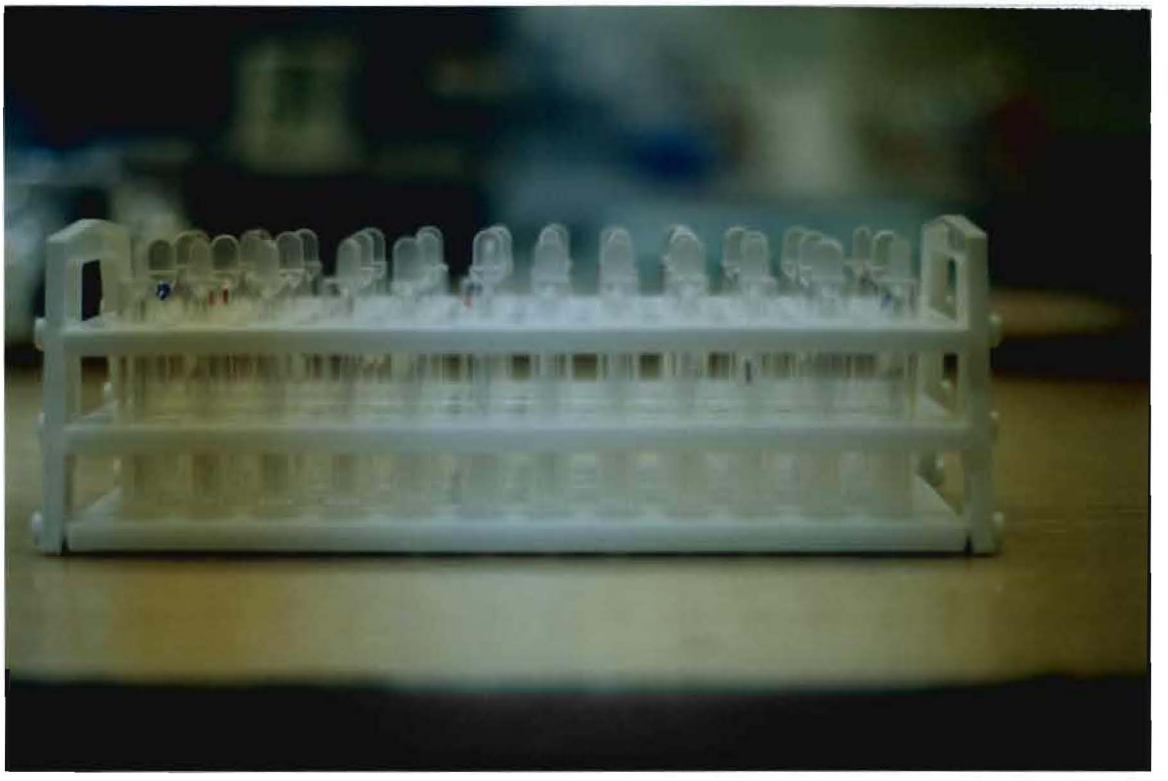


Figure 9. A clear solution is seen as the dipsticks and the enzyme conjugate are incubated at room temperature.



remaining two wash basins at room temperature. The dipsticks were blotted dry on absorbent paper and placed in the appropriate test tubes containing the substrate-chromogen solution. The samples were incubated again at room temperature for 30 minutes. A blue coloration appeared if *Listeria* was present (Figure 10). The dipsticks were removed and discarded after the incubation period had expired. Stop solution (250 μl) was added to each sample, including the blank reagent, and a yellow coloration appeared as the samples were mixed (Figure 11).

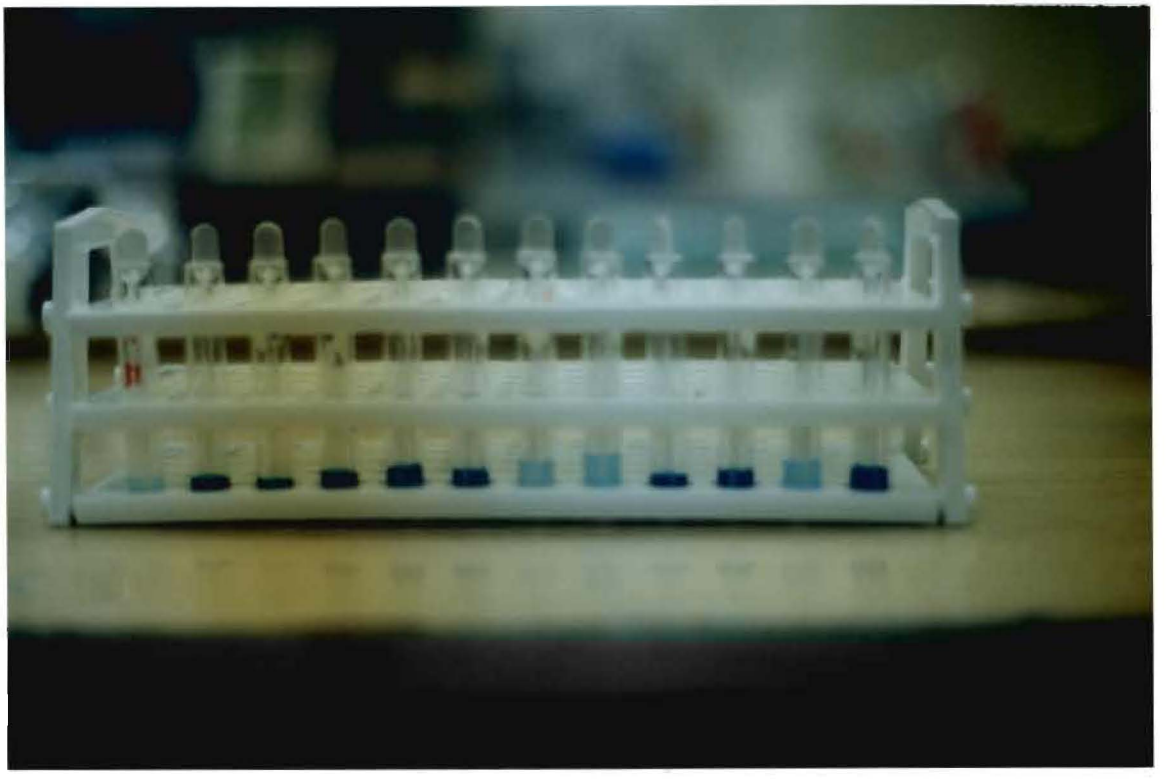
Absorbance was determined with the Gene-Trak photometer at 450 nm. The blank reagent test tube was placed into the reference position of the photometer and the negative control was placed into the sample position. The negative control indicated an absorbance less than or equal to 0.15 OD against the blank reagent. The negative control was removed and the positive control was read against the blank reagent. The positive control indicated an absorbance greater than or equal to 1.00 OD. In order to read the samples, the negative control was placed into the reference position. Each sample was read against the negative control. If a sample was less than 0.10 OD, it was considered to be negative for the presence of *Listeria.* If a sample was greater than 0.10 OD, it was determined to be positive for *Listeria.* All positive samples were streaked on agar plates for confirmation.

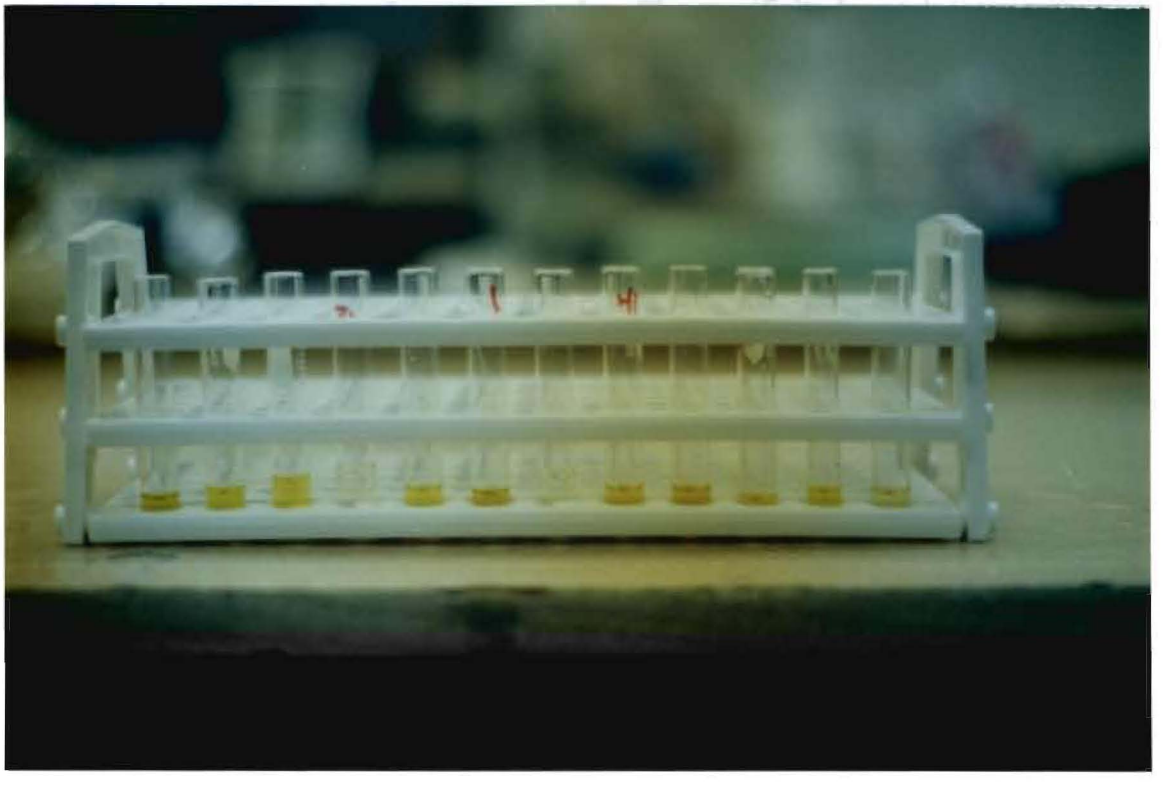
Streaking Agar Plates

The four different medias used for streaking included lithium chloride-phenylethanol-moxalactam (LPM), McBride's medium (MB), modified Oxford medium

Figure 10. Blue coloration appears if *Listeria monocytogenes* is present because of the reaction of the substrate-chromogen binding to the rRNA-detector probe complex.

Figure 11. After the sulfuric acid stop solution is added, the reaction is interrupted and a yellow coloration appears if *Listeria* is present.





(MOX), and trypticase soy agar-yeast extract medium (TSA-YE) (Difco Laboratories, Detroit, MI). LPM, MB, and MOX are selective media for *Listeria,* whereas TSA-YE is a non-selective medium. All plates were streaked from the UVM *Listeria* 10 ml broth samples used in the ELISA preparation procedures. A sterile cotton swab was used to streak the plates, which were then incubated at 35^0C for 48 hours to ensure adequate time for growth if present (Figure 12). LPM was also used for the Gene-Trak preparation procedures. Cotton swabs were dipped into the modified Fraser broth and streaked upon the LPM plates, which were then placed in the incubator at 35^0C for 48 hours.

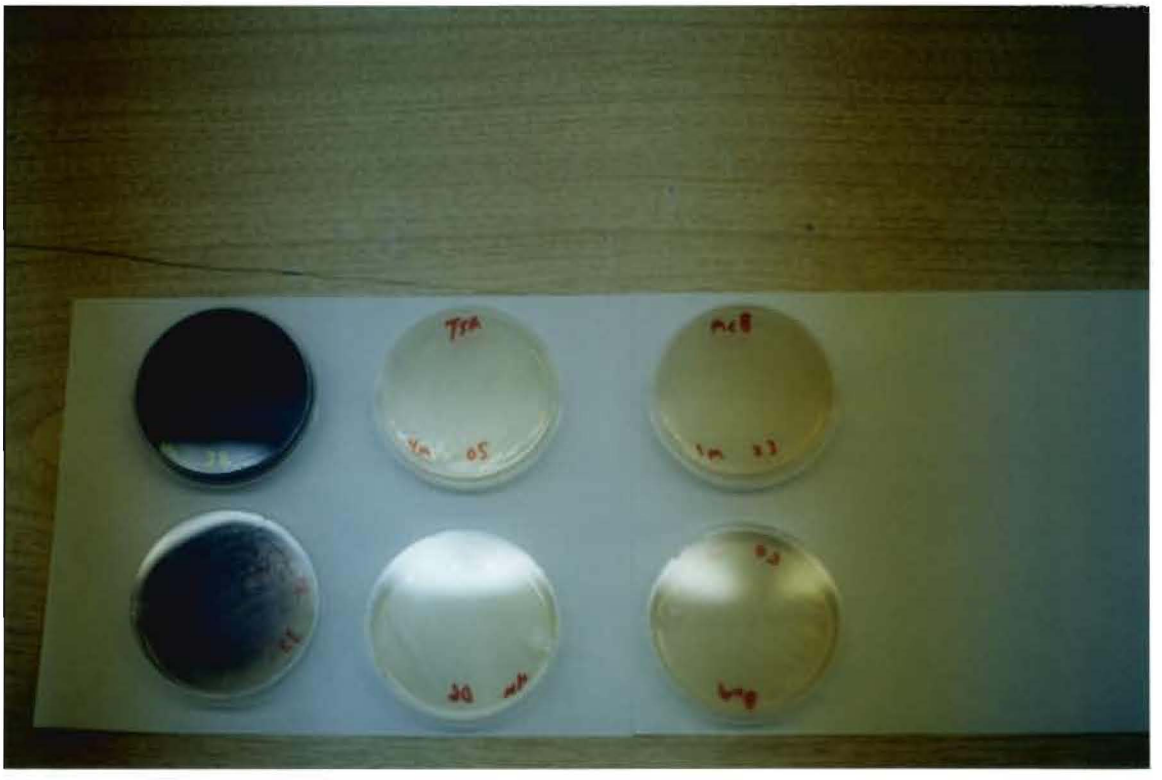
Procedure Analysis

Sensitivity for each assay was determined by the number of method positives divided by the total number of positive samples. The specificity for each assay was calculated by the number of method negatives divided by the total number of negative samples.

Statistical Analysis

A Chi square analysis was used to determine if a significant difference existed between the ELISA and DNAH assay in identifying *Listeria monocytogenes* in powdered dairy products after exposure to freezing. The Chi square analysis was calculated by using the following 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).

Figure 12. From left to right. Modified Oxford medium (MOX) is a selective agar that turns black in the presence of *Listeria monocytogenes* from the hydrolysis of esculin. Trypticase soy agar-yeast extract (TSA-YE) is a non-selective agar that allows for growth of many organisms including *Listeria monocytogenes*. McBride's agar is a selective agar that shows small white colonies if *Listeria* is present.



CHAPTER 3

RESULTS

ELISA vs. DNAH

Thirty powdered dairy products (cheddar cheese, milk, and lactose) were tested by the ELISA and DNAH assay at three different periods: samples at room temperature, sample exposure to freezing for one month, and sample exposure to freezing for four months. Twenty-five of the thirty samples were artificially inoculated with *Listeria monocytogenes*. The remaining five samples were not inoculated to serve as a control and to determine specificity for each assay.

The ELISA identified 25 positive and five negative samples for the testing period at room temperature. Table 1 shows the positive and negative results for each powdered food product tested (cheddar cheese, milk, and lactose). Ten positives and three negatives were identified for the cheddar cheese products. Five positive and two negatives were determined for the powdered milk products, whereas all five lactose samples were found to be positive. Appendices A-C show the absorbance for each powdered dairy sample tested with the ELISA at room temperature. These readings were higher than normal but were validated by a significant separation in absorbance values observed between positive and negative samples. Positive sample determination was based on darkness of coloration after the stop solution was added. Samples were visually compared to the positive and negative controls. Positive samples were dark yellow, and negative samples were lighter in color or exhibited no color at all.

Table 1. Results determined by the ELISA for all powdered dairy products tested at room temperature.

	Number per Method				
Product	Samples	Positive	Negative	False Positive	False Negative
Cheddar Cheese	18	15	3	0	0
Milk	7	5	2	0	0
Lactose	5	5	0	0	0

For samples tested after exposure to freezing for one month, the ELISA accurately determined all 25 positive samples and five negative samples. Table 2 shows the results for each powdered food product. Again, 10 out of 13 samples were determined positive in cheddar cheese products while five out of seven and five out of five samples were identified as positive for powdered milk and lactose products, respectively. Appendices D-F list the absorbance rates for each sample tested after exposure to freezing for one month by the ELISA. The absorbance for the positive and negative controls were within the proper limits, thus making this test valid.

After sample exposure to freezing for four months, the ELISA identified all 25 positive samples. However, it only identified four negative samples, thus giving one false positive result (Table 3). The results for the ELISA for each powdered dairy product are also provided in Table 3. The absorbance for each sample tested is shown in Appendices G-I. In Table 3, sample number 13 had an absorbance of 0.434 which was greater than the absorbance limit (0.300 OD) to be considered a negative result. The absorbance for the positive and negative controls were again within the proper limits.

As seen in Table 4, the ELISA demonstrated 100% sensitivity by identifying all positive samples for each time interval tested. The specificity was 100% with the ELISA for identifying all negative samples tested at room temperature and after freezing for one month. However, since the ELISA only determined four out of the five negative samples after freezing for four months, thus giving one false positive, the specificity was only 80% (Table 4). Sensitivity was determined by the number of method positives divided by

Table 2. Results determined by the ELISA for all powdered dairy products after one month of freezing.

	Number per Method				
Product	Samples	Positive	Negative	False Positive	False Negative
Cheddar Cheese	18	15	3	0	0
Milk	7	5	2	0	0
Lactose	5	5	0	0	0

Table 3. Results determined by the ELISA for all powdered dairy products after four months of freezing.

		Number per Method			
Product	Samples	Positive	Negative	False Positive	False Negative
Cheddar Cheese	18	15	2	1	0
Milk	7	5	2	0	0
Lactose	5	5	0	0	0

Table 4. Sensitivity and specificity of the ELISA for all three testing periods.

Time of Sample Testing	Sensitivity %[1]	Specificity %[2]
Room Temperature	100	100
One Month Exposure	100	100
Four Months Exposure	100	80[3]

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

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

[3] One false negative was determined.

the total number of sample positives. Specificity was calculated by the number of method negatives being divided by the total number of sample negatives.

The DNAH assay identified 24 out of 25 positive samples at room temperature (Table 5). All 10 cheddar cheese samples and all five powdered milk samples were identified correctly. However, there was a false negative in the lactose group (Table 5), which means this sample absorbance was below the positive determination absorbance rate. Appendices J-L list the absorbance for each sample tested at room temperature. After the initial testing, samples numbered 25 and 44 were both considered false negatives. After retesting these samples, sample number 25 was identified as being positive while sample 44 was still determined as a false negative (Table 5). The absorbance for the positive and negative controls were within the proper limits, thus making this test valid.

For the samples exposed to freezing for one month, the DNAH assay identified 23 out of 25 positive samples (Table 6). Again, all cheddar cheese and powdered milk samples were identified correctly. However, only three out of five lactose samples were determined to be positive, thus resulting in two false negatives. The absorbance for each of the dairy products is listed in Appendices M-O. Samples numbered 43 and 44 were retested but still produced false negative results. The absorbance for the positive and negative controls were again within the proper limits.

The DNAH assay identified all 25 positive samples that had been exposed to freezing for four months (Table 7). One false positive was determined in the powdered milk samples. The absorbance for each dairy sample is listed on Appendices P-R.

Table 5. Results determined by the DNAH assay for all powdered dairy products tested at room temperature.

	Number per Method				
Product	Samples	Positive	Negative	False Positive	False Negative
Cheddar Cheese	18	15	3	0	0
Milk	7	5	2	0	0
Lactose	5	4	0	0	1

Table 6. Results determined by the DNAH assay for all powdered dairy products tested after sample exposure to freezing for one month.

		Number per Method			
Product	Samples	Positive	Negative	False Positive	False Negative
Cheddar Cheese	18	15	3	0	0
Milk	7	5	2	0	0
Lactose	5	3	0	0	2

Table 7. Results determined by the DNAH assay for all powdered dairy products tested after sample exposure to freezing for four months.

	Number per Method				
Product	Samples	Positive	Negative	False Positive	False Negative
Cheddar Cheese	18	15	3	0	0
Milk	7	5	1	1	0
Lactose	5	5	0	0	0

The samples numbered 31 and 34 were determined as false negatives after initial testing. After retesting these samples, both were identified as being positive for *Listeria* (Appendix Q). The absorbance for the positive and negative controls were again within the proper limits.

As seen in Table 8, the sensitivity for the DNAH assay when testing samples at room temperature was 96%; one false negative had been determined during testing. For samplesexposed to freezing for one month, the sensitivity was 92% with two samples identified as being false positives. The sensitivity for the DNAH assay was 100% for samples that had been exposed to freezing for four months. The specificity was 100% for both the room temperature and one month testing periods. The specificity was only 80% for the sample testing after four months of freezing because one negative sample was identified as a false positive (Table 8). Table 9 compares sensitivity and selectivity percentages for the ELISA and DNAH assay for all three testing periods.

Table 10 shows the overall comparison between the ELISA and DNAH assay. The ELISA identified all 75 possible samples, whereas the DNAH assay identified only 72 positive. Both assays produced one false positive. However, the DNAH assay determined a total of three false negatives, whereas the ELISA showed none (Table 10). A Chi square analysis of total positive samples identified between the ELISA and the DNAH assay had a value of 0.44 (Table 10). A comparison of the ELISA and DNAH assay of total positive samples verses total negative samples provided a Chi square value of 3.06 (Table 11). Table 12 shows the comparison of samples identified at -10^0C and -70^0C by the ELISA and DNAH assay. There was a total of 25 positive samples tested at

Table 8. Sensitivity and specificity of the DNAH assay for all three testing periods.

Time of Sample Testing	Sensitivity %[1]	Specificity %[2]
Room Temperature	96[3]	100
One Month Exposure	92[4]	100
Four Months Exposure	100	80[5]

[1] Sensitivity was determined as stated before.

[2] Specificity was determined as stated before.

[3] One false negative was determined.

[4] Two false negatives were determined.

[5] One false positive was determined.

Table 9. Comparison of sensitivity and specificity percentages for the ELISA and DNAH assay for all three testing periods.

Time of Sample Testing	Sensitivity %[1]		Specificity %[2]	
	ELISA	DNA	ELISA	DNA
Room Temperature	100	96	100	100
One Month Exposure	100	92	100	100
Four Months Exposure	100	100	80	80

[1] Sensitivity was determined as stated before.

[2]Specificity was determined as stated before.

Table 10. Comparison of the ELISA and DNAH assay on total number of positive samples determined for all three testing periods.

Time of Sample Testing	Positive Samples	ELISA Positive	DNAH Positive
Room Temperature	25	25	24[1]
One Month Exposure	25	25	23[2]
Four Months Exposure	25	25[3]	25[3]
Overall Total	75	75	72

$*\chi^2=0.44$

$\chi^2_{0.05},df=2,=5.99$

χ^2_is not significant

[1] A false negative was determined.

[2] Two false negatives were determined.

[3] A false positive was determined.

* Chi square analysis was calculated by 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.

Table 11. Comparison of the ELISA and DNAH assay for identification of total positive samples verses total negative samples.

Assay	Total Positive Samples	Total Negative Samples
ELISA	75	0
DNAH	72	3

$*\chi^2=3.06$

$\chi^2_{0.05},df=1,=2.84$

χ^2 is significant, $P<0.05$

* Chi square was determined as stated before.

Table 12. Comparison of the ELISA and DNAH assay in identifying *Listeria monocytogenes* in differing frozen environments.

Assay	Freezing for One Month Positive Samples at -10^{0}C	Positive Samples at - 70^{0}C
ELISA	13	12
DNAH	13	10

$*\chi^2=0.167$

$\chi^2_{0.05,df=1,}=2.84$

χ^2 is not significant

Assay	Freezing for Four Months Positive Samples at -10^{0}C	Positive Samples at - 70^{0}C
ELISA	13	12
DNAH	13	12

$*\chi^2=0.00$

$\chi^2_{0.05,df=1,}=2.84$

χ^2 is not significant

* Chi square was determined as stated before.

each period. Thirteen samples were placed in a -10^{0}C environment and 12 samples were placed in a -70^{0}C environment.

For the one month testing period, the ELISA identified all positive samples, whereas the DNAH assay identified all 13 positive samples from the -10^{0}C environment, but only determined 10 of the 12 positive samples from the -70^{0}C environment (Table 12). Both assays identified all positive samples in the four month testing period in each frozen atmosphere. A Chi square analysis on the comparison of samples identified by the ELISA and DNAH assay at the different temperatures of -10^{0}C and -70^{0}C showed a value of 0.167 (Table 12).

Comparison of Plating Agars

All four plating agars showed growth from samples tested at room temperature. For the samples tested after one month of freezing, results varied for the four media. Table 13 lists the total plates that showed growth for each dairy product group. For the 15 cheddar cheese samples, MOX and McBride's agars showed growth on 14 plates, and TSA-YE and LPM agars showed growth from all 15 samples. All plates showed growth for all powdered milk samples tested. For the five lactose samples, MOX and McBride's agars only showed growth for two of the samples while TSA-YE and LPM agars showed growth from five and three samples, respectively (Table 13). The overall results showed MOX and McBride's agars producing growth on 21 out of 25 samples. LPM agar showed growth on 23 plates, and TSA-YE produced growth on all 25 positive samples tested. Appendices S-T list positive or negative growth on each agar for each sample.

Table 13. Summary comparison of four different plating agars for growth of *Listeria monocytogenes* in all samples tested after one month of freezing.

Product	Total Positive Samples	MOX Plates	TSA-YE Plates	LPM Plates	McBride's Plates
Cheddar Cheese	15	14	15	15	14
Milk	5	5	5	5	5
Lactose	5	2	5	3	2
Overall Total	25	21	25	23	21

The results of growth on all four media for the samples tested after exposure to freezing for four months is given on Table 14. Out of 15 positive samples for cheddar cheese product, MOX showed growth on 11 plates, whereas LPM and TSA-YE showed growth for all 15 samples. McBride's agar only produced growth for one sample tested.

For the powdered milk samples, MOX, LPM, and TSA-YE showed growth for all five positive samples. MOX, LPM, and TSA-YE also showed growth for all five positive samples in the lactose product. McBride's agar didn't produce any growth for any lactose sample. The overall results determined growth for all 25 positive samples on LPM and TSA-YE agars. MOX agar showed growth for 21 samples, and McBride's agar only showed growth on two plates (Table 14). Appendices U-V list positive and negative growth of each sample exposed to freezing for four months on the four different plating agars.

Table 14. Summary comparison of four different plating agars for growth of *Listeria monocytogenes* in all samples tested after four months of freezing.

Product	Total Positive Samples	MOX Plates	TSA-YE Plates	LPM Plates	McBride's Plates
Cheddar Cheese	15	11	15	15	1
Milk	5	5	5	5	1
Lactose	5	5	5	5	0
Overall Total	25	21	25	25	2

CHAPTER 4

DISCUSSION

ELISA vs. DNAH

Over the past decade, there has been an increase in research involving *Listeria monocytogenes* prompted by foodborne listeriosis outbreaks in the early 1980s, its role in food contamination, and its ability to cause disease (Schlech, 1991). Research has shown *Listeria monocytogenes* can be resistant to heating (Farber et al., 1992) and freezing (El-Kest and Marth, 1991b), and can grow in a wide range of pH conditions (Schuchat et al., 1991; Hill et al., 1995) and NaCl concentrations (Hudson, 1992).

There has also been extensive research in identification assays and media for determining the presence of *Listeria monocytogenes* in food products. Culture-based methods developed by the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) have been the standard protocols in identifying *Listeria* species (Walker et al, 1990). In 1988, Mattingly et al. described a rapid diagnostic test for the detection of *Listeria monocytogenes*. This assay uses monoclonal antibodies that are specific for protein antigens found on all *Listeria* species (Butman et al., 1988). This test is known as an enzyme-linked immunosorbent assay or ELISA. Comparisons between the ELISA and cultural methods have been conducted, and studies show that the ELISA produces quicker results and is a good alternative to the cultural methods (Curiale et al., 1994a; Walker et al., 1990).

Another test developed recently is the deoxyribonucleic acid hybridization assay (DNAH). This assay uses synthetic DNA probes complementary in sequence to the ribosomal ribonucleic acid (rRNA) of *Listeria* (Klinger et al., 1988). Comparisons between the DNAH assay and cultural methods has determined that the DNAH assay is also another good alternative in identifying *Listeria monocytogenes* in food products (Url et al., 1993; Bottari et al., 1995).

In this study, comparisons were made between the ELISA and the DNAH assay. Thirty powdered dairy samples (cheddar cheese, milk, and lactose) were used in this experiment. Twenty-five of the samples were inoculated with *Listeria monocytogenes,* and the remaining five were used as a control. The samples were tested at three different time intervals: room temperature, after one month of freezing, and after four months of freezing. The samples were divided equally and placed in either -10^0C or -70^0C frozen environments. Ray (1979) showed that *Listeria* is injured by freezing, but the organism can repair itself, regain the capacity to multiply under favorable conditions, and become pathogenic or disease-causing. Therefore, identification methods must not only identify healthy, virulent cells, but also identify injured cells resulting from freezing and other harsh conditions.

In this study, the ELISA identified all 25 positive samples at room temperature (Table 1), whereas the DNAH assay only identified 24 positive samples (Table 5). The unidentified positive sample was a lactose product, and this false negative result could have resulted from low organismal presence. Martin and Katz (1993) stated that both the DNAH assay and the ELISA require at least 10^5-10^6 organisms/ml before detection.

Murphy et al. (1996) showed that non-distribution throughout a food product can be misleading in the analysis of food contamination. The false negative result from the DNAH assay could have been caused by low distribution of *Listeria monocytogenes* in the sample tested.

After sample exposure to one month of freezing at -10^{0}C or -70^{0}C, the ELISA identified all 25 positive samples (Table 2). The DNAH assay determined only 23 positive, thus resulting in two false negatives (Table 6). Again, the false negatives were obtained from the lactose products. Low numbers of organism in the sample tested or injured cells, caused by freezing temperatures (-70^{0}C), not being resuscitated by the enrichment procedures before performing the assay could explain the false negative results.

Both the ELISA and the DNAH assay identified all 25 positive samples at the four-month testing period (Tables 3 and 7). However, both the ELISA and the DNAH assay only identified four out of the five negative samples, thus resulting in a false positive (Tables 4 and 8). This could be due to improper testing technique (Organon Teknika Corporation, 1994) or sample contamination.

The ELISA proved to be more accurate than the DNAH in the overall total of samples tested. The ELISA identified all 75 positive samples, whereas the DNAH assay only determined 72 positives, thus resulting in three false negatives (Table 10). The sensitivity for the ELISA was 100% for each testing period and the specificity was 100% for the room temperature and one-month-exposure to freezing periods. With the one false positive determined in the four-month testing period, the specificity for the ELISA was

only 80% (Table 9). The sensitivity for the DNAH when testing at room temperature was 96% with one false negative (Table 9). The sensitivity rate for the one-month testing period was 92%, again because of false negative results. For the four- month sample testing period, the sensitivity was 100% (Table 9). The specificity for the DNAH assay was 100% for the room temperature and one-month periods. Also, because one false positive, the DNAH assay specificity for the four-month period was only 80% (Table 9).

Table 10 shows a Chi square analysis with a value of 0.44 for the comparison of the ELISA and DNAH assay in the identification of total samples. This value indicated that there was not a significant difference in identification of total samples between the ELISA and DNAH assay. There was also no significant difference seen between each individual testing period. However, when comparing total positive samples verses total negative samples, the Chi square value of 3.06 indicated a significant difference between the ELISA and DNAH assay (Table 11). Therefore, in food microbiological analysis, mis-identification of *Listeria monocytogenes* in food samples could be detrimental to public health.

There was no significant difference seen between the ELISA and DNAH assay for identification of positive samples in the differing frozen temperatures of -10^0C and -70^0C (Table 12). A total of 25 positive samples tested at each period were separated equally into each frozen environment with thirteen samples in -10^0C and 12 samples in -70^0C. For the one month testing period, the ELISA identified all positive samples. The DNAH assay, however, identified all 13 positive samples from the -10^0C environment, but only determined 10 of the 12 positive samples from the -70^0C environment (Table 12). Both

assays identified all positive samples in the four month testing period in each frozen atmosphere. It should be noted that a greater sample size might lead to a significant difference between the ELISA and DNAH assay.

Conclusions about the comparison of the ELISA to the DNAH assay were fourfold. First, the ELISA proved to be the more effective assay by identifying all positive samples. Second, the ELISA procedures were easier to follow and testing time was faster. Third, both the ELISA and DNAH assay methods were able to demonstrate the resuscitation of injured *Listeria* and identify the organism in powdered dairy products. The DNAH assay might not be suitable for testing lactose products because of the high false negative rate in this experiment. However, low numbers of organisms resulting from poor distribution within the in the samples tested would serve as one explanation for the false negatives. Finally, there were no differences seen between samples tested from the -10^{0}C or -70^{0}C environment; the *Listeria monocytogenes* cells were able to survive and be resuscitated during enrichment procedures before analysis with the ELISA and DNAH assay at both temperatures.

It should be noted that both the ELISA and DNAH assay methods can reduce the analysis time of determining food products for contamination. However, no one method is 100% accurate (Martin and Katz, 1993). It is difficult to obtain true representative food samples (Murphy et al., 1996). Problems with obtaining samples from processed food plants include non-uniform distribution of *Listeria* throughout the food and small sample size, which can be misleading in the analysis of an entire food product (Murphy et al., 1996).

Comparison of Plating Agars

There are several selective and non-selective plating agars used to identify *Listeria monocytogenes* in food products. A selective agar inhibits the growth of competitive flora and allows growth of the selected species, whereas a non-selective agar allows growth of all organisms (Speck and Ray, 1977). The selective agars used in this project were modified Oxford medium (MOX), lithium chloride-phenylethanol-moxalactam (LPM), and McBride's agar. The non-selective medium was trypticase soy agar-yeast extract (TSA-YE).

In this study, all four plating agars showed growth from the samples tested at room temperature. In samples tested after one month of freezing, TSA-YE showed growth on all 25 samples that tested positive for *Listeria monocytogenes* (Table 13). However, in sample 43 (a lactose product), a gram stain on colonies showed no presence of *Listeria monocytogenes*; however, gram negative rods and cocci were observed (Appendix T). Because TSA-YE agar is non-selective, competitive flora might have reduced or eliminated the growth of *Listeria monocytogenes*. In Appendix T, growth of *Listeria* colonies was not observed on the selective agars for sample 43. Again, low numbers of organism in the sample tested or injured cells, caused by freezing temperatures (-70^{0}C), not being resuscitated by the enrichment procedures could explain the lack of growth on all plating agars. MOX and McBride's agars produced growth on 21 plates, whereas LPM showed growth on 23 plates (Table 13).

For the four-month testing period, TSA-YE and LPM agars demonstrated better results with growth on all 25 plates (Table 14). MOX agar produced growth from 21

samples, but McBride's agar only showed growth on two plates (Table 14). A gram stain was performed again from colonies on the TSA-YE plates. In Appendix U, the gram stain did not show the presence of *Listeria monocytogenes*; gram negative rods and cocci were observed, indicating sample contamination.

In this study, TSA-YE showed the most bacterial growth; however, *Listeria monocytogenes* was not always present. Biochemical tests should be used to further explore the presence of *Listeria.* Also, *Listeria* might be present in the food sample, but the competitive flora reduced or eliminated the presence of *Listeria* on non-selective agar plates. LPM agar showed the most growth out of the selective agars for *Listeria.* However, MOX agar provided easier determination of *Listeria* being present due to the blackening of the agar caused by hydrolysis of esculin. This is supported by the research of Westöö and Peterz (1992). They showed that Oxford agar allowed for greater recovery than LPM agar. Paranijpye et al. (1992) also concluded that visualization of color change makes MOX medium better than LPM. In this study, McBride's agar showed poor growth; therefore, it might not be a suitable agar for plating when testing freeze-injured cells of *Listeria monocytogenes* from powdered dairy products. It should be noted, however, that no single medium is suitable for recovery of all different food types and methods of injury (Pini and Gilbert, 1988; Lammering and Doyle, 1989).

CHAPTER 5

SUMMARY

Listeria monocytogenes is a human pathogen commonly found in food products such as meats, poultry, and dairy products. There has been an increase in disease, thus resulting in new media and techniques in identifying *Listeria* in food products. Culture-based methods are slow in identification of contamination, whereas the enzyme-linked immunosorbent assay (ELISA) and the deoxyribonucleic acid hybridization assay (DNAH) are good alternatives and are more rapid than the cultural methods.

Listeria monocytogenes is able to survive heating, freezing, a wide pH range, and other harsh conditions. These conditions injure the bacterium, yet it possesses the capability to repair itself, multiply, and regain virulence. Identification of injured cells in food products is important for public health.

This study compared the ELISA and the DNAH assay by testing dairy products inoculated with *Listeria monocytogenes* and exposed to a frozen environment. The ELISA provided the best results by identifying all positive samples. The DNAH assay misidentified three positive samples in the overall testing results. The ELISA was also an easier and quicker method to perform.

This study showed that there were no significant differences in the identification of positive samples from the -10^0C or -70^0C frozen environments. However, there was a significant difference seen between the ELISA and DNAH assay when comparing total positive samples verses total negative samples. *Listeria monocytogenes* cells were able to

survive freezing and be resuscitated during enrichment procedures before analysis with the ELISA and DNAH assay. In food microbiological analysis, mis-identification of *Listeria monocytogenes* in food products could be detrimental to public health.

This study compared four plating agars used in identifying *Listeria monocytogenes*. Trypticase soy agar-yeast extract (TSA-YE) allowed growth on all plates streaked but *Listeria* was not always present due to the growth of other competitive flora. Lithium chloride-phenylethanol-moxalactam (LPM) and Modified Oxford medium (MOX) showed the best results. However, MOX plates were easier in determining the presence of *Listeria* because of the blackening of the media caused by hydrolysis of esculin. McBride's agar showed poor growth from samples that had been exposed to freezing and is not suitable in identifying injured *Listeria*.

CHAPTER 6

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APPENDICES

Appendix A. Absorbance for powdered cheddar cheese samples tested at room temperature with the ELISA. Rates were higher than normal but still valid. Positive and negative samples were determined visually by final coloration.

Sample #	Absorbance (OD)	Positive/Negative
1	*.***[1]	+
2	*.***	+
3	*.***	+
4	*.***	+
5	*.***	+
6	*.***	+
7	*.***	+
8	*.***	+
9	*.***	+
10	*.***	+
11	0.760	-
12	1.226	-
13	0.586	-
21	1.882	+
22	*.***	+
23	*.***	+
24	*.***	+
25	*.***	+

[1] A sample is considered positive if the absorbance is *.*** because of the rate is greater than the Microplate reader can indicate with a number value.

Appendix B. Absorbance for powdered milk samples tested at room temperature with the ELISA. Rates were higher than normal but still valid. Positive and negative results were visually determined by final coloration.

Sample #	Absorbance (OD)	Positive/Negative
31	*.***[1]	+
32	*.***	+
33	*.***	+
34	*.***	+
35	*.***	+
36	1.293	-
37	1.231	-

[1] The *.*** again indicates a high absorbance as stated before.

Appendix C. Absorbance for lactose samples tested at room temperature with the ELISA. Rates were higher than normal but still valid. Positive and negative results were determined visually by final coloration.

Sample #	Absorbance (OD)	Positive/Negative
41	*.***[1]	+
42	*.***	+
43	*.***	+
44	*.***	+
45	*.***	+

[1] The *.*** again indicates a high absorbance as stated before.

Appendix D. Absorbance for powdered cheddar cheese samples tested with the ELISA after sample exposure to freezing for one month.

Sample #	Absorbance (OD)	Positive/Negative
1	*.***[1]	+
2	0.770	+
3	0.820	+
4	*.***	+
5	*.***	+
6	1.096	+
7	0.921	+
8	*.***	+
9	0.578	+
10	0.440	+
11	0.230	-
12	0.243	-
13	0.248	-
21	0.342	+
22	1.233	+
23	0.310	+
24	*.***	+
25	*.***	+

[1] The *.*** again indicates a high absorbance as stated before.

Appendix E. Absorbance for powdered milk samples tested with the ELISA after sample exposure to freezing for one month.

Sample #	Absorbance (OD)	Positive/Negative
31	*.***[1]	+
32	*.***	+
33	*.***	+
34	*.***	+
35	*.***	+
36	0.238	-
37	0.213	-

[1] The *.*** again indicates a high absorbance as stated before.

Appendix F. Absorbance for lactose samples tested with the ELISA after sample exposure to freezing for one month.

Sample #	Absorbance (OD)	Positive/Negative
41	*.***[1]	+
42	*.***	+
43	0.447	+
44	0.836	+
45	0.516	+

[1] The *.*** again indicates a high absorbance as stated before.

Appendix G. Absorbance for powdered cheddar cheese samples tested with the ELISA after sample exposure to freezing for four months.

Sample #	Absorbance (OD)	Positive/Negative
1	*.***[1]	+
2	*.***	+
3	*.***	+
4	*.***	+
5	*.***	+
6	*.***	+
7	*.***	+
8	*.***	+
9	*.***	+
10	*.***	+
11	0.156	-
12	0.174	-
13	0.434[2]	-
21	*.***	+
22	*.***	+
23	*.***	+
24	*.***	+
25	*.***	+

[1] The *.*** again indicates a high absorbance as stated before.

[2] Absorbance was greater than 0.300 OD, thus resulting in a false positive.

Appendix H. Absorbance for powdered milk samples tested with the ELISA after sample exposure to freezing for four months.

Sample #	Absorbance (OD)	Positive/Negative
31	*.***[1]	+
32	*.***	+
33	*.***	+
34	*.***	+
35	*.***	+
36	0.163	-
37	0.174	-

[1] The *.*** again indicates a high absorbance as stated before.

Appendix I. Absorbance for lactose samples tested with the ELISA after sample exposure to freezing for four months.

Sample #	Absorbance (OD)	Positive/Negative
41	*.***[1]	+
42	*.***	+
43	*.***	+
44	*.***	+
45	*.***	+

[1] The *.*** again indicates a high absorbance as stated before.

Appendix J. Absorbance for powdered cheddar cheese samples tested at room temperature with the DNAH assay.

Sample #	Absorbance (OD)	Positive/Negative
1	0.33	+
2	0.49	+
3	1.50	+
4	1.25	+
5	1.19	+
6	1.39	+
7	1.59	+
8	1.27	+
9	1.35	+
10	1.48	+
11	0.04	-
12	-0.04	-
13	-0.04	-
21	1.67	+
22	1.86	+
23	1.31	+
24	1.38	+
25	1.09[1]	+

[1] First testing resulted in a false negative. A positive result was confirmed after retesting.

Appendix K. Absorbance for powdered milk samples tested at room temperature with the DNAH assay.

Sample #	Absorbance (OD)	Positive/Negative
31	1.26	+
32	1.31	+
33	1.14	+
34	1.28	+
35	0.63	+
36	-0.00	-
37	-0.02	-

Appendix L. Absorbance for lactose samples tested at room temperature with the DNAH assay.

Sample #	Absorbance (OD)	Positive/Negative
41	0.78	+
42	1.47	+
43	1.02	+
44	-0.02[1]	+
45	1.45	+

[1] First testing resulted in a false negative. After retesting, absorbance still showed a false negative.

Appendix M. Absorbance for powdered cheddar cheese samples tested with the DNAH assay after sample exposure to freezing for one month.

Sample #	Absorbance (OD)	Positive/Negative
1	1.61	+
2	0.77	+
3	1.43	+
4	1.14	+
5	1.61	+
6	1.19	+
7	1.55	+
8	1.65	+
9	0.88	+
10	1.69	+
11	-0.01	-
12	-0.01	-
13	-0.01	-
21	0.72	+
22	1.87	+
23	1.89	+
24	0.82	+
25	1.88	+

Appendix N. Absorbance for powdered milk samples tested with the DNAH assay after sample exposure to freezing for one month.

Sample #	Absorbance (OD)	Positive/Negative
31	1.89	+
32	1.81	+
33	1.93	+
34	1.99	+
35	1.78	+
36	0.04	-
37	0.05	-

Appendix O. Absorbance for lactose samples tested with the DNAH assay after sample exposure to freezing for one month.

Sample #	Absorbance (OD)	Positive/Negative
41	1.94	+
42	1.92	+
43	-0.02[1]	+
44	-0.03[1]	+
45	0.74	+

[1] Initial testing and retesting determined a false negative.

Appendix P. Absorbance for powdered cheddar cheese samples tested with the DNAH assay after sample exposure to freezing for four months.

Sample #	Absorbance (OD)	Positive/Negative
1	1.77	+
2	1.86	+
3	1.78	+
4	1.85	+
5	1.38	+
6	1.67	+
7	1.53	+
8	1.68	+
9	0.47	+
10	1.59	+
11	0.06	-
12	0.00	-
13	-0.02	-
21	1.50	+
22	1.13	+
23	1.60	+
24	1.12	+
25	1.53	+

Appendix Q. Absorbance for powdered milk samples tested with the DNAH assay after sample exposure to freezing for four months.

Sample #	Absorbance (OD)	Positive/Negative
31	1.88[1]	+
32	1.48	+
33	1.56	+
34	1.79[1]	+
35	1.07	+
36	0.08	-
37	0.13[2]	-

[1] Initial testing resulted in a false negative. Retesting confirmed a positive result.

[2] The absorbance was above 0.10 OD and was determined as a false positive.

Appendix R. Absorbance for lactose samples tested with the DNAH assay after sample exposure to freezing for four months.

Sample #	Absorbance (OD)	Positive/Negative
41	1.14	+
42	1.38	+
43	1.06	+
44	1.70	+
45	1.02	+

Appendix S. Comparison of four different plating agars for growth of *Listeria monocytogenes* in powdered cheddar cheese samples tested after freezing for one month.

Sample #	MOX	TSA-YE	LPM	McBride's
1	+[1]	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	-[2]
7	+	+	+	+
8	+	+	+	+
9	+	+	+	+
10	+	+	+	+
21	-	+	+	+
22	+	+	+	+
23	+	+	+	+
24	+	+	+	+
25	+	+	+	+

[1] A positive indicates good growth (>300 colonies).

[2] A negative indicates poor growth (<20 colonies) or no growth.

Appendix T. Comparison of four different plating agars for growth of *Listeria monocytogenes* in powdered milk (31-35) and lactose (41-45) samples tested after freezing for one month.

Sample #	MOX	TSA-YE	LPM	McBride's
31	+	+	+	+
32	+	+	+	+
33	+	+	+	+
34	+	+	+	+
35	+	+	+	+
41	+	+	+	+
42	+	+	+	+
43	-	+[1]	-[2]	-
44	-	+	-[2]	-
45	-	+	+	-

[1] Gram stain did not show the presence of *Listeria monocytogenes*. Gram negative rods and cocci were observed.

[2] No growth of *Listeria monocytogenes* was observed and thus resulted in a false negative when tested by the DNAH assay.

Appendix U. Comparison of four different plating agars for growth of *Listeria monocytogenes* in powdered cheddar cheese samples tested after freezing for four months.

Sample #	MOX	TSA-YE	LPM	McBride's
1	-	+[1]	+	-
2	+	+	+	-
3	+	+	+	-
4	+	+	+	-
5	-	+	+	+
6	-	+	+	-
7	+	+	+	-
8	+	+	+	-
9	+	+	+	-
10	-	+	+	-
21	+	+	+	-
22	+	+	+	-
23	+	+	+	-
24	+	+	+	-
25	+	+	+	-

[1] Gram stain did not show any presence of *Listeria monocytogenes*. Gram negative rods and cocci were observed.

Appendix V. Comparison of four different plating agars for growth of *Listeria monocytogenes* in powdered milk (31-35) and lactose (41-45) samples tested after freezing for four months.

Sample #	MOX	TSA-YE	LPM	McBride's
31	+	+	+	+
32	+	+	+	-
33	+	+[1]	+	-
34	+	+	+	+
35	+	+	+	-
41	+	+	+	-
42	+	+	+	-
43	+	+	+	-
44	+	+	+	-
45	+	+	+	-

[1] Gram stain verified the presence of *Listeria monocytogenes* with the presence of gram positive bacilli.

Robert Brent Lee
Signature of Graduate Student

Billie L. Johnson
Signature of Major Advisor

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July 30, 1997
Date

Acquisition of *Listeria monocytogenes* from Powdered Dairy Products Using ELISA and DNA Gene Probe Assays Prior to, and Following, Freezing of Samples at -10^{0}C and -70^{0}C

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